Winter School on
Advances in Molecular and Serological Tools in Fish Disease Diagnosis

9-29 November 2013

Dr. P. Jayasankar
Director

Dr. S.S. Mishra
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& Co-course Director

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Organised by :

Fish Health Management Division
Central Institute of Freshwater Aquaculture
P.O. - Kausalyaganga, Via-Bhubaneswar-2

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(Indian Council of Agricultural Research)
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CURRENT AND FUTURE MOLECULAR DIAGNOSTIC TESTS FOR USE IN THE AQUATIC MICROBIOLOGY LABORATORY

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Introduction

Nucleic acid based diagnostics gradually are replacing or complementing culture based, biochemical and immunological in routine microbiology laboratories. The main function of all diagnostic bacteriology laboratories is the detection and identification of microorganisms in a variety of samples of freshwater, brackish water and marine origin. Similar to conventional test, the first generation deoxy ribonucleic acid assay determined only a single analysis. Recent improvements in detection technologies have paved the way for the development of multiparameter assays using macro arrays or microarray, while the introduction of closed tube real time polymerase chain reaction systems has resulted in the development of rapid microbial diagnostics with a reduced contamination risk. The use of these new molecular technologies is not restricted to detection and identification of microbial pathogens but also can be used for genotyping, allowing one to determine antibiotics resistance or to perform microbial fingerprinting.

Phenotype-based Methods

A first indication of the presence of nature of an organism can be obtained by direct microscopic examination of the specimen. Different staining procedures routinely are used, with the Gram stain being the most common. However, mostly the final confirmation of bacterial infections is based on culturing the pathogen in selective growth medium followed by characterization of the organism based on phenotypic criteria. The requirements for standardization, quality, efficiency, and reduced labour cost have led to the introduction of automated systems into the bacteriology laboratory for the isolation and identification of microorganisms. Continuously monitored blood culture systems such as BACTEC 9000 (BD Diagnostics, Sparks, MD) or BacT/ALERT (Bio Merieux, Marcy I'Etoile, France) are standard laboratory equipment. For identification and drug susceptibility testing, both manual and automated systems are well established. All systems are based on the miniaturization of conventional methods to reduce the volume of reagents, increase the user-friendliness, and shorten the time to obtaining a result. Manual sytems such as API (Bio Merieux) or Crystal ID (BD Diagnostics) require skilled users, whereas the stand alone systems, Vitek (BioMerieux), Phoenix (BD Diagnostics), or Micro Scan WalkAway (Dade Behring, Deerfield, IL), Generate results automatically Turnaround time from receipt of the sample in the laboratory to delivery of the report to the physician for culture based methods requires minimally 24h, but an average of 48 h or more usually are needed.

Rapid detection and identification of microorganisms without culture was made possible by the development of immunoassays, which are based on the specific binding between an antigen and its corresponding antibody. The assays either detect the presence of specific antibodies raised in response to an antigen or detect the substance with antigenic properties itself. The technique is being used in many applications in bacteriology and is available in many different formats, such as enzyme immunoassays, immunofluorescence assays, latex agglutination assays, line immunoassays or, more recently, lateral-flow immunassays. Rapid identification of microorganisms in clinical samples such as cerebrospinal fluid or urine by direct antigen testing is specific, easy to perform, and

economical, whereas screening for specific antibodies is used mostly for serodiagnosis and allows the differentiation of acute or past infections(2). Although the different assay formats are continually being improved, antigen detection still suffers from a lack of sensitivity and, as a result of the time lag before seroconversion, serology can be ineffective for early diagnosis of infection.

Genotype-Based Methods

Both culture based identification and immunological assays use the phenotypic characteristics of the microorganism. However, identification criteria such as colony morphology or production of certain antigens (e.g. toxins) can change or be influence by nutritional or environmental conditions and may lead to misinterpretation of results and subsequent misidentification of the organism. Nucleic acid-based identification methods make use of the more stable genotypic characterstics of the microorganism. Conserved regions or genes in the bacterial genome can be exploited for bacterial genus or species identifcation and used to define taxonomic relationships, whereas genes encoding virulence factors or toxins can be useful for defining the pathogenicity of the organism.

Direct hybridization assays using labeled oligonucleotide probes currently are used for culture confirmation or for direct detection of organisms in clinical or food samples. Probe based assays show a high degree of specificity because, when using stringent reaction conditions, a positive hybridization signal is correlated directly with the presence of the organism. A disadvantage of direct hybridization based assays is the need for a relatively large number of target cells.

This lack of sensitivity can be partially circumvented by using ribosomal ribonucleic acid (rRNA) as a target molecule. Examples are the Accuprobe assays (Gen-Probe Inc., San Diego, CA), which commonly are used in clinical microbiology laboratories, or GeneTrak dipstic assays (NeoGen, Lansing, MI), which mainly are applied for the identification of foodborne pathogens.

Fluorescent *In situ* hybridization (FISH) is an attractictive method for the rapid detection and identification of bacteria or fungi directly from slide smears. This technology has the speed and ease of use of conventional staining methods combined with the specificity of molecular methods. Hybridization with fluorescent labeled probes that target rRNA is performed on smears, and results are observed by fluorescence microscopy. An interesting new development is the use of peptide nucleic acid (PNA) probes in FISH applications. PNA molecules have a neutral peptide like backbone unlike the negatively charged sugar phosphate backbone present in deoxyribonucleic acid (DNA). This unique property allows PNA probes to bind strongly and rapidly to their target sequence. This bond is resistant to nucleaseases and proteases, and the hydrophobic nature of the molecule increases its ability to enter the organism through the hydrophobic cell wall. PNA FISH can be used for rapid species identification of positive blood cultures and also is being applied to investigate the bacterial diversity in environmental samples.

The introduction of nucleic acid amplification technologies that enable the multiplication of a few target molecules to a detectable level has provided new tools for rapid, specific, and sensitive detection, identification, and resistance testing of microorganisms starting from sample material without culturing.The Polymerase chain reaction (PCR) is the technique most used in research or diagnostic laboratories. The literature describes a number of home-brew PCR assays for analyses of the most important bacterial and fungal species based on species specific DNA fragments, toxin encoding

genes, virulence factors, or broad range primers targeting ribosomal RNA genes. Initially, in vitro diagnostic companies focused on the development of amplification based assays for virological applications. Commercial assays only slowly entered the bacteriology laboratory and at present, mainly are designed for detection and identification of slow growing or uncultivable organisms. Alternative DNA amplification techniques such as the legase chain reaction (LCR) have been used for bacterial diagnostics(9). For RNA amplification, techniques such as nucleic acid sequence based amplification (NASBA) and transcription mediated amplification (TMA) are well established in the field. Specific and sensitive detection of RNA can be advantageous, especially in food applications. PCR is based on the detection of intact viable cells and, therefore, a positive reaction may arise from either dead or live cells. Specific amplification of messenger RNA can be used for the detection of living pathogenic or spoiler organisms in food samples.

The analysis of the amplified nucleic acid easily can be performed by agarose gel electrophoresis by suing fragment length as an indicator for identification. The specificity of the primers used in the amplification reaction determines the accuracy of the test result. A lack of purity of the nucleic acid extract or the presence of background DNA can influence specific annealing of the primers, resulting in a specific amplification, which can lead to misinterpretation of the results. Therefore, most of the amplification based assays make use of hybridization probes, immobilized onto a solid support or in solution, for specific detection and identification of the amplified material.

Array Based Technologies

Standard solid phase hybridization uses labeled probes in solution to detect immobilized unlabeled amplified material or target DNA. However, most solid phase hybridization assays are based on the reverse hybridization principle. These methods use specific hybridization probes attached to a solid support to detect labeled amplified material or target DNA in solution. Initially, in assays for detection and identification of single parameters, specific probes were immobilized in the wells of a microtiter plate. Later, advantage was taken of the possibility of using reverse hybridization to investigate the reactivity of an amplification product with multiple probes in a single test run. Macroarrays of fixed multiple probes at specific laocations on nitrocellulose or nylon membranes were developed, with the different probes being applied as dots (dot-blots) or as lines (line probe assay (LiPA). Innogenetics, Gent, Belgium).

A LiPA strip consists of membrane onto which synthetic oligonucleotide probes are bound as parallel lines, Precise probes design, combined with stringent hybridization and wash conditions, allows the hybridization of all oligonucleotides with the required specificity. The target region is labeled during the amplification reaction by using biotinylated primers or by incorporating biotin labeled nucleotides. After the hybridization procedure, specific hybrids formed are visualized by an enzyme based colorimetric procedure. Aside from applications in viral genotyping and human genetics, LiPA is an easy to use tool for the development of probe panels for identification of bacteria or fungi.

Given the complexity involved in detecting all relevant pathogens in a variety of biological sample, the use of a single or a limited number of target genes for all bacteria or fungi of interest is key to the successful design of a multiparameter DNA probe based identification and detection system. The availability of target genes that meet the requirements of broad range amplification and specific probe design is limited. One of these target areas in the microbial genome is the spacer region between the genes codin g for r RNA sequences flanking this intervening region allows the design of asingle primer set for simultaneous amplification of all bacteria or fungi present in a sample.

LiPA strips with spacer probes are used for the identification of pathogens in biological sample, as well as for culture confirmation in cases where convention techni3ques are time consuming, cumbersome, or unreliable. Figure 1 shows the results obtained with a commercial assay, INNO-LiPA Mycobacteria v2.0 (Innogenetics), that was designed for the rapid identification of clinically relevant mycobacterial species.

Research efforts have focused more recently on the development of microarrays. As their name implies, microarrrays are miniaturized versions of macroarrays, with the main difference being in the size of the probe spots. The spot sizes in microarrays are usually less than 200 to 300µ in diameter. Specialized equipment is required for applying the probes to the solid support, and imaging systems are needed for readouts. Whereas macroarrays are limited to fewer than 100 probes, DNA chips can vary from low –density arrays carrying a few hundred to a thousand probes, to high density arrays containing tens of thousands of sports. The two main technologies used for the manufacture of microarrayss are spotting onto the solid support usning specialized dispensers and *in situ* synthesis on the array. The first approach uses pre-synthesized oligonucleotides that are deposited onto the surface of the array followed by fixation of the probes. Solid supports mostly used here are glass, silicone, or plastic slides. In the second method, the oligonucleotide probes are synthesized base-by-base directly onto the surface at predefined locations. The best known example of this technology is the Affymetrix™ system (Affymetrix Inc, Santa Clara, CA) that uses photolithography for the production of high density GeneChip® arrays.

The use of microarrays is well established in research projects for gene expression studies, but their use in diagnostic applications for microbiology is still in its infancy. Diagnostics companies also are developing DNA chips as illustrated by the recent introduction of an Affymetrix™ array Food-Expert-ID® (BioMerieux, Marcy I'Etoile, France).

High specificity, high sensitivity, a rapid turnaround, and user-friendliness are the most important requirements for microbial diagnostic assays. The PamGene 5D Pulse™ (PamGene BV,'s-Hertogenbosch, The Netherlands) microarray technology incorporates unique features that enable it to meet these objectives. The use of a three dimensional membrane structure, which provides a 500- fold increase in reaction surface compared with a flat two dimensional surface, allows more material to bind and enhances the probe target material through the reactive membrane maximizing the reaction kinetics. The ability to measure temperature variation and continuous monitoring of the reaction using fluorescence technology allows melting curve analysis instead of end point detection.

Suspension arrays are based on the coupling of oligonucleotide probes to microbeads that are color coded using different ratios of two fluorescent dyes. A third dye is used for generating labeled target DNA, which is subsequently hybridized in suspension with a set of different beads, each carrying a different probe. The bead mixture is sorted by flow cytometry based on their internal colors and hybridized samples produce a fluorescent signal.

Real-Time Fluorescent PCR Technologies

The introduction of homogeneous or real time PCR assays based on solution phase hybridization methods, which combine fluorescent labeled probes with amplification technologies, have contributed strongly to the acceptance of molecular assays in the microbiology laboratory. Several different probe technologies that are compatible with different real time thermocycling instruments currently are available. Most of these technologies are based on the principle of fluorescence resonance energy transfer (FRET).

This process occurs when two fluorophores are in close proximity to each other and the energy from an excited donor moiety is transferred to an acceptor moiety.

The so-called hydrolysis probes make use of the 5′ to 3′ exonuclease activity of *Taq* DNA Polymerase. An oligonucleotide probe is labeled with a fluorescent reporter dye at the5′ end and a fluorescent quencher dye at the 3′ end. Upon hybridization of the probe molecule to the target DNA during amplification, the 5′ nuclease activity of the enzyme cleaves the probe, separating the reporter dye from the quencher dye, resulting in a measurable increase in fluorescence. PCR thermal cycling results in a measurable increase influorescence. PCR thermal cycling results in an exponential amplification of the target DNA and, subsequently, of fluorescence intensity. This technology is also known as the TaqMan® system, and many applications in bacteriology are described in literature or can be purchased as commercial kits for use on real time instruments from Roche Molecular Diagnostics (Pleasanton, CA) or Applied Biosystems (Foster City, CA.

Molecular beacons are oligonucleotide probes that emit a fluorescent signal only when hybridized to their target molecules. The oligonucleotides are able to form a stem-and-loop structure. The probe-specific sequence is located in the loop, whereas the stem is formed by two complementary sequences unrelated to the target sequence. A reporter dye is attached to the end of one arm and a quencher is attached to the end of the other arm of the stem. In closed formation, no fluorescent signal is produced, but upon hybridization, a conformational change forces the arm sequences apart and moves both dyes away from each other resulting in a fluorescent signal. Several microbiological assays using molecular beacons combined with PCR or nucleic acid sequence-based amplification have been developed for home-brew use or as commercial diagnostics products. Examples of the latter are the IDI-Strep B™ assay, a qualitative test for the rapid detection of Group B *Streptococcus* DNA in vaginal/rectal specimens, and the IDI-MRSA ™ assay, used in the direct detectin ofnasal colonization by methicillin-resistant *Staphylococcus aureus* (MRSA; both from GeneOhm Sciences, San Diego, CA). Both these in Vitro diagnostic assays should be performed on the SmartCycler® instrument (Cepheid, Sunnyvale, CA).

The hybridization probe format was developed for use in the Lightcycler®instrument (Roche Applied Science, Penzberg, Germany) and commonly is referred to as LightCycler® chemistry(LC). Two separate oligonucleotide probes are designed to hybridize next to each other on the target DNA. The donor probe carries a 3′ label, usually 6-carboxy-fluorescein which, upon excitation, will transfer its energy to the 5′ LC dye on the acceptor probe. Any increase in fluorescene during amplification can be monitored in specific channels corresponding to the LC dye use. The recently introduced LightCycler® 2.0 instrument allows multiparameter detection using four defferent dyes, LC Red 610, 640,670 and 705. Hybridization probes can be used for mutation detection based on melting curve analysis. Mismatches in the target region will cause one or both probes to dissociate at a lower temperature than the identical target sequence. Multiple amplicons or polymorphic sites can therefore be identified in the same detection channel based on different melting peaks. Numerous protocols using hybridization probes for quantitative PCR, genotyping, or mutation detection in different fields, including bacteriology, have been published .

An example of the advantage of melting curve analysis is illustrated by the LightCycler® Staphylococcus Kit M GRADE(Roche Diagnostics GmbH, Mannheim, Germany). Differentiation of *S.aureus* and coagulase-negative *Staphylococcus* spp. (CoNS) is based on melting curve analysis after PCR amplification using specific primers devived from the internal transcribed spacer region. *S. aureus* will form a unique melting peak at approx 62°C

to 57° C, whereas CoNS give melting peaks at approx 46° to 57°C . This manufacturer also produces similar kits designed for the detection and differentiation of *Enterococcus faecium* and *E. faecalis* or for the specific detection of *Pseudomonas aeruginosa, Candida albicans,* MRSA, or vancomycin-resistant enterococci. Recently, amultiparameter assay (LightCycler® SeptiFast Test; Roche Diagnostics) was launched for routine applications in critical care medicine. This assay detects the 25 most important bacterial and fungal species causing bloodstream infections (Sepsis).

Although TaqMan probes, molecular beacons, and hybridization probes are used most widely in real time amplification, several alternative probe or primer systems have been developed, for instance, Scorpions® (DxS Ltd. Manchester, UK) or minor groove binding probes (Epoch Biosciences, Bothell, WA.

Sequencing-Based Technologies

Owing to rapid technological developments in equipment and reduction of cost per reaction, DNA sequencing has become established in the routine laboratory. The use of PCR based sequencing reactions and the replacement of slab gel for capillary based electrophoresis instruments has made automation possible. Sequencing currently is being used for the identification of organisms that are difficult to identify using conventional methods or to detect and identify uncultivable organisms. The target studied the most for this application is the ribosomal DNA (rDNA) gene. These rDNA sequences are cornerstone of studies on taxonomy and phylogenetic relationships between bacterial or fungal species. Thousands of ribosomal sequences are available in public as well as private databases and can be used for producing alignments and subsequent species assignment. Other conserved regions in the microbial genome, such as the ribosomal spacer region, the *rpo*B gene, the *gyr*B gene, or eleongation factor Tu, can be used as alternative targets for closely related taxa .

Although Sanger-based dideoxy sequencing is the most widely used method for obtaining genetic information, other technologies such as Pyrosequencing ™ (Biotage AB, Uppsala, Sweden) or mass spectrometry are promising alternatives. Pyrosequencing ™ or sequencing by synthesis is applied mainly for single nucleotide polymorphism (SNP) anyalysis or characterization of short sequences (maximum 100bp). It is based on an enzyme cascade system with real time monitoring or light produced as a result of incorporation of nucleotides. Sequence data are represented in a pyrogram, and the peak heights reflect the number of nucleotides integrated. The method is accurate, easy to use, and data can be obtained within 1 h after target amplification,. Rapid results are an advantage for diagnostic applications where time to result is essential, such as diagnosis of neonatal sepsis.

Initially, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was applied for the analysis of whole bacteria or proteins. Sequencing of bacterial nucleic acids using MALDI-TOF MS has been introduced and further improved during the last few years. Different methods to produce base specific cleavage of nucleic acids have been described. The cleavage products are analyzed by MALDI-TOF MS, and the resulting mass patterns are compared with reference spectra for sequence determination. At this time, mass spectrometry for microbial genotyping is still in its infancy and mainly limited to academic centers or large reference laboratories. However, MALDI TOF MS has the potential to become the method of choice for high throughput testing. The technology gives accurate results, is fast, automatable, and cost efficient.

Microbial Typing

In addition to detection and identification of the microorganism to the genus or species level, a more in depth characterization of the isolates is an important task for the microbiology laboratory. Epidemiological typing not only is important for infection control or monitoring of outbreaks but also for microbial population genetics or studies on pathogenesis. Phenotypic typing methods such as sero-, phage-, pyocin-, and antimicrobials-susceptibility typing are relatively simple and inexpensive techniques, but their discriminatory power is often of little value, especially when the organism in question undergoes physiological changes during chronic infections. Although these techniques are often a first alert for an emerging outbreak, more rapid and higher performance systems for bacterial typing and epidemiological use are available today as the result of the advent of molecular technologies.

The most commonly used techniques for strain characterization are either based on macrorestriction analysis of total genomic DNA or make use of PCR. Based methods for genome typing. Well known examples are pulsed-field gel electrophoresis (PFGE) and ribotyping, both being established methods for bacterial subtyping in the reference laboratory. PFGE generates DNA fingerprints based on the separation of large DNA fragments after digestion of chromosomal DNA using rare-cutting restriction enzymes. In ribotyping, complex RFLP patterns are reduced to a limited number of fragments by hybridization with a rDNA probe. This method allows the identification of organisms to subspecies level, and the technique has been made available commercially in the RiboPrinter® Microbial Characterisation System (Dupont Qualicon, Wilmington, DE.

Alternative techniques use PCR to amplify a number of fragments of differing length to generate DNA fingerprints. Random amplification of polymorphic DNA, or RAPD, uses short random primers at low annealing temperature to generate amplicons across the entire genome. The criticism has been expressed that RAPD lacks reproducibility between laboratories because of the use of non stringent PCR condition. This variability can be reduced by suing primers directed towards specific repetitive sequences distributed throughout the genome such as enterobacterial repetitive intergenic consensus(i.e., ERIC)-PCR, BOX-PCR, or transcription DNA-PCR.

Fingerprinting using a combination of restriction enzyme analysis and PCR is known as the amplified fragment-length polymorphism method (AFLP). This involves restriction of the genomic DNA followed by ligation of double stranded adapters and subsequent amplification of a subset of the restriction fragments. The AFLP primers match their target sequences perfectly, that is , the adapter and restriction site sequence and a small number of selective nucleotides adjacent to the restriction sites. AFLP is a reliable and robust technique that provides an excellent performance in terms of reproducibility and resolution and has become a standard in molecular typing.

Next to these DNA fragment size based typing techniques, DNA sequencing has become an attractive method for the characterization of isolated strains. A multilocus sequence typing is based on sequence comparison of DNA fragments derived from a set of housekeeping genes. Allelic variants are assigned for each house keeping gene based on sequence difference and the alleles obtained for each of the investigated loci define the sequence type of the isolate. Sequence data are unambiguous and have the advantage of being submitted to data base accessible worldwide for further epidemiological studies. Additionally, the enormous progress made in high throughput sequencing and bio informatics has opened the door for whole genome sequence comparison and resulted in the

new discipline of comparative genomics. The number of published microbial whole genome sequences is expected to increase exponentially, and further advancements in bio-informatics will enable scientists to compare nonvirulent and virulent strains, to study pathogenesis, and to study evolutionary relationships between microbes and their hosts. New microbial genotyping procedures that are based upon the use of sets of SNPs with predefined levels of resolution are being developed.

SNP-based genotyping, in combination with the detection of clinically relevant mobile genes, can result in new diagnostic assays providing both epidemiological fingerprints and information on virulence and resistance. The *Pseudomonas aeruginosa* Array Tube (Clondiag GmbH, Jena, Germany) is such a molecular assay for strain discrimination and pathotyping. The first part of each array detects SNPs from conserved gene regions of *P. aeruginosa* allowing strain typing, whereas the second part comprises probes representing relevant pathogenicity markers and gene islands.
Future improvements in nanotechnology will boost the generation of comparative data on bacterial genomes. This information can provide new insight into microbial diversity as well as assist in discovering new tools to fight infectious diseases.

Promising Applications and Some Concerns

In view of the progress made as a result of the application of molecular biological tools in the field of microbiology in general, and in medical microbiology in particular, some aspects are being questioned and re-evaluated, although not necessarily rejected. Some of these are the "species" and "clone" concept, and "Koch's postulates" as well as the definition of the "gold standard". Especially in specific applications like bloodstream infections and endocarditis (47-49). In the next few paragraphs, we will try to frame examples of specific applications of molecular diagnostics and some aspects of concern.

Diagnosis of Bloodstream Infection

Bloodstream infections (BSIs) are a major cause of morbidity and mortality worldwide. It has been shown that an increased time to detection of BSIs was associated with longer hospital stay and higher cost(55). The detection of bacterial or fungal growth in blood cultures currently is considered as the gold standard. Despite improvements in growth media and instrumentation, blood culture is too slow and has a poor diagnostic sensitivity and specificity. Molecular techniques for pathogen identification in positive blood cultures by FISH or PCR are able to generate faster and more specific results than conventional methods. Most attractive are amplification based methods for direct microbial detection in whole blood, which can potentially lead to an increased diagnostic sensitivity, specificity, and shorter time to result and should result in a significant clinical benefit to the patient as well as savings on global hospitalization costs(56). Although first studies showed promising results, more information is needed on the clinical relevance of detecting DNA instead of living bacteria and the interpretation of positive PCR vs negative blood culture results. The use of DNA free reagents and equipment, together with strict procedures to avoid laboratory contamination, is a critical factor for the success of this application.

Determination of Bacterial load

Quantitative PCR is an established technique in the field of gene-expression studies or viral load testing. Since the successful introduction of real time PCR instrumentation in the laboratory, the quantitative detection of bacteria for diagnostic purposes also is attracting much interest, for example, in dental health care (58). The determination of bacterial load has the potential to allow monitoring of antimicrobial therapy and it should enable discrimination among infection., colonization, or possible contamination.

Molecular Resistance Testing

Modern medicine is facing an increasingly important problem, namely the threat from multidrug resistant is a complex and multifactorial phenomenon that requires a multidisciplinary approach if it is to be kept under control. This approach will be a challenge given that the phenomenon of antibiotic resistance can be viewed as a typical emergent characteristic of a dynamic, highly complex, and self organizing system evolving at the edge of chaos. However, new molecular technologies offer promising tools in the fight against antibiotic resistance. Not only is rapid bacterial detection, identification, and resistance testing a significant advance, but powerful typing methods for monitoring microbial populations are also a basic requirement for containment of multidrug resistant strains.

Currently, the main advantage of molecular resistance testing is a shorter time to result and improved accuracy, both of which are relevant particularly in cases of life threatening diseases such as meningitis and sepsis, for which rapid detection, identification, and resistance testing is important, or for fastidious organisms such as *Mycobacterium tuberculosis*. Despite these benefits, molecular methods can only screen for known genes or polymorphisms and will not detect emerging or new resistant strains.

Conclusion

After the successful introduction of molecular diagnostic technologies in fish genetics and virology, the number of diagnostic bacteriology protocols in medicine, food, and environmental testing has increased exponentially during the last decade. Conventional microbiology will not be replaced in the immediate future, but multi parameter identification of the most important pathogens using array based detection technologies or closed tube, rapid real time PCR based assays are becoming commonplace in today 's laboratories. Improvements in molecular microbial typing methods will bring epidemiological tools closer to the routine laboratory.

Further molecular research and development of more sophisticated technologies will increase knowledge of microbial genomes and create enormous opportunities to investigate the diversity and pathogenicity of microbes and the relationship with their host. The introduction of these new technologies naturally will not only have an impact on the global organization of the microbiology laboratory but also on the training and distribution of the available resources.

FISH AND SHELLFISH DISEASES- AN OVERVIEW

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Fish and shellfish aquaculture plays a pivotal role in the national economy of the country for its contribution to the food basket, employment generation it has been established as an industry in the last decade. The total fish production during 2001-2002 was 6.13 million tonnes with marine and inland fisheries contributing 46.2% and 53.8%, respectively to the total. Fish is a cheap source of animal protein and current per capita consumption of fish in India is around 9 kg per annum as compared to 11 kg recommended by World Health Organisation (WHO). The contribution of fisheries sector to the gross domestic product (GDP) and agriculture GDP has been estimated to be 1.2 and 4.2%, respectively (Ayyappan and Birader, 2004).

Due to intensification of aquaculture practices with improved technologies, diseases of microbial etiology of economic significance have surfaced in rearing and grow out pond. Currently, infectious disease is the single most devastating problem in shrimp and fish culture and presents ongoing threat to other aquaculture sectors. In Asian countries, losses due to disease problems have been estimated to be more than US$ 3000 million. Most disease problems may be caused by bacteria, viruses, fungi or parasites. Annual losses due to diseases in carp culture in Andhra Pradesh are estimated at about Rs 40 million. Viral disease in shrimp culture has become a stumbling block and the economic loss was estimated at about Rs 9450 million in 1995-96. So suitable and approximate health management measures involving development of diagnostics and vaccines are necessary. For disease diagnosis, forecasting and planning of sound aquaculture practices.

Aquatic Animal Diseases

In the context of aquaculture, disease may be broadly defined as any condition that leads to sub-optimal production. A definition from the medical literatures state that: a disease is the sum of the abnormal phenomena displayed by a group of living organisms in association with a specified common characteristic or set of characteristics by which they differ from the norm of their species in such a way as to place them at a biological disadvantage (Campbell *et al*, 1979). Aquatic animal diseases can be caused by:

a) primary pathogens without involvement of environmental stress;
b) primary pathogens as a result of environmental stress;
c) opportunistic pathogens as a result of environmental stress; and
d) poor environmental conditions without involvement of pathogens.

The degree to which an animal is susceptible to pathogens and the occurrence of clinical diseases largely depends on three main tactics: The status of the host organism, the environment and pathogens. When one or more factors are unfavourable the host must adopt its physiology or behaviours to compensate. These adaptive responses, stress, impair normal physiological functioning and reduce the host's chance of survival. In particular, chronic stress lowers the resistance of fishes to infectious agents. Specific examples of things can cause stress (stressors) are listed in table 1.

Table 1. Different types of stressors

Chemical stressors	Biological stressors	Physical stressors	Procedural stressors
i) Poor water quality-low dissolved oxygen, ii) Pollution iii) Diet composition-types of protein, aminoacids iv) Nitrogenous and other metabolic wastes accumulation of ammonia or nitrite	i) Population density crowding ii) Other species of fish-aggression, territoriality, lateral swimming space requirements iii) Microorganisms-pathogenic and nonpathogenic iv) Macro organisms-internal and external parasites	i) Temperature ii) Light iii) Humidity iv) Dissolved gases v) Dissolved solids	i) Handling ii) Shipping iii) Disease treatments

Different types of fish and shellfish diseases of India

In India, the increase in aquaculture production particularly the expansion into intensive and semi intensive methods of production has been coupled by increase in fish and shellfish resulting from high stocking densities and stress conditions that favour the occurrence and spread of infectious diseases. It is very essential to identify these infectious diseases in fish and prawn even in the initial stage and control the same before it spreads.

Bacterial diseases

Bacterial diseases are frequently encountered in eggs, fry, fingerlings of fish and prawn causing heavy mortality. These microorganisms are essentially opportunistic pathogens which invade the tissues of a fish host rendered susceptible to infection by stress factors or bacteria belonging to 8 genera are associated with fish diseases. Summary of bacterial diseases encountered in fish is given in table 2.

Table 2. Common bacterial diseases recorded from cultivable species of fish

Disease	Causative agents
1. Columnaris disease	*Flavobacterium columnare* (=*Flexibacter/Cytophaga columnaris*)
2. Bacterial gill disease	**Flavobacterium branchiophilum**
3. Pseudomoniasis/Psedomonas septicemia	*Pseudomonas* sp. particularly *Pseudomonas fluorescens*
4. Aeromoniasis	**Aeromonas hydrophila**
5. Edwardsiellosis or Ephysematous putrefactive disease (EPD)	**Edwardsiella tarda**
6. Vibriosis	**Vibrio anguillarum** *Vibrio parahaemolyticus* *Vibrio alginolyticus*
7. Eye disease of Catla catla	**Aeromonas liquefaciens**
8. Infectious Dropsy	**Aeromonas hydrophila**

Fungal diseases

Fungal infection of fish by Oomycetes commonly known as water moulds are widespread in freshwater and represent the most important fungal groups affecting wild and cultured fish. Other major groups of fungi involved belong to Acbyla, Aphanomyces, Branchiomyces, Dictyuchus, Fusarium, Leptomities, Pythium and Saprolegnia. Among these saprolegnic is considered as a dreaded disease once since it affects almost all the species of fish and even fish eggs. Usually fungal diseases occur as secondary infections arising from conditions such as bacterial infections, poor husbandry, infestations by parasites and social interactions. However, there are several reports of oomycetes as primary infectious agents of fish. Some of the common fungi associated with fish diseases are given in table 3.

Table 3. Common fungal diseases recorded from cultivable species of fish

Disease	Causative agents
1. Saprolegniasis	*Saprolegnia parasitica* (common name: Fish moulds)
2. Branchiomycosis (Gill rot)	Branchiomyces demigrans Branchiomyces sanguinis
3. Ichthyophoriasis	*Ichthyophorus* sp.

Prorozoan diseases

Ciliates, Mastigophora and Myxosporidia are three main phyla responsible for various protozoan diseases of fish and prawn. Most of the organ systems are infected by these parasites and majority of the groups of protozoan parasites can cause mortality in fish. Among the ciliates, *Ichthyopthirius multifillis* causes large scale mortalities of freshwater fish. Its counter part, *Cryptocaryon irritans* is of marine origin and causes severe mortalities among the marine ornamental and other commercial fishes. Some of the common protozoan diseases found frequently in fishes are given in table 4.

Table 4. Common protozoan diseases recorded from culturable species of fish

Disease	Causative agents
1. Ichthyophthiriasis (White spot disease)	Ichthyophthirius multifilis
2. Trichodiniasis	*Trichodina* sp.
3. Myxosporodiosis	*Myxosporidian* sp.

Helminth diseases

Worm diseases caused by the groups Monogenia, Digenia and cestode. In fishes causing their growth retardation and mortality. In many cases these parasites in association with other parasite groups causing serious damage to the host fishes. Among monogenetic trematodes, Gyrodactylus sp. and Dacty;ogyrus sp. are most important as sometimes they causes extensive danage. Some of the common helminth diseases in fishes are listed in table 5.

Table 5. Common Helminth diseases encountered in culturable species of fishes

Disease	Causative agents
1. D.is	*D.* (Gill fluke)
2. Gyrodactylosis	*Gyrodactylus* (Skin fluke)
3. Black spot disease	*Diplostomum* sp.
4. Acanthocephalum disease	*Acanthogyrus acanthogyrus* *Pallisentis* sp. Acanthosentis antespinus
5. Ligulosis	Ligula intestinalis
6. Sanguinicolasis	*Sanguinicola* sp. *(Blood fluke)*

Crustacean diseases

Crustacean parasites are frequently found to infest fishes and prawns. These are ectoparasites and mainly 3 groups- Branchiura (*Argulus* sp.), Copepoda (*Learnea* sp. *Erguillus* sp.) and Isopoda (*Isopod* sp.) are responsible for fish and prawn diseases. Two crustacean parasites Learnea sp (anchorworm) and *Argulus* sp. (fish louse) are most widespread and commonly found parasitizing major carp species sometimes causing large-scale damage in nursery, rearing and stocking ponds. However in general low infestations in crustacean parasites do not cause serious damage to fish/prawn except irritation and localized ulceration. Some of the common crustacean diseases in fish are cited in table 6.

Table 6. Common crustacean diseases encountered in culturable species of fish

Disease	Causative agent
1. Lernaeosis	*Lernaea* sp.
2. Argulosis	*Argulus* sp.
3. Ergasilosis	*Ergasilus* sp. *Murguli cola* sp.

Viral diseases in fishes

In India fish diseases due to viruses have not been yet reported, however some major fish disease reported in the world are

i) Infectious pancreatic necrosis (IPN)
ii) Viral haemorrhagic septicaemia (VHS)
iii) Infectious haematopoietic necrosis (IHNV)
iv) Epizootic haematopoietic necrosis (EHN)
v) Spring viremia of carp (SVC)
vi) Channel catfish virus (CCV)
vii) Viral encephalopathy and retinopathy

Major shrimp diseases

Shrimp aquaculture has become one of the fastest developing industries in recent year. It is a multidisciplinary activity involving culture of food organisms, seed procurement, stocking, feeding, and health management. Due to intensification of culture practices, disease outbreaks are frequently encountered in shrimp culture. The most important predisposing factors leading to disease outbreaks in shrimp culture are:

i) adverse environmental conditions
ii) high stocking density with limited water exchange facilities
iii) nutritional deficiency
iv) accumulation of unutilized feed followed by its putrefaction by the native heterotrophic microorganisms
v) inadequate aeration
vi) heavy algal bloom in the pond
vii) physical injury
viii) presence of virulent pathogens in high count and
ix) stocking of weak, not good quality shrimp larvae in the pond.

There are many less known stress factors causing outbreak of diseases in shrimp farms. Various etiologic agents such as viruses, bacteria, fungi, parasites, algal toxins, nutritional deficiency or adverse environment may cause the diseases. Some of the common shrimp diseases are listed in table 7.

Table 7. Some common shrimp diseases of India

Viral diseases of shrimp

Disease	Species affected
1.Monodon type baculovirus (MBV) disease	*Penaeus monodon* (post larva and adult)
2.Yellow head disease	*Penaeus monodon*
3. Baculovirus penaie	Penaeid prawns
4. Infectious hepatopancreas haematopoietic necrosis	*Penaeus monodon* *Penaeus indicus* *Penaeus senisulcatus*
5. White spot disease	*Penaeus monodon* *Penaeus indicus* *Penaeus merguiensis,* *Penaeus setiferous* *Penaeus stylirostes*

Bacterial diseases of Shrimp

1. Necrosis of appendages	*Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Flavobacterium* spp.
2. Vibriosis	*Vibrio alginolyticus, V. parahaemolyticus, V. anguillarum*
3. Luminescent bacterial disease	*V. harveyi*
4. Filamentous bacterial disease	*Leucothrix mucor*
5. Brown spot disease(Shell diseas or Rust disease)	*Vibrio* spp., *Aeromonas* spp., *Flavobacterium* spp.
6. Bacterial septicaemia	*Vibrio* spp.

Parasitic diseases of shrimp

Diseases	Causative agents
1. Larval mycosis	Oomycetous fungi such as Legenidium spp., Sirolpidium spp., Haliphthorous spp.
2. Protozoan fouling	Protpzoans such as Vorticella, Zoothemnium, Epistylis, Aceneta and Ephelota
3. Cotton shrimp disease	Microsporidia such as Agmosoma, Ameson and Pleistophora
4. Bopyrid parasitic infwestation	Bopyrid parasite, Epipenaeon spp.
5. Enterozoic Cephalin gregarine infection	Protozoan such as Cephalolobus spp.

Environmental diseases of shrimp

Diseases	Causative agents
1. Chronic soft shell syndrome	Environmental factors such as pH, salinity, temperature, organic matter
2. Black gill disease	Environmental as well as microbial agents
3. Cramped tail disease	Not Known

Freshwater prawn diseases

In fresh water prawn several diseases have been identified both in the hatcheries and grow-out systems, but many of them have an unidentified etiology. But most of the diseases appear to be due to poor hygienic conditions, inadequate water exchange, poor feeds or low oxygen levels. Protozoans, bacteria, fungi, coelenterates etc. appear to cause diseases to

freshwater prawns. But most of them are considered as opportunistic rather than obligate pathogens. During recent years, attention of aquaculturists, fishery entrepreneurs and fisheries research organizations have gone towards diseases in farmed prawns. Fortunately, so far diseases and mortality problems in *Macrobrachium* culture have been less in comparision to its penaid counterpart. Following are some of the common diseases of *Macrobrachium* species which are frequently encountered during different stages of their life history.

Larval Midcycle disease (MCD): This is commonly called MCD of *Macrobrachium* larvae, generally affects zoea IV-XI stages. Diseased prawns show marked reduction in food consumption and weak swimming and often spiral movement.

Bacterial necrosis: Bacterial necrosis cause 100% mortality in larval stage 4 to 5, but the older stages are more resistant. The etiology of disease is suspected to be bacterial in origin.

Epibiont fouling disease: Affects all the species of Macrobrachium throughout the life cycle. Numerous causes have been attributed including rod shaped bacteria, filamentous bacteria, filamentous algae (Lyngbya sp.), fungi (Aphanomyces sp.), Achyla sp. Peritrich protozoans (Epistylis sp., zoothaminium sp., Vorticella sp.), barnacles etc.

Black spot disease: Black spot is one of the most prevalent diseases of Macrobrachium. The disease is caused by a chitinoclastic bacteria usually Beneka sp., however, *Pseudomonas* and *Aeromonas* have also been isolated.

Branchiostegite melanisation: It affects juveniles and adults of cultured/captured Macrobrachium sp. Etilogy and treatment is unknown.

Idiopathic muscle necrosis: A non-infectious disease causes necrosis of muscles. No definite proof of cause is known. However stressful conditions like increase in ammonical nitrogen (0.2) and pH (8.0), IMN was observed.

Isopod parasitic disease: The bopyrid isopod, probopyrus has been found to attack the juvenile, M. rosenbergii and M. eequidus etc. The affected prawns are normally in the size range of 25-55mm.

White muscle syndrome: A viral disease of M. rosenbergii, recently represented in Nellore district in Andhra Pradesh, which causes huge economic loss. The disease is caused by a nodavirus and affect the post larva and juvenile stage.

Principles of disease treatment and control

The essential principles of disease treatment and control are to:

i) establish an accurate diagnosis;
ii) select an appropriate and environmentally responsible treatment;
iii) evaluate management practices within the farm and determine if future outbreaks could be prevented by changes in procedure or design.

Establish an accurate diagnosis

Accurate diagnosis of a disease is often incorrectly described as complicated and costly. Disease diagnosis is not solely a laboratory test. A laboratory test may confirm the presence of a specific disease agent or it may exclude its presence with a certain level of certainty. Incorrect diagnosis can lead to ineffective or inappropriate control measures. Disease diagnostics should be made as a continuum of observations starting as the firm and

intact, commencing prior to disease event. The different levels of disease diagnostics which can be undertaken when investigating a disease situation are as follows.

a) Level I: Farm/Production site observations, record keeping and health management should strongly emphasized, as this forms the basis for triggering the other diagnostic levels (II and III).
b) Level II: It includes the specializations of parasitology, histopathology, bacteriology and mycology, which require moderate capital and training investment and which generally speaking cannot be conducted at the farm or culture site.
c) Level III: It comprises the types of advanced diagnostic specialization which requires significant capital and training investment. Level III technology will become field accessible in the near future.

Select an appropriate and environmentally responsible treatment

A holistic approach should be adopted to treatment that considers the interaction between the host, the environment and pathogens. Clearly the most approprite treatment for the disease will depend on the diagnosis determined by a fish health specialist and also on the specific management practices and resource of the farm.

Prevention – controlling disease through effective management

The most important approach to disease control is managing the culture unit to reduce disease predisposing conditions. This is best achieved through the use of realistic stocking densities, preventing the introduction of pathogens, maintenance of good water quality, avoiding stress and through the provision of adequate nutrition.

Diagnostic tools

Some of the specific diagnostic tools for diagnosis of specific pathogens are available in the Indian market.

- Spot agglutination kit: Spot agglutination kit are available in CIFA for detection of bacterial gill disease, Edwardsiellosis and Aeromoniasis disease.
- PCR for detection of shrimp pathogen: PCR is widely used for detection of shrimp pathogens such as white spot syndrome virus (WSSV) and Vibrio sp. By using two step PCR, a large number of apparently healthy *Penaeus monodan* post larval stages were screened for the presence of WSSV. Only 5% apparently healthy PL gave positive reaction in one step PCR whereas 48% showed positive reaction in two step PCR.

EXOTIC PATHOGENS AND STRATEGIES FOR COMBATING DISEASES IN INDIAN AQUACULTURE

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Introduction:

India is the second largest food producer in the world. Fish and marine merchandise form an important constituent of India's food production. The Indian sub-continent has a promising future in producing marine merchandise as it has 8041 km of coastal line, 3 million hectares of reservoirs and 1.2 million hectares of brackish water. India is presently the third largest producer of fish and is playing an important role in global fisheries. The Indian fisheries sector has grown tremendously since 1950s to the present annual production levels of over 7 million tonnes of fish and shellfish from capture fisheries and aquaculture. India currently produces nearly 5% of the world's total fish production, and about 7% of the total aquaculture production. The potential for growth is immense and the country is on the threshold of massive development in fisheries and aquaculture. Given the abundance of resources with potential and the national importance attributed, aquaculture in India is poised for great expansion in the near future. At the national level, the Government of India is planning to develop a road map for enhancing fish production from the present level to 10 million tonnes by 2012; explore avenues to meet domestic demand; and to increase export earning potential in terms of value-added products and ornamental fish trade. The export industry is poised to achieve 5 billion US$ level by 2012 with 75% contribution from value-added products (GOI Report). The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. Thus, the aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. Disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries. This situation can be attributed to a variety of multi-faceted and highly interconnected factors such as the increased globalization of trade in live aquatic animals and their products; the intensification of aquaculture through the translocation of broodstock, postlarvae, fry and fingerlings; the development and expansion of the ornamental fish trade (Bondad-Reantaso et al., 2005; Subasinghe et al., 2001). Aquatic animal health management programmes carried out in different parts of the globe have been reviewed with respect to efficacy of disease diagnosis, prophylaxis and control, inherent problems with national legislation and international/regional codes, and the effectiveness of programmes on education, training and extension services. The need for effective communication at all levels of the production system is also discussed. The roles of government at the regional and international levels, along with actions needed to address gaps in effective health management have been elaborated.

Transboundary aquatic animal diseases:

Advances in live aquatic animal trade, facilitated by improved transportation efficiency, are now recognized as having played a pivotal role in the introduction and spread of pathogens and diseases into many aquaculture systems (Subasinghe et al., 2001). Although local pathogens combined with other factors, such as poor husbandry and

inadequate water quality, are the most common causes of disease outbreaks in fish farming, the introduction of 'exotic' pathogens through international trade in live aquatic animals and their products continues to be associated with new epizootics. The epidemic spread and devastating impacts of aquatic animal diseases such as epizootic ulcerative syndrome (EUS) in freshwater fish; viral nervous necrosis (VNN) in marine fish; white spot syndrome virus (WSSV) in penaeid shrimps; white tail disease (WTD) in *Macrobrachium rosenbergii* and the emerging Taura syndrome virus (TSV) in *Penaeus vannamei*; in Asia have clearly demonstrated the vulnerability of aquaculture systems to infectious disease emergencies (Mohan et al., 2008). In Scottish Atlantic-salmon farms serious problems have been caused by the emergence of sea lice, furunculosis (Austin and Austin, 1997), infectious pancreatic necrosis (IPN) and infectious salmon anaemia (ISA) (Murray and Peeler, 2005). Koi herpes virus (KHV) has recently been introduced to Indonesia and Japan. In Japan, it has caused mass mortalities not only among farmed carp but also wild carp in many rivers and lakes. The KHV incursion is a good example of an infection which initially spread from a cultured ornamental fish (koi carp) to cultured food-fish (common carp) and then into wild populations of carp. This clearly shows that cultured fish can be a source of major disease transfer to wild populations, a risk that the aquaculture industry (e.g. salmon culture) has tried to down play in the past. (Bondad-Reantaso et al., 2005).

White spot syndrome virus (WSSV) remains an important pathogen of farmed shrimp (*Penaeus monodon*) and has now been recovered from wild penaeid shrimps. The shrimp viral disease epidemics are classical examples of the range and distance that aquatic animal pathogens can travel alongside the movement of their hosts. One shrimp viral disease alone, white spot disease (WSD) remains the most serious pathogen of cultivated shrimp in the world. The Asian pandemic occurred in the 1990s, affecting many shrimp producing countries in Asia (Bangladesh, Indonesia, Japan, India, Malaysia, Philippines, Sri Lanka, Thailand and Vietnam) (Bondad-Reantaso et al., 2005). WSD has been officially confirmed in the Americas, Mexico and Brazil. The major mode of transfer has been through the movement of infected live animals (postlarvae and broodstock). A second important shrimp disease, Taura syndrome (TS), caused by the Taura syndrome virus (TSV) which was previously reported only from the Western Hemisphere, is now becoming widespread in Asia (Bondad-Reantaso et al., 2005). Examples of infection of cultured stocks via wild stock reservoirs for shrimp diseases have been described by Ruangsiri and Supamattaya (1999), Rajendran et al. (1999) and Dixon (1999) for marine finfish disease.

The Office International des epizootics (OIE), the world organization for animal health, develops codes and guidelines for minimizing the risk of disease transmission as a result of international movement of animals and their products. The IOE is to facilitate international trade in animals and animal products, including aquatic animals, on the basis of health control and preventive measures. OIE also provides a disease reporting mechanism for its member countries, making essential information available for use in quarantine policy formulation and decision making. To harmonize the health guarantees for international trade in aquatic animals and their products, OIE has prepared an International Aquatic Animal Health Code and a companion document "Diagnostic Manual for Aquatic Animal Diseases (Swaminathan et al., 2006). The Office International des Epizooties (OIE) currently lists 35 pathogens/ diseases of finfish, molluscs and crustaceans that fit three major criteria in terms of consequence, spread and diagnosis (OIE 2000). It is generally accepted that the current version of the IOE codes does not readily apply to developing countries In this regard, the NACA/FAO and OIE list of diseases reportable in the Asia-Pacific Region were developed to reflect the Asian situation. These lists include all OIE listed diseases as well as a number of other serious diseases that occur in the Asia-Pacific region. The Quarterly Aquatic Animal Disease Reporting System (QAAD) in the Asia-Pacific, jointly developed by

the Network of Aquaculture Centres in Asia-Pacific (NACA), the Food and Agriculture Organization of the United Nations (FAO) and OIE, is one of the key activities of NACA's regional aquatic animal health management programme. The goal of the regional disease reporting is to reduce the economic impact of aquatic animal diseases and minimize the risk of regional and international spread of pathogens. (Swaminathan, 2006).

Currently, the NACA/ FAO lists 32 diseases, including many of the OIE-listed diseases as given below (NACA/FAO, 2005) and the details are presented inTable1.

Diseases prevalent some parts of the region

A. Fin fish diseases

1. Epizootic haematopoietic necrosis (EHN)
2. Infectious haematopoietic necrosis (IHN)
3. *Oncorhynchus masou* virus disease (OMVD)
4. Spring Viremia of carp
5. Viral haemorrhagic septicemia
6. Viral encephalopathy and retinopathy (VER)
7. Infectious pancreatic necrosis (IPN)
8. Epizootic ulcerative syndrome (EUS)
9. Bacterial kidney diseases (BKD)
10. Red seabream iridoviral disease
11. Enteric septicemia of catfish

Non –OIE listed diseases relevant in the region

12. Epitheliocystis
13. Grouper iridoviral disease
14. Infection with koi herpes virus

B. Crustacean diseases

OIE listed diseases:

1. Taura syndrome
2. Yellow head disease (YH Virus) (Gill associated virus)
3. Infectious hypodermal and haematopoeitic necrosis (IHHN)
4. White spot disease (WSD)
5. Spherical baculovirus (*P.monodon* type Baculovirus)
6. Tetrahedral baculovirus (*Baculovirus peaei*)
7. Spawner mortality syndrome (" Midcrop mortality syndrome")(SMVD)

Non OIE listed diseases relevant in the region

8. White tail disease of *Macrobrachium rosenbergi*
9. Baculovirus midgut gland necrosis (BMN)
10. White tail disease (MrNV and XSV)

Table – 1. Details of important NACA/ FAO and OIE notifiable and significant diseases of Fish and shellfish

I-Fish diseases	Causative agent	Species affected	Prevalence/ Region
Epizootic haematopoietic	EHNV, ESV, &ECV,(Ranavirus)	Red perch, Rainbow trout,	Australia, Europe

necrosis (EHN)	Iridovirus group	Sheathfish, Catfish	
Infectious haematopoietic necrosis (IHN)	IHNV a Rhabdo virus	Rainbow trout, Pacific salmon,Atlantic salmon	North America, Europe
Oncorhynchus masou virus disease (OMVD)	Oncorhynchus masou virus (OMV), Type II Herpes virus	Salmonid fishes	Japan, Eastern Asia,
Spring viraemia of carp (SVC)	SVCV a Rhabdo virus	Common carp, grass carp, Silver carp, sheathfish,	Europe
Viral haemorrhagic septicaemia (VHS)	VHSV, Novi-rhabdovirus, Family : Rhabdoviridae	Rainbow trout, Brown trout, Pike, Turbot, Pacific salmon	Europe, North America,
Channel catfish virus disease (CCVD)	Channel catfish virus(CCV), (Ictalurid herpes virus1)	Channel catfish (Itctalurid punctate)	USA
Viral Encephalopathy and reticulopathy/ Viral nervous necrosis (VNN)	VERV/ VNNV, Nodavirus, family: Nodaviridae	Seabass, Stripped jack, Groupers, Puffer, Flatfish	World wide except Americas & Africa
Infectious pancreatic necrosis (IPN)	IPNV Birnavirus, Birnaviridae family	Salmonids, Rainbow trout, Brook trout, brown trout, Atlantic salmon	North and South America, Europe and Asia
Infectious salmon anaemia (ISA)	ISAV, Orthomyxo- like virus	Atlantic salmon, Rainbow trout	Canada, Norway, U.K.
Red sea bream Iridoviral disease (RSIVD)	RSBIV, Iridovirus	Marine fish, Red sea bream, Yellowtail, Sea bass, Grouper	Japan, Thailand
Epizootic Ulcerative syndrome/red spot disease/mycotic granulomatosis	*Aphanomyces invadans/A. piscicida/ A.invaderis* with secondary Rhabdoviruses and Gram negative bacteria	Most farmed freshwater and estuarine fish susceptible but Tilapia, milk fish and Chinese carp are resistant	Most Asian countries, Australia, USA
Bacterial kidney disease	*Renibacterium salmoninarum, Corynrform* bacteria, Genus: Renibactrium	Pacific salmon,	North America, Japan, Western Europe, Chile
Enteric septicaemia of catfish	*Edwardsiella ictaluri*, Family, Entreobactericeae	Channel catfish, Clarius batrachus, several ornamental fishes, Salmonids	USA, Thailand
Piscirickettsiosis	Piscirickettsia salmonaris	Salmonids,	Chile, Ireland, Norway, Canada

Non-OIE listed but relevant to the region			
Infection with Koi herpes visus	Koi herpes virus or carp nephritis and gill necrosis virus, Family- Herpesviridae	Common carp(cyprinus carpio) and koi (Cyprinus carpio koi)	Europe, china, Japan, Africa, USA,
II.Shellfish diseases			
White spot disease/ White spot syndrome	WSSV)/ WSV : Under WSSV complex or Whispo virus (New genus)	Penaeid shrimp	Most Asian countries, USA
Yellow head viral Disease	YHV/ Lymphoid organ virus (LOV) and Gill-associated virus (GAV), Coronaviridae family	Penaeid shrimp	Most Asian countries, Americas and Australia
Taura Syndrome	TSV, Picorna- viridae family	*P.vennamei, P.stylirostis*	Americas,Hawaii, Mexico
Nuclear polyhedrosis/ Tetrahedral baculovirosis/ Baculovirus penaei disease	Baculovirus penaei (BP), PvSNPV (variable strains involved)	*P.vennamei, P.penicillatus, P.aztecus, P.duorarum*	Western hemisphere- North and South Americas, Hawaii,
Spherical baculovirosis/ Monodon-type baculovirus disease	*Penaeus monodon*- type baculovirus (MBV), PmSNPV	Cultured and wild penaeid prawns and shrimp	Indo-pacific region, SE-Asia, Australia, Africa, Middle east, Americas, Tahiti, Hawaii, Caribbean
Infectious hypodermal and hematopoietic necrosis / Runt deformity Syndrome (RDS)	IHHNV, 22nm size, Family- Parvoviridae	*P.vennamei, P.stylirostis, P.semisulcatus, P.japonicus*	Peru, Mexico, Hawaii, Indo-Pacific region, SE-Asia, Middle-East, Australia
Spawner isolated mortality virus (SMV)disease/ Mid-crop mortality syndrome,	SMV, Parvovirus, 20nm size, Family Parvoviridae	*P.monodon*	Queensland (Australia), Philippines
Non-OIE listed but relevant to the region			
Necrotising hepatopancreatitis	Obligate intracellular Alpha Proteobacteria	*American Penaeids: P. vannamei, P. azetecus, P. stylirostris*	Peru, Venezuela, Brazil, Coasta Rica, Texas
Baculoviral midgut gland necrosis (BMN)	BMNV, PjNOB I, Type C Baculovirus	*P.japonocus, P.monodon*	Japan, Korea, Philippines, Australia, Indonesia
White Tail Disease (WTD)	Two viruses, M.rosenbergii noda virus (MrNV) and	*Giant Freshwater Prawn, M. rosenbergii,* larval	India, China, Thailand Taiwan, West Indies

	extra small virus (XSV), family-Nodaviridae	stages mostly affected	

Source: Swaminathan et al., (2006)

Strategies for combating diseases in Indian aquaculture:

Presently in Asia-Pacific region, status of IOE and FAO/NACA listed diseases of aquatic animals is monitored by the Quarterly Aquatic Animal Disease (QAAD) reporting System based on national reporting system established in various countries participating in the world fish health programme (Swaminathan et al., 2006). The national coordinator of each country is responsible for collation of the disease information available in the country on a regular basis and submits it to the regional coordinator at NACA in Bangkok. The regional coordinator in turn prepares the regional summary and distributes it to national coordinators, OIE regional centres and associated organizations. India has to be prepared for not only the correct disease reporting system but also for correct diagnosis of exotic pathogens. There are general strategies currently being implemented in the Asia-Pacific region as also in other regions of the world, and apply to all infectious diseases (Bondad-Reantaso et al., 2005).

i) International codes : A number of international codes of practice, agreements, and technical guidelines exist and are aimed, at least in part, at providing a degree of standardization for the protocols used to minimize the risks of disease associated with movements of aquatic animals. These include OIE's Aquatic Animal Health Code and the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES). The OIE develops normative documents relating to rules that its member countries can use to protect themselves from diseases without setting up unjustified sanitary barriers. The main normative documents produced by the OIE for aquatic animals are the Aquatic Animal Health Code (Aquatic Code) and the Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual). The aim of the Aquatic Code is to assure the sanitary safety of international trade in aquatic animals (fish, molluscs and crustaceans) and their products. The code provides details of health measures to be used by the veterinary or other competent authorities of importing and exporting countries so that the transfer of pathogenic agents for animals or humans is minimized but unjustified sanitary barriers are avoided. The Aquatic Code provides general and disease specific provisions that OIE Member Countries can adopt to prevent and control aquatic animal disease (Mohan et al., 2008; NACA/FAO, 2005; OIE, 2000; Swaminathan et al., 2006).

ii. Regional guidelines: Because many countries in a region like in Asian-pacific, share common social, economic, industrial, environmental, biological and geographical characteristics, a regionally adopted health management programme is considered a practical approach (Bondad-Reantaso et al., 2005; Mohan et al., 2008). The Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy (FAO/NACA, 2000, 2001) was based on a set of guiding principles developed through a regional project of FAO and implemented by the Network of Aquaculture Centres in Asia-Pacific (NACA) using a consultative process that involved representatives from 21 participating governments and technical assistance from regional and international experts on aquatic animal health. The Technical Guidelines describe a number of health management considerations aimed at minimizing the risk of disease spread via aquatic animal movements and were developed to i) assist countries in the Asia-Pacific to move live aquatic animals in a way that minimizes

the disease risks associated with pathogen transfer and disease spread, both within and across boundaries; ii) enhance protection of the aquatic environment and biodiversity, as well as the interests of aquaculture and capture fisheries; iii) provide a mechanism to facilitate trade in live aquatic species and avoid unjustifiable trade barriers based on aquatic animal health issues; and iv) implement relevant provisions of FAO's CCRF and other international treaties and agreements applicable to the Asian region (Bondad-Reantaso et al., 2005; NACA/FAO, 2005).

iii) National strategies on aquatic animal health Management: The national strategic plan usually identifies the roles and responsibilities of different stakeholders at the state and national levels. National programmes and legislation are also being implemented in many countries, particularly in developed regions, and these include some good examples of successful fish health control policies and programmes with effective diagnostic accreditation programmes, as well as quality assessment and quality control (QAQC) procedures (Mohan et al., 2008; NACA/FAO, 2005). These aquatic animal health programmes emphasise good management, adherence to strict hygiene practices and sanitation standards, and general layout of farm premises and site selection, as well as strict quarantine protocols with biosecure facilities (Subasinghe et al., 2001). The national strategic plan normally provides the basic framework and principles on which to implement a comprehensive health management strategy. The strategic plan covers most of the issues that needs to be implemented at the farm/state/national level. Canada offers another good example of controls such as quarantine, disinfection of eggs, and disease history documentation, which has reduced disease risks associated with the introduction and transfer of Atlantic salmon (*Salmo salar*). Since 1977, Canada has had federal legislation to protect the country's fisheries resources, embodied in the Fish Health Protection Regulations (FHPR) and Manual of Compliance (Subasinghe et al., 2001). Thailand has a good model for establishing strong relationships between government and industry sectors from a developing country standpoint. In an effort to maintain its status as the number one shrimp producer in the Asia-Pacific Region, the government instituted a number of mechanisms to provide support to the industry. These include re-investing profit from shrimp exports into improved aquatic animal health capabilities, establishing a code of conduct for responsible shrimp farming and intensifying support to shrimp farmers (Subasinghe et al., 2001). The various processes and examples of national strategies are provided by Bondad Reantaso (2004) and Bondad-Reantaso et al., (2005).

iv) Disease Surveillance and reporting system: Surveillance is defined as a systematic collection, analysis and dissemination of health information of a given population of aquatic animals and is an ongoing process involving handling of health information from different sources, including surveys (Mohan et al., 2008). To produce meaningful reports on the disease status of a farm, zone or country, some systematic processes of gathering information about the geographical distribution of important diseases and pathogens must be in place. This process is known as disease surveillance and reporting (Swaminathan et al. 2006). It is an effective and fundamental foundation for any method of disease prevention and control. However, many factors complicate accurate disease reporting. The surveillance applies to detection of the emergence of a new disease situation as well as to monitoring the status of established disease agents. The surveillance programme involves inputs from personnel trained in disease recognition in the field. The programme is supported by reporting and diagnostic procedures to ensure accurate and rapid identification of pathogens. Disease reporting and information sharing can go a long way in minimizing the impact of serious aquatic animal health emergencies. By international agreement, diseases listed by the OIE should be reported by member countries and are subject to specified health

measures that are intended to limit disease spread and assure sanitary safety of international trade in aquatic animals and their products (Mohan et al., 2008).

v). Diagnostics, prophylaxis and therapy:

Successful health management programme depends on prompt, efficient and accurate diagnosis of disease and pathogen. At present the only diagnostic methods that are accepted in those countries where aquatic animal health control programme are implemented are based on either on isolation of the pathogen followed by is specific identification or on the demonstration of pathogen-specific antigens using immunodiagnostic tests (Swaminathan et al., 2006). Because of the scale of resource expertise and infrastructure required (e.g. training, facilities, resources) for disease diagnostics, FAO/NACA (2000) recommended the use of three levels of diagnostics. All three levels of diagnostic specialization are necessary for confirmation of newly or rarely encountered disease. The level of diagnosis was categorized based on the activity and techniques involved in the process in to level I, II, and III.

(a) Level I: It involves inspection of production site/ farm and observation of the cultured animal and culture environment, clinical examination of animals. It includes gross pathology. It is essential for farm level monitoring and also for making presumptive diagnosis which in some cases sufficient to provide adequate health management decision. Level I observation provide vital information that support more meaningful interpretation of findings of level II & level III.

(b) Level II: It includes laboratory observations using parasitology, bacteriology, mycology and histopathology, which require moderate capital and training investment. It is required for disease with clinical signs caused by both infectious and non-infectious etiologies. It is essential for pathogens/ etiological factors which could not be properly diagnosed in level-I gross pathology and light microscopy observations of samples like lesions, smears, cultures and squashes.

(c) Level III: It comprises the types of advanced diagnostic laboratory observations using virology, electron microscopy, molecular biology and immunology, which requires significant capital and training investment. Level III diagnosis are required for problematic pathogens and essentially required for those that could not be easily diagnosed at level I & II. Level III diagnosis requires accurate attention, refined background knowledge on pathogen, host and pathology (Swaminathan et al., 2006).

The three levels have broad-scale application to disease detection and diagnostics. Level III diagnostic support is usually based on referral laboratories. The baseline aim for initiating diagnostic capability is Level I. Therefore, organizations are encouraged to move from one level to the next as capacities are improved and as resources become available (Bondad-Reantaso et al., 2005).

The application of molecular technologies (Level III) in aquaculture health has advanced rapidly. These tools include both immunoassay and DNA-based diagnostic methods, e.g. fluorescent antibody tests (FAT), enzyme-linked immunosorbent assays (ELISA), radio-immunoassay (RIA), in situ hybridization (ISH), dot blot hybridization (DBH) and polymerase chain reaction (PCR) amplification techniques. They are currently

used to screen and/or confirm diagnosis of many significant exotic pathogens of cultured finfish e.g. channel catfish virus (CCV), infectious hematopoeitic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV), viral nervous necrosis virus (VNN) and bacterial kidney disease (BKD)), as well as shrimp diseases (e.g. WSSV, YHV, infectious hypodermic and haematopoeitic necrosis virus (IHHNV) and Taura syndrome virus (TSV) world (Lightner, 1996; Lightner and Reed, 1998; Tang et al., 1997). These molecular techniques (immunoassays and nucleic-acid assays) provide quick results, with high sensitivity and specificity at relatively low cost, and are particularly valuable for infections which are difficult to detect (e.g. subclinical infections) using standard histology and tissue-culture procedures. Molecular tools are also useful for research into the pathology and immunology of specific infections. They can be used with non lethal sampling and are valuable to monitor challenge experiments under controlled laboratory conditions (Subasinghe et al., 2001; Tang et al., 1997)

Select an appropriate and environmentally responsible treatment

A holistic approach should be adopted for treatment that considers the interaction between the host, the environment and pathogens. Clearly, the most appropriate treatment for the disease will depend on the diagnosis determined by a fish health specialist and also on the specific management practices and resource of the farm.

Efficacy of disease prophylaxis and control

While chemotherapy will perhaps remain one of the main strategies for controlling transmissible diseases in the foreseeable future, especially in finfish, there is increasing recognition of its limitations in terms of aquaculture situations, host species and effectiveness against certain pathogen groups. In some cases, rather than providing a solution, chemotherapy may complicate health management by triggering toxicity, resistance, residues and occasionally, public health and environmental consequences (OIE, 2000). Vaccination is another established, proven and cost-effective method for controlling certain infectious diseases in cultured animals. Vaccines reduce the severity of disease losses, reduce the need for antibiotic use, leave no residues in the product and do not induce pathogen resistance. There are currently many commercially available vaccines for finfish diseases, and a few more are under development (Subasinghe et al., 2001). In Japan, for example, the use of injection vaccines is now widespread among maricultured animals, proved effective against bacterial (e.g. *Lactococcus garvieae* infection of yellowtail) and viral infections (e.g. iridoviral infection of red sea bream) and has changed the patterns of disease occurrences. In conjunction with good health management and good husbandry practices, there is great potential for the use of vaccine technology for specific use in Asian aquaculture (Bondad-Reantaso et al., 2005).

Prevention – controlling disease through effective management

The most important approach to disease control is managing the culture unit to reduce disease predisposing conditions. This is best achieved through the use of realistic stocking densities, preventing the introduction of pathogens, maintenance of good water quality, avoiding stress and through the provision of adequate nutrition.

v) Quarantine and health certification

Quarantine is defined as maintaining a group of live aquatic animals in isolation with no direct contact with other aquatic animals, in order to undergo observation for a

specified length of time and, if appropriate, conducting tests and treatment, including proper treatment of the waste waters. Quarantine process involves pre-border, border and post-border activities, including pre-movement certification, movement, confinement on arrival, checking during confinement, releases, and subsequent monitoring as appropriate. The purpose of applying quarantine measures is to facilitate trans-boundary trade in living aquatic animals, while minimizing the risk of spreading infectious diseases (Bondad-Reantaso et al., 2005). An effective system of quarantine measures also increases protection of surrounding resources (e.g., harvest fisheries, non-exploited species and other components of the environment) (Mohan et al., 2008). Health certificates are documents issued by some of the exporting countries attesting to the health status of a consignment of live aquatic animals. A health certificate is a legal document which is used especially for the purpose of applying quarantine measures in trans-boundary trade of live aquatic animals and their products, for minimizing the risk of spread of infectious diseases. Health certification is also one of the strategies aimed to protect the natural environment and native fauna from the deleterious impacts of exotic species and/or diseases. Because of the diversity of species, the purposes for which the aquatic animals are being traded (import-export, local market), and other variable factors, health certificates should be comprehensive and be able to accommodate all the required information. Model health certificates are provided in the OIE Code (Bondad-Reantaso et al., 2005; Mohan et al., 2008).

Conclusion

Aquaculture is the fastest growing food production sector in the world, providing a significant supplement to, and substitute for, wild aquatic organisms. However, disease is a primary constraint to the growth of many aquaculture species and is now responsible for severely impeding both economic and socio-economic development in many countries of the world. A good understanding of biosecurity and disease causation is essential for developing and implementing farm-level plans and husbandry measures to respond to disease emergencies. Using epidemiological approaches, it is possible to identify pond- and farm-level risk factors for disease outbreaks and develop intervention strategies. Better management practices (BMPs) should be simple, science-based, cost-effective and appropriate to their context if farmers are to adopt and implement them. As part of a regional initiative by NACA to control aquatic animal diseases, effective extension approaches to promote the widespread adoption of BMPs have been developed in India, Indonesia, Vietnam and Thailand, and have proved their worth (Mohan et al., 2008). It is emphasized that strong national policies in disease management, health certification, seed certification, quarantine and import- export regulation should be strictly promulgated. As per Bondad-Reantaso et al., (2005), only through responsible trade, with the partnership and responsibilities shared between importing and exporting organizations/countries, the risks of serious aquatic animal pathogen introduction and spread would be minimized

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IMMUNE MECHANISM OF FISH

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Fish immunology is one of the important branches of fish pathology and has similarity with the higher vertebrates, but fish immune system is less developed in comparison to higher vertebrates. Immune system is able to discriminate between self and non-self substances and plays an important role in maintaining the normal health. It protects from a variety of diseases by detecting and neutralizing the disease causing agents including viruses, bacteria and fungi. This process involves interaction between target cells and both cellular and humoral elements of immune system. The cells that mediate immunity in fish include lymphocytes and phagocytes.

Fish have many non-specific (Table-1) and specific (humoral and cell mediated) immune mechanisms to resist infectious diseases, but some times this natural immunity is not sufficient against microbial infections. Fish produce antibodies in mucosal tissue like gut and gills. Humoral and cellular immune response are two functional components of the immune system. Protection against disease producing microorganisms is manifested as a classical immune response, which produces antibody, effectors cells, several cytokines and metabolites. Aquatic environment consists of a great variety of bacteria and most of them are neutralized by innate immune system i.e. non-specific immune system. Phagocytosis is the principle mechanism for the destruction of extra cellular bacterial pathogens. Non-specific cell mediated immune responses appear to play a very important role in providing protection against commonly encountered pathogens

Table1. Non- specific defense mechanism in fish against bacteria

Humoral	Cell Mediated
(a) Inhibitors	(a) Neutrophils
i. Transferrin	i. Respiratory burst
ii. Lectin	ii. Lysozyme
iii. Antiproteases	
iv. Antibacterial peptides	
(b) Lysins	(b) Macrophages
i. Proteases	i. Hydrolytic enzyme
ii. Lysozyme	ii. Respiratory burst
iii. C-reactive protein	iii. NO
iv. Complement	

Physical barrier

The epithelial surfaces viz. skin, gills and gut are examples of these physical barriers but also contain and release antimicrobial agents. In fish, like other vertebrates, there are at least three barriers viz. mucous secretion (Trypsin, Lysozymes, Agglutinins), mucosal lining cells and blood cells (especially granulocytes and monocytes) to combat infectious agents. The main role of these barriers is to scavenge the endogenous and exogenous harmful substances and eliminate them from the host (fish) body. The organs that are involved in scavenging activities include skin, kidney, liver, spleen, etc. The non-specific immune

mechanisms become highly active during microbial invasion and lead to more efficient clearance and destruction of pathogens and other harmful substances (Dalmo *et al*.1997).

Non-specific immune system

a) Humoral Defense

Fish have varied non-specific immune responses. Humoral factors inhibit nonspecifically the growth of microbial pathogens and are found in serum, mucus and egg of fish. But pathogens also modulate either by increasing or suppressing the immune responses of fish to antigenic stimuli. Bony fish have only one type of immunoglobulin (IgM) but they can develop an adequate humoral antibody against various antigens.

Transferrin

Transferrin of fish has similar biochemical characteristics like those of higher vertebrates. It is an iron binding protein that limits the amount of free iron in the bloodstream and causes survival of pathogens in body extremely difficult. Hence, it is an essential element of body to counter the establishment of infection by most pathogens by chelating the metal and making it unavailable for the bacterial use. The amount of transferrin in host blood is therefore an important parameter in deducing the condition of a pathogen-susceptible host. In most species of fish transferrin genes are polymorphic in nature and are responsible for the secretion of fish transferrin.

Lectins

Lectins (hemagglutinins) are proteins of non-immune origin that agglutinate cells and / or precipitate glycoconjugates. They exhibit diverse roles including morphogenesis, polyspermy, blocking serum screening by the liver and defense system against microorganisms

Lysozyme

In vertebrates, lysozyme plays an important role in the innate immune response that helps to degrade peptidoglycan layer of the cell wall of Gram-negative and Gram-positive bacteria after lysis of the outer lipopolysaccharide layer by the action of complement. Presence of lysozyme has been reported in mucus, lymphoid tissue, plasma and other body fluid of most of the fishes and plays an important role in defense mechanism against the pathogen invasion.

Creactive protein (CRP)

In fish, CRP is a serum component that increases rapidly upon exposure to bacterial endotoxins. CRP has also been reported in skin mucus and ova. CRP reacts with phosphorylcholine molecules, which is a widespread component of surface structures of microorganisms. It has to activate the complement system similar to Ig and thereby activates phagocytic and lytic defenses.

Complement

Complement is a group of serum proteins that circulate in an inactive state. A variety of specific and non-specific immunological mechanisms can convert the inactive forms of complement proteins into an active state with the ability to damage the membranes of pathogenic organisms, either destroying the pathogens or facilitating their clearance. Complement may function as an effector system and refers to a series of interactions initiated by two distinct pathways - the alternative complement pathway (ACP) and the classical complement pathway (CCP). The result of complement activation by both the

pathways is the same i.e. the formation of the lytic membrane attack complex (MAC). The MAC is a complex of complement molecules that polymerize to form a lytic hydrophobic plug that penetrates the bacterial cell membrane to form pores. Lysozyme is thought to play an important role in complement lysis by breaking apart the outer peptidoglycan layer and thus exposing the membrane so the MAC can attach and create pores.

The CCP is considered to be a component of the specific immune system because it is activated by an antigen-antibody complex while the ACP is considered to be part of the nonspecific immune system because it is activated by a variety of compounds and surfaces such as the LPS found in the cell membranes of Gram-negative bacteria, rabbit erythrocytes, zymosan, and inulin. During the sequence of events that culminates in complement mediated lysis, many by-products are formed and some of these substances have important roles in the inflammatory immune response as opsonins, anaphylatoxins, neutrophil and macrophage chemoattractants. The bactericidal activity of the ACP may be related to the sialic acid content of the bacterial cell membrane.

Complement activity in fish appears to be comparable to that seen in mammals. Both ACP and CCP pathways exist, with complement activity detected in the serum and skin mucus. The ACP pathway appears to have a greater role in the protection of fish, as it is more active in the early stages of infection and at low temperatures when antibody production is suppressed. Alternative complement pathway bactericidal activity requires Mg^{+2} plus factors B (glycoprotein) and D (serine protease). Fish complement, in general, exhibits highest activity between 15^0C and 25^0C and can remain active at temperatures as low as 0-4^0C, which is in contrast to mammalian complement with an optimal temperature of 37^0C (Lobb and Hayman 1989). Fish complement has been found to be heat sensitive, with complete inactivation at 45^0C for 20 min.

b) Cellular Defense

A variety of leukocytes (monocytes/macrophases, granulocytes and nonspecific cytotoxic cells) are involved in nonspecific cellular defences of fish. These cells can be isolates from blood, lymphoid organ and peritoneal cavity. Functionally, the macrophages can act, as accessory cells for lymphocyte responses, are avidly phagocytic, can secrete nitrogen and oxygen free radicals and kill a variety of pathogens although differences do exist between macrophages from different sources.

Phagocytic cells

Elimination of microorganisms in the blood stream is accomplished through engulfment and digestion by the neutrophils and macrophages. In fish, neutrophils and macrophages are polymorphic dominant white cells and have many functional properties similar to that of mammal. An array of granulocytes viz. primary granulocytes consisting of lysozyme and secondary granulocytes containing lactoferrin and lysozyme have proved to be formidable for microbes and play a significant role in antibacterial defense mechanism and promote the cell-mediated immunity (CMI) by phagocytosis . It can be divided in to three main phases; attachment of the particle to cell surface, ingestion involving the formation of phagosome and breakdown of the particle within the phagosome. The principle cells involved in phagocytosis are neutrophils and macrophages. Generally, phagocytic cells act as accessory cells that are required for lymphocyte response in fish.

Respiratory burst

The respiratory burst appears to hold promise as a bio indicator of fish health. Invading microorganisms are killed by free oxygen and nitrogen radicals; either

extracellularly without prior phagocytosis or intracellularly after phagocytosis. This antigen-nonspecific immune function is executed by macrophages and granulocytes. The respiratory burst of professional phagocytes involves reduction of oxygen to the anionic radical, superoxide.

Nonspecific cytotoxic cells (NCCs)

Nonspecific cytotoxic cells in fish may be phylogenetic precursor of mammalian natural killer cells (NKC). They are part of the defense against environmental pathogens and provide resisitance against tumor developlment. The catfish NCCs are small granular lymphocytes that are commonly found in pronephros, spleen and rarely in the blood (Fischer *et al.*, 1998). In addition to catfish, NCC-like activity has been seen in other fish species like rainbow trout, carp, and tilapia. Indeed, in kidney 15-20% of leukocytes are considered to be NCCs. Recently, a "function–associated molecule" (FAM) has been described on NCCs, which is involved in recognition of target cells and also a marker for the isolation and identification of fish.

Inflammation

Inflammation is a local reaction brought about by high number of macrophages and granulocytes. The final component of internal immunity to consider is inflammation. A variety of non-specific substances, which may be protein or glyco-protein in nature, are commonly present in mucous, serum and eggs of fish. These substances inhibit the growth of bacteria and other microbes. So they play an important role during microbial infections viz. vasodilatation, increased vascular permeability, action of blood clotting, and inflammation of phagocytic cells into the injured tissue, which otherwise would lead to severe inflammation and eventually death.

Cytokines

The development of an effective immune response involves lymphoid cells, inflammatory cells and hematopoieitc cells. The complex interactions among these cells are mediated by a group of secreted low-molecular –weight proteins that are collectively designated as cytokines to denote their role in cell to cell communication. Cytokines assist in regulating the development of immune effector cells and some cytokines possess direct effector function of their own. The effector phages of both natural and specific immunity are in large part mediated by cytokines. The effector cytokines are mostly produced by mononuclear phagocytes and therefore often called monokines. Although monokines can be elicited directly by microbes, they can also be secreted by mononuclear phagocytes in response to antigen-stimulated T-cells, i.e. as part of specific immunity. Most cytokines in specific immunity are produced by activated T lymphocytes and such molecules are commonly called lymphokines. T-cells produce several cytokines that serve primarily to regulate the growth and differentiation of various lymphocyte populations and thus play important role in the (activation phage) of T-cell dependant immune responses. Other T-cell derived cytokines function principally to activate and regulate inflammatory cells such as mononuclear phagocytes, neutrophils and eosinophils. Two cytokine induced enzymes that have a key role in inflammation and which have been sequenced in fish are the cyclooxygenase enzyme responsible for prostaglandin production and inducible nitric oxide synthase that generates nitric oxide. The evolutionary relationship of innate immune molecules identified in bony fish includes selected chemokines (CC and CXC chemokines and their receptors), cytokines (IL-1, IL-8, interferons, TGF-β, TNF-α), acute phase proteins (SSA, SAP, CRP, alpha2M and the complement components C_3-C_9, MASP, MBL, Bf), NK cell receptors and molecules upstream and downstream of the toll signaling pathways. Some cytokines function principally to regulate the growth and differentiation of lymphocytes

and mediate the activation phase of specific immune responses. T cells, especially antigen specific CD4+ T lymphocytes, produce most of these cytokines. Such T cells provide help for both cell mediated and humoral immune response, in large part through the secretion of cytokines. The cytokines that act primarily to regulate lymphocyte themselves are IL-2, IL-4 and transforming growth factor-beta (TGF-β).

Specific Immunity

Although T-cell subsets have yet to be demonstrated, it is well established that fish possess lymphocyte populations analogous to B and T cells, nonspecific cytotoxic cells (similar to NK cells), macrophages and granulocytes. Teleost immune systems have been shown to possess lymphocyte subpopulations that are differentially responsive to B-and T-cell mitogens, acute allograft rejection reaction (MLR) and the cooperative interactions between T cell and macrophages requisite for an in vitro antibody production. Much work has been done on humoral immunity and the knowledge of antibodies has greatly accumulated in fish immunology. In contrast, little is known about cell-mediated immunity inspite of accumulating evidence that cell-mediated immunity and /or local immunity play an important roles in the protection against pathogens achieved by vaccines, particularly when delivered by the immersion method.

Elasmobranchs and teleosts are the most primitive groups possessing the major histocompatibility complex (MHC)/T cell receptor system. To date MHC genes including class IA, β2 m, class IIA, class IIB have been reported from more than 25 species of teleosts and elasmobranches. However, information on allelic polymorphism of fish MHC class I genes has been described in many species.

Specific Immune Response

Fish possess cellular and humoral immune responses, and central organs whose main function is involved in immune defense. Teleost lymphoid tissues are not as defined in fish as those of higher vertebrates. Fish lack a bone marrow and lymph nodes. The major lymphoid tissues in teleosts are anterior (or head) kidney, spleen, thymus and mucosa-associated tissue. The anterior kidney is the principal immune organ responsible for phagocytosis, antigen processing and formation of IgM and immune memory. The lymphoid tissues are composed of a reticular cell framework supporting migratory and non-migratory cell populations. The cell populations are responsible for the nonspecific and specific immune defences and include lymphocytes, monocytes, macrophages, granulocytes and thrombocytes.

Lymphocytes have been divided into immunoglobulin-positive B cells and immunoglobulin-negative T cells. The lymphocyte heterogeneity has been investigated by using monoclonal antibodies and by functional tests of cell cooperation. The identification of T-cells has to date been indirect and typically based on their lack of surface immunoglobulin or their morphology.

Acquired or specific system plays an important role in the protection against recurrent infections by generating memory cells (cell mediated immunity) and specific soluble and membrane bound receptors (humoral defense) such as T cell receptors and immunoglobulins. The specific immunity is responsible for quick and efficient elimination of the specific pathogens. It also serves to improve and enhance the efficiency of natural immune mechanisms and to remember the infection next time.

Fish are the most primitive vertebrates to posses adaptive immune system (lymphocytes, Ig, MHC and TCR). The specific immune response in fish is sluggish (up to 12

weeks) in comparison to the instant and relatively temperature independent innate immune response. The reason is the intrinsic inefficiency of the acquired immune response of fish due to its evolutionary status and poikilothermic nature. This results in a limited antibody repertoire, affinity maturation and memory and a slow lymphocyte proliferation.

Humoral immunity

Teleost fish display a primary and secondary humoral response upon antigen administration, although in contrast with mammals, a shift in immunoglobulins class is absent. Following a first antigen contact, B-memory cells retain the capacity to be stimulated by the antigen. A secondary antibody response in fish is faster and results in higher Ig titers in the serum, than the primary response. In carp, the ratio between secondary and primary antibody responses never reached the high levels found in mammals (10-20 fold in carp and up to 100-fold in mammals).

The Ig in bony fish consists of heavy (H) and light (L) chains and hence is similar to that in other vertebrates. The fish immune system contains five types of immunoglobulins: IgM, IgD, IgZ, IgT and IgH. The major immunoglobulin of fish blood serum is tetrameric IgM-like molecule. The H chain of the piscine IgM shows homology with the μ chain of mammals and consists of eight light and eight heavy chains. Fish mucus contains monomeric Ig.

Cellular Immunity

Cell mediated immunity is a function of T cells in mammals. These cells act directly as T killer cells or indirectly via antigen-stimulated release of lymphokines which activate and recruit other cell types particularly macrophages into executing the response. Cell mediated killing is the most important defense mechanism in combating viral infections. *In vivo* manifestation of cell-mediated immunity (CMI) are delayed type hypersensitivity, transplantation and tumor immunity whereas, *in vitro* tests are specific contact cytotoxicity, mixed leukocyte reaction, macrophage migration inhibition, macrophage activation and antigen-stimulated blastogenesis of lymphocytes.

Teleosts also exhibit T-cell responses based on functional criteria like proliferation induced by T-cell mitogens, response in the mixed-leukocyte reaction, and function as helper cells in antibody production against thymus-dependent antigens, allograft rejection and secretion of lymphokines. Specific cell-mediated cytotoxicity has been documented for different fish species infected by viruses and intracellular bacteria.

Elasmobranchs and teleosts are the most primitive groups possessing the major histocompatibility complex and T cell receptor system. T-cell subsets have not been demonstrated in teleosts. Classical division of T cells into cytotoxic (Tc) and helper (Th) subpopulations is probably relevant to fish, based on the presence of these functional activities and MHC class I and II molecules. Specific cellular reactions in immune fish, as indicated by inhibition of macrophage migration and delayed type hypersensitivity with associated T-cell like activity has also been detected in fish.

Conclusion

In the last decade considerable progress has been made in describing and understanding the immune system of fish. Our knowledge on fish immune system can be exploited for evaluation of the health status of fish under different conditions. The increased awareness of the fish immune system has led to evolve a number of techniques for fish disease diagnosis and prophylaxis.

IMMUNOASSAYS IN DETECTION AND QUANTIFICATION OF PROTEINS

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Immunoassays are biochemical tests used to detect or quantify a specific substance, the analyte, in a blood or body fluid sample, using an immunological reaction. Immunoassays are highly sensitive and specific. Their high specificity results from the use of antibodies and purified antigens as reagents. An antibody is a protein (immunoglobulin) produced by B-lymphocytes (immune cells) in response to stimulation by an antigen. The "Immuno" refers to an immune response that causes the body to generate antibodies, and "assay" refers to a test. The assay takes advantage of the specific binding of an antibody to its antigen. The antibody: antigen complex is also known as an immuno-complex. Immunoassays are different from other types of laboratory tests, such as colorimetric tests, because they use antibody antigen complexes to generate a signal that can be measured.

Berson and Yalow described the first immunoassay in 1959. Their work resulted in their receipt of the Nobel Prize in Medicine in 1977. Since this introduction, immunoassays have evolved considerably. There have been several milestones that have led to the proliferation of modern immunoassays. The development of monoclonal antibodies from mouse hydridoma cells by Millstein and Kohler (Nobel Prize in 1984) enabled the production of high quantities of antibodies with well-characterized epitope specificity. Immunoassays have been in use since the early 1960's when radioimmunoassays were used to quantify insulin in plasma samples. Since their introduction, immunoassays have been used to detect and quantify hundreds of types of molecules both native to living organisms, such as hormones, and foreign molecules, such as pharmaceuticals. The molecules detected by immunoassays vary widely in size, chemical and physical properties, and biological activity. The crux (essential point) of an immunoassay is the natural response of a living organism to a foreign agent. Immunoassays may be qualitative (positive or negative) or quantitative (amount measured). An example of a qualitative assay is an immunoassay test for pregnancy. Pregnancy tests detect the presence of human chorionic gonadotropin (hCG) in urine or serum. Measuring the signal produced by the indicator reaction performs quantitative immunoassays.

Antibodies and Antigens

An antigen is the substance that the body is trying to "fight off" (eliminate or reduce) by mounting an immune response. An antibody is a protein that is produced by the body in response to an "invading" (foreign) substance. Antibodies are produced as part of the body's immune response to protect itself. Antibodies (Ab) are a type of protein called immunoglobulins. The most common one is immunoglobulin G (IgG). IgG is a protein composed of two main structural and functional regions one is Fab region that contains the antigen (Ag) binding site that varies between different antibodies and Fc region is the Region of constant structure within an antibody class.

Antibodies are used extensively as diagnostic tools in a wide array of different analyses. Monoclonal and recombinant antibodies provide a never-ending source of molecules and can produce endless possibilities for novel genetic constructs. Antibodies are

still very much in vogue and are now also being used in microarray analysis of the proteome using protein chips. Antibodies are of two types such as monoclonal and polyclonal. Monoclonal antibodies are often used as they only usually bind to one site of a particular molecule, and therefore provide a more specific and accurate test, which is less easily confused by the presence of other molecules. The antibodies picked must have a high affinity for the antigen (if there is antigen available, a very high proportion of it must bind to the antibody). In immunoassays, both monoclonal and polyclonal antibodies are used for detecting antigens, each with specific strengths for particular applications. The choice of an assay is determined by the nature, quantity and source of the antigen.

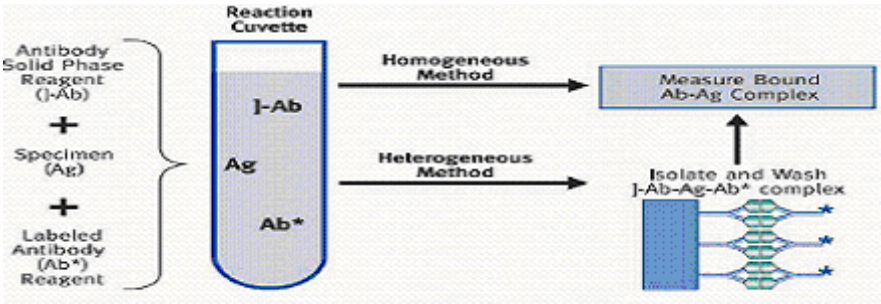
Types of immunoassays

Immunoassays can be divided into those that involve labelled reagents and those which involve non-labelled reagents. Those which involve labelled reagents are divided into homogenous and heterogeneous (which require an extra step to remove unbound antibody or antigen from the site, usually using a solid phase reagent) immunoassays. Heterogeneous immunoassays can be competitive or non-competitive.Because homogeneous assays do not require this step, they are typically faster and easier to perform.

All immunoassays require the use of labeled material in order to measure the amount of antigen or antibody present. A label is a molecule that will react as part of the assay, so a change in signal can be measured in the blood or reagent solution. Examples of a label include a radioactive compound and an enzyme that causes a change of color in a solution, or a substance that produces light. Immunoassay technologies utilize different formats to distinguish the bound antigen-antibody complex from the free unbound label.

Homogeneous and heterogeneous immunoassay

Immunoassay methods that require separation of bound Ab-Ag complex are referred to as heterogeneous immunoassays. Those that do not require separation are referred to as homogeneous immunoassays. Homogeneous methods have been generally applied to the measurement of small analyte such as abused and therapeutic drugs. Since homogeneous methods do not require the separation of the bound Ab-Ag from the free Ag, they are generally much easier and faster to perform. The first homogenous immunoassay (no separation step required) was the Enzyme Multiplied Immunoassay Technique (EMIT), EMIT was also one of the first immunoassay that made use of non-isotopic labels. Other non-isotopic labels became available such as chemiluminescence's to improve the analytical sensitivity of immunoassays.

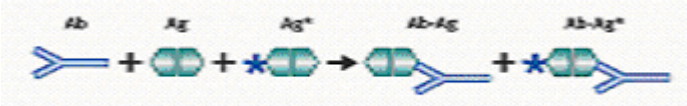


Competitive and Noncompetitive Immunoassays

In competitive formats, unlabelled analyte (usually antigen) in the test sample is measured by its ability to compete with labeled antigen in the immunoassay. The unlabeled antigen blocks the ability of the labeled antigen to bind because that binding site on the

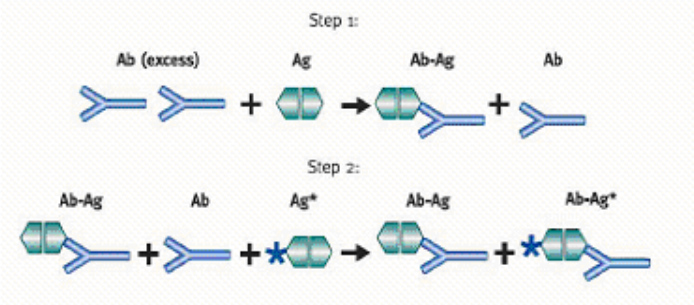
antibody is already occupied. Thus, in a competitive immunoassay, less label measured in the assay means more of the unlabeled (test sample) antigen is present. The amount of antigen in the test sample is inversely related to the amount of label measured in the competitive method.

One step competitive format-



In the one step competitive format, both the labeled antigen reagent (Ag*) and the unlabeled specimen (or test sample analyte) compete for a limited amount of antibody. In the two-step competitive format, the antibody concentration of the reaction solution is present in excess in comparison to the concentration of antigen. Antibody reagent is first incubated with specimen containing antigens of interest; then in the second step, labeled antigen is added. Remember that in the competitive format, less bound labeled antigen indicates more antigen present in the test sample. Two-step competitive assay formats provide several fold improved assay sensitivity compared to one step assay formats.

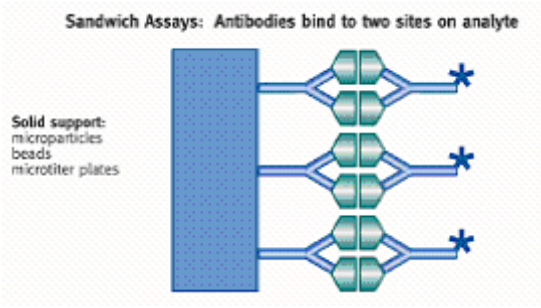
To-step competitive format-



In noncompetitive immunoassays, also referred to as the "sandwich assay," antigen in the unknown is bound to the antibody site, then labeled antibody is bound to the antigen. The amount of labeled antibody on the site is then measured. Unlike the competitive method, the results of the noncompetitive method will be directly proportional to the concentration of the antigen. This is because labeled antibody will not bind if the antigen is not present in the unknown sample.

Noncompetitive assay formats generally provide the highest level of assay sensitivity and specificity and are applied to the measurement of critical analyte and this format is referred to as a “sandwich” assay because analyte is bound (sandwiched) between two highly specific antibody reagents. Noncompetitive assay formats can also utilize either one step or two step methods, as with the competitive assay. The two step assay format employs wash steps in which the sandwich binding complex is isolated and washed to remove excess unbound labeled reagent and any other interfering substances. The two-step noncompetitive format usually offers the highest specificity and sensitivity than the two-step competitive assays.

Noncompetitive assay



Detection methods

Detecting the quantity of antibody or antigen can be achieved by a variety of methods. One of the most common is to label either the antigen or antibody. The label may consist of an enzyme (see enzyme immunoassay (EIA)), colloidal gold (lateral flow assays), radioisotopes such as I-125 Radioimmunoassay (RIA), magnetic labels (magnetic immunoassay - MIA) or fluorescence substances . Other techniques that detect antigen antibody include agglutination, nephelometry, turbidimetry and Western Blot. Immunoassays have a particularly important role in the diagnosis of many Infectious diseasesof all animals including humanbeing.Basing on lebelling materials the immuneassays are of several types theses are -:

- Enzyme immunoassay (EIA)
- Fluorescence immunoassay (FIA)
- Radioimmunoassay (RIA)
- Magnetic immunoassays (MIA)
- Chemiluminescent immunoassay (CLIA)
- Fluorescence Polarization immunoassays (EPIA)
- Kinetic Interaction of Microparticles in Solution (KIMS)

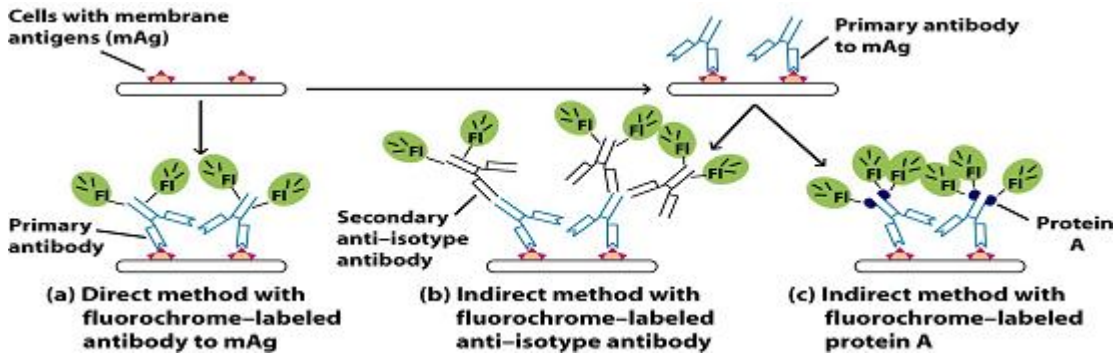
Among these assays, Radioimmunoassays was first developed but it needs specific facilities and the half-life of radioisotope is not long so Enzyme immunoassay is at present most popular. The goal of development of immunoassay is improvement of sensitivity and automation.

Enzyme immunoassay (EIA)

EIA is discussed in separate chapter

Fluorescence immunoassays (FIA)

In these assays, antibodies or other reagent proteins are "tagged" or labeled with fluorescent dyes. These fluorescent reagents can then be used to stain samples mounted onto microscope slides and the slides can be examined using a fluorescent microscope. Typical fluorescent labels include FITC, TRITC, PE, rhodamine etc. Cells in suspension can also be stained fluorescently and then analyzed by fluorescence-activated cell sorting or FACS analysis.

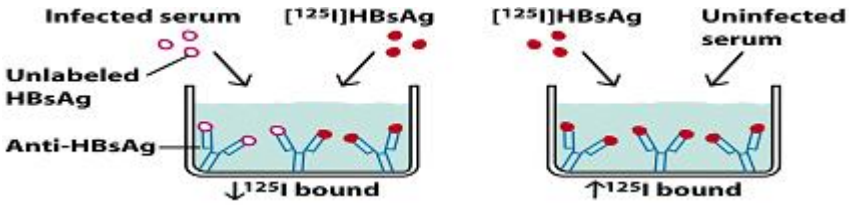


Magnetic immunoassay(MIA)

Magnetic immunoassay (MIA) is a novel type of diagnostic immunoassay utilizing magnetic beads as labels in lieu of conventional enzymes (ELISA), radioisotopes (RIA) or fluorescent moieties (fluorescent immunoassays). This assay involves the specific binding of an antibody to its antigen, where a magnetic label is conjugated to one element of the pair. The presence of magnetic beads is then detected by a magnetic reader (magnetometer) which measures the magnetic field change induced by the beads. The signal measured by the magnetometer is proportional to the analyte (virus, toxin, bacteria, cardiac marker,proteins etc.) quantity in the initial sample.

Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a scientific method used to test antigens (for example, hormone levels in the blood) without the need to use a bioassay. RIA technique is extremely sensitive and extremely specific, it requires a sophisticated apparatus, radioactive isotopes and is also costly. It also requires special precautions, since radioactive substances are used. Therefore, today it has been largely supplanted by the ELISA method, where the antigen-antibody reaction is measured using colorometric signals instead of a radioactive signal.



Chemiluminescent immunoassay (CLIA)

Chemiluminescent immunoassays utilize a chemiluminescent label. Chemiluminescent molecules produce light when they are excited by chemical energy. A light detector measures these emissions.

Antibody microarray

An antibody microarray is a specific form of protein microarrays, a collection of capture antibodies are spotted and fixed on a solid surface, such as glass, plastic and silicon chip for the purpose of detecting antigens. Antibody microarray is often used for detecting protein expressions from cell lysates in general research and special biomarkers from serum or urine for diagnostic applications.

The theoretical background for protein microarray-based ligand binding assays was initially developed by Ekins et al. in the late 1980s. According to the model, antibody microarrays not only would permit simultaneous screening of an analyte, but would also be

more sensitive and rapid than conventional screening methods. Interest in screening large protein sets only arose as a result of the achievements in genomics by DNA microarrays and the Human Genome Project. The first array approaches attempted to miniaturize biochemical and immunobiological assays usually performed in 96-well microtiter plates. 96-well antibody arrays were first created with 144 elements each for "standard enzyme-linked immunosorbent assays" (ELISA).Filter membranes were also initially used because of their superior protein binding capacity. They were mostly probed with antibodies using ELISA techniques. Filter based arrays were constructed but the limitations were the low resolution and considerable background making it difficult to use them in applications with limiting sample quantities such as protein expression profiling of tumor biopsies.

Immunoprecipitation

The simplest immunoassay method measures the quantity of precipitate, which forms after the reagent antibody (precipitin) has incubated with the sample and reacted with its respective antigen to form an insoluble aggregate. Immunoprecipitation reactions may be qualitative or quantitative.

Immunonephelometry

The immediate union of antibody and antigen forms immune complexes that is too small to precipitate. However, these complexes will scatter incident light and can be measured using an instrument called a nephelometer. The antigen concentration can be determined within minutes of the reaction.

Immunoassays in Aquaculture

Infectious disease is currently the major devastating problem and an ongoing threat to aquaculture. In addition, there is increasing concern over the consequences of newly emerging diseases in aquaculture. Fish in culture are susceptible to a wide range of bacterial, viral, parasitic and fungal infections and losses through disease currently make a significant impact on the quality and volume of fish production. Disease control is therefore a vital component of successful aquaculture. Conventional methods of controlling such diseases, such as chemotherapeutants, are ineffective for many new pathogens (notably viruses). In recent years there have been significant advances in the tools and techniques available for the identification and characterizations of a wide range of fish pathogens. Rapid detection and identification of pathogens is necessary for effective disease management and this in turn leads to a reduction in the use of antibiotics and chemicals in the environment. Moreover, the movement of infected fish needs to be restricted to prevent the spread of disease, thus, regular screening for the presence of pathogens is vital. Monitoring the immune response of fish to infection or following vaccination can also provide useful information for health management.

Among several techniques the molecular techniques are receiving increasing attention for pathogen screening and identification. In addition, these techniques are providing significant insights into pathogenesis (disease development), showing strong potential for disease control and prevention programs, as well as for treatments of diseases (e.g., DNA vaccines).

One of the most urgent needs for aquaculture health management is establishment of standards for quantitative assessment of health status in the broad range of species under culture. For this immunoassays are the best methods for simple and predictive health tests. Besides screening for pathogens, techniques of immunoassays can be used to ascertaining other health parameters, including haematocrits, leucocrits, blood cell differentials, neutrophills oxidative radical production, Myeloperoxidase activity, phagocytic functions

and to know the causative agents. Such techniques can be applied to quantitative protein, immunoglobulins, lysozyme, cortisol and ceruloplasmin analysis from plasma samples. Methods such as agglutination tests to assay antibody after immunization can now be supplemented with immunoassays, such as fluorescent antibody test (FAT) and enzyme-linked immunosorbent assay (ELISA). ELISPOT can be used to accurately quantify numbers of immunoglobulins or non-specific antibody-secreting cells and are used in immunodiagnostic.

The sensitivity and specificity of many immunoassays for identifying fish pathogen have been improved by the use of monoclonal antibodies. Monoclonal antibodies (mAbs) provide ideal tools for detection of pathogens and a variety of mAbs and tests have been developed for use in aquaculture. Polyclonal antibodies are also convenient tools for pathogen detection although their use is limited due to product standardization and cross reactivity with related pathogens. Immunological methods such as immunohistochemistry (IHC) and the indirect fluorescence antibody test (IFAT) enable rapid specific detection of pathogens in tissue samples without the need to first isolate the pathogen. Novel methods of antibody production (e.g recombinant antibody technology) will extend the existing range of probes available for the future and application of these in multiplex testing systems where a variety of pathogens or immune response molecules (e.g, cytokines, lysozyme, IgM) can be detected simultaneously will make a significant impact on disease screening programmes in aquaculture.

Immunoassay has already made a significant impact on disease management in aquaculture besides these immunoassays are use in expression and quantification of hormones and proteins, detection of pollutant in aquatic environment.

Advantages of immunoassays

- Applications in all areas of the laboratory
- Good sensitivity; large menu of tests
- Easily automated with applications on many instrument platforms
- In most cases, there is no sample preparation required; efficient; cost effective
- Immunochemical methods provide rapid, sensitive, and cost effective analyses for a variety of environmental contaminants. These methods are highly sensitive to specific antibodies, and provide analytical systems capable of detecting very low levels of chemicals. This high sensitivity is due to the powerful catalytic ability of enzymes. These methods are also highly selective due to the extraordinary discriminatory capabilities of antibodies.
- Immunoassay methods are fast and relatively easy compared to conventional methods.
- Immunoassays are inexpensive when compared to traditional GC/MS and liquid chromatography.
- Immunoassays are a very efficient screening tool. Detection limits are suitably low for environmental work, and sample preprocessing is not required with its abundant losses. Recovery of analyte from spiked sample can be better with immunoassays than with conventional methods.

Limitations of immunoassays

In spite of its numerous advantages in helping make significant progress in research, enzyme immunoassays cannot replace conventional methods altogether, and are best used in combination with them. Immunoassays are most useful if we understand their limitations. These are:

- Depending on the reagent, they may have dynamic range

- Cross-reactivity and specificity
- Interferences from non-structurally-related compounds
- Interpretation of results should always occur in this context
- Some immunoassays are not highly selective, and they may respond to families of Compounds rather than to individual compounds. Because of the design of the immunoassay, sample contaminants that might Interfere with the antigen-antibody reaction can produce positive readings when samples are indeed negative (called false positive results). Conventional methods, can then have be used as a backup test for positive samples to be sure sample contamination is not skewing your results.
- Lower molecular weight molecules often lack specific antigens, and sometimes there are cross linking problems.

MONOCLONAL ANTIBODIES PRODUCTION AND ITS USAGE FOR FISH HEALTH MANAGEMENT

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Antibody is a specialized serum protein (mostly gamma globulins) produced in response to an antigen, which has the ability to combine specifically with that antigen. Antibodies (Abs) have two very useful characteristics; they are extremely specific and they can confer protection against disease. The importance of antibodies for fish health management measures such as diseases diagnosis, identification, stereotyping, antigen characterization, epidemiology and vaccine development is well known. The serum of immunised or infected animals contains an array of antibodies those are specific for a variety of antigens (i.e., polyclonal antibodies). Polyclonal antibodies are convenient tools for pathogen detection, although their use is limited due to product standardization, limited quantity, unwanted background reactions, in ability to discriminate antigen at epitope level and cross reactivity with related pathogens**.** Monoclonal antibody (MAb) technology, on the other hand allows the production of large amounts of homogenous antibodies on a continual basis and are more superior over polyclonal antibody in a number of aspect presented in Table 1.

Table 1. Comparison between conventional antiserum and monoclonal antibody

Features	Conventional antiserum (Polyclonal antibodies)	Monoclonal antibodies
Determinant	Several	Single
Specificity	Variable with animal and bleed	Standard
Cross-reactions	Unexpected cross-reactions	Too specific
Affinity	Variable with bleed	May be specific during cloning
Yield	Up to 1 mg/ml	Up to 100 mg/ml in tissue culture; Up to 20 mg/ml in ascites fluid
Contaminating immunoglobulins	Up to 100%	None in culture; 10% in ascites fluid
Purity of antigen	Required	Desirable, but not essential
Cell culture requirement	Nil	Well equipped and expensive laboratory set up
Method	Simple	Tedious, expensive and needs extra care
Ethical issues	Needs frequent bleeding, hence torture of animal	No ethical problems
Antibody composition	Heterogenous	Homogenous

Fish in culture suffer a wide range of diseases, including bacterial, viral, parasitic and fungal infections. Disease control is therefore an essential component of successful fish cultivation. Prevention from infection in the first place is the optimal strategy for disease control and fish vaccination has been shown to have great potential. For this rapid diagnosis, which can be achieved using antibody-based tests, is required so that infections can be treated quickly and with the correct medication. Both polyclonal antibodies (PAbs) and

MAbs, either individually or in combination, provides useful reagents for such tests. However, MAbs assure a reliable source of antibodies and very often achieve greater sensitivity than PAbs as described above. Monoclonal antibodies have made a significant contribution to the diagnosis and treatment of many clinical and veterinary diseases, and over the last decade they have been introduced as tools to assist in fish health management.

Production of monoclonal antibodies

Monoclonal antibodies production was first introduced in 1975 by Kohler and Milstein, for which they were awarded the Nobel Prize for medicine in 1984. Monoclonal antibodies are typically made by fusing myeloma cells with the spleen cells from a mouse that has been immunized with the desired antigen. However, recent advances have allowed the use of rabbit B-cells. Monoclonal antibody production is initiated by fusing the differentiated B lymphocyte(or plasma cells that have the capacity of producing antibodies of perticular specificity and identical) with the immortal myeloma cells because plasma cells are short lived and can not grown in culture. Originally, Kohler and Milstein used sendai virus as fusion agent and later polyethylene glycol is used, but the success rate is low so a selective medium is used in which only fused cells can grow. This is because myeloma cells have lost the ability to synthesize hypoxanthine-guanine-phosphoribosyl transferase (HGPRT), an enzyme necessary for the salvage synthesis of nucleic acids. The absence of HGPRT is not a problem for these cells unles the de novo purine synthesis pathway is also disrupted. By exposing cells to Aminopterin, they are unable to use the de novo pathway and become fully auxotrophic for nucleic acids requiring supplementation to survive. The selective culture medium is called HAT medium because it contains Hypoxanthine, Aminopterin, and Thymidine. This medium is selective for fused (hybridoma) cells. Unfused myeloma cells cannot grow because they lack HGPRT, and thus cannot replicate their DNA. Unfused spleen cells cannot grow indefinitely because of their limited life span. Only fused hybrid cells, referred to as hybridomas, are able to grow indefinitely in the media because the spleen cell partner supplies HGPRT and the myeloma partner has traits that make it immortal (as it is a cancer cell). This mixture of cells is then diluted and clones are grown from single parent cells on microtitre wells. The antibodies secreted by the different clones are then assayed for their ability to bind to the antigen (with a test such as ELISA or Antigen Microarray Assay) or immuno-dot blot. The most productive and stable clone that secret the desier monoclonal antibody is then selected for future use.

Application of MAbs for fish health management

Cultured fish are susceptible to a wide range of diseases. Losses through diseases are currently making a significant impact on the quality and volume of fish produced in aquaculture systems. Disease control is therefore, a vital component of successful aquaculture, and rapid detection and identification of pathogens are crucial for effective disease management. Moreover, the movement of infected fish needs to be restricted to prevent the spread of disease. Thus, regular screening for the presence of pathogens is vital. Monitoring the immune response of fish to infection or following vaccination can also provide useful information for health management. MAbs are widely used in the diagnosis, stereotyping and analysis of antigens in microbial pathogens in medical and veterinary sciences. Currently, they are also used in disease research and health management in aquaculture systems of developed countries. But, their application in commercial aquaculture systems in developing countries is yet to be implemented at any significant level. Understanding a pathogen itself is a main step in disease management. In fisheries, monoclonal antibodies have been applied for the study of fish immunology and infectious diseases. In last few decades, a large number of MAbs to fish immunoglobulins (Igs) have

been raised for most of the commercially cultured fish species. These MAbs have been used for identification of heavy and light chain variants in fish Ig and for studies on the ontogeny of B-lymphocytes. Techniques for the measurement of fish Ig and specific antibodies (Abs) have been greatly improved through use of MAbs. MAbs have been obtained against several leucocytes surface antigens and are being used as markers for different subsets of leucocytes: neutrophils, non- specific cytotoxic cells and cells responsible for mixed leucocyte reaction. The sensitivity and specificity of many immunoassays for identifying fish pathogens have been improved by the use of MAbs. New methods have been developed to detect low levels of pathogens in adult carriers and in eggs, thus helping to prevent the dissemination of fish diseases. MAbs to fish pathogens are also being used for passive immunization studies as well as for conformational probes in the development of genetically engineered vaccines. ELISA with MAbs do not have problems of non-antibody mediated precipitates induced by C-reactive proteins, as encountered in agglutinin and precipitin tests for bacterial pathogens, and in addition provide the advantage of greater sensitivity and large scale testing.

Therefore, there is a vast the application of Mabs in fish and shellfish health management. Several applications of Mabs are:

1. Vaccine development

Antibodies not only provide useful tools for monitoring the immune response in fish following vaccination, but also are invaluable in the identification of protective antigens. This includes one and two-dimensional gel electrophoresis of pathogen antigens followed by western blot analysis using fish sera from infected fish for the development of traditional vaccines. Moreover, antibody probes are extremely useful in the development of recombinant vaccines as MAbs, polyclonal antibodies and fish sera can be utilised to screen the expressed products from pathogen DNA libraries for potential vaccine antigens.

2. Lymphocyte characterization

Monoclonal antibodies raised against different T and B cell markers and fish immunoglobulin heavy chains are being used for identification, ontogeny and activity of 'T' and 'B' cells. They are also being used for identification and characterisation of B and T cell markers.

3. Detection of pathogens

A combination of methods is usually required for a definitive diagnosis of disease, and immunodiagnostic techniques complement both traditional and molecular methods very well. Once the specificity of the antibody probe has been confirmed, MAbs provide ideal tools for rapid detection of pathogens. A variety of different immunodiagnostic tests based on MAbs have been developed for use in aquaculture and they are widely being used in the diagnosis, serotyping and analysis of antigens of microbial pathogens in medical and veterinary sciences. Currently, many Mabs are commercially available and they are being used in disease research and health management in aquaculture systems of developed countries. However, their application in commercial aquaculture systems in developing countries is yet to be implemented. Immunological methods such as the indirect fluorescence antibody test (IFAT) or fluorescence antibody test (FAT) and immunohistochemistry (IHC) enable rapid and specific detection of pathogens in tissue samples without the need to isolate the pathogen. IFAT is the current method of choice for detection of *R. salmoninarum* in kidney imprints of rainbow trout (*Oncorhynchus mykiss*), *Flavobacterium psychrophilum* infection and detection and identification of infectious salmon anaemia (ISAV) in salmon *(Salmo salar)*.

Serology also provides tool for monitoring the immune response of fish following vaccination and therefore, assists in the development of vaccines as new aquaculture species are cultured and new diseases emerge. Such tests for fish require anti-fish species IgM MAb probes and reagents are now commercially available to perform serological testing in a variety of fish species including trout, salmon, cod (*Gadus morhua)*, halibut *(Hippoglossus hippoglossus)*, turbot (*Scophthalmus maximus)*, grouper *(Epinephelus* sp.*)*, catfish *(Clarias* sp.*)*, tilapia *(Oreochromis niloticus)*, snakehead *(Channa striata)*, giant gourami *(Osphronemus goramy)*, Asian sea bass *(Lates calcarifer)*, European sea bass (*Dicentrarchus labrax),* sea bream (*Sparus aurata)*, sturgeon (*Acipencer* sp.) and carp (*Carassius* sp.*)*. The ELISA using these MAb probes provides a convenient method for testing large numbers of samples and the sampling of fish is non-destructive, requiring only a serum sample. This type of ELISA has many applications and may be utilised for the screening of fish populations suspected of having a particular disease (in culture or in the wild), for brood stock health testing, verification of post-vaccination antibody response and vaccine efficacy.

4. MAbs to Fish Immunoglobulins

MAbs specifically reacting with Ig of fish provides a powerful tool for monitoring antibody production following infection or vaccination, and for immunoblotting analysis of antigens recognized by the immune system. These reagents help in evaluation of potential vaccines in terms of protection and monitoring of antibody production, thereby leading to successful development of vaccines for fish. Presently, MAbs against serum Ig are available for several marine and freshwater fish species such as Atlantic salmon, Carp, Puffer fish, Sea bass, Rainbow trout, Tilapia and Turbot. Recently two MAbs specific to heavy chain of Ig of *Labeo rohita* have been developed. D7 MAb has been used as diagnostic reagent in ELISA and immuno-cytochemical assays to demonstrate its application for sero-surveillance and for immunological studies in rohu. This MAb was found to be suitable for the detection of pathogen specific antibodies in serum of immunized fish by an indirect ELISA. It was also used in flow cytometry to demonstrate the percentage of Ig bearing cell in different lymphoid tissues of fish.

5. For detection of viral pathogens of fish and shrimps

Mabs are use for detection of several viral pathogens of fish and shellfish. MAbs were produced from white spot syndrome virus (WSSV), isolated and purified from *Penaeus monodon* and were used to illustrate antigenic similarity of isolates from Indonesia and Malaysia to the Taiwan isolates have demonstrated a unique variant of Taura syndrome virus from Belize, which could not be detected by specific MAbs. MAbs against Taura syndrome virus (TSV), yellow-head virus (YHV) isolated from naturally infected shellfish. MAbs have been used in detection and differentiation of yellow head complex viruses. This indicates the potential application of MAbs in aquaculture for disease diagnosis.

6. Use of MAbs as Fish Leucocyte Markers

Presently, numbers of MAbs are available against leucocytes of humans, laboratory animals as well as livestock. These have been extremely useful in the study of normal and diseased conditions. In teleost also few attempts have been made to develop MAbs against leucocytes. Most of the MAbs obtained were also positive for common carp Ig by ELISA. MAbs recognizing T cells in *Cyprinus carpio* and *Ictalurus punctatus* have already been produced. MAb specific for neutrophilic granulocytes and non-specific cytotoxic cells have also been produced for channel catfish. These MAbs have been used to study immune response in fish.

Conclusion

Immunodiagnostics have already made a significant impact on disease management in aquaculture, however, there is potential for rapid advancements in the future to bring fish immunodiagnostics more in-line with veterinary immunodiagnostics for domestic animals. Understanding a pathogen itself is a main step in disease management. Therefore, there is scope for the application of Mabs in fish and shellfish health management. A large range of commercial MAbs is now available for detection of fish pathogens and many new ones are in the pipeline and this provides much needed standardised reagents. In addition, the availability of standardised anti-fish species IgM antibodies enables serology to be performed for a large variety of fish species. Novel methods of antibody production (e.g., recombinant antibody technology) will extend the existing range of probes available for the future and application of these in multiplex testing systems where a variety of pathogens or immune response molecules (e.g., cytokines, lysozyme, IgM) can be detected simultaneously will make a significant impact on disease screening programmes in aquaculture.

GENE EXPRESSION CONSTRUCTS AND CLONING: AN OVERVIEW

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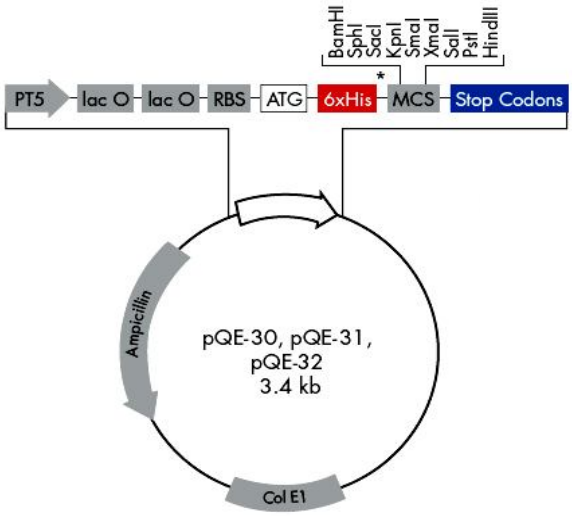
Major steps in expression construct cloning

- Selection of the appropriate vector and scanning for the restriction sites
- Identification of the gene of interest and its restriction enzyme-scanning
- Creation/synthesis of insert through PCR/RE digestion
- Ligation and transformation
- Selection of the clone by RE-digestion/PCR/hybridization
- Screening of best clones synthesizing the protein of interest
- Characterization of the protein

Selection of the appropriate vector

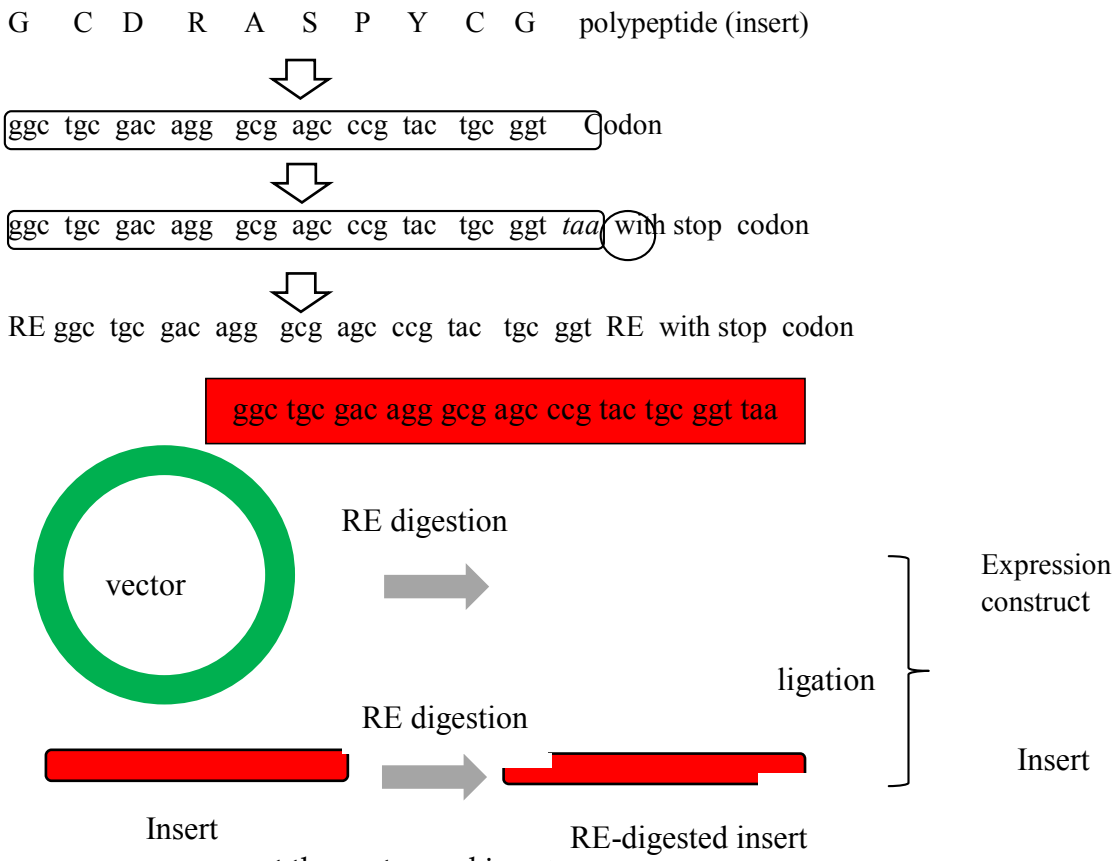
Selection of the appropriate vector is mostly dependent on the type of cell used for protein expression. Generally, CMV promoter containing vector is used for attached (fibroblastic) cell type and SV40 promoter for floating cell types. In addition to it, availability of restriction enzymes in the multiple cloning sites (MCS), the tag attached and their direction (N or C-terminal) also determines the selection of expression vector. Example: pcDNA3, pSG5-EGFP, PQE-30,31,32 etc.



Design of the gene

During designing the gene, codon usage must be considered as all codons do not function equally. Un-optimized codons may lead to lower level of expression. The codon usage reflects availability of tRNA in the competent cells like *E.coli.* Attention need to be paid for the usage of the stop codon as TAG (amber stop codon) may likely to be read by some strains of bacteria (e.g : XL1-Blues).

Making the gene expression construct for the expression of GCDRASPYCG polypeptide



- Digest the vector and insert with the same RE
- Treat the plasmid DNA with shrimp alkaline phosphatase
- Schematic representation of the gene expression construct

Once the functional construct is ready, it needs to be transformed into *E. Coli* and will be cultured in presence of appropriate antibiotic viz., ampicillin (100 µg/ml), kanamycin (50µg/ml), tetracyclin (50-100 µg/ml) etc. After 16-20 h of culture (under vigorous shaking), plasmid will be isolated and will be DNA sequenced for verification and confirmation of the appropriate gene sequence.

Protein expression system

In addition to the cell free system, various bacteria, yeast, insect and mammalian cells systems are used for protein expression. Each system has its own advantage and disadvantages. Most commonly used protein expression system is bacteria. It has the advantage of faster growth and easy to culture, but has the limitations in expression of eukaryotic protein. Post translational modifications (SS bonds, glycosylation etc) are deficient in bacteria, and there is the accumulation of lipopolysaccharides (LPS) along with the protein.

DEVELOPMENT OF DIAGNOSTICS AND CONTROL METHODS FOR THE FISH ECTOPARASITE, *ARGULUS SIAMENSIS*

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Introduction

Parasitic lifestyle comes with a lot of benefits that include availability of large resources of food and living habitat generally due to the small size of the parasite compared to its host. Also a host provides a relatively protected microhabitat compared to highly variable external environment. Parasitism however, involves negative effect on the organism exploited by a parasite and also antagonistic mechanisms against the parasite exerted by the host. This leads to a co-evolutionary arm race between the host and parasite. Parasitic form of life has proved to be a highly successful strategy in terms of evolution. At least half of all living organisms are obligate parasites or their existence resemble parasitism at some point in their ontogeny.

Fish appear to be lucrative hosts for a large diversity of parasites including protozoans, monogeneans, leeches, molluscs and crustaceans. Amongst these the crustacean ectoparasites are particularly notorious, being ubiquitous and generalist parasites with the potential of causing huge fish population losses. The branchiurans are a group of crustacean ectoparasites of fishes commonly known as the 'fish lice'; wherein *Argulus* Muller, 1785 is the largest and the most diverse genus. The members of the genus *Argulus* are obligate ectoparasites of freshwater fishes, feeding on skin, mucous and blood of the fish hosts. These parasites depend upon the fish host only for nutrition and are able to feed on multiple host species. Owing to the damage this group of parasites can inflict upon their host, it is now well understood that parasitic lice can play a significant role in economic success of aquaculture organizations.

The genus *Argulus* contains about 129 valid species and occurs around the world except in Antarctica, in marine and estuarine (n = 44) and freshwater habitats (n = 85). The species of this genus reported so far from India are *A. indicus* Weber, *A. giganteus* Ramakrishna, *A. bengalensis* Ramakrishna, *A. siamensis* Wilson, *A. siamensis peninsularis* Ramakrishna, *A. puthenveliensis* Ramakrishna, *A. siamensis* sub sp. Sundari Bai, *A. fluviatilis* Thomas and Devaraj, *A. cauveriensis* Thomas and Devaraj, *A. japonicus* Thiele, *A. quadristriatus* Devaraj and Ameer Hamsa and *A. mangalorensis* Natarajan. However, *Argulus siamensis* is the most prevalent species of the Indian subcontinent having been found to parasitize fish in most of the major aquaculture zones of India. The following discussion particularly pertains to the diagnosis and control of this major fish pest.

Distribution of *Argulus siamensis*

A. siamensis has been reported only from the Asian continent. In a nationwide survey of Argulosis conducted under NFBSFARA funded project at CIFA, Bhubaneswar, *A. siamensis* was reported from 13 different locations covering 8 states of India. Based on the survey it was found to be the most prevalent species of the genus *Argulus* in the Indian subcontinent. Furthermore, *Labeo rohita* appears to be preferred or rather susceptible host for this parasite. *L. rohita* being the highest contributor to freshwater aquaculture among the major Indian carps, the economic implication of *A. siamensis* further magnifies.

Morphological identification of *A. siamensis*

A. siamensis can be identified based on the description of major species of the genus *Argulus* provided by Meehean (1940). The most distinguishing morphological characteristics of this species are as follows:

- The carapace is ovate almost reaching the 4th swimming appendage in female and overlapping abdomen in male.
- Abdomen is elliptical, longer than broad with deep anal sinus and acute tips.
- The respiratory areas consist of one anterior club-shaped area curved around posterior area but slenderer, and both end at the same level.
- The 1st antenna is stout with long lateral hook, a slight anterior knob and spine at base which is long and blunt.
- The 2nd antenna is long with small transparent spine at its base.
- The 2nd maxilla has a broad basal plate with three broad blunt teeth. A pair of spines is present near midline of body which are small and blunt.
- The suction discs have 4-5 imbricate plates and the basal plate is considerably longer than the others.
- The swimming appendages are provided with flagella. The 4th pair of swimming leg in males has a peg like a short fingerlike papilla originating a short distance back from the distal edge of the basis. A posterior bilobed process with a scant lateral prolongation is also present. In 3rd pair of swimming legs, coxa is rounded posteriorly where the socket is located, with a meager swelling anteriorly. In female parasites the 4th swimming leg has a posterior boot shaped lobe and a toe drawn into long fingerlike tapering process.

Molecular methods developed for diagnosis of *A. siamensis*

Development of effective control measures for any parasite depends on its identification because even subtle differences in physiology and ecology of the parasites seen within species influence control strategies. Identification of parasites, particularly crustaceans poses a serious problem for the researchers throughout the world. The traditional method used for identification of *Argulus* sp. is generally based on morphological distinctions. However, these characteristics can only be distinguished by experienced taxonomists in adult parasites of both sexes, mostly in live/well-preserved male specimens. This method poses serious problems in case of closely related species. Due to wide variety of species under this genus and non-availability of key to identify all the species, it is very difficult to identify the parasite at species level. However, molecular techniques provide more accurate schemes for the identification of *Argulus* sp. by overcoming problems associated with phenotypic similarity.

In an attempt to study the genetic diversity of *Argulus* sp. collected from 13 different locations representing the major aquaculture zones of India by RAPD-PCR; two species specific primers were developed for *A. siamensis* and *A. japonicus* using the unique DNA bands generated by amplification with RAPD primers. The primer specific for *A. siamensis* amplifies a band of size 235 bp from genomic DNA of the parasite and does not show cross amplification with other species. A species specific primer for *A. japonicus* was also generated in the same study amplifying a band of 299 bp. Also, PCR-restriction fragment length polymorphism (RFLP) based screening assays are fast, simple and can be highly discriminatory depending on the target gene. An assay was developed for the characterisation of the two major *Argulus* species *A. siamensis* and *A. japonicus*, using PCR of the 18s ribosomal DNA, followed by a single digestion reaction using restriction endonucleases at our laboratory.

Control and prevention of *A. siamensis*

It is estimated that a loss to the tune of 29,524.40 INR/ha/year occurs due to argulosis outbreak in Indian major carp farming and 48% of the water bodies under carp culture are affected by this disease. The extent of financial loss caused by this parasite makes its control and prevention highly important.

Some parameters act as pre-disposing factors making fish more susceptible to parasitic infections. Stress due to handling, capture, confinement, overcrowding, suboptimal environment etc. can result in compromised immune system making the fish more susceptible to parasitic infection. Also, the high temperature of the Indian sub-continent narrows the time gap between generations resulting in overlapping of cohorts and abundance in parasite numbers. Moreover, it is known that *A. siamensis* is transmitted biologically in pond system by snails and water weeds. Though like every other parasitic diseases, argulosis can be defended against by good fish husbandry and stock management. However, it is not always possible to avoid these factors and hence control methods are opted for large scale fish production.

There are numerous chemical treatments available for the control of these *Argulus*, but none of them have been able to control *Argulus* infestation effectively. Most commonly, these parasites have been controlled by dip baths in organophosphates or synthetic pyrethroids which are now being discouraged due to their damaging effect on the immune system of the host, environment and wildlife. The use of chitin inhibiting treatment for argulosis in form of oral administration of diflubenzuron has also been reported. Some biological control methods used for control of parasite numbers include use of novel egg laying boards and cleaner fish like Wrasse. However, the need for new age therapeutants with higher efficacy against parasite and less damaging effect on environment has led to the use of avermectins like doramectin and ivermectin for control of argulosis. Both doramectin and ivermectin provide protection against infection of *A. siamensis* for a period of 17–18 days post-administration and could be used as prophylactic agents. However, dependence on one group of therapeutants may lead to resistance against the drug in the parasites. The eradication of these ectoparasites is not practical at present stage due to non-availability of any preventive vaccines against the parasite. Therefore, the development of integrated management programmes is a more realistic prospect for the control at present.

Nevertheless the development in our knowledge about the physiology of the parasite at molecular level has paved way for control approaches that hold promise for future. In the recent few years there has been a growing understanding of the pathology of this parasite and many studies have been undertaken to study the effect of this parasite on the immune system of the host. A major step with regards to studies on this parasite has been the transcriptomic analysis of *A. siamensis*, which would lead to availability of the genomic information of the parasite and a better understanding of the proteins and metabolic pathways which are critical to the development of potential vaccine candidates. With the increasing repertoire of knowledge about the parasite, the development of newer control approaches becomes possible to tackle the problem of argulosis resourcefully.

DIAGNOSIS OF *DACTYLOGYRUS* (MONOGENEA; PLATYHELMINTHES) INFECTING CYPRINIDAE

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Introduction:

Fish are indispensable source of proteins for humans. Development of aquaculture during the last decades has resulted in much greater attention being paid to problems posed by parasites and their importance for fishery leading to constraints in the productivity of aquaculture. Besides direct losses caused by mortality, parasites may have considerable impact on growth and behaviour of fish, their presence may also reduce marketability of fish (Crowden and Boom, 1980; Lom and Dyková, 1992; Williams and Jones, 1994; Kumaraguru et al., 1995; Woo, 1995). Diseases affect the normal health conditions and cause reduction of growth, abnormal metabolic activates and even death. The disease problems in fish culture frequently occurred as a result of the lack of controlled systems in the farms either at broodstock level or at fry and fingerling stages. Parasitism is one of the most serious problems for cultured fish (Scholz, 1999). Several species of monogenea have been implicated in the mortality of cultured fish (Thoney and Hargis, 1991). They could either have already occurred in farm or brought into the farm by an introduced fish. Despite of considerable progress in fish parasitology in the last decades, major gaps still exist in the knowledge of taxonomy, biology, epizootiology and control of fish parasites.

Among parasites infecting fishes, the monogeneans play an important role as pathogen causing severe diseases. This is because they affect those organs and tissues which are vital to the normal functioning such as gills and skin, the organs of respiration and barrier of defence respectively. In majority of cases, they cause dual type of injury to their hosts, i.e., through their hooks and other organs of attachment. Very little attention has been paid by workers to study the fresh water monogeneans in general. *Dactylogyrus* species, commonly known as gill flukes (belonging to Class: Monogenea, Phylum: Platyhelminthes) are ectoparasites living on the gills, present the largest group of fish parasites and major importance in the pathology of fishes (Woo, 2006). Young fishes are subjected to the risk of infection with these parasites which might cause diseases and mortalities among fry in hatcheries, and also among larger fishes (Amlacher, 1970). These parasites are commonly found on cyprinid fish throughout Asia, Europe and North America (Beverley- Burton, 1984).

The genus *Dactylogyrus* is the largest helminth genus with more than 900 species. It represents the most dominant genus among the monogenea with regards to host distribution and location. Till now 30 species of *Dactylogyrus* and 5 species of *Paradactylogyrus* are identified in different locations of India by different authors. The genus *Dactylogyrus* was described by Diesing in 1850 and *Paradactylogyrus* was described by Thapar in 1948 based on its morphological characteristics.

Table 1: Showing different *Dactylogyrus* Species abstracted from India

Species	Author	Host
1. *D. anchoracanthus*	Kulkarni, 1970	Cirrhinus,Chela
2. *D. angularis*	Gussev, 1973	Barbus, Puntius
3. *D. bacailai*	Agrawal and Singh, 1982	Oxygaster
4. *D. barbi*	Gussev, 1973	Barbus
5. *D. batae*	Jain, 1959	Labeo

6. *D. bati*	Triphati, 1959	Labeo
7. *D. boli*	Triphati, 1959	Raimas
8. *D. brevicardus*	Gussev, 1973	Barbus
9. *D. brevifucatus*	Kulkarni, 1970	Cirrhinus
10. *D. brevitignus*	Gussev, 1973	Barbus, Puntius
11. *D. brevitubus*	Gussev, 1973	Labeo
12. *D. bucinus*	Gussev, 1973	Barbus
13. *D. calbasi*	Jain, 1957; Gussev, 1973	Labeo
14. *D. catlaius*	Thapar, 1948	Catla, Labeo
15. *D. cauveryi*	Tripathi, 1959	Barbus, Puntius
16. *D. chagunionis*	Tripathi, 1959	Barbus
17. *D. chauhani*	Gussev and Musselius, 1975	Cirrhinus
18. *D. chitravansii*	Gussev, 1973	Labeo
19. *D. circumphallus*	Venkatanarsaiah, 1981	Chela
20. *D. cirrhini*	Jain, 1960	Cirrhinus
21. *D. cotius*	Jain, 1957; Gussev, 1973	Rohiee
22. *D. crucitrabus*	Gussev, 1973	Cirrhinus
23. *D. dubii*	Gussev, 1973	Barbus
24. *D. fotedari*	Gussev, 1973	Labeo
25. *D. glossogobi*	Jain, 1960	Glossogobius
26. *D. hyderabadensis*	Kulkarni, 1972	Barbus
27. *D. indicus*	Jain, 1957	Barbus, Puntius
28. *D. kalyanensis*	Musselius and Gussev,1973	Catla
29. *D. kontii*	Tripathi, 1959	Labeo
30. *D. labei*	Musselius and Gussev,1973	Labeo
31. *P. catlaius*	Thapar, 1948	Catla
32. *P. bati*	Tripathi, 1959	Labeo
33. *P. thapari*	Agarwal, 1980	Labeo
34. *P. indicus*	Singh & Rastogi, 2000	Catla
35. *P. himalayansis*	Singh et al., 2003	Catla, Labeo

Identification:

Dactylogyrus species are usually identified using morphological characteristics, principally those of the attachment organs, but also in more detailed studies, the protonephridia (Malmberg, 1970). Some species are easily and quickly differentiated from each other. However, others are very similar morphologically and differentiation requires detailed analysis of the characters used in identification. These characters can also vary depending on environment, host species, and water temperature (Mo, 1991a; 1991b; 1991c). As a result, a high level of experience is required for accurate identification of *Dactylogyrus* species. In addition to the variability in morphology, problems for identification can be encountered during preparation of the parasites for microscope examination. Preparation of slides can be time consuming and difficult, the characters used in identification needing to be orientated properly for reliable diagnosis. This is especially difficult in the case of preserved specimens, a situation that is usually necessitated during the collection of samples for diagnostic laboratories. Developments in molecular techniques have resulted in their application to parasite diagnostics. Molecular criteria can be more objective than morphological criteria for species identification, and can easily be performed.

Morphology:

They are gill parasites that are highly specific to freshwater fishes. They attach themselves to their host gill by special attachment organs called opisthaptor which is

positioned posteriorly on their body (Park et al., 2007). They are nearly transparent flatworms that have elongated bodies equipped with four eye spots on the head and a pair of dorsal anchor as well as 14 marginal hooks, two anchors and one to two connective bars on their haptor part which they use to latch onto their host. Other characteristics of the *Dactylogyrus* include the appearance of four eye-spots and two needle-like structures and spindle-shaped dactylogyrid-type seminal vesicles. These parasites appear to have preference for attaching to the middle sections of the gill filaments (Turgut et al., 2006). They are usually 300 - 2000μm long.

Life cycle:

Dactylogyrus feeds on the blood and mucus of the host, which can place significant stress on the fish's body and can cause secondary infections. The adult worm infecting a vertebrate host lays eggs, from which ciliated larvae (oncomiracidia) hatch out to infect vertebrates, usually of the same species. The rate at which they lay eggs and hence become sources of infestation to other fish varies according to the water temperature - the higher the temperature, the faster the egg-laying, up to at least 20 eggs per hour in warm water temperatures in the range of 22 – 24^{0}C. These eggs on release float out from the gills into the environment, and hatch into the next, larval stage. Again the time taken to hatch depends on water temperature, but can vary between 20 days in cool water to as short as 4 days at higher temperatures. The newly hatched larvae can attach to the next host after about 4 days, and 10 days or so after that the larvae will have matured into the egg-laying stage and the cycle starts all over again. Since no intermediate host is required, this is what is known as a direct life cycle, and one infested fish has the capability of infestion large numbers within a very short time in warm conditions.

They are highly host specific. It is thought that some mechanical or chemical stimuli are responsible for the ability of the parasite to discern between species of fish and find their preferred host (Buchmann et al., 2002). Most *Dactylogyrus* parasitize Cyprinids species, although certain species of *Dactylogyrus* are adapted to more advanced fish families. Furthermore commercial and aquaculture exploitation of Cyprinids demands for examination of these species for potential pathogen (Gibson et al., 1996).

Symptoms:

Cyprinidae that are infected by *Dactylogyrus* may have symptoms that include inflamed and pale colouration of gills, excessive mucous secretions and accelerated respiration. The infected fish also becomes lethargic, swims near the surface, and its appetite decreases. Additionally the infected fish may hold its gill covers open and scratch its gills on rocks. In severe infections, *Dactylogyrus* can cause hemorrhaging and metaplasia of the gills which can lead to secondary bacterial infections and death. Heavily infected fish are also anorexic and can be found gasping for air and exhibiting abnormal behavior such as jumping out of the water.

Molecular description:

Many of its species of *Dactylogyrus* are yet to be described. A morphological identification method has been developed by Malmberg (1970) based on the hard parts of the posterior attachment organ. The classical morphological analysis is important but it may lead to an underestimation of the number of species. To resolve the problems of identification of *Dactylogyrus*, molecular methods were inspired from the molecular techniques introduced by Cunnigham et al., 1995 for identification of Gyrodactylus.

Molecular tests developed for the diagnosis of *Dactylogyrus* parasites have targeted regions of the ribosomal RNA (rRNA) gene array, also known as ribosomal DNA, or rDNA. This array consists of three genes for ribosomal RNA; the 28S large subunit, the 18S small

subunit, and the 5.8S, interspersed with spacers (Fig. 5). The internal transcribed spacer regions (ITS1 and ITS2) and the external transcribed spacer (ETS) at the 5' end of the 18S gene are transcribed, but the intergenic spacer (IGS) is not. These arrays occur in tandem repeats throughout the genome and as such provide a high number of targets for amplification by polymerase chain reaction (PCR). For molecular identification DNA has to be extracted from a single parasite so that species level identification will be possible.

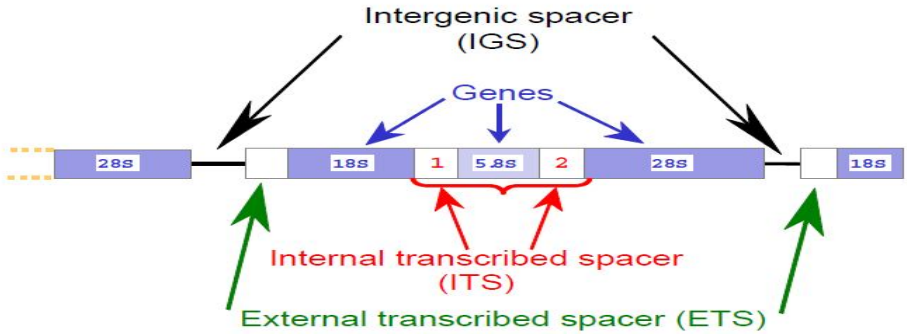

Figure 5. Genes for ribosomal RNA; the 28S large subunit, the 18S small subunit, and the 5.8S, interspersed with spacers.

Out of the 35 species of *Dactylogyrus* and *Paradactylogyrus* identified in India, only 3 species has the molecular identification with their respective accession numbers and rest are yet to be identified.

Table 2: Showing accession number of different *D.* and *ParaD.* Species abstracted from India

Species	Author	Acession number
1. *D. anchoracanthus*	Kulkarni, 1970	
2. *D. angularis*	Gussev, 1973	
3. *D. bacailai*	Agrawal and Singh, 1982	
4. *D. barbi*	Gussev, 1973	
5. *D. batae*	Jain, 1959	
6. *D. bati*	Triphati, 1959	
7. *D. boli*	Triphati, 1959	
8. *D. brevicardus*	Gussev, 1973	
9. *D. brevifucatus*	Kulkarni, 1970	
10. *D. brevitignus*	Gussev, 1973	
11. *D. brevitubus*	Gussev, 1973	
12. *D. bucinus*	Gussev, 1973	
13. *D. calbasi*	(Jain, 1957; Gussev, 1973)	
14. *D. catlaius*	Thapar, 1948	KC687091
15. *D. cauveryi*	Tripathi, 1959	
16. *D. chagunionis*	Tripathi, 1959	
17. *D. chauhani*	Gussev and Musselius, 1975	
18. *D. chitravansii*	Gussev, 1973	
19. *D. circumphallus*	Venkatanarsaiah, 1981	
20. *D. cirrhini*	Jain, 1960	
21. *D. cotius*	(Jain, 1957; Gussev, 1973)	
22. *D. crucitrabus*	Gussev, 1973	
23. *D. dubii*	Gussev, 1973	
24. *D. fotedari*	Gussev, 1973	
25. *D. glossogobi*	Jain, 1960	
26. *D. hyderabadensis*	Kulkarni, 1972	

27. *D. indicus*	Jain, 1957	
28. *D. kalyanensis*	Musselius and Gussev,1973	
29. *D. kontii*	Tripathi, 1959	
30. *D. labei*	Musselius and Gussev,1973	JX566720
31. *P. catlaius*	Thapar, 1948	KF269531
32. *P. bati*	Tripathi, 1959	
33. *P. thapari*	Agarwal, 1980	
34. *P. indicus*	Singh & Rastogi, 2000	
35. *P. himalayansis*	Singh et al., 2003	

In the absence of molecular data for the rest of the species of *Dactylogyrus* and *Paradactylogyrus.*, a thorough analysis of taxonomic affinities of a group must await for a detailed molecular coverage in India.

Table 3: Showing accession number of different *Dactylogyrus* and *Paradactylogyrus* Species abstracted from all over the World.

28S ribosomal RNA gene (Partial)

Species	Accession Number
1. *D. lamellatus*	JX524549
2. *D. extensus*	JX524548
3. *D. anchoratus*	JX524547
4. *D. inversus*	AY548928
5. *D. kikuchii*	AY548929
6. *D. petruschewskyi*	AY548927
7. *D.* sp. 2	JX960642
8. *D.* sp. 2 AC-2012	JQ926200
9. *D.* sp. 1 AC-2012	JQ926198
10. *D.* sp. YY	EF100538
11. *D.* sp. LY1	EF100537
12. *D. quanfami*	EF100536
13. *D. pekinensis*	EF100535
14. *D. parabramis*	EF100534
15. *D. hypophalmichthys*	EF100532
16. *D. gotoi*	EF100531
17. *D. extensus*	AY553629
18. *D. cryptomeres*	AJ969947
19. *D. hemiamphibothrium*	AJ969946
20. *D. inexpectatus*	AJ969945
21. *D. sphyrna*	AJ969943
22. *D. nanus*	AJ969942

18S rRNA gene (partial), ITS1 and 5.8S rRNA gene (Partial)

	Species	Accession Number
1.	*D. petenyi*	EF582621
2.	*D. carpathicus*	EF582618
3.	*D. crivellius*	EF582617
4.	*D. zandti*	AJ564165
5.	*D. wunderi*	AJ564164
6.	*D. vranoviensis*	AJ564163
7.	*D. vistulae*	AJ564162
8.	*D. vastator*	AJ564159
9.	*D. tuba*	AJ564158
10.	*D. squameus*	AJ564156
11.	*D. sphyrna*	AJ564155
12.	*D. similis*	AJ564153
13.	*D. rutili*	AJ564152
14.	*D. rarissimus*	AJ564151
15.	*D. ramulosus*	AJ564150
16.	*D. prostae*	AJ564148
17.	*D. propinquus*	AJ564147
18.	*D. parvus*	AJ564146
19.	*D. nanus*	AJ564145
20.	*D. nanoides*	AJ564144
21.	*D. minor*	AJ564143
22.	*D. malleus*	AJ564142
23.	*D. lamellatus*	AJ564141
24.	*D. izjumovae*	AJ564140
25.	*D. intermedius*	AJ564139
26.	*D. inexpectatus*	AJ564138
27.	*D. hemiamphibothrium*	AJ564137
28.	*D. fraternus*	AJ564136
29.	*D. formosus*	AJ564135
30.	*D. folkmanovae*	AJ564134
31.	*D. finitimus*	AJ564133
32.	*D. fallax*	AJ564132
33.	*D. falcatus*	AJ564130
34.	*D. extensus*	AJ564129
35.	*D. ergensi*	AJ564128
36.	*D. dyki*	AJ564127
37.	*D. dulkeiti*	AJ564126
38.	*D. distinguendus*	AJ564125
39.	*D. difformoides*	AJ564124
40.	*D. cryptomeres*	AJ564123
41.	*D. crucifer*	AJ564122
42.	*D. cornu*	AJ564119
43.	*D. cornoides*	AJ564118
44.	*D. chranilowi*	AJ564117
45.	*D. chondrostomi*	AJ564116
46.	*D. carpathicus*	AJ564115

47.	*D. caballeroi*	AJ564114
48.	*D. borealis*	AJ564113
49.	*D. auriculatus*	AJ564112
50.	*D. anchoratus*	AJ564111
51.	*D. amphibothrium*	AJ564110
52.	*D. alatus*	AJ564109
53.	*D. achmerowi*	AJ564108
54.	*D. dulkeiti voucher* LZDD-138	KC876020
55.	*D. arcuatus voucher* LZDF-145	KC876019
56.	*D. formosus vouche*r LZDA-114	KC876018
57.	*D. intermedius voucher* LZDI-018	KC876017
58.	*D. vastator voucher* LZDV-005	KC876016
59.	*D. sp*. 2 AC-2012	JQ926199
60.	*D. falciformis*	FN391583
61.	*D. eucalius*	EU643634
62.	*D. ctenopharyngodonis*	EU643633
63.	*D. inexpectatus*	EU643632
64.	*D. extensus*	AY553619
65.	*D. anchoratus*	AJ490161
66.	*D. difformis*	AJ490160
67.	*D. minor*	AF294952

Immunity against infection:

As shown in the table 4, the production of oxygen radicals by phagocytic cells, measured by the NBT assay, was significantly high in fish infected by 100- 200 gill flukes as compared to fish infected with 7- 40 parasites. Similar result is observed in case of WBC but an opposite result is noticed for RBC and haemoglobin, in which the value is significantly low in fish with heavy infection as compared to the less infected and control fish.

When the serum is tested for total protein, albumin, globumin, A:G ratio, lysozyme and bactericidal test, a similar kind of result is found for each parameter i.e., the value is significantly decreasing in heavily infected fish in comparisoin to the less infected and control fish. Thus, all these values lead to a conclusion that this infection ultimately results in loss of non specific and specific immunity with increase in the number of parasites.

Table 4: Result for blood immune parameters against gill fluke during preliminary (7- 40 parasites) and heavy (100- 200 parasites) infection.

Sl.No.	Parameter	Number of Parasites	Result
1	Hb (g %)	7- 40	= normal (≥ 7.0)
		100- 200	↓ (≤ 5.5)
2	RBC (* 10^6 million cells)	7- 40	= normal (≥ 9.0)
		100- 200	↓ (≤ 7.0)
3	WBC (* 10^4 cells)	7- 40	↓ (≤ 17.0)
		100- 200	↑ (≥ 22.0)
4	NBT	7- 40	↓ (≤ 0.7)
		100- 200	↑ (≥ 1.0)
5	Total Protein (mg/ml)	7- 40	= normal (≥ 35.0)
		100- 200	↓ (≤ 26.0)

6	Albumin (mg/ml)	7- 40	= normal (≤10.0)
		100- 200	↓ (≤ 7.0)
7	Globulin (mg/ml)	7- 40	= normal (≥20.0)
		100- 200	↓ (≤ 15.0)
8	A: G	7- 40	= normal (≥0.6)
		100- 200	↓ (≤ 0.4)
9	Lysozyme (Unit)	7- 40	= normal (1.0)
		100- 200	↓ (≥ 3.0)
10	Bactericidal	7- 40	= normal (≥ 90 %)
		100- 200	↓ (≤ 60%)

Control:

Control of many important parasitic diseases is still far from being satisfactory and further research is needed. A very promising area of future research seems to be studies on immunity in parasitic infections, use of molecular technology in diagnostics and development of new vaccines against the most pathogenic parasites. Despite the availability of chemotherapy for *Dactylogyrus*, many side effects of chemical parasiticides have been found, such as resistance, environmental contamination, and toxicity (Goven et al. 1980; Marshall 1999). It has prompted a search for new alternatives.

Figure 1: Opisthohaptor with a pair of anchors, ventral bar and apparatus marginal hooks.

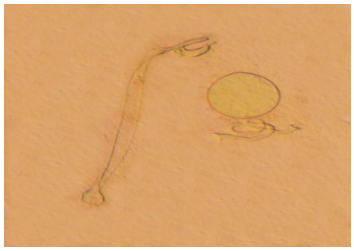

Figure 2: Enlarged view of the copulatory organ, vaginal and ovary

Figure 3: Whole mount of *Dactylogyrus* parasite stained with ammonium picrate glycerine (APG)

Figure 4: Infected gills

IMMUNITY OF BROOD AND YOUNG FISHES

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A healthy mother can give birth to a healthy offspring is an old say since it is widely acknowledged that a mother and maternal environment can have a profound effect on the phenotype of the offspring. The overall health and immune status of brood fish is very important not only for breeding performances but also for the health condition of offspring. Availability of healthy and disease resistant fish seeds is key to the development of aquaculture industry. But many times, the target is not achieved due to low availability of healthy and sufficient fish seeds. One of the main reason of heavy mortalities of larvae and young ones at this stage is due to lack of specific immunity and competence to fight against pathogen thereby causing major managemental problem and economic loss every year. Therefore, maternal derived immunity is very much required for fish embryos and larvae to survive against microbial infection since they are most vulnerable from birth to the development of indigenous antibodies. This phenomenon is not only observed among different vertebrates such as mammals, reptiles, avian, fishes but also in the invertebrates as well.

Fish offspring, like other vertebrates, are dependant on the maternally derived nutritional and immune factors for their sustainability. Most fish embryos that develop externally are exposed to an environment full of microbial pathogens, which can have a decisive impact on the survival and performances of offspring. Significant mortality is routinely recorded at larval and post larval stages of fishes throughout the world. Because of delayed maturation of lymphoid organs and immunocompetence in fish and little or only limited ability of developing fish embryos and larvae to synthesize specific antibodies after certain weeks of hatching, maternally derived immunity is very essential in this early phase of life.

Immunity of fish

The piscine immune system is comparatively simple and undifferentiated. The major lymphomyeloid organs are thymus, head kidney and spleen. The ontogeny of the lymphomyeloid organs start developing within few hours of post fertilization but the sequence of development differs markedly among the freshwater, estuarine and marine species. Thymus is the first organ to become lymphoid, followed by kidney and spleen. Though, the lymphoid organs and T- and B-lymphocytes appear early in embryogenesis, achievement of immunocompetence takes few to several weeks depending upon the fish species.

The immune system of fish has evolved with both innate immunity and acquired immunity. Each type of immunity can be either cell mediated or humoral. The cell mediated innate mechanisms include physical barriers such as skin, epithelial tissue lines, gill, mucus layers and specialized cells such as macrophages, granulocytes, and natural killer cells and several soluble immune-reactive molecules such as lysozyme, lectins (carbohydrate recognition), antibacterial lytic enzymes, transferrin (iron binding protein) and components of the complement system and interferon are mainly responsible for providing non-specific innate immunity.

On the other hand, fish can display typical vertebrate adaptive immune responses characterized by immunoglobulin, T-cell receptors, cytokines, and major histocompatibility complex molecules. The cell-mediated response in fish is similar to that in mammals and relies on the presence of accessory cells (macrophages) to present antigen to T-cells. Majority of Teleosts possess a single class of Immunoglobulin (Ig) namely Ig M which is tetrameric.Besides some other types of Ig i.e. IgD, IgZ, IgT and IgH have been reported in some fishes. The tetramer structure of fish Ig M is composed of four monomeric units, two identical heavy (H) chains (70 kD) and two light (L) chains (25 kD). Disulphide bonds and non-covalent combinations of units may coordinate the assembly of the subunits.

Immunity of young fish

Immunity of both innate and adaptive type is transferred from mother to offspring in vertebrates. In Teleosts also, both innate and adaptive immune factors like Ig/antibody, complement factors, α macroglobulin, serum amyloid A, lectins, and serine protease like molecules are commonly transmitted to the offspring.

During the early stage of vitellogenesis, the maternal Ig M is transferred through the yolk, to the immature ovaries possessing early yolk globule stage oocytes. Its transfer follows this in a sequential manner to reproductively active mature ovaries, eggs and finally yolk sac larvae. Development of power to mount specific immunity against pathogens starts only after three weeks of age in most of the species including Indian major carps before which the fish is either dependent on nonspecific immunity or maternally derived immunoglobulins. The cells responsible for the active immunity are lymphocytes those are matured in the kidney after three weeks to become capable to respond to an antigen/pathogen.

Immune factors of brood fish and their role in offsprings

Innate immune factors such as lectin like agglutinins; C-reactive proteins and lysozyme are indispensable for fish egg/larvae to fight against infectious agents. The maternally transferred Ig M and complement components provide defense to the newly hatched ones against infectious agents. Ig/Antibodies can opsonize bacteria followed by their degradation and eradication by phagocytic cells. The presence of Ig/antibodies in eggs may also act in concert with complement components and thus facilitate binding of opsonized bacteria to complement receptors on phagocytes.

In several fish species, though the maternal transfer of complement components are recorded, their functional role in fish embryos is yet to be established. However, complement components are believed to play an active role in the protection of offsprings in the early developmental stages. Likewise, complement components can either attract innate immune cells for phagocytosis though not present before hatching or activate the C3 - C9 complement for lysis. Similarly, the protective role of lectins with antibacterial activity in early phase of life in teleosts is reported.

Among different bacteriolytic enzymes, lysozyme is usually recorded in high concentration in the eggs of fish. Its role in preventing the vertical transfer of pathogens is reported in fish. Besides these factors, other maternally derived immune factors like α macroglobulin and serum amyloid A exerts scavenging action against both endogenous and exogenous proteases and tissue regeneration, respectively, during development.

The maternal derived immunity provides defense not only against the harmful agents present in the environment during embryo and larval stages but also protects them

from vertically transferred pathogens. Therefore, like their mammalian counter parts, in teleosts, passive transfer of immunity is responsible for high survivability.

Factors affecting the maternal transfer of immunity

The overall health status and immunity of brood fishes to various environmental conditions can significantly contribute to the breeding performances, quality seed production and protection of offspring. But the immunity in brood fishes is often affected by various biological factors such as age, maturation, reproduction, nutrition, and environmental conditions. Adverse environmental conditions and stress conditions like handling, crowding, and water pollution greatly affect the immune system of any fish. These factors can suppress the immunity of brood fishes thereby causing more vulnerable to various types of microbial infections during breeding season.

Vaccination of brood fish

Vaccination of mother starts along with the development and maturation of eggs preferably one month before breeding and followed by a booster injection 10-15 days before breeding to ensure optimum maternal antibody transfer through the egg. The transferred antibodies protect the young ones up to three weeks of age when specific active immunity fails to develop. The preferred route is intraperitoneal route using a mineral oil as adjuvant.

Bath immunization of young fishes

It is very difficult to vaccinate the young ones. So bath or immersion vaccination is preferred. The vaccination is done with fish above three weeks of age since before that the immunity of young fish fails to develop. They are kept in bath tank containing correct quantity of antigen for 30 minutes and one hour for spawns and fry, respectively. This method is found to be successful and effective in young fishes where no other method is possible. Fish vaccinated by this method remain competent up to one month and resist the challenge infection by the pathogen.

Fish vaccines

Fish vaccines in general fall into three major categories viz. killed whole cell vaccine, live-attenuated vaccine and recombinant DNA-based vaccine. Killed whole cell vaccines have been developed for some pathogenic fish viruses. Live attenuated vaccine is a suspension of attenuated live pathogens that are able to replicate inside the host and induce protective immune response but unable to cause disease. They mimic the actual infection by the pathogens and hence a small dose of vaccine is enough to induce long lasting protective immune response. These live attenuated vaccines can induce both humoral and cell-mediated immune response. Recombinant protein vaccines are produced in prokaryotic cells. Efficacy of these vaccines has been appreciably improved using adjuvant, immunostimulant or vaccine carriers. However, it is still affected by the routes of vaccine administration. In general, injection is better than immersion and oral administration. Nevertheless, one has to consider the duration of protection by economic routes of administration. To date, there are only six commercially available fish vaccines; five formalin killed vaccines of bacterial origin and one recombinant vaccine against infectious pancreatic necrosis virus. However, all the vaccines are made from the pathogenic strains of the temperate zone and hence may not be able to exert the same effectiveness in protection against strains that have different antigenicities in the tropics. This shows that much needs to be done to improve the efficacy of the existing vaccines and to develop new vaccines to several important infectious diseases affecting fish, particularly in the tropics.

Conclusions

In aquaculture practices, fish breeding is done by purely looking into the apparent growth conditions of brood fish, which in turn affect the seed production, and mortality of spawn and larvae. Looking into the existence of heritability transmission of innate resistance against infection and maternal transfer of immunity to offspring, the mass mortality in the early phase of life can be prevented. This concept can further be explored for reducing mortalities at larval/post larval stages by adopting the variation in disease resistance and immune status among the families through selective breeding programme and/or large scale brood stock immunization programme. Determination of immune status of brood stocks under different conditions is an essential component to analyze the relationship before reproduction and defense mechanism. Additionally, immunity is required to be maintained at its peak level during vitellogenesis and oogenesis, so that optimum immunity could be transferred.

PROTEOMICS AND ITS APPLICATIONS IN DISEASE DIAGNOSIS

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Introduction:

Proteomics is a recent field in molecular biology that involves the study of all proteins present in a cell or tissue or whole organism. It includes protein identification and quantification, their structure and function as well as their interactions with other biomolecules. The study of proteomics gained importance after the genomic sequences of human and other organisms were deciphered. It is estimated that the human body may contain over two million proteins, coded for by only 20,000 - 25,000 genes. The entire protein content in a given cell, tissue, or organism is defined as the PROTEOME. Wilkins coined the word 'proteome - the PROTEin complement expressed by a genOME'. Proteome depicts the protein complement of the genome and represents the end product of the genome. The genome is thus a mere blueprint for a vastly complex network of interactions principally mediated by proteins. The genome though remains constant for all cells in an organism, the proteome is dynamic and differs between cells in response to various developmental stages and external factors. Proteins being the ultimate effectors of most biological activities in a system, the proteomic study assumes significance compared to genome analysis or gene expression (mRNA) profiles that do not evaluate post transcriptional or post translational modifications. Further, the abundance of mRNA expression profiles does not always correlate with protein expression profiles. All these characteristics make the protein expression analysis much more complex but quite informative.

Protein and peptide separation – gel based techniques:

A proteomic study involves a large variety of different protein molecules to be studied simultaneously and hence requires high throughput methodologies for analysis. Two-dimensional gel electrophoresis (2-DE) is one of the most widely used techniques to obtain data on protein expression levels under various conditions. In 2-DE the protein samples are first separated by isoelectrofocusing in one dimension followed by SDS-PAGE in 2nd dimension resulting in a pattern of spots. Thus all proteins are separated according to their two independent properties first by isoelectric point (pI) and second by molecular size. The development of immobiline pH gradient (IPG) strips provides a precise and stable pH gradient for the first dimension IEF that helps in generation of a more reproducible 2D maps. The 2D gels are then stained and the protein spots are analyzed by commercial 2D softwares. Each spot represents one polypeptide and the intensity of spot indicates the quantity of the particular protein present in the sample. The protein spots of interest can be excised from the gel, subjected to in-gel digestion with trypsin and the resulting fragments are exposed to mass spectrometry (MS) analysis for identification of the protein. Several thousands of proteins can be successfully resolved on a single gel using this technique. These gels can also provide additional information such as isoelectric point (pI) and molecular weight of the proteins. However, low sensitivity, narrow linear dynamic range of detection by staining methods and poor reproducibility of 2-DE have made the high-throughput quantitation of proteins considerably challenging.

The problem of comparison of images across 2-DE gels has been overcome by difference gel electrophoresis (DIGE) where cyanine dyes, Cy3 and Cy5, are used for

differential labeling of the same protein from different samples. These dyes are designed to be charge- and size-matched so that charge state of the labeled proteins are maintained and it allows precise co-migration of differentially labeled protein species within the same 2-D gel. However, due to spectrally distinct fluorophores of Cy3 and Cy5 it allows detection of the signal from one sample without appreciable contribution from the other. Cy2 dye is used to label an internal standard, which consists of a pooled sample comprising of equal amounts of control and treatment samples. DIGE allows the analysis of two or more protein samples simultaneously on a single two-dimensional gel, which is not possible with other 2-DE techniques. Relative protein abundance of samples can be readily obtained by simple comparison of their fluorescence intensities using a fluorescence gel scanner.

In addition to fluorescent labels, radioactive isotopes have also been employed for labeling procedures and subsequent protein detection and identification in gel-based proteomics. Isotopic labeling techniques offer a less destructive means for the protein of interest without compromising on the detection sensitivity. Coupling of 2-DE with mass spectrometry (MS) in recent years has facilitated the identification of various protein complexes. In spite of the significant advancements in gel-based methods, detection of low abundance, hydrophobic proteins has proved challenging, due to which many gel-free proteomic approaches are emerging.

Protein and peptide separation – LC (liquid chromatography)-based techniques:

Protein separation using liquid chromatography and electrophoresis has shown tremendous progress to enable resolution and identification of digested peptides of a proteome. Procedures applicable for such complex resolution exercises of several thousands of proteins/peptides are mainly high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Ion exchange, strong cation exchange (SCX), reverse phase (RP), size exclusion and hydrophobic interaction are the various modes of HPLC available while capillary zone, isoelectric focusing (IEF), affinity and micellar electrophoresis are the different types of CE commonly used. However, it is quite well known that no single chromatographic or electrophoretic separation strategy is by itself sufficient to resolve a complex mixture of peptides. A combination of two of more separation procedures, which must be orthogonal and compatible with each other, is therefore employed to improve the overall resolution of complex proteome digests. One such successful combination method developed and widely used is the Multidimensional Protein Identification Technology (MuDPIT), which makes use of strong cation exchange chromatography (SCX) in one dimension and reverse phase HPLC in the other dimension. Combinations such as RP-HPLC with capillary zone electrophoresis (CZE), affinity and RP chromatography and several others have been developed, each with their own advantages and limitations. The ultimate objective of these separation protocols is to reduce the sample complexity in order to facilitate efficient and accurate identification and quantitation by mass spectrometry.

Mass Spectrometry:

Mass spectrometry coupled with various protein separation technologies, has emerged as one of the most powerful and widely used tools for proteomics studies. The basic components of a mass spectrometer are an ionization source, a mass analyzer and an ion detector. Matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the most commonly used ionization methods in proteomics. These soft ionization methods have made possible the analysis of large biomolecules like proteins and peptides. While MALDI requires the sample of interest to be in a crystalline state, ESI can

directly ionize molecules in solution thereby, enabling on-line coupling of chromatographic separations with MS. The generated ions are then separated by the mass analyzer based on their mass-to-charge (m/z) ratios and directed into the detector either by electric or magnetic fields. Time-of-flight (TOF), ion trap, quadrupole, fourier transform ion cyclotron resonance (FT-ICR) are the mass analyzers that have found wide applications in proteomics. In addition, a combination of mass analyzers known as hybrid analyzers such as TOF-TOF or Q-TOF are also increasingly being used to provide improved mass range, mass accuracy and resolution.

MALDI coupled to the time-of-flight analyzer is frequently used for the protein identification strategy called peptide mass fingerprinting. This is based on the fact that proteins having unique sequences also often have distinct patterns of cleavage sites for a given enzyme. The cleaved protein therefore yields peptides that reflect this unique distribution, allowing the matching of the experimental digest with a theoretical digest stored in databases, thus aiding the identification of a protein. However, this is not a very high-resolution method and it often fails when you are looking at related proteins or isoforms. Therefore, it is still important to get actual sequence information and this can be obtained through de novo sequencing. De novo sequencing can be achieved by using two MS procedures performed in tandem (MS/MS) usually generated by an ion trap, MALDI TOF-TOF, or Quadrupole TOF mass spectrometer. For this, peptides are typically selected one-at-a-time by the first stage MS for collisional dissociation and then the fragments are analyzed by a second MS stage to obtain sufficient peptide sequence information for identification using a computer search of an appropriate protein or genome database.

Bioinformatics in proteomics:

Bioinformatics plays a major role in the study of proteomics as well as for the management of large amount of data generated by such studies that require fast and profound analyses and data mining. This helps in understanding the protein sequence, structure, function, regulation, expression and modification.

Several protein databases have been created to provide information related to protein characterization, domain and families, sequences, biophysical and catalytic properties, function and applications, identification and quantification, annotations, expression and profiling, 2D/3D structure and homology models, protein-protein and protein-ligand interactions, pathways and networks, post translational modifications, structure predictions etc. These databases help in extracting relevant information from large data sets and understanding the statistical significance of protein identification results. The development of protein databases is important for discovery of signature candidates for biomarkers, drug targets, therapeutics, and mapping protein interaction networks.

Besides generation of protein databases, several bioinformatics tools are also available for MS data analysis and identification of proteins. Sequence databases are searched to identify proteins, which match the peptide mass fingerprints generated by MALDI-MS. The growth of databases along with genome sequencing projects adds to the great accuracy of the protein identification. Once the complete genome for an organism is available, all the fingerprint patterns can be predicted and detected accurately.

Applications of proteomics:

The scope of application of proteomics is wide extending from disease diagnosis, drug discovery, microbiology, biochemistry, physiology etc. and is rapidly expanding towards newer fields of applications. It has provided a powerful means to identify proteins that are expressed/ altered under experimental conditions and hence, a target for intervention, therapeutics and drug discovery. Clinical researchers use proteomics to compare normal versus disease samples, disease versus treated samples, find molecular markers in body fluids for diagnosis, monitor diseases and their treatments, determine and characterize post-translational modifications.

Most of the work in proteomics concentrated on mammalian organisms particularly human. Of late a reasonable amount of research has also been carried out on various fish species. It has been applied to the study of transgenic fish, anti-freeze proteins and to identify the effects of particular environmental conditions like starvation, temperature, anoxia, salinity, toxicity etc. on the proteome. A liver proteome of rainbow trout has been generated to study the effects of starvation and use of plant derived proteins in the diet, ultimately to understand the protein metabolism in fish. Other studies include the use of proteomics in diagnosing the effects of nutritional and environmental fluctuations on the proteins in fish, including the effects of endocrine disrupting compounds. A substantial amount of research has also undergone into the quality assessment of seafood; both fresh and, after processing and storage.

Proteomics in disease diagnosis

Proteomics can be applied to screen clinical samples for diagnosis, for prognosis and for therapeutic monitoring of diseases. Proteomics thus provides the opportunity to understand the protein expression pattern in healthy and diseased states that can be used to discover new biomarkers for clinical diagnosis. Any change in the proteome of appropriate body tissue or fluid indicates a disease state and forms a basis of diagnosis. A great amount of research is directed towards search for protein biomarkers particularly for diagnosis of human cancers so that timely intervention would help to increase the survival rate. Considerable research efforts are also on the important cardiovascular and neuro-degenerative diseases. Proteomics also shows a great promise in studying the microbial diseases through proteins expressed by a pathogen of interest or by infected animal and may lead to development of diagnostics in future. Besides studying the protein changes in infected organisms, it may be possible to detect the progression of the diseases. Advanced proteomic studies such as protein microarray are being attempted in serum for detecting the presence of antibodies against microbial antigens from serodiagnosis standpoint. However, most of these studies are in experimental stages and much work is necessary to develop validated assay systems for disease diagnosis.

INNATE HOST DEFENSE MECHANISMS OF FISH AGAINST BACTERIA AND VIRUSES

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Introduction

Fish are in intimate contact with their environment, which can contain very high concentrations of microorganisms. Many of the microorganisms are saprophytic, some are pathogenic and both are very capable of digesting and degrading the fish's tissues. However, under normal conditions the fish maintains a healthy state by defending itself against these potential invaders by a complex system of innate defense mechanisms. These mechanisms are both constitutive and responsive (i.e. preexisting or inducible) and provide protection by preventing the attachment, invasion or multiplication of microbes on or in the tissues. Their importance is three fold. Firstly the protection is non specific and specific and does not depend upon recognition of the distinctive molecular structure of the invading species. Secondly, there is no or only little time lag for them to act. Even the inducible defenses, like inflammation, are relatively quick to respond (1-2 days) and thus give pathogens little time to establish themselves. Thirdly, they are relatively temperature independent. For ectothermic vertebrate, these characteristics are very important because the specific immune defenses take a considerable time to respond and are very temperature dependent. For temperate species like salmonids, antibody production takes atleast 4-6 weeks even at optimum temperature and when many pathogens can kill the fish within a few days of infection, the protection afforded by the specific response is important only in previously immunized fish. Thus it is to be expected that the innate defenses might be more important in fish than in endothermic vertebrates and indeed some of them like lysozyme and complement appear to be more potent in fish than in mammals.

Bacterial disease

The innate defense mechanisms of fish against bacteria include production of broad spectrum antimicrobial substances and acute phase proteins, non-classical complement activation, release of cytokines, inflammation and phagocytosis. While these mechanisms provide potent defenses against invasion against saprophytic environmental bacteria, pathogenic bacteria have evolved means of avoiding many of them. In some cases, on-immunised fish appear to co-exist with highly virulent bacterial pathogens in a carrier state, without showing any signs of morbidity., so presumably the innate mechanisms of defense provide some degree of protection. However, disease out breaks and mortalities often result from the fish being stressed and presumably these defense mechanisms being compromised. Very little is known or understood about such interactions.

Integumental innate defenses

Many observations suggest the importance of innate defenses in the integument of fish. For instance it is well known that rainbow trout are virtually resistant to immersion challenge by *Aeromonas salmonicida* while Atlantic salmon are very susceptible. However, there is little difference in the LD50 when these fish species are injected with the bacteria.

Mucus

The protective role of skin mucus has been demonstrated by carefully removing mucus with a swab and then challenging turbot or ayu with *Vibrio anguillarum* and inducing increased mortality. The precise mechanisms of this protection are not known and are probably multifactorial. Mucus is continually being produced and sloughed from the integumental surface thus physically trapping and preventing bacteria from attaching to the epithelium and having an opportunity to invade the fish's tissues. Mucus also contains many substances with antibacterial activities and these may play important roles.

Anti-bacterial peptides

These substances have been identified from mucus secretions of a number of fish species but little is yet known about their ability to kill fish pathogenic bacteria. Pathogenic strains of *A. salmonicida* are less susceptible to cecropin P1 (an antibacterial peptide derived from the pig.) than non pathogenic strains lacking the A layer, but they were, nevertheless, killed by higher concentrations. The gene encoding the antibacterial peptide pleurocidin has been cloned from the winter flounder and shown to be predominantly expressed in skin and intestinal tissue and the gene is first expressed as earl as 13 days post hatch. Thus these peptides may provide an simportant line of defence before development of the specific immune response in larval fish. Synthetic pleurocidin has recently been shown to protect coho salmon from infectin by *V. anguillarum.*

Proteases

Trypsin-like proteases and cathepsin L and B prteases have been found in skin mucus of a number of fish species. The ability of these enzymes to lyse formalin killed *V. anguillarum* has led to the suggestion that they may play a role in defense against bacteria but their action on live bacteria does not appear to have been studied yet.

Lectins

Once bacteria have made contact with their fish host, many pathogenic species can adhere to the mucus and the epithelial cells by surface molecules known as adhesions. Frequently, these interactions involve binding of carbohydrates. Once the bacterium has attached to the host cell, the latter is induced to endocytose the bacterium which can then grow and spread in the host to produce disease.

Lectins are a group of proteins with different specificities for binding carbohydrates. They have been found in salmon eggs, serum and mucus. These lectins are Ca2+ dependent and can agglutinate a number of fish bacterial pathogens. Their role in defence is still unclear but in mammals they can have opsonic and complement activating properties. It is possible that an important role of lectins in fish mucus is to bind to the carbohydrates on the surface of bacteria which are involved in attachment to the integumental cells. This could block attachment and subsequent invasion of the host, but such experiments have not yet been done.

Lysozyme

This enzyme can attack the peptidoglycan layer of bacterial cell walls causing them to lyse. Lysozyme has been found in fish mucus, serum and ova. Peritoneal macrophages and blood neutrophila containlysozyme and the latter are though to be the source of serum lysozyme. The production of lysozyme by Atlantic salmon macrophages in vitro is enhanced in the presence of yeast glucan or bacterial lipopolysaccharide. Fish lysozme occurs in two forms and one of these appears to be much more bactericidal than lysozyme of higher

vertebrates. There are several reports of lysozyme isolated from fish serum and ova, being bactericidal even for important fish pathogens like *A. salmonicida* and *A, hydrophila.*

Systemic innate humoral defenses

If bacteria are successful in crossing the integumental defenses there are a number of plasma proteins which may prevent further spread of the infection. Some of the proteins mentioned above as present in the mucus also occur in higher concentration in the serum e.g. lysozyme and lectins. However, probably the most important of the serum defense factors is the complement system because of its activating effects on the cellular defenses.

Complement

The complement of teleost fish can be activated directly by lipopolysaccharide (LPS), which is a major constituent of the cell wall of Gram-negative bacteria. This is the so called alternative complement pathway (ACP) and results in lysis of the cell membrane of many non-virulent bacteria. However, the species of bacteria that cause disease in fish are resistant to being killed by this mechanism, though some can be killed when the complement is activated by the classical (antibody-mediated) pathway, e.g. *V. angullarum* serogroup O1. Nevertheless, complement has another important innate defense function. During its activation on the bacterial cell wall, two components are important for recruiting phagocytes. The C5a component is released from the complement complex and is potent chemotaxin for macrophages and neutrophils.these cells have receptors for the C3b component which remains attached to the bacteria, which are then more readily pahgocytosed. The ACP activities very high in fish serum compared with mammals suggesting this pathway is very important in the defense mechanism of fish.

Lectins

As mentioned above, lectins have been isolated from the serum of a number of fish but evidence fo their role in defense is only recently coming to light. A mannose-binding lectin, isolated from the serum of Atlantic salmon, has been shown to have opsonising activity for a virulent strain of *A. salmonicida* and furthermore, lectin coated bacteria induced the macrophages to produce an enhanced respiratory burst and were more susceptible to being killed by the macrophages.

An *N-acetyle-* galactosamine binding lectin has been isolated from the serum of blue gourami. This lectin was shown to have opsonising activity and lectin treated virulent *Aeromonas hydrophila* cells were killed in the presence of complement. Furthermore, supernatants obtained from lectin stimulated macrophage cultures exhibited significant bacterial killing activities.

Pentraxin: C-reactive protein (CRP) and serum amyloid protein(SAP)

These serum proteins are usually acute phase proteins in mammals but in fish they appear to be consitutively expressed and may show only a slight increase or decrease in concentration during inflammatory response. Pentraxins are capable of binding to a number of polysaccharide structures in the presence of Ca2+ ions. Their role in defense is not well understood but in mammals they are capable of activating complemtn and phagocytes hve receptors for them. While there are many reports of the presence of pentraxins in the serum, mucus and ova of fish there is only one report of CRP binding to bacteria (*V. anguillarum)* and activating complement with enhanced pahgocytosis. Form the widespread occurrence of pentraxins in fish and the relatively high concentrations in serum(50-300µg/ml;, it is to be expected that they play an important role in defense mechanisms and more research is merited.

Bacterial growth inhibitors

Transferrin and the hypoferraemic response. Bacteria, like other cells require iron as a co-factor for many enzyme systems but in the host the availability of iron is highly restricted by being bound to the high –affinity iron-binding protein, transferring, in the plasma. Most bacteria are thereby unable to grow in the host tissues. However, pathogenic bacteria have evolved several ways of overcoming this defense by producing high affinity iron sequestering mechanisms of their own. Nevertheless, as a further step to reduce the availabilityof iron to pathogenic bacteria, vertebrate show a hypo ferraemic response. In mammals, the LPS of Gram-negative bacteria results in the release of interleukin-1 (IL1) from macrophages and this stimulates neutrophils to release lactoferrin. The latter removes iron from plasma transferring forming lactoferrin. The latter removes iron from plasma transferring forming lactoferrin-iron complexes that are rapidly sequestered by the liver. In fish there is no evidence to date of the existence of lactorferrin but the presence of an iron bingid activity at low pH in lysates of Atlantic salmon leucocytes suggests that it is present. However, the ability of fish to express the hypoferraemic response follwing injection of LPS has been clearly demonstrated. Furthermore, bovine lactoferrin has been shown to enhance the respiratory burst of rainbow trout macrophages in vitro and to increase resistance to bacterial and infection after oral administration.

A novel role of transferring in fish has recently been reported. During mixed lymphocyte reactions ormitogenic stimulation of goldfish kidney leukocytes, proteolytic fragments of transferin were released and these fragments, but not the full length transferring, were able to induce the production of nitric oxide by LPS stimulated goldfish macrophage cultures. Thus, as monocytes infiltrate inflammatory sites, transferrin- derived peptides may initiate the differentiation of these cells into mature tissue macrophages with enhanced bactericidal properties.

Anti proteases

For bacteria to invade and grow in the fish tissues they need to digest host proteins as a source of amino acids. Fish plasma contains a number of protease inhibitors, principally a1-anti-protease, a2-anti-plasmin and a2-macroglobuling (a2M) which may play a role in restricting the ability of bacteria to invade and grow in vivo. However, once again it appears that highly adapted pathogenic bacteria have evolved evasive mechanisms. For example, the highly toxic serine protease produced by *A. salmonicida* is resistant to a1-anti-protease. This is regarded as a universal serine protease inhibitor and represents over 80% of the anti-protease inhibitor activity in salmonid plasm. Nevertheless, the *A. salmonicida* serine protease is inhibited by a2M. The ability of salmonid plasma to inhibit this bacterial protease has correlated with between species differences in susceptibility to furunculosis and within species differences in rainbow trout.

Systemic innate cellular defenses

Inflammatory response and phagocytes

If bacteria gain entry into the tissues of the fish an inflammatory response is induced with the ultimate influx of phagocytes which have potent bactericidal properties. The inflammatory response in the peritoneal cavity of rainbow trout following the intraperitonal injection of bacteria has been studied in detail. The resting peritoneal cavity contains a population of leucocytes comprised of macrophages (about 40%), lymphocytes (about 55%) and neutrophils (about2%). Following injection of bateria the macrophage population rapidly pahgocytosed the bacterial cells. Over the next 24-48 h, there was a marked increase (500-fold) in the number of neutrophils in the cavity and a more modest increase in the

number of macrophages (eight-fold increase by 96h). By 48h, neutrophils were about five times more numerous that macrophages. Neutrophils were also highly phagocytic if any bacteria were remaining at the time they entered the peritoneal cavity from the circulation. Once the bacteria were cleared this response subsided and the resting population level of peritoneal cells was re-established by about 15 days. Of particular interest in these studies was the observation that following the infiltration of neutrophils into the peritoneal cavity many of the macrophages now contained myeloperoxidase and glycogen granules derived from the neutrophils. Resting macrophages do not contain these substances and the authors speculated that the macrophages could make use of these neutrophil components to enhance their own bactericidal activity. This possible cooperation between macrophages and neutrophils at the site of an in vivo inflammatory response has been omitted from all the experiments dealing with the ability of these cells to kill bacteria in vitro and some of the data describing the resistance of some pathogenic bacteria to macrophage killing e.g. virulent *A. slamonicida* and *V. anguillarum* serogroup O2a may have under estimated the killing power of macrophages and neutrophils combined.

The control of inflammation

The initiation of inflammation is highly complex and multifactorial. A number of blood enzyme system, including the clotting system the kinin system and the complement system play a major role and while little is known of the details in fish it is clear that thy share many similarities to their mammalian counterparts. For example, during the activation of the complement system by bacteria (directly by the alternative pathway or indirectly by lectins or CRP) the anaphylactic factors C3a and C5a are produced. In mammals, these factors induce the release of vasoactive amines (histamine or 5-hydroxytryptamine; 5-HT) from platelets and mast cells. In fish, thrombocytes and eosinophilic granular cells (EGCs) probably play an equivalent role, though histamine does not appear to be present in fish and the observed degranualtion of EGCs by bacterial products may result in the release of 5HT. The amines induce local vasodilatation and extravasation of neutrophils and monocytes into the infected site. The C5a component of the activated complement also has chemotactic activity for fish phagocytes and thus they accumulate at the site of infection. This influx of phagocytes is further stimulated by cytokines and eicosanoids. In mammals, bacteria and LPS stimulate macrophages to secrete interleukin-1 (IL1) which sequentially stimulates the release of eicosanoids, which have pro-inflammatory and chemotactic activity. In fish, a similar process is apparent as LPS has been shown to induce IL-1 production by trout leukocytes and the production of eicosanoids (with leukocyte chemotactic activity) by a variety of leukocytes has been reported.

Phagocytosis

The phagocytosis of bacteria by fish macrophages and neutrophils first requires attachment of the bacteria to the surface of the phagocyte. This may involve hydrophobic interactions or sugar/lectin interaction. However, the most active promoter of phagocytosis is the C3 component of complement, which is bound to the bacterial surface LPS directly via the alternative pathway or indirectly via lectins or CRP. The mannose binding lectin of Atlantic salmon is able to opsonise *A. salmonicida* even in the absence of complement.

Phagocyte bactericidal mechanisms

Fish macrophages and neutrophils both produce bactericidal reactive oxygen species (ROS) during the respiratory burst on contact with or during phagocytosis of bacteria. Neutrophils contain large amounts of myeloperoxidase, which in mammals is involved in the production of bactericidal hypohalite ions (principally from iodine) and presumably the

same can occur in fish. Another bactericidal mechanism, is the production of nitric oxide (NO) and subsequent peroxynitrite and hydroxyl ions by fish macrophage. An interesting observation is that following ip. Injection of bacteria (Renibacterium salmoninarum) into rainbow trout, the kidney showed expression of nitric oxide synthase (the enzyme which produces No) but a more rapid and sustained response was detected in the gill tissue. This indicates that the gill tissue is capable of mounting a strong bactericidal response, but the cell type (presumably macrophages) has not yet been identified.

Conclusion

It is plainly evident that innate mechanisms of defense play a vital role in preventing bacterial diseases in fish, yet there is still much to understand. An interesting situation is in the embryos and larvae offish which do not have a specific immune system,. When larvae of Atlantic salmon were challenged with *Yersinia ruckeri* 2 weeks after hatching, mortalities reached only 8%. As the fish aged they became more susceptible with mortalities reaching 13% when challenged at 4 weeks post-hatch and 60% when challenged from 6 weeks onwards. A similar greater resistance of very young salmonids to furunculosis has also been noted. This strongly suggests that some unidentified factors originating from the mother provided considerable protection against bacterial challenge, and this declined as the larvae aged. Eggs are rich in several non-specific defense factors, like lectins and lysozyme (source of the later probably being the ovary). And macrophages differentiate early in embryonic development. It is important to know more about these defense mechanisms and if the health status of the mother might affect the survival rates of her eggs and larvae. Such considerations are important for both farmed and wild fish population

Viral diseases

Viral diseases cause very significant losses in aquaculture. Some viruses mainly affect young fish e.g. Infectious Pancreatic Necrosis Virus (IPNV) in salmonids, while others cause mortality in fish throughout their life e.g. viral Hemorrhagic Septicemia virus (VHSV) and Infectious Salmon Anemia virus (ISAV). Many fish viral pathogens produce a persistent carrier state in the host. These fish can shed virus into the environment and be a source of infection to other fish. Low levels of virus can often be isolated from the kidney of carrier fish and evidence indicates that some viruses e.g. IPNV, can replicate in and be released from the kidney macrophages without being cytolytic. Little is known about the mechanisms involved in viral carrier states in fish and how the viruses evade the host defenses. The innate defenses against viruses in fish may be categorized as constitutive or responsive. A constitutive innate anti viral response in fish comprises the action of non specific cytotoxic cells to virus infected cells, and aresponsive mechanism in interferon production, which is non specifically inducible by virus infection. In some cases, e.g. defense against the rhabdoviruses, VHSV and IHNV, complement appears to play an essential role in virus neutralization mediated by the specific antibody response. Other constitutive defenses may include factors like lectins (which could bind to glycosylated residues on the surface of viruses), and non-specific lysins(which could lyse the enveloped viruses) but evidence for these is lacking in fish. For viruses to replicate in fish cells they first have to attach to the surface of the cell, enter the cell membrane and engage the cell's biochemisty for nucleic acid and protein production. All of these stages will require molecular recognition and if these are lacking the cell will be refractory to viral replication. Very little is known bout virus/host recognition for viral diseases of fish though some progress is being made with VHS and ISA.

Interferons and Mx proteins

The type I interferon system is a rapid and powerful antiviral defense mechanism in vertebrates. Interferons (IFNs) are pH resistant cytokines which are produced by many cell types in response to a viral infection. Viral double stranded RNA (dsRNA) induces the production of IFN and the synthetic dsRNA at some time in their replication and it appears that animals have evolved an ability to recognize these molecules and to respond to them by this innate mechanism. In mammals, the mode of action of IFN is to induce the expression in host cells of a number of proteins which inhibit the translation of viral mRNA. These proteins include 2′,5′-oligoadenylate synthetase, protein kinase P1 and MX Proteins. IFN-like activity has been demonstrated in a number of fish species but to date neither the protein nor the genes have been isolated. However, the genes encoding the Mx protein have been cloned for several fish species, including rainbow trout, Atlantic salmon and Atlantic halibut. Detection of the expression of Mx gene mRNA by RT-PCR or of Mx protein using labeled antibodies have been used as a sensitive method for detection of INF responses in fish.

Production of IFN like activity and Mx gene/ protein expression in fish has been demonstrated in vitro and in vivo in response to a number of viruses as well as poly I: C (see Table1). Protection against infection of fish and fish cell lines against several viruses has been demonstrated by injection of, or exposure to IFN containing serum or supernatants, respectively. Rainbow trout appear to have three Mx genes and proteins while Atlantic salmon and halibut appear to have two. The molecular weight of the trout Mx proteins is about 70kDa and of salmon about 76 kDa.

INF-like activity		Mx Protein	
In response to	Expressed in	In response to	Expressed in
VHSV	Rainbow trout		
IPNV	Rainbow trout		
IHNV;poly I:C	Rainbow trout HK macrophage		
VHSV (active and inactivated)	Rainbow trout blood and kidney leucocytes		
VHSV	RTG2		
IPNV	FHM		
IPNV	RTG2		
Poly I:C	Atlantic salmon HK macrophages (by 24h)	INF from Atlantic salmon marophages Poly I:C	Macrophages (by 48h) AS cells; CHSE Macrophages; AS cells (but not CHSE)
		Poly I:C injected salmon	Hepatocytes
		Poly I:C injected salmon	Liver, gills, HK (after 2 days)

Protection in fish and fish cell lines against viral infections by interferon like activity

Protection in	To	By
Rainbow trout	VHS	Serum from trout infected with VHSV 2 days previously
Rainbow trout	VHS	Supernatants from VHSV stimulated trout leucocytes

Chum/sockeye salmon	IHN	Injection of poly I:C
Atalantic salmon	ISA	Injection of Poly I:C
Cell culture		
RTG2	IPNV	Supernatants from VHSV stimulated trout leucocytes
CHSE	IHNV	Supernatants from Rainbow trout HK leucocytes stimulated with poly I:C
CHSE	IPNV	Supernatants from Atalantic salmon macrophages stimulated with poly I:C

Interferon production occurs very rapidly after virus infection (within 2 days in rainbow trout injected with VHSV; and occurs in very young fish (rainbow trout fry of less than 0.2gm, 600 degree days. In isolated Atlantic salmon macrophages stimulated with poly I:C, peak interferon production occurred within 24 h and peak Mx protein production after 48h. In Atlantic salmon injected with poly I:C, Mx protein was produced by various tissues within 2 days and for at least 14 days. Thus, IFN mediated antiviral defense mechanisms are able to respond during the early stages of a viral infection and this has led many authors to believe that IFN responses provide some degree of protection until the specific immune defenses are able to respond. However, serum IFN levels in rainbow trout following experimental infection with VHSV do not correlate with resistance and more closely reflect the level of viraemia i.e. it is the most susceptible fish which sow the highest IFN responses.
Recent work with DNA vaccines against VHS and IHN has shown that they not only stimulate the production of antibodies and protection but they also induce the expression of Mx genes in the muscle tissues of the rainbow trout at the site of injection . Interestingly, the control DNA plasmid lacking the viral glycoprotein gene insert did not induce any of these responses. Work in the author's laboratory has confirmed this. Furthermore, protection against VHS can be found as early as 1 week after DNA vaccination, a time when Mx genes were already being expressed, while antibodies to the VHSV were not detected until between 4 and 8 weeks post vaccination. It thus seems likely that DNA vaccines may induce a rapid protection against viruses, which is mediated by the innate non–specific IFN responses while long-term protection is mediated by the specific immune responses.
Although it is well established in higher vertebrates that natural killer (NK) cells are important in limiting the spread of virus by lysing infected cells in the early stages of infection, very little work has been done to investigate this aspect in fish. However, it is apparent that such mechanisms do exist in fish. Kidney leucocytes of normal rainbow trout and Atlantic salmon, while showing some *in vitro* cytotoxicity for RTG-2 as AS cells (tissue culture cell lines derived from rainbow trout and Atlantic salmon, respectively),, were significantly more cytotoxic when the cell lines were infected with IPNV. Because NK-like activity is shown by leukocytes in assays using allogenic cells, even when the target cells are not infected with virus, clearer evidence for anti-viral cytotoxic cells was provided using a channel catfish autologous system. Here, autologous catfish lymphoid cell lines, which were experimentally infected with Channel Catfish virus, were killed by peripheral blood leukocytes from the same (non-immunized) individual fish, while non-infected autologous target cells were not. Furthermore, the results of these workers indicated that the cytotoxic cells responsible for killing allogeneic targets were a different population from those responsible for killing viral infected targets. These workers also showed that blocking late viral gene expression in the infected target cells with acycloguanosine did not affect the level of cytotoxicity indicating that expression of early viral gene products was sufficient to render the target cells susceptible to lysis. Thus, the defensive, power of these cytotoxic cells

is clear, as they are capable of destroying infected cells even before the entire viral genome has been expressed to produce new infective particles.

Miscellaneous innate anti-viral defense

Glucan-induced

Rainbow trout injected ip with glucan were more resistant to immersion challenge with IHNV than saline injected controls. This increase in resistance took about 3 weeks to develop and lasted for up to 58 days. The mechanism of this resistance is not understood but it did not correlate with serum neutralizing antibody production which was, paradoxically, lower in the serum of surviving glucan injected fish than the controls.

Complement

The ability of salmonid antibodies to neutralize the rhabdoviruses, VHSV and IHNV, is dependent on the presence of complement. Results indicate that the classical complement pathway is involved but attempts to demonstrate the involvement of C3 have not been successful and the full mechanism of the role of complement is still unknown. It is possible that the requirement for complement is related to the enveloped nature of rhabdoviruses and depends on the membranolytic activity of complement lysing the virus particle. Non-enveloped viruses e.g. IPNV do not require complement for antibody to neutralize them.

Genetic resistance

There are strong indications that genetic traits are involved in resistance of rainbow trout against VHS. Using gynogenetic reproduction it has been possible to obtain highly resistant families of trout after only two generations. Brook trout (*Salvelinus fontinalis*) are highly resistant to VHS and this resistance is transmitted to triploid hybrids between rainbow trout dams and brook trout sires. When hybrids and rainbow trotu were challenged with VHSV by either immersion or injection, mortalities were much lower in the hybrids. Surprisingly, while viraemia was lower in the hybrids, so was the interferon responses. Interferon responses. Interferon levels reflected the level of viraemia, both of which were high in the susceptible rainbow trout, as well as the few moribund and dead hybrids. Thus, there was no correlation between resistance and interfereon responses, in fact the opposite was apparent. Other attempts to identify the mechanism of resistance against VHS have focused on comparisons of allelic variations in the complement component C3 as well as MHC between resistant and susceptible families of rainbow trout but once again with no success. The basis of this genetically determined VHS resistance in rainbow trout has thus still to be elucidated.

Host/ virus compatibility

The integumental tissues of fish are important as the portal of entry of viruses and they may be considered as a barrier to infection in resistant fish or as a primary site of attachment and replication in susceptible fish. This hypothesis was tested in two strains of rainbow trout, one which was susceptible to VHS by both immersion and injection challenge and another which was only susceptible to injection challenge. A strain of rainbow/brook trout hybrid, which was resistant to both routes of challenge, was also studied. Pectoral fins, gills and skin were excised and immersed in a VHSV suspension for 1h. To assess differences in viral adsorption, the tissues and supernatant were titred for virus. Titres in the tissues were very low, indicating that only very few particles had attached to the tissues, and no differences were detected between the tissues from different fish. This was confirmed in the supernatants, which showed no decrease in the original titre. Replication of virus in the excised tissues was assayed after 3 days of culture. Viral multiplication in all the tissues excised from susceptible trout was very high while there was no indication that virus replication occurred at all in any of the hybrid tissues. In trout that were susceptible to

injection challenge only, replication of VHSV occurred only in the skin. A possible interpretation of this is that the pieces of skin used were composed not only of epidermal layers that could be resistant to viral replication, but also inner layers of dermis and blood vessels in which virus could have been able to replicate. Thus, the susceptibility of different host cells to support viral replication may reflect different levels of resistance in fish to VHS.

Recently, progress has been made in understanding the mechanisms of how ISAV attaches and enters Atlantic salmon cell lines. This virus attaches to sialic acid residues on the surface of the host cell, leading to endocytosis of the virus by the cell. Fusion of the virus with the host cell membrane takes place in the endosome and is dependent on low pH. The viral genome is then released into the cytosol of the cell. It is known that large structural diversity occurs in sialoglycoproteins and certain types of sialoconjugates appear to be unique to salmonids. This is relevant because it is known that such host cell surface receptors are major determinants of host range and tissue tropism for many viruses of higher vertebrates. Thus, the precise nature of the type of sialic acid residues recognized by ISAV may provide a means of identifying more or less resistant salmon for selective breeding purpose.

Conclusions

It is particularly evident from studies on VHS in rain bow trout that innate defenses can play a very important role in resistance to viral infections but we have virtually no understanding of the mechanisms involved. While few studies have yet been performed to correlate resistance with innate defense responses, serum interferon responses and differences in C3 and MHC genes do not appear to explain differences in resistance to VHS. No studies have yet been performed to find correlates with anti viral cytotoxic cells.

It has long been recognized that many viral diseases have a limited host range even amongst very closely related viruses. For example, marine strains of VHSV are not virulent or have low virulence in rainbow trout though virulence can be increased by repeatedly passaging the virus through rainbow trout. It seems possible that innate differences in susceptibility of fish to different viruses resides in differences in the ability of viruses to attach to enter or replicate in different fish host cells. It would, therefore, be of interest to study the ability of different fish cells isolated in vitro (especially superficial tissues that are likely to be the first site of viral adsorption and multiplication), to support viral replication and produce interferon and Mx protein. Finally, much more work is required to identify the molecular receptors on the surface of susceptible fish cells which may allow means of modulating their structure of availability to the virus of identifying resistant strains of fish for selective breeding.

IN SITU HYBRIDIZATION

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Nucleic acid hybridization or reassociation is a process in which complementary single stranded (*ss*) nucleic acid anneals into double stranded (*ds*) nucleic acid. Complementary nucleic acid strands have extremely high avidity and, in stringent conditions, exceptionally high specificities. Conveniently, energy released during hybridization is large enough to obtain stable hybrids but not too large to dissociate the hybrid by a simple procedure.

Hybridization (reassociation) is based on the existence of hydrogen bonds between the two strands of the DNA:DNA, DNA:RNA or the RNA:RNA duplex and base stacking within the strands. These hydrogen bonds are situated so that adenine specifically pairs with thymidine (or uridine in RNA) and guanidine with cytosine. Zippering of two complementary strands, by base pairing, requires matching sequences, explaining its high specificity.

Hybdrogen bonds can be disrutpted (denaturation' or 'melting'of the duplex) or reformed ('renaturation' or 'reannelaling' of *ss* into *ds* nucleic acid) repeatedly by simple procedures without significantly altering the molecular integrity of either strand. Thus, the reannealing reaction is reversible, predictable and controllable. Whenever the target can be represented by a nucleic acid, hybridization is an ideal approach for detection. Such methods use a probe, a labeled nucleic acid, which can be hybridized specifically with the target nucleic acid. After selective removal of the nonannealed probe, the radioisotopic or nonradioisotopic label introduced in the duplex by the probe, can be detected.

History and development of hybridization

A cursory glance at the literature of over 30 years of hybridization development shows clearly that many difficulties had to be overcome (see table).

Complementary pairing of one strand to a second strand, resulting in a *ds* (duplex) helix structure of DNA, was proposed by Watson and Crick (1953) and forms the basis of hybridization. Their model accounts for (i) the finding by E. Chargaff that for all DNA's investigated A+C=G+T or G+A=C+T (hence G/C= A/T=1); and (ii) the X-ray diffraction patterns obtained from DNA fibers by M. Wilkins.

Table: Milestones in the history of development of nucleic acid probes

Understanding of basic principles	
Denaturation and renaturation	1957
Duplex formation between homopolymer strands	1957
Specific hybridization of nucleic acids	1960
Kinetics of duplex formation	1968
Thermodynamic principles of duplex formation	Mid-1970s
Sequence-dependence of thermodynamic stability of duplex	Mid 1980s

Principles of triple helix formation	1990
Development of hybridization techniques DNA binding to nitrocellulose	 1963
Prevention of binding of *ss* DNA to notorcellulose	1966
In situ hybridization	1969
Southern hybridization	1975
Colony hybridization	1975
Northern hybridization	1979
Dot/spot hybridization	1979
Sandwich/capture hybridization	Mid1980s
Alternative solid phases for hybridization	Mid 1980s
Hybridization on paramagnetic beads	=1990
Branch capture hybridization	=1990
Development of probe synthesis Cloning procedures for DNA Single stranded DNA probes	 1973 1978
Automatic oligonucleotide synthesis	1980
Single stranded RNA probes	1983
Polymerase chain reaction	End 1980s
Isothermal amplification	=1990
Ligase chain reaction	=1990
Labeling procedures of probes	
Nick-translation labeling	1977
Biotinylation of probes	1981
Labeling by random-primer extension	1983
Immunodetection of haptenated probes	1984
Enzyme-labeled probes	1984
Amplification (PCR,IAS) with concurrent labeling	=1990
Chemiluminescent probes	=1990

In situ hybridization of probes to nucleic acid in cell preparations has been useful in the study of the functional organization of nucleic acids. Advances in the last 10 years have simplified and optimized all stages of the procedures. The small sample size (down to a single cell) requires a small amount of probe and several targets can be detected simultaneously. Probes smaller than those used in filter hybridization are most efficient and synthetic oligonucleotides are becoming increasingly popular for in situ hybridization.

ISH allows the detection of genes or their expression in cells and complements studies on extracted nucleic acid by filing the gap between cell and molecular biological methods in the study of gene localization, translocation, amplification, transcription and transcript processing. This histological technology has several unique features which enables it to answer important biological questions such as (i) the localization of particular nucleic acids in single cells or the mapping of particular sequences on condensed chromosomes (possibly due to an increased topographical resolution by nonradioactive detection methods and an increased knowledge on the mechanisms and meaning of banding of mitotic or

meiotic chromosomes or even on interphase chromatin; (ii) simultaneous detection of two or more target nucleic acids in a single cell (by variant detection methods);(iii) distinguish subsets of cells or follow cell development with respect to the target nucleic acid (e.g., to determine whether only subsets of cells express a given gene or whether many cells have a low expression or a few cells a high expression) (iv) detect both particular nucleic acids and proteins encoded by them in the same cell by complementary techniques (ISH and immunohistochemistry).

ISH has a very high detectability, down to 1-20 copies/cell. mRNA can often be detected where the expressed protein cannot or when it is not expressed, if care is taken to prevent RNA degradation. ISH is not standardized, since standardized tissue does not exist, and can be accomplished in any of several approaches, the steps or which are not always parallel (see table e.g., sectioning after/before embedding). Optimization of ISH for a given tissue requires finding the optimal combination of the different steps, controls, buffers and probes. Most often, fixation is the crucial step.

Nature of Probe

Small probes often reach the target more efficiently than large probes due to its cellular localization, yet very small probes do not form stable hybrids. Short probes also require a higher specific activity (cpm/µg) to obtain the same amount of cpm/mol and will have a suitable shelf life which is exponentially shorter (not a problem for ^{3}H). on the other hand, unhybridized large probes diffuse with more difficulty from the cells during posthybridization washes resulting in higher background levels, particularly in the case of nonradioactive probes. The ideal length is about 100-500 bases.

The abundance and stability of the target will dictate the specific activity required. RNA probes, oligomers or cDNA probes are the most frequent choice. PCR probes and in vitro transcription allow better control of the probe. The use of oligomer probes to different regions of the target provides an excellent control for the specificity of the hybridization, especially if the target is a member of a gene family. The increased specificity of oligomers is often at the cost of detectability. Nuclease treatments can indicate whether an ISH signal was not due to a nonspecific adsorption to cellular component.

RNA probes have several advantages since they (i) are single stranded, (ii) have high thermal stability, (iii) allow a background reduction simply by posthybridization RNase treatment, (iv) are easily produced in defined form and in large quantities and (v) permit a larger probe size range to be used than with DNA probes. The stability may be lower than that of DNA probes as well as the tendency of increased secondary structures may make DNA probes a viable alternative.

Among the radioisotopes, ^{35}S is often superior for ISH due to the relatively high specific activity, the high autoradiographic efficiency, low nonspecific adherence, reasonable half life both for the radioisotope and the probe and is safer than ^{124}I or ^{32}P. However, the phage RNA polymerase do not use [^{35}S] UTP as efficiently as UTP oxidation of S may lead to high background (include DTT or 2-mercaptoethanol!0 and resolution with this isotope is much less defined.

Nonradioactive probes have become popular since they allow superior definition, are rapid, safe and cheaper. Nonradioctive probes also permit different targets to be detected simultaneously in the same cell (e.g., probes with different enzymes, enzyme/FITC, rhodamine (red) and coumarin (blue) fluorescence, etc.).

Biotinylated probes can be detected with avidin, streptavidin or modified avidin. Replacing phosphate buffered saline by SSC during hybridization reduces the background of unmodified avidin (fluoresceinated) making it superior to modified avidin for single copy detection.

Requirement of tissue preparation

The material to be investigated may be : (i) whole, small animals or organs; (ii) large pieces of tissues, directly from the organism or maintained as explants; (iii0 cell suspensions of cells grown in vitro, which do not require sectioning, if rendered permeable to facilitate the access of the probes to the target nucleic acid. Whole animals can be studied if precautions are taken to prevent shattering of the sections during cutting. Cells from suspensions or in vitro cultures and even small pieces of tissue teased from freshly dissected tissue can be permeablilized simultaneously with the fixation. This treatment should not (i) modify the target nucleic acid, (ii) (locally) elute the target nucleic acid, (iii) allow the redistribution of the target nucleic acid and (iv) change the morphological features of the tissue preparation. When a cellular monolayer is used, best results are obtained when the layer is only about 70% confluent. Morphologic analysis is optimum when the cells are flat and adhere tightly to the support. Moreover, a low cell density improves the signal/noise ratio and avoids cell overlapping.

The pH of the formamide solutions used in hybridization is very important for the preservation of cellular morphology and optimal hybridization. Concentrated formamide has a pH of about 4.5 (note that pH measurement of undiluted formamide is incorrect), but after dilution with SSC (pH7) and EDTA to 70% formamide, 2 x SSC and 1mM EDTA it has a pH of 9.5 chromosome preparations denatured with this solution have a poorly conserved morphology whereas titration of the solution to pH7 with HCI produced bot good morphology and increased specific hybridization. Moreover, if $DeSO_4$ is sused, autoclaving this accelerator reduces background.

Positive and negative hybridization controls

Controls should include determinations of the detectability, specificity and reproducibility of the probe. The detectability and specificity can be established on slot/dot or Northern blots. At least 1 pg of radiolabeled probe should be readily detectable. Moreover, if positive cells are available, reproducibility of the technique should be tested.
Negative controls for both background and specificity are required. Background levels can be established using blank slide, omitting the probe, or using negative cells or only the detection system.

Cytological procedures

From large pieces of tissue, paraffin sections of fixed tissue or cryostat sections of fresh or fixed tissues are prepared. Many laboratories prefer either one or the other. Cryosectioning is rapid and relatively easy but morphology may be better preserved with paraffinsectionin. Methacrylate sections are used rarely for ISH. The sensitivity of ISH on paraffin-embedded tissue sections of on cryosetions has rarely been compared but does not seem to differ greatly. This may depend on the type of tissue and the nature of the target nucleic acid.

Permeabilization and fixation of tissues and cells

Optimal fixation is the key element of successful ISH. Advances in tissue preparation and fixation now allow the study of a wide range of material. Two types of

fixatives are used; (i) 'Precipitant fixative' which remove lipids from the tissues and precipitate proteins; (ii) 'cross-linking' fixatives which cross link macromolecules. Initially, precipitating fixatives (e.g., Carnoy's fixative, acetic acid/ethonal) were used since cross linking fixatives were expected to make cells impermeable for the probes. Several research groups have reported that cross linking fixatives (e.g formaldehyde, glutaraldehyde) yield superior results, particularly with mRNA due to a better retention of the target nucleic acid and a better preservation of the morphology of the cell. This conviction is strongly challenged by other groups implying the absence of a universal fixative. The effects of the fixatives on preservation of cellular morphology, preservation and accessibility of target and relative hybridization efficiency depend on the cell type. Optimal fixatives have to be determined empirically for each tissue and optimal fixation is the most important step in ISH. Good starting points for mRNA detection are 50% methanol and 50% acetone for 20 min at -20°C (precipitating fixative) or, most often, 4% Para formaldehyde in PBS for 20 min at 4°C (Cross linking fixative) for small tissues and longer for larger tissues.

For the detection of cellular DNA, precipitant fixative (e.g., 3:1 methanol/acetic acid)are generally used. Formaldehyde adversely affects the denaturation of DNA targets and should be avoided whenever possible. The most frequently used precipitant fixatives are alcohol-or acetone based.

For the detection of RNA, freshly prepared formaldehyde is a versatile fixative since it cross links proteins less than glutaraldehyde, permeabilizes cell membranes and provides an improved penetration of the probes due to the limited cross linking. Moreover, formaldehyde has the added advantage of its compatibility with other fixatives. Fixation with formaldehyde is highly pH dependent and is extremely slow at pH 6.5.

The best fixative for the preservation of cellular morphology is glutaraldehyde. Unfortunately, glutaraldehyde has several drawbacks for ISH: extensive cross linking reduces permeability, it is reactive with nucleic acids and introduces reactive groups which may enhance background staining. Reactive groups may be blocked, however, by treatment with 50 mM NH_4Cl before hybridization. Due to the potency of glutaraldehyde, subsequent treatment of the sample with a protease is generally beneficial for the accessibility of target nucleic acid, as is the use of short probes. This method requires thus a delicate compromise and is only chosen when other fail.

Sectioning and mounting of cells and tissues

Sections cut from frozen or embedded tissues are most frequently used for ISH, although single cell suspension, such as hematopoietic cells, can also be mounted using nuclease free physiological buffers.

In addition, humid chambers are needed to prevent evaporation during the hybridization steps. Any tight fitting box with a plastic lid (to prevent the formation of large condensation drops; no glass!) can be used with a paper towel with a small amount of buffer (same ionic strength as on preparation to prevent distillation) on the bottom.

ISH is often possible on routinely processed formalin fixed paraffin embedded tissues. Retrospective studies of routinely processed human material are therefore feasible. Protease treatments prior to the hybridization may be necessary since the duration of fixation in autopsy material is often unknown. Sections of the same block have then to be examined to establish the optimum degree of protease digestion.

Hybridization procedure:

Tissue section or cultured cells are used and probes are detected, after hybridization and washes, by autoradiography or nonradioactive methods. Usually, very low probe concentrations, e.g., 50 ng/ml, are used to prevent background accumulation. However, when hybridization is optimized background levels increase only marginally with much higher probe concentrations.

Prehybridization treatments

Accessibility of the target can often be improved by different techniques. They all intend to improve the diffusion of the probe to the target and to prevent nonspecific trapping of probe in tissues. Mild detergent treatments, proteinase K or pronase digestion, heat and mild HCI treatments have been suggested. These treatments can also include nonspecific blocking with the usual prehybridization methods. Permeabilization can sometimes be improved by rinsing with 0.1% Triton X-100 in PBS for 5 min. Often, however, pretreatments are unnecessary or even detrimental after paraformaldehyde fixation but remain beneficial after glutaraldehyde fixation. Although pretreatment can be critical to the accessibility of the probe, it can be disruptive of cellular morphology, it can lead to RNA degradation and it may cause cells to lift from the support. Pretreatments should therefore be optimized by serial two fold dilutions/incubation times.

Hybridization to DNA

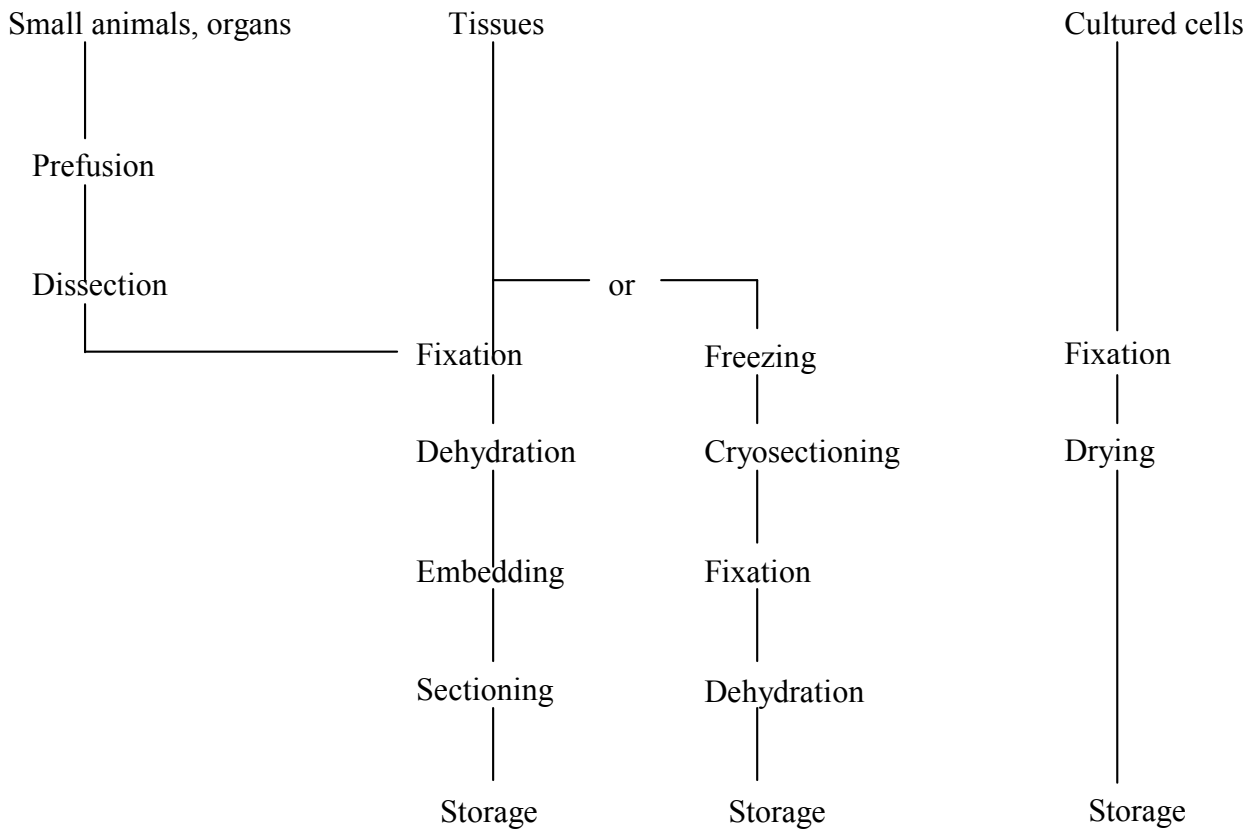
For DNA, fixation with a precipitant fixative is far superior to formaldehyde. RNA may yield false signals if DNA has to be detected since (i) the number of RNA transcripts exceeds the number of corresponding DNA sequences, (ii) the target RNA is *ss* and (iii) RNA is mostly located in the cytoplasm which the probe has to pass. Therefore, cells are treated with DNase-free RNase, followed by inactivation of RNase. A large number of variants have been described (50-95% formamide, 0.1-2xSSC, 60-100°C; (i) for simultaneous denaturation usually 50% formamide in hybridization solution with probe for 5 min at 70-80°C and then overnight at 37°C (ii) for denaturation of only target higher formamide and lower SSC concentrations can be used (often without any significant difference in denaturation efficiency but with considerable differences in the preservation of morphology.

Posthybridization and detection of probes

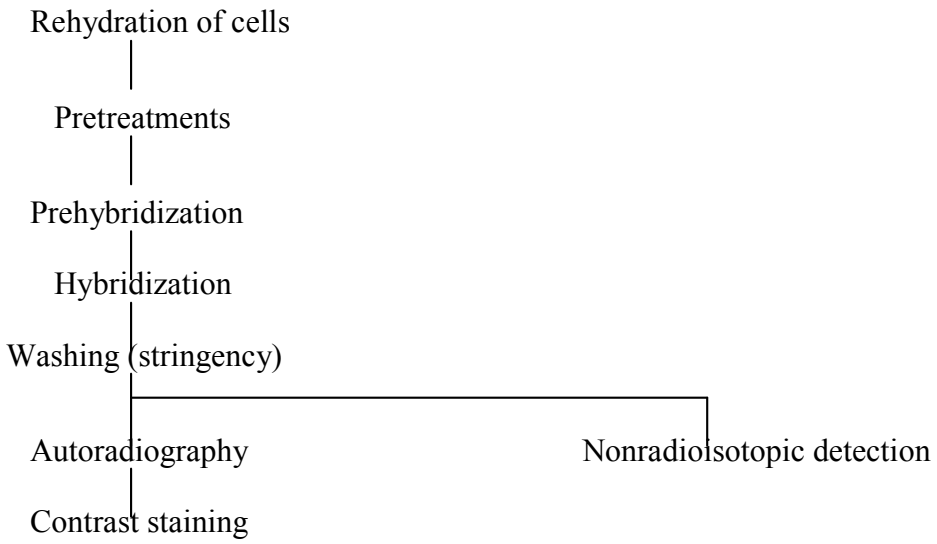
Extensive posthybridization washes are usually not necessary or do not make a difference. Standard methods or a few rinses in 2 XSSC suffice. Radiolabeled probes are detected in an autoradiographic emulsion or stripping film, by film or using a scintillation counter. Haptens in the probes are detected using streptavidin, for biotin, or antibody conjugates. The size of a biotinylated probe is critical to keep noise at a low level and the signal/noise ratio is often between 10 and 100. Acetic anhydride treatment before hybridization may reduce nonspecific adherence of these probes. Simultaneous detection of different probes is possible with different fluorescent tags, enzymes, radioisotopes or combinations thereof.

Steps in in situ hybridization

Cytological procedures



In situ Hybridization Procedures



VIRAL DISEASES OF FISH AND THEIR DIAGNOSIS

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Introduction

Generally, aquaculture plays an important role in economy as harvests from natural waters have declined or, at best, remained static in most countries. Fish and shrimp, the main aquaculture product sources, have gained the most attention. Many factors can cause losses in yields of fish products among these infectious diseases in fish and shrimp is the biggest threat to the fishery industry. Among various causative agents of fish and shrimp diseases such as bacteria, fungi, parasites and so on, viruses are one of the most destructive pathogens. Viral diseases cause serious problems in every aspect of aquaculture causing 100% mortality in both wild and cultured fish. Until now, approximately 60 different viruses have been detected in fish. Among these 25 viral diseases (caused by 29 different fish viruses), which have been classified according to virus families. Among them infectious pancreatic necrosis virus (IPNV), channel catfish virus disease (CCVD), infectious hematopoietic necrosis (IHN), infectious salmon anemia (ISA) and lymphocystis disease (LCD) are the emerging viral diseases. Several countries in every year faces severe loss due viral diseases such as Canada have experienced severe losses due to infectious salmon anemia virus (ISAV) and infectious haematopoietic necrosis virus (IHNV). Wild stocks and aquaculture facilities in Europe, Scandinavia, regions of the Indo-Pacific and Mediterranean as well as the United States of America have also been affected by serious viral infections. High mortality rates and severe economic losses emphasize the need for a better understanding of the fish viral diseases and their diagnosis in order to better combat diseases.

Types of fish viruses

Mainly two types of viruses are responsible for fish diseases these are presented in following table

DNA virus of fish		**RNA virus of fish**	
Family	**Species**	**Family**	**Species**
1.Herpesviridae	Channel catfish virus Carp pox Papillomatous eel Epizootic epidermal papillomatous in European smelt Koi herpes virus Cyprinid herpes virus	1. Picornaviridae	Smelt virus Taura syndrome virus
2. Iridoviridae	Lymphocystis disease of fish Parvoviridae Infectious hypodermal haematopoitic necrosis virus Hepatopancreatic like	2. Reoviridae	Golden shiner virus Grass carp haemorrhagic disease reovirus Trench reovirus Reo III virus of shrimp Reo IV virus of shrim

	virus		
3 Nimaviridae	White spot disease virus/systemic ectodermal mesodermal baculovirus.	3 Birnaviridae	Infectious pancreatic necrosis virus
4Adenoviridae	Cod adenovirus Dab adenovirus Sturgeon adenovirus	4 Rhabdoviridae	Viral haemorrhagic septicemia Infections haematopoitic necrosis Penaeid shrimp rhabdovirus
		5 Orthomyxoviridae	
		6 Paramyxoviridae	Phocid distemper virus Chinook salmon paramyxovirus

Viral diseases in fish

DNA viral diseases

Lymphocystis Disease

- Caused by Iridovirus
- Observed in most freshwater and saltwater species.
- Clinically, fish are presented with variably sized white to yellow cauliflower-like growths on the skin, fins, and occasional gills. Occasionally, this virus may go systemic with white nodules on the mesentery and peritoneum.
- Histopathology: Fibroblast undergoes cytomegaly with many basophilic cytoplasmic inclusion bodies and a thick outer hyalin capsule. The inflammatory response is variable but is usually a chronic lymphocytic inflammatory infiltrate.
- The disease gains entry through epidermal abrasions. The virus infects dermal fibroblasts.The disease is self limiting and refractory to treatment. Nodules may last several months and cause infected fish to be susceptible to secondary bacterial infections. Reinfection can occur

Herpesvirus salmonis (Herpesvirus disease of Salmonids)

- Caused by Herpesvirus
- Disease is observed primarily in the fry of rainbow trout.
- Clinically the fish are lethargic with prominent gill pallor. Mucoid fecal casts are commonly observed trailing from vent.
- Lesions:
  Exophthalmus and ascites
  Low hematocrit and numerous immature erythrocytes
  Hemorrhage in eyes and base of fins
- Histopathology:
  Multifocal areas of necrosis of the myocardium, liver, kidney, and posterior gut (leading to cast formation)

Syncytial cells involving the acinar cells of the pancreas is considered to be a pathognomonic sign.

- Transmission of the virus is believed to be direct.
- Control is by avoiding exposing susceptible trout to the virus. If the disease occurs, raising the water temperature to 15°C or more will minimize losses.

Channel Catfish Virus

- Caused by Herpesvirus
- Observed in fry or fingerling channel catfish (less than 10-gram weight) during the summer when water temperatures are above 22°C.
- Clinically these fish usually show erratic swimming or spiraling followed by terminal lethargy. Mortality is very high.
- Lesions:
  Hemorrhage at the base of the fins and skins;
  Ascites; exophthalmos; and pale gills;
  Kidneys swollen and pale with hemorrhage;
  Spleen is enlarged and dark red;
  Gills usually pale;
- Histopathology: Multifocal areas of necrosis and hemorrhage are observed in the posterior kidney, liver, intestines, and spleen.
- Infection is direct with transmission of the virus in the water or feed. Piscivorous birds, snakes, or turtles may mechanically carry the virus from pond to pond. Transovarian transmission has not been conclusively demonstrated but is suspected. Survivors are persistently infected and become carriers for life.
- Control of the disease is by sanitation, purchasing of virus free brood stock and lowering water temperature to less than 19°C during an outbreak to lessen the mortality.

Epithelioma papillosum (Fish Pox)

- Herpesvirus cyprini (Cyprinid herpesvirus 1)
- Non-fatal disease is observed in carp and other cyprinids
- Lesions: Elevation of the epidermis with the formation of white to yellow plaques over the body of the fish. Healed lesions usually turn black.
- Histopathology: There is epidermal hyperplasia with the epithelial cells occasionally demonstrating intranuclear inclusion bodies.
- Transmission is unknown, however, it is probably direct.

Papillomatosis of eels(Cauliflower Disease)

- First isolated from eels at German Baltic coast in 1910. Since then it has been reported from Denmark, Holland, Belgium and many other places.
- Disease develops a greyish white plaque of epidermal hyperplasia, which can occur anywhere on the body as verrucose lesion, which increases in size and acquires a pinkish tinge. Mature lesion is usually greyish in colour. The term caulifilower disease is given because of the papillomatous growth, which frequently obstruct the mouth. The lesion develops from the basal layer of the dermis. Ultrastructure examination reveals presence of virus-like particles in the affected cells. As the characteristic papillomatous growth has not so far been reproduced experimentally, there is controversy regarding the etiological factor as virus.

Koi Herpesvirus

- Caused by Koi herpesvirus of family Herpesviridae
- Koi Herpesvirus Disease is a viral disease of common carp *Cyprinus carpio*, including all its ornamental varieties such as koi, ghost koi etc. The virus is highly contagious and may cause up to 100% mortality. KHV has already caused severe fish losses to ornamental wholesalers, retailers and carp fishery owners and continues to pose a significant threat to anyone dealing with or keeping common carp.
- There are a variety of clinical signs associated with KHV disease: infected fish may be lethargic and move away from the shoal. They may display erratic behavior, often gathering at water inlets or points of oxygenation, show loss of balance, loss of mucus resulting in dry, rough patches, sloughing of mucus, and sunken eyes. The gills are frequently affected displaying necrotic patches of dead tissue.
- The disease is transmitted by fish to fish contact and it is also possible that the disease can spread through contaminated water, nets and other equipment. Transmission of the virus through ova cannot be ruled out. The virus is thought to be shed through faeces, and urine, and also possibly via gills and skin.
- There is no treatment for KHV and any fish that recover from the disease may carry the virus and act as a source of infection. Controls may include applying a movement restriction to the site, culling of stocks and disinfections of the facility.
- KHV Disease was first recorded in Israel in 1998 followed by large-scale mortalities in China, Indonesia, and USA; so far the occurrence of koi herpes virus infection in India has not been reported except some seroincidences.

RNA viral diseases

Infectious Hematopoietic Necrosis (IHN)

- Rhabdovirus
- The disease is observed in the fry of trout (rainbow) and salmon (Chinook and sockeye) with mortality up to 100%.
- Clinical signs and lesions:
  Fish become lethargic or hyperactive.
  The fish become dark due to increase in pigmentation.
  Exophthalmus, abdominal distension, and fecal cast.
  Hemorrhage on skin and viscera primarily at base of fins, behind the skull, and above the lateral line.
  Anemia with pale gills.
  Surviving fish may develop scoliosis.
- Histopathology: There is prominent necrosis of hematopoietic tissue including melanomacrophages of the kidney, red pulp of the spleen and hepatic parenchyma. Necrosis of the submucosal eosinophilic granular cells is considered pathognomonic for IHN. (This lesion is observed in other systemic viral diseases.) Intranuclear and intracytoplasmic inclusions are occasionally observed in acinar and islet cells of pancreas.
- The virus is transmitted by direct contact with infected survivors or by feeding contaminated feed. The virus is probably shed in contaminated semen and eggs. The disease is most severe at 10oC and rare at temperatures above 15°C.

Infectious Pancreatic Necrosis (IPN)

- Caused by Birnaviridae
- The disease is observed in salmonoid, which was first decribed in 1940 caused mortality up to 100%. The disease in and the virus has been isolated from N.

America, Europe and Japan and demonstrated in trout, Atlantic salmon and coho salmon.

- Clinical signs and lesions:
  Fish become lethargic or hyperactive.
  Affected fish produce chronic infection, darker in colour and show characteristic show characteristic spiral swimming fbehaviour sassociated with nervous signs and exophthalmus.
  The virus is demonstrable in Kidney, faeces and sex products.
- Pathology: At nercropsy the abdomen is distended containing pancreatic tissue, which may show areas of punctate haemorrhage. Liver and spleen is often swollen and pale. Stomach and intestine contain catarrhal exudates. Areas of congestion and necrosis are frequently seemed in the abdominal cavity. Intestine may contain solid yellow material.
- Histopathologically: There is focal or generalized necrosis of the pancreatic acini characterized by rupture of the infected cells with release of zymogen granules, shrinkage and basophilia of cytoplasm, pyknosis of nuclei and perinuclear hallow. E.M. shows memberace bound areas of assembly of virus.
- Transmission: IPN occur either vertically through egg or horizontally through water, faeces or sexual secretion. The most important reservoir of infection is the infected fish.
- Control: There are no means of treating infected fish or eliminate virus. Avoidance is theonly effective control measure. Eradication of virus from infected farms has been successful when all infected fish was slaughtered. A vaccine is available.

Viral Hemorrhagic septicemia

- Caused by Rhabdovirus
- Widespread and very contagious viral disease of rainbow trout. This is a serious disease of trout in Europe. Affects both salmonids in fresh water and seawater. Disease occurs in temperatures below 14°C.
- Two forms of the Disease --- Acute and Chronic
  Acute disease: High mortality in affected fish. Fish have pale gills, dark body coloration, ascites, exophthalmus and erratic swimming behavior (spiraling). Hemorrhage is a common finding in the eyes, skin, serosal surfaces of the intestines and muscles. Necrosis of the hematopoietic and lymphoid elements of the anterior kidney and congestion and necrosis of the hepatic parenchyma are histopathologic findings.
  Chronic disease: See a slower prolonged mortality. Fish become lethargic, have pale anemic gills, darken skin coloration, exophthalmus, and distention of the abdominal cavity. Internal organs are commonly involved with splenomegaly, hepatomegaly, and swollen kidneys.
  Turbot, sea bass, and Atlantic salmon are commonly affected
- Transmission is believed to be direct with contact of carriers and contaminated water and feed. Vertical transmission via the egg is not reported.

Spring Viremia of Carp (SVC)

- Caused by several subtypes of Rhabdovirus carpio.
- Disease occurs in carp and other cyprinids.
- Clinical Signs and Lesions:
  Loss of coordination and equilibrium.
  Exophthalmus and abdominal distension (ascites).
  Inflamed and swollen vent.

Edema and hemorrhage in many organs.
In SBI see pronounced inflammation and hemorrhage of swim bladder.

- Transmission: Virus shed in feces and found in contaminated eggs.

Infectious Pancreatic Necrosis (IPN)

- Caused by Birnavirus
- Affects most salmonids primarily rainbow trout and brook trout. IPN has also been implicated in disease among several nonsalmonid fish.
- Clinical signs and lesions:
  IPN is characterized by a sudden explosive outbreak with high mortality.
  Affected fish become dark and rotate their bodies while swimming.
  Diseased fish usually have distended abdomens and exophthalmus.
  The presence of a gelatinous material in the stomach and anterior intestine is highly suggestive of IPN; mucoid fecal casts are common.
  Infected fish commonly have a low hematocrit and hemorrhage in gut, primarily in the area of the pyloric ceca.
- Histopathology: Histologically, there is necrosis of the pancreatic acini, gut mucosa, and renal hematopoietic elements. A moderate inflammatory infiltrate is usually observed around the pancreatic acini. Hyalin degeneration of skeletal muscle is also observed.
- Virus can be transmitted vertically in the eggs.

Swim-bladder Inflammation (SBI)

- Caused by Rhabdovirus
- Affects most young cyprind fishes
- Clinical signs and lesions:
  Affected fish become dark and most remarkable feature is the degeneration of swim bladder. The first chamber becomes congested and cloudy due to oedema followed by haemorrhages and necrosis.
- Transmission: Mode of transmission is not fully known.
- Control: Good nutrition may prove to be useful along with antibiotic treatment.

Pike Fry Virus Disease(PFVD)

- Caused by rhabdovirus
- Affects most young cyprind fishes
- Clinical signs and lesions:
  Affected fish show loss of equilibrium and marked distention of the cranium along with exophthalmus and haemorrhages of the gills, skin and petechiae of muscle.
- Transmission: Vertical as well as horizontal transmission
- Histopathology: The most distinctive feature is marked hydrocophalus and congestion of menigeal and cranial vessels. Ventricles of the brain show distention with clear transudate compressing the optic tectum. Renal glomeruli and tubules show marked necrosis and vascular organs reveal haemorrhages.
- Control: Good nutrition may prove to be useful along with antibiotic treatment.

Grass carp reovirus/Haemorrhagic disease of grass carp

- Caused by reoviridae.
- Affects most young cyprind fishes
- Clinical signs and lesions: The virus causes haemorrhagic condition in grass carp and maortlity goes upto 80%. The disease causes serious losses in China.

- Transmission: The virus is transmitted by water and enters the fish through mouth. The virus can be isolated from spleen and kidney.
- Control: The disease is controlled by a inactivated vaccine.

Diagnosis of viral diseases

Viral diseases in fish can cause moderate to high mortalities yet be difficult to diagnose. Unfortunately, in some situations, viruses can be used as a scapegoat without any real validity. It is critical to rule out water quality, nutrition, system management issues, other environmental or social stressors, and, of course, other infectious agents (parasites, bacteria, fungi). However, it is not uncommon to have mixed infections, and in some instances, viruses can be a primary or underlying cause for a disease problem. Fish mortality due to viral infection is difficult to monitor in wild stocks due to insufficient monitoring techniques and procedures. In many cases, the only recourse is to quarantine and destroy infected stock. In many fish species, in particular, the hundreds of ornamental fish, the ability to isolate virus is severely impeded by the lack of cell lines and other diagnostic tools (e.g., antibodies for serology; PCR) for each species and/or for each potential viral pathogen. For the numerous "undiscovered viruses," clinical signs, histopathology, and electron microscopy are the best options for considering causation.

The viral diseases can be diagnosed by the following ways:

- Clinical symptoms/lesions
- Histopathology/detection of inclusion bodies
- Detection of viral antigen in tissue/cell
    - Detection of inclusion bodies through H&E staining.
    - Viral interference test
    - Haemadsorption test
    - Fluorescent antibody and immunoperoxidase staining
- Detection of antiviral antibodies in the serum
- Electron microscopy

Different techniques for viral diseases diagnosis

Molecular techniques for the rapid diagnosis of viral diseases

Molecular techniques have enabled significant advances in virus identification and epidemiology. A wide variety of genome organisation is found in the viruses; they may contain DNA or RNA and can be single- or double-stranded. There are no sequences that are highly conserved, as the rRNA genes are, which can be exploited in design of 'universal' virus primers or probes. However, degenerate primers can be useful and within genera and use for the detection and characterization of virus. Virus isolation are confirmed by PCR of the viral isolates. Even without isolation of the virus PCR can be used to detect the virus in fresh, infected tissues. Detection of several viruses can be carried out by PCR amplification of the glycoprotein or nucleoprotein genes, by probe hybridization. PCR and probes are also employed in classification and examination of different isolates, along with nucleotide sequencing, nuclease fingerprinting and the RNAse protection assays. By nested PCR the specificity and sensitivity (to detect organism present in a very low copy number) can increase. RT-PCR is used for detecting RNA viruses. Molecular diagnostics are likely to be important in the study of emerging viral diseases. Already, analysis has begun of the genomes of iridovirus, nodavirus, alphavirus and herpesvirus. Viral pathogens are particularly influential in shrimp culture. Methods for molecular diagnosis of white spot baculovirus and bacilliform virus have been developed. New developments such as design of PCR conditions that can detect several pathogens at one time in a multiplex reaction will improve time and cost-efficiency of molecular techniques. It has been predicted that in the

middle of the 21st century DNA-based diagnostic will replace protein-based methods for detection and characterization of fish viral pathogens.

***Serological* techniques**

The definitive diagnosis of viral disease in fish has traditionally been accomplished through the direct isolation using culture procedures followed by serological identification of the causative agent(s). The serological techniques most commonly used for identification and detection of fish antibodies against viral disease agents is the serum neutralization and immunofluorescent techniques. Other techniques such as the enzyme linked immunosorbent assays (ELISA), immunoblotting or counter-current immunoelectrophoresis, virus neutralization test, immunoperoxidase test etc. have been less commonly used. However, ELISA techniques for the detection of antibodies are now widely used throughout the world for the diagnosis and monitoring of infectious diseases in veterinary medicine and are currently being examined for monitoring of fish viruses. Antibodies against fish rhabdoviral pathogens were first reported over 20 years ago in rainbow trout infected with viral hemorrhagic septicemia virus (VHSV) or infectious hematopoietic necrosis virus (IHNV). Recently that IHNV was discovered in Europe. Seroepizootiological tests with high specificity would be advantageous over virus isolation procedures because IHNV and VHSV cause similar cytopathic effects and replicate in common cell lines and hosts. It has also been demonstrated that fish populations previously exposed to a virus could be identified by a antibody detection method when they were negative by virus isolation. Another important attribute of serological testing is that it can be accomplished without sacrificing valuable fish. Additional serological testing in parallel with virus isolation should be implemented to generate information about the usefulness of serological techniques for virus detection.

Conclusion

Viruses are microorganisms, which are extremely difficult to study because of their small size and inability to live outside their host tissue. Viral diseases in fish can cause moderate to high mortalities yet be difficult to diagnose. Therefore identification of viruses and investigation of viral diseases is most promising concept and requires proper characterization of viral diseases, highly specialized diagnostic methods equipments, and proper control measures to prevent transmission.

RAPD-PCR PRINCIPLES AND ITS APPLICATION IN MICROBIAL GENOME STUDIES

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RAPD assay and its principle

This method termed Randomly Amplified Polymorphic DNA (RAPD) fingerprinting allows detection of DNA polymorphism by randomly amplifying multiple regions of the genome through PCR using arbitrary primer (s) designed independently of the target DNA sequence. This technique was first devised by Williams *et al.* (1990) and Welsh and McClelland (1990). They showed that DNA amplification takes place by the combination of three steps denaturation, annealing and extension. First the target DNA containing the sequence to be amplified is heat denatured at 94°c to separate its complimentary strands. In the next step, the temperature is lowered to 36°c so that primer can form hydrogen bonds or anneal to the DNA on both sides of the target sequence. Since the primers are present in excess, the target DNA strands will almost always anneal to the primers rather than to each other. Moreover, when the primer is short (e.g. 10 mer in size), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientations. Finally temperature is again raised to 72°c so that the enzyme, *Taq* DNA Polymerase, added extends the primers and synthesizes copies of the target DNA sequence. The profile of amplification products depends on the template primer combination. The amplification products are then resolved on agarose gel or Polyaclyamide gel electrophoresis (PAGE) and polymorphism based on presence and absence of amplified bands serve as dominant genetic markers, which are inherited as a Mendelian fashion. Arbitrary nature of the nucleotide sequence makes it possible to detect DNA polymorphism in the absence of specific nucleotide sequence information, avoiding much labour intensive training and work.

Two modification of detecting RAPD markers have been described as DNA Amplification Fingerprinting (DAF) and Arbitrarily Primed Polymerase chain Reaction (AP-PCR). DAF uses short random primers of 5-8 bp and visualizes the relatively greater number of amplification products by polyacrylamide gel electrophoresis and silver staining. AP-PCR uses slightly longer primers (such as Universal M13) and simplification products are radioactively labelled and resolved by PAGE. Data obtained from these studies could be used to assess the genetic diversity and such studies could be used to monitor trends in occurrence of pathogenic strains, to identify possible sources of infection, to assist in gene mapping, to aid in individual, to study population genetics of species and to serve as characters in molecular phylogenetic studies.

Very little work has been done to investigate the nature of the RAPD amplicons and what give rise to observed polymorphism. There is also confusion existing with regard to RAPD primer-template interaction. Sommer and Tautz,(1989) established that first three bases from the 3'end of the primer must match perfectly in order for the DNA polymerase to bind and extend the primer. While Caetano-Anolles, (1993) concluded perfect homology to at least the 3'first five or six nucleotides were required.

Applications of RAPD in Microbes

This technique has successfully been used to characterize several bacterial species such as *Streptococcus spp. Helicobacter pylori Legionella pneumophila* and *Pseudomonal aeruginos, bacillus cereus, Renibacterium salmoninarum, yersinia ruckeri, Aeromonas hydrophila* and *A slamonicida.* Genomic analysis using RAPD has been performed for several fungi and yeast as well. This technique had proved to be useful for obtaining fingerprints of microorganism for epidemiological investigations in medicine or food science.

Lately the use of individual RAPD bands to prepare specific probes in hybridization reaction has also been reported. The advantage of PCR over other culture based conventional methods is that some of the pathogenic microorganism are difficult to culture e.g. *Renibacterium salmoninarum* but can be detected using PCR.

RAPD as a tool for typing and characterization

Recently, genotyping has successfully been employed for identification and classification of microorganism. RAPD assay has been proved to be suitable for identifying a variety of microorganisms, such as *Escherichia coli* and *Helicobacter pylori*, *Saccharomyces spp., Bacillus spp.* Etc. Though many conventional techniques are available for typing bacterial species like biochemical tests etc. its ability to discriminate the strains have always been questioned. Methods are available to characterize bacterial strains like phage typing, whole cell protein profiling, outer membrane profiling, isozyme electrophoresis, but they are all time consuming difficult to perform and not completely reliable. Even the methods developed for typing strains based on digestion of genomic DNA with restriction enzyme and analysis by electrophoresis,

DNA fingerprinting techniques such as Restriction Fragment Length Polymorphis, (RFLP) and Random Amplification of Polymorphic DNA (RAPD) provide alternative approach to distinguish genotypic variants. RAPD analysis is a PCR based alternative method to the use of species-specific DNA sequences for isolates or strain differentiation. The technique can be very useful tool in providing needed strain specific information. It has been possible using this technique for fast screening of the commonly occurring useful isolates. With strain specific primer it was possible to distinguish a probiotic strain of *Lactobacillus* spp from gastrointestinal tract strain. To increase the amount of diagnostic fragments, it is better to use two primers in a PCR. RAPD –PCR method applied to *Propionobacter* sp. provided profiles consisting of a few bands that allowed immediate visual comparison of the amplification patterns and identification at the species level. It has been found RAPD to be very efficient for discriminating as well as revealing the degree of relatedness of isolates of *Pediococcus* sp.

Interlaboratory comparison of RAPD patterns may not always be applicable since the RAPD patterns may not always be applicable since the RAPD pattern can be influenced by many technical factors. The reproducibility problem could be overcome with the use of longer random primers, which would allow more stringent conditions to be used but may reduce the number of products and therefore discriminatory power of this technique.

Assessment of genetic diversity may be useful in epidemiology, breeding for disease resistance, etc. Data from genetic diversity studies could be used to monitor trends in the occurrence of pathogenic strains to identify the possible source of infection, to assist in gene mapping, to aid in individual strains identification, to study population genetics of species, and to serve as characters in molecular phylogenetic studies.

It also allowed the characterization of the bacterial association of a natural starter for Parmeson cheese production and growth kinetic analysis of different *Lactobacillus* sp. populations. They also suggested, since RAPD 16sr DNA sequencing were demonstrated to be an efficient tool for the analysis of these communities, the combined use of these molecular techniques should be applicable for ecological study of bacterial communities in other fermentation.

Probe development using RAPD

In aquaculture, bacterial infections are mostly diagnosed using conventional microbiological methods like biochemical and/or serological test. But biochemical methods are time consuming and reliability of serological techniques can sometimes be handicapped by the lack of specificity of the antisera used in the test. Recently attempts have been made through molecular techniques to solve this diagnostic problem. One attempt has been directed towards development of specific probe using RAPD technique, since it generate molecular genetic markers at the level of genus, species or even strain.

It is possible to design the gene probe depending on the type of information required. If a particular taxonomic group is to be identified, the probe must be directed towards a gene on region of a gene that is conserved throughout a particular species or genus. For the detection of all members of a genus or specie ribosomal RNA has been a popular target because it contains both conserved and variable regions. If a pathogenic organism is to be detected, the probe is usually targeted to a virulence factor gene responsible for causing disease.

Argenton *et al*. (1996) combined sequentially two variants of the PCR for development of specific prove against *Yersinia ruckeri,* one , the PCR with a random primer at low stringency or RAPD, and the other, the PCR with specific primers at high stringency. The use of specific primer (23-24 mer) random primer resulted in better amplification patterns than those obtained with 10 mers.

The probe, in addition to its use as a diagnostic tool, might be useful in microbial ecology research as a tool for quantifying these groups in the environment over time or in soil after ecological disturbances and are useful to study genetic element within a complex genomes.

Much attention needs to be paid while interpreting RAPD-PCR profiles since single RAPD band may contain multiple fragments of a similar size which are not distinguished by gel electrophoresis but differ in nucleotide sequence. For any RAPD marker to be used as diagnostic tool by wider group of researchers. It is necessary to characterize it more thoroughly through isolation, cloning and sequencing to generate either probes or specific primers for future application.

ELISA AND OTHER ENZYME IMMUNOASSAYS

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Introduction

During the last two decades there has been a phenomenal increase in the number and variety of immunodiagnostic tests. One of the reasons for this is, the development and perfection of methods, which use labeled antigens or antibodies, resulted very high levels of sensitivity and specificity. Among different labels fluorescent labels are very useful for the rapid identification of organisms responsible for infectious diseases as well as for the measurement of antibody levels. Similarly isotopes in radioimmunoassay are also found to be suitable, especially as labels on antigens, and acts as a method of choice for sensitive assays on both large and small molecular weight substances. However, both immunofluorescence and RIA have their limitations. Immunofluorescence is tedious, time consuming, and not easily automated so it can be used for only small batches of tests. In contrast, RIA is particularly suitable for large-scale operations but the short shelf life of the reagents, the safety of laboratory personnel, the radioactive waste problem, the requirement of building special laboratory facilities, and the procurement of expensive sophisticated equipments, and the strict regulatory controls on the use of isotopes have tended to exclude RIA from many small laboratories. These considerations have led workers to search for alternative labels for antibodies or antigens. Different substances have been proposed for this role, for example, bacteriophages, metals and free radicals but at present the most promising new labels are enzymes. These can be linked to antibodies or antigens such that the complexes have both immunological and enzymatic activity. Degradation of chromogenic or fluorogenic substrate by the enzymes creates an colored reaction, which enables accurate and sensitive detection of the presence of the enzyme which in turn detects the presence of antibody in the test samples.

Assay systems involving use of antigens, haptens, or antibodies labeled with an enzyme have recently been applied to the measurement of substances in biological fluids and these assay systems have been given various names: enzyme-, enzymic-, enzymatic-, and enzymoimmunoassay (EIA), enzyme-linked immunoassay, enzyme-labeled immunoassay, enzyme-coupled immunoassay, immunoenzymatic assay and enzyme linked immunosorbent assay (ELISA). Enzyme immunoassay (EIA) has become household names for medical laboratories, manufacturers of *in vitro* diagnostic products, quality assessment. The EIA techniques are based on the principle of immunoassay with an enzyme rather than radioactivity at the reporter label. Enzyme immunoassays are more superior to radioimmunoassay and described below.

Advantages of enzyme immunoassay

- Specific and sensitive assays of wide applicability
- Equipment required is relatively cheap and is widely available
- Reagents are relatively cheap and have a long shelf life
- Manipulations are simple
- Assays may be very rapid
- A separation step may not be required

- The variety of labels available may allow multiple, simultaneous assays to be performed
- Potential for automation
- No radiation hazards

Enzyme Labels used in EIA

Enzymes are used as suitable labels because their catalytic properties allow them to act as amplifiers, and many enzyme molecules can catalyze the formation of more than 105 product molecules per minute. To be suitable as a label an enzyme must have some characteristics and these are

- Available cheaply in high purity
- High specific activity
- Stable under assay and storage conditions
- 4.Soluble
- Assay method that is simple, sensitive, rapid, and cheap
- Absent from biological fluids
- Substrates, inhibitors, and disturbing factors absent from biological fluids

Different enzymes that have been used in EIA

Enzymes	Source	Substrates
1. Glucose-6phosphate dehydrogenasea 2. Glucose oxidase 3. Peroxidase 4. Acetyl cholinesterase 5. Alkaline phosphatase 6. Glucoamylase 7. Lysozyme 8. β 3-Galactosidase 9. Malate dehydrogenasea	1. Mitochondria 2. *Leuconostoc mesen teroides* 3. Fungal 4. Horse-radish 5. Calf intestinal mucosa and *E. coli* 6. Rhizopus nivens 7. Egg white 8. *E. co/i* 9. Pig heart	1. Glucose-6-phosphate 2. Glucose 3. Hydrogen peroxide (H2O2), Ortho-phenylene-diamine(OPD), ABTS (2,2'-azino-bis-[3-ethylbenthiazoline-6-sulfonic acid],TMB. 4. Acetylthiocholine 5. PNPP (paranitrophenylphosphate) 6. Starch 7.Polysaccharides,N-acetylglucosamine 8.β-galactoside,beta-D-galactosylamine. 9. Oxaloacetate

All the labels are available commercially. Horseradish peroxidase and alkaline phosphatase have been the most commonly used while lysozyme and glucose-6- phosphate dehydrogenase are used in the homogeneous EIAs that are commercially available. No one enzyme is ideal, but each has its strong points.

Types of Enzyme-Immunoassay

Enzyme-Immunoassay is classified based on the requirement of free and bound reactants. These are

1. Heterogeneous enzyme-immunoassay
    a. Competitive
    b. Non- Competitive
    c. Two-site immunoenzymomatric assays
    d. Double antibody immunoenzymomatric assays

2. Homogenous enzyme-immunoassay
    a. Enzyme-multiplied immunoassays
    b. Substrate-labeled immunoassays
    c. Coenzyme-labeled immunoassays
    d. Enzyme-enhancement immunoassays

Several types of EIA have been used, and these are

1. Competitive EIA for antigen.

Labeled antigen competes with unlabeled antigen for binding to a limited quantity of antibody. The antibody-bound antigen is separated from the free antigen by the use of solid phase antibody or a second antibody with specificity for the first. The enzyme activity in either the bound or free fraction is determined and related to the concentration of the unlabeled antigen.

2. Immunoenzymometric assay for antigen.

Antigen reacts with excess-labeled antibody and after incubation excess solid-phase antigen is added. The solid-phase antigen reacts with the free-labeled antibody remaining and, after separation of the solid-phase; the enzyme activity associated with soluble antigen is measured and related to the concentration of antigen.

3. Sandwich" EIA for antigen.

This procedure requires the antigen to have at least two binding sites. Antigen reacts with excess solid-phase antibody, and after incubation followed by washing, the bound antigen is treated with excess labeled antibody. After further washing the bound label is assayed, and this provides a direct measure of the amount of antigen present. This assay is analogous to the "two-site" immunoradiometric procedure. A variation of this method (double sandwich EIA) involves a third antibody. This antibody carries the label and reacts with unlabeled second antibody already bound to the antigen. As before, the amount of antigen is found by measuring the amount of bound label.

4. EIA for antibody.

Antibody binds to excess solid-phase antigen and, after incubation followed by washing, labeled second antibody with specificity for the first antibody is added. The bound label is assayed after further washing and it provides a direct measurement of the amount of specific antibody present. The system may also be used to assay antigens.

5. Immunoperoxidase assays (IPA)

Immunoperoxidase assays (IPA) have been used for the detection of antigens of different pathogens in tissue sections. In this test the enzyme labeled antibody was directly applied on tissue section. Then after washing the tissue is incubated in the appropriate enzyme substrate that creates colored reaction showing the presence of antigen. This test is used mostly in case of viral diseases because virus isolation from tissue samples is frequently unsuccessful due to autolysis of the tissues. It has advantage over immnuoflourescence technique because morphological features of cell as well as the specificity of immunologic reactions can be studied by conventional light microscopy.

6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay, also called ELISA, is a biochemical technique mainly used to detect the presence of an antibody or an antigen in the infected and suspected fish tissue and blood sera. This test is applied when other conventional tests fail to detect. Since its invention by Engvall and Perlman in 1971, the test is being widely used and even is a day-to-day test for diagnosis of diseases of human beings, animals, birds, and fishes. In ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substrate is added that the enzyme can convert to some detectable signal. Performing an ELISA involves at least one antibody with specificity for a particular antigen and this antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized the primary antibody is added, forming a complex with the antigen. The primary antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Older ELISAs utilize chromogenic substrates, but newer assays employ fluorogenic substrates enabling much higher sensitivity. This tests are particularly useful when analysing large numbers of samples and when extreme sensivity is required and since ELISA is a micrassay, the method is extremely economical in the use of reagents. It dose, however required specialised equipment (an ELISA reader) and sensivity is problem when tissue samples are used as source of antigen. This is usefull for the detection of clinical diseases but is limited in its application to sub-clinical infection, although in some case it has been reported to be as sensitive as IFAT. The ELISA is the first and widely used screening test for HIV.

Types of ELISA

Different types of ELISA are Direct ELISA, indirect ELISA (detect antibody in sample), sandwich ELISA (detect antigen in sample), Competitive, ELISA and Multiplex ELISA.

- Direct ELISA is a method of directly labeling the antibody itself. The microwell plates, which are coated with a sample containing the antigen and the labeled antibody, are quantitated using a chemiluminiscent, colorimetric or fluorescent end-point. This method avoids the secondary antibody to cross-react with the antigen sample components. However, direct ELISA consumes more time to carry out and requires expensive proposition.
- Indirect ELISA is a two-step method that uses a primary and secondary labeled antibody. The primary antibody is incubated with the antigen followed by the incubation of the secondary antibody. But, this may give a nonspecific signal result because cross-reaction with the secondary antibody may occur. This is done to quantify the antibody in the sera of infected and vaccinated animals.
- Sandwich ELISA measures the amount of antigens between a two given antibody layers. The antigens contain at least two antigenic spots since at least two antibodies act in the sandwich. In performing this assay, the first antibody is purified and bound to a solid phase attached to the bottom of the plate. Then an antigen is added and allowed to combine with the bound antibody while the unbound products are

removed. Finally, the second antibody is bound to the antigen completing the sandwich ELISA.

- Competitive ELISA is an assay that does not require a "matched pair" antibodies. In this method, one reagent must be paired to a detection enzyme (horseradish or peroxidase) and then the enzyme is linked to another antibody. With competitive ELISA an opposite relationship between the signal and the concentration of the analyte in the sample is obtained.
- Blocking/Inhibition ELISA is done to detect the antigen or antibody and is done as competitive ELISA, where the unknown antigen/antibodies are treated first followed by addition of standard antigen/antibody conjugated with enzyme. The binding of antigen/antibody is blocked by the presence of unknown antigen/antibody and a reduced color reaction is observed.
- In avidin-biotin ELISA the specific binding between avidin (an egg-white protein) and biotin (a water soluble vitamin) has been exploited. Avidin a tetramer containing four identical subunits, each of which contains a very high affinity-binding site for biotin. The binding is not disturbed by extremes of salt, PH or chaotropic agents such as guanidine hydrochloride. The avidin-biotin system is well suited for use as a bridging system in association with antigen-antibody reactions. The biotin molecule can be easily coupled to either antigens or antibodies and avidin can be conjugated to enzymes. This method is extremely sensitive as compared to other ELISA tests.
- In Dot- or paper ELISA an antigen/antibody is absorbed to the nitrocellulose paper and other steps are same like other ELISA methods.

7. Enzyme-linked immunosorbent spot (ELISPOT) assay

The Enzyme-linked immunosorbent spot (ELISPOT) assay is a common method for monitoring immune responses in humans and animals. It was developed by Cecil Czerkinsky in 1983. The ELISPOT assay is a modified version of the ELISA immunoassay. ELISPOT assays were originally developed to enumerate B cells secreting antigen-specific antibodies, and have subsequently been used for the identification and enumeration of cytokine-producing cells at the single cell level. The ELISPOT assay allows visualization of the secretory product of individual activated or responding cells. Each spot that develops in the assay represents a single reactive cell. Thus, the ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information.

8. Western Blotting

In this technique, the immunogenic component of different proteins of the pathogen is determined. The protein are separated on SDS-PAGE and then they are transferred onto the nitrocellulose paper either through diffusion (passive) or by means of electricity (active). The nitrocellulose paper is then processed as per dot-ELISA priniciple. In positive case, immuno dominat proteins are seen as coloured bands on nitrocellulose paper.

Enzyme Immunoassays in Aquaculture

One of the most urgent needs for aquaculture health management is establishment of standards for quantitative assessment of health status in the broad range of species under culture. For this enzyme immunoassays are the best methods for simple and predictive health tests. Besides screening for pathogens, techniques of immunoassays can be used to ascertaining other health parameters, including haematocrits, leucocrits, blood cell differentials, neutrophills oxidative radical production, Myeloperoxidase activity, phagocytic functions and to know the causative agents. Methods such as enzyme-linked immunosorbent assay (ELISA), ELISPOT can be used to accurately quantify numbers of

immunoglobulins or non-specific antibody-secreting cells and are used in immunodiagnostic.

At first in aquaculture ELISA was used to measure antibody titers in the serum samples and later monoclonal antibody based ELISA is used for the diagnosis of bacterial kidney disease and other several fish diseases. This technique has been found to be highly sensitive because very low quantity of antigen can be detected within few hours. Rapid identifications of *F. psychrophilum* and *F. branchiophilum* have been achieved by ELISA methods. The direct ELISA for the rapid detection of specific antibody has also been used and indirect ELISA was used for antibody detection test. Indirect ELISA has been used for successful detection of bacterial antigens in fish tissue and found to be more sensitive than conventional methods of detection of this organism. Enzyme immunoassay has already made a significant impact on disease management in aquaculture besides these immunoassays are use in expression and quantification of hormones and proteins, detection of pollutant in aquatic environment.

Conclusion

The knowledge about several enzyme immunoassays and their suitability in different laboratory and field condition as well as their accuracy for detecting causative agents are required diagnosis and better aquatic animal health management programmes. So the diseases surveillance and monitoring system can be done effectively to boost the aquaculture industry in the country.

ANTIOXIDANT DEFENCES AND FISH HEALTH

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It is needless to mention that for a better management of fish health it is essential to have absolute comprehension of fish physiology. In this respect, oxygen metabolism in aquatic organisms deserves special attention. Oxygen contain of aquatic ecosystem is a dynamic one and continuously influenced by biotic and abiotic factors. This considerably influences oxidative metabolism of aquatic organisms. Reactive oxygen species (ROS) are by-products of oxygen metabolism in all most all aerobes. Their metabolism is greatly influence by various internal and external factors. It is now well established fact that maintenance of redox homeostasis in cells is an index of good health. This needs a stringent regulatory mechanism for metabolism of reactive oxygen species. Fish is no exception to it. Being aquatic in nature, its chances for exposure to changing oxygen content of water, to various pathogens and finally to pollutants is much more than terrestrial species. Identification of abiotic and biotic agents that adversely affect metabolism of reactive oxygen species in fish and their proper and timely management may be a good alternative for better health of fish. For the purpose, it is necessary to have a good understanding of oxygen metabolism in aquatic organisms in general and ROS in particular. In addition, identification of early biochemical changes in this context deserves special attention because they may serve as bioindicators of health status of fish.

Although formation of earth occurred around 4.5 billion years back, aerobic organisms evolved around 500 million of years. All aerobic organisms whether they are aquatic or terrestrial in nature use oxygen for their respiration to generate energy. Cellular respiration takes place in mitochondria where oxygen is reduced to water by four complexes of electron transport system. During this process energy rich ATP molecules are synthesized by complex V. These complexes are situated in inner mitochondrial membrane. Electrons are flown to electron transport chain of mitochondria from NADPH and $FADH_2$ as by-products of tricarboxylic acid cycle or beta-oxidation of fatty acids. This Oxygen molecule is tetra-electronically reduced to water molecule in electron transport chain of mitochondria during cellular respiration. About 1-5% oxygen is not fully reduced during above biological process and is considered as progenitor of reactive oxygen species. Usually generation of ROS from oxygen occurs in complex I and complex III of electron transport chain of mitochondria. Besides, several biochemical reaction of different cellular components such as cytoplasm, microsomes, peroxisomes also contribute in formation of ROS.

Reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide are highly reactive in nature. Reactive oxygen species have four general characteristics. They are as follows: (i) highly reactive in nature, (ii) non-specificity regarding their substrates (iv) very short half life and finally (v) autocatalytic in nature. Being highly reactive and non specific in nature, it is presumed that ROS could oxidize any biological molecules present in their vicinity and affects its functional ability. They oxidize membrane lipids and form lipid peroxides. They form protein carbonyls from proteins. They also attack nucleic acid and may cause mutation or deletion. Such oxidation of biomolcules finally leads to impairment of cellular functions. A state defined as oxidative stress (OS). Aerobes have developed through evolution a defence mechanism to protect itself from dreadful effects of ROS. This defence system of aerobes is known as antioxidant defence system (ADS) which

comprises both enzymatic as well as non-enzymatic components. Enzymatic system contains a cascade of enzymes. The first member of this cascade is superoxide dismutase which dismutates superoxide radicals to hydrogen peroxide. Hydrogen peroxide is neutralized by two enzymes. One of them is catalase and the other one is glutathione peroxidise. Antioxidant enzymes are highly conserved in nature. They are reported from microorganisms to human beings. Although enzymatic antioxidant defence system is very effective, its concentration in cell is comparatively very low. Non-enzymatic system includes small molecules such as reduced glutathione (GSH), ascorbic acid, vitamin E, carotenoids, uric acid etc. Any process that maintains the redox environment of a cell or its compartments is defined as redox homeostasis. Maintenance of redox homeostasis is essential for maintaining health of an organism. When redox homeostasis of an organism is disturbed in favour of proxidant condition in aerobes either by increase production of ROS or failure of cellular antioxidant defences, it leads to diseases. . This happens to aerobes when they are exposed to pathogenic microbes or organisms or to environmental contaminants. In fact several pathophysiological states of different organs of mammals as well as fish are related to ROS directly or indirectly. Recently several studies have clearly demonstrated that ROS are also important for various cellular functions. Several physiological processes in cells are efficiently regulated by ROS. They play important role in fertilization, development, differentiation, apoptosis and aging processes of organisms. They protect cells from microorganism invasion. Their important roles in signal transduction and gene regulation by influencing transcriptional factors are now well established in mammals and gaining attention in aquatic organisms.

Although much information on antioxidant defences of mammals is known, our knowledge of antioxidant defences in fish with reference to fish health is scanty. Fish is an aquatic organism. It dwells in an ecosystem which has oxygen concentration around 30 times less in comparison to terrestrial animals. Therefore, it is believed that oxygen consumption and generation of ROS will be lesser in fish than terrestrial vertebrates under normal physiological conditions. Further, ambient oxygen concentration in water varies depending upon several biotic and abiotic factors such as phytoplanktons, temperature, salinity pH etc. Therefore, generation of ROS in fish is presumed to change with its oxygen metabolism depending upon the availability of environmental oxygen. Such change will influence fish health by modulating several biochemical pathways.

In the last two decades, several papers have published from different laboratories of India on antioxidant defences and oxidative stress in fish. They have enriched our knowledge on impact of pesticides, pollutant water or heavy metals on antioxidant defences and oxidative stress parameters of fish. However, not much information is available on Indian fish species regarding physiological significance of antioxidant defences relevant to development, differentiation, ecology. Also our knowledge on regulation of antioxidant defences in fish with reference to hormones is scanty. Similarly, not much is known about regulation of signal transduction pathway and regulation of various genes by ROS in fish. It will be interesting to know how fish maintains its redox homeostasis under different physiological and pathological states. Enrichment of our knowledge on antioxidant defences of fish will definitely help us in maintaining their health both in natural and culture conditions.

DNA SEQUENCING

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DNA sequencing refers to the structural determination of a given DNA fragment in terms of number of nucleotides. Basically, two methods are used for sequencing a DNA fragment. One is di-deoxy chain termination method by Sanger et al. and the other one is chemical degradation method by Maxam and Gilbert both developed in 1977. Traditionally, manual DNA sequencing has been performed based on the Sanger's ddNTP mediated chain termination method, which involves radioisotopic labeling. Over the past few years, novel DNA sequencing and automated detection methods involving replacement of radioisotope labeling with fluorescent dyes have been developed. These greatly reduce time and effort required for manual sequencing and data entry. Specific instruments are designed to perform the *in situ* imaging of fluorescent dye-labeled DNA fragments during electrophoresis. The following protocol is for sequencing DNA using a capillary based automated DNA sequencer.

General protocol for sequencing of double stranded DNA using BigDye Terminator V1.1/3.1 version.

Cycle sequencing reaction set- up

Ready reaction mix……..4µ
BigDye sequencing Buffer……..2µ
Primer……..3.2 pmol
Template……..200ng
Deionized water……..q.s.
Total Volume……...20 µl
Mix well and spin briefly.

- Place the tube in a thermal cycler and set to the correct volume.

	Temperature °C	Time
• Initial Denaturation	96	1 min
• Denaturation	96	10 sec
• Annealing	50	5sec
• Extention	60	4 min

- Repeat for 25 cycles and store the sample at 4°C till loading.

Purification of Extension product.
Ethanol/EDTA Precipitation.

2 µl of 125mM EDTA
62 µl of 100% Ethanol
16 µl of Deionized water
20 µl of PCR reaction product

Total 100 µl

- Gently vortex, Keep 15 min at room temperature and Centrifuge in 14000g for 20 min in 4°C
- Extract the supernatant leaving the Visible/ invisible pellet.
- Add 250 µl of 70% ethanol to the tube and vortex briefly.
- Centrifuge at 14000g for 10 min at 4°C.

- Aspirate the supernatant and dry the sample in vacuum for 10 min.
- Add 25 µl of Hi-Di-Formamide.
- Thoroughly vortex.
- Short Spin it.
- Take 12.5 µl in PCR tube.
- Heat in thermal cycler at 95 ^{o}C for 5 min.
- Quickly chilled the sample on ice.
- Load in Autosampler.

APPLICATION OF LACTIC ACID BACTERIA AS PROBIOTICS IN AQUACULTURE

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Introduction

Aquaculture is one of the fastest developing growth sectors in the world and Asia presently contributes about 90% to the global production. It is expanding into new areas, intensifying and diversifying through the trade of aquaculture commodities, live animals or products. Acknowledging the challenges that this relatively new industry may face in coming years, there is a need to prepare plan for its sustainable growth. For this purpose rapid conduction of experiments on fish, crustaceans and molluscs are going on globally to enhance their growth pattern, living environmental condition. However, disease outbreaks are constraint to aquaculture production thereby affecting both, economic development of the country and socio-economic status of the local people in many countries of Asia-Pacific region. Disease control in aquaculture industry has been achieved by following different methods using traditional ways, synthetic chemicals and antibiotics. However, the use of such expensive chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of residues, development of drug resistance, immunosuppressants and reduced consumer preference for aqua products treated with antibiotics and traditional methods are ineffective against controlling new diseases in large aquaculture systems (Sahu *et al.*, 2008; Watson *et al.*, 2008) .Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the aquaculture systems there by to maintain the health of the cultured organisms. In this context, microbial intervention can play a vital role in aquaculture production, and effective probiotic treatments may provide broad spectrum and greater nonspecific disease protection (Balcazar *et al.,* 2008; Flores**,** 2011). Hence, the research of probiotics for aquatic animals is increasing with the demand for environment friendly aquaculture and the use of microbial probiotics in aquaculture is now widely accepted.

The research of probiotics for aquatic animals is increasing with the demand for environment friendly aquaculture. The probiotics were defined as live microbial feed supplements that improve health of man and terrestrial livestock. The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continous intrusion of microbes coming from water and food.The first probiotics tested in fish were commercial preparations devised for land animals. Though some effects were observed with such preparations, the survical of these bacteria was uncertain in aquatic environment.Fish are continuously exposed to a wide range of microorganisms present in the environment.

Lactic acid bacteria

Lactic acid bacteria are characterized as Gram-positive, usually nonmotile, nonsporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism. Members of this group containing both rods and cocci and they are generally catalase-negative and they lack cytochromes. Lactic acid bacteria are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins.

Different species of lactic acid bacteria (LAB) have adapted to grow under widely different environmental conditions and they are wide spread in nature.LAB are commonly found in the gastrointestinal tract of various endothermic animals and their role within the digestive tract has been extensively investigated and reviewed but only few studies have demonstrated that LAB are part of the normal intestinal microbiota in fish. A better understanding of factors affecting the population level of lactic acid bacteria in the digestive tract will be of interest for commercial aquaculture.

Lactic acid bacteria in the gastrointestinal tract of fish

The gut is sterile until hatching but soon after hatching, the fish comes in contact with the environment and live food that leads to successive colonization by a variety of microbes.It is generally considered that Gram-positive bacteria including lactic acid bacteria are numerically dominant members of the normal microbiota in the gastrointestinal tract of endothermic animals at their early life stage.However, only very few investigations have isolated lactic acid bacteria from the gastrointestinal tract of larval and juvenile fish.

Lactobacilli have complex nutritional requirements, needing to be supplied with carbohydrates, amino acids, peptides, fatty acids or fatty acid esters, nucleic acid derivates and vitamins. However, identification of these bacteria with a large number of tests is tedious and time consuming.This may be the probable reason why *lactobacillus* spp. Isolated from fish intestine seldom are identified to species level.It is reported that lactic acid bacteria are part of the normal microbiota of Cyprinidae, Escocidae and Percidae and that lactic acid bacteria are present in several fish species at larval, fry and fingerling stages. Seasonal variations of lactic acid bacteria in fingerlings of pond fish also exhibited in *Cyprinus carpio*, *Aristichthys nobilis* and *Hypophthalmichthus molitrix*, with highest population in the middle of summer. Today, it is well documented that lactobacilli are part of the native intestinal microbiota of Atlantic cod, Atlantic salmon, Rainbow trout, Wolffish and Arctic char.

Although some lactic acid bacteria strains can colonise the digestive tract of larvae and fry, living bacteria from the diet may be lost from the digestive tract within few days after the intake has stopped.This has not been well established and further research is needed.Other lactic acid bacteria like *streptococcus* spp. and *leuconostoc* spp. Have also been reported to be isolated from gastrointestinal tracts of different fishes.

Factors affecting lactic acid bacteria in the gastrointestinal tract

Although some lactic acid bacteria strains can colonize the digestive tract of the host, from feed and food preparations, are in most cases lost from the gastrointestinal tract within a few days after the intake has stopped. Those lactic acid bacterial strains those are normal inhabitants of the digestive tract, must possess the ability to survive in this environment and must be able to adhere to the exposed surface of the epithelial cells.

Dietary poly unsaturated fatty acids affect the attachment sites by modifying fatty acid composition of the intestinal wall and help adherance of lactobacilli to the intestinal wall. Similarly, chromic oxide helps in maintaining lactic acid bacteria population there by declining Gram –ve bacteria. Salinity and stress too play roles in determining the populations of such bacteria in fish intestinal tract.

Mode of action

The term "probiotics" has been used in several ways and it was first used by Lilly and Stillwell(1965) who proposed the definition " Substances produced by one protozoan

that stimulated the growth of another". Later parker (1974) defined probiotics as " organisms and substances which contribute to the intestinal balance" but fuller (1989) gave a more precise definition " A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". The beneficial effects of probiotics are well established for terrestrial animals, whose flora is composed of about 90% of facultative lactic-acid producing bacteria.

Colonisation is a way where in a probiotic microbe can persist for a long time due to a multiplication rate higher than the expulsion rate. Lactic acid bacteria colonize very early in the gastrointestinal tract of terrestrial animals and they belong to their dominant or subdominant flora. It is possible to induce an artificial dominance of *lactobacillus* by adding a strain into the enrichment medium of live food organism.Even this can be achieved by incorporating the probiotic to feed.

Adhesion is acknowledged as the first step of a microorganism in the process of colonization; therefore, it is essential that lactobacilli chosen for probiotic preparations should adhere well to the gastrointestinal epithelium. Adhesion of lactic acid bacteria in endothermic animals is complicated by the fact that they have been shown to exhibit host specificity in the gastrointestinal tract. Not much is known of the adhesions involved in attachment of lactic acid bacteria to the tissues of the gastrointestinal tract of fish. However, *in vitro* tests indicate that *Carnobacterium* sp. are able to adhere to intestinal mucous of rainbow trout, but no specific adhesion mechanism is reported to be involved.Competition for adhesion receptors with pathogens might be the first probiotic effect.

Colonisation of the fish gigestive tract by bacteria capable of producing a lactic acid fermentation, may inhibit the proliferation of putrefactive microbes in that site, thus protecting the host from diseases caused by toxinx generated by proteolytic bacteria. Lactobacilli can produce compounds that inhibit the growth of microorganisms. Bacteriocins are the specific compounds but hydrogen peroxide and organic acids including lactic acid are also effective. Bacteriocins are bactericidal or bacteriostatic peptides that are mostly active against bacteria closely related to the producer but reports are there to indicate that some bacteriocins also act against gram negative pathogens.

The effect of lactic acid bacteria as growth promotor in fish has also been studied.The possible competition for nutrients with pathogens in the digestive tract or the hypothetical stimulation of the immune system are the other factors in favour of lactic acid bacteria as fish probiotics.

Lactic Acid Bacteria as Immunostimulants in fish:

It has been found that (Gatesoupe, 1994) the *Lactobacillus* or *Carnobacterium* strain isolated from rotifers increased the resistance of turbot larvae against a pathogenic *Vibrio* sp. Further, Gildberg et al.(1997) demonstrated that *Carnobacterium divergens* decreased the mortality rate of atlantic cod fry challenged with *Vibrio anguillarum* but not the mortality of salmon fry challenged with *Aeromonas hydrophila*. Robertson et al (2000) reported that another strain of Carnobacterium, administered to fingerlings and fry of Atlantic salmon, reduced the mortality caused by Aeromonas salmonicida, Vibrio ordallii, and Yersinia ruckeri but not V.anguillarum.

It has also been reported that probiotics supplementation in feed has been able to enhance non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of resistance to *E.tarda* infection in *Oreochromis niloticus*.

Villamil et al.,(2002) had studied for the first time the effects of several lactic acid bacteria on the nonspecific immune response of turbot (*Scophthalmus maximus*). In the case of *Lactococcus lactis*, in vivo experiments to determine its effect on nonspecific immune functions were also performrd, as well as experiments to determine its capacity for binding turbot intestinal mucus.

The use of lactic acid bacteria in fish culture has been associated in some cases with an improvement in disease resistance and fish survival; however, the immune mechanisms that may be related to this process was rarely studied.It was observed that *Lactococcus lactis*, an exogenous lactic acid bacteria, is able to modulate the turbot immune system both in vitro and in vivo.

There are few studies on the suitability of dairy and human derived lactic acid bacteria as fish probiotics with varying degree of success.However while all such studies reflect upon the acquiring of abilities to withstand challenge to some fish pathogens, emphasis on detailed information on immunomodulation is lacking. Although the role of Lactic Acid Bacteria as immune modulators improving non specific defenses is well known for mammals, this role, as far as is known, still has to be shown for fish in many more trials.

Selection of probiotics

The principal purpose of the use of probiotics is to produce a proper relationship between useful microorganisms and the pathogenic microflora of digestive organs and their environment. Hence, a successful probiotic is expected to have a few specific properties.

1. Antagonism to pathogens, which is one property of probiotic bacteria. Probiotic should stimulate the immunity of the host by increasing the number of erythrocytes, macrophages and lymphocytes. One sign of antagonistic properties against bacteria is the production of antimicrobial substances such as organic acids, hydrogen peroxide, siderophores and lysozyme.
2. Benefits to the host animals in some ways. In order to have a beneficial effect in the form of a growth promoter or to protect fish against bacterial pathogens, the strains should produce important substances, for example vitamins such as biotin and vitamn B12.
3. The capability of surviving or colonizing the gut of an aquatic organism by adhesion.Similarly, the presence of a dominant bacterial strain in high densities in culture water indicates its ability to grow successfully under the general conditions, and one can expect that this strain will compete efficiently for nutrients with possibly harmful strains.
4. Adhesion is one of the most important selection criteria for probiotic bacteria because it is considered a prerequisite for colonization.
5. Applied microorganisms should be stable for long periods under storage as well as in field conditions.
6. Probiotic microorganisms will, of course, have to be non-pathogenic and nontoxic in order to avoid undesirable side-effects when administered to aquatic organisms.
7. Probiotics should be of animal species origin. This criterion is based on ecological reasons, and takes into consideration the original habitat of the selected bacteria. Many workers believe these bacteria have a better chance of out-competing resident bacteria and establishing themselves at a numerically significant level in their new host. In addition, the existence of a dominant bacterial strain in high densities in

culture water indicates its ability to grow successfully under the prevailing conditions, and one can expect that this strain will compete efficiently for nutrients with possible harmful strains.

Conclusion

In particular, there are a very few reports on the presence of *Lactobacillus* in the intestines of freshwater fish inhabiting river environments and from fish reared in aquaculture units with water recirculation systems, whereas other groups of bacteria have been better studied. Knowledge on the presence of *Lactobacillus* as a natural flora in fish intestines may lead to further applications to improve health of edible species (Bucio *et al*, 2006). Moreover the use of commercial probiotics in fish is relatively ineffective as most commercial preparations are based on strains isolated from non-fish sources that are unable to survive or remain viable at high cell density in the intestinal environment of fish during the active growth phase of the fish. Hence, there is elegant logic in isolating putative probiotics from the host in which the probiotic is intended for use. Such strains should perform better because they have already adhered to the gut wall of the fish and, thus, are well-adapted to compete with pathogens (Ghosh *et al*,2007). Probiotic strains that are already adapted, through natural processes, to the dynamics of an aquaculture production system will probably lessen any farm management environmental manipulation practices required to achieve the desired probiotic effect in the final product. Introducing such specifically intended probiotics is bound to favour an increase in the application of probiotics.

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IMMUNE FUNCTIONS IN CRUSTACEANS

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Introduction

The immune system of crustaceans has been studied in detail in only a limited number of species. There are some immune response systems that have been investigated intensively, such as the prophenoloxidase activating system (proPO), clotting, and phagocytosis. These investigations have led to unifying concepts based on molecular data from a variety of species. However, a comprehensive mechanistic approach to unravel the defense system of invertebrates is only possible when complete genomes, expressed genes and suitable disease challenge models are available. The crustaceans belong to the Arthropoda, which includes the insects. Genomes of two representatives of the latter namely the fruit fly (*Drosophila melanogaster*) and the mosquito (*Anopheles gambiae)* has been completely sequenced. The availability of the complete genome now allows a genome wide analysis of the immune responses in these insect species using standardized pathogen challenges. Expression profiling of immune responses in insects may be instrumental in unraveling more expediently the immune functions in crustaceans, especially of *Penaeus monodon,* as it has been suggested that this species is closely related to insects.

Identification of immune response genes in *Drosophila* and *Anopheles* has been performed in several ways. One approach uses cell lines, which are treated with LPS. RNA is extracted from the treated and untreated cell cultures at different time points. RNA is converted into cDNA and at the same time labeled with fluorescent dyes. The labeled cDNA and at the same time labeled with fluorescent dyes. The labeled cDNA pools are hybridized with a micro-array, containing all expressed genes of the organism used in the experiment. After hybridization, the micro arrays are washed and the relative fluorescence of each gene is determined. In this way gene expression can be determined as unaffected, up regulated or down regulated compared to the control treatment. The other makes use of *in vivo* challenges with pathogenic micro organisms, such as Gram positive, Gram-negative bacteria or a fungus. These experiments have lead to the identification of different immune genes involved in the responses to different pathogen challenges.

Expression profiling after challenge with gram-positive and gram-negative bacteria, and fungi identified two major pathways: Toll and Immunodeficiency (Imd). These pathways in insects comprise four steps 1. recognition, 2. regulation, 3. intracellular signal transduction, and 4. cellular and humoral responses. These pathways, including the cellular and humoral immune responses have been reviewed in detail. The first step involves Pattern Recognition Receptors (PRR), which have been identified throughout the animal kingdom and play an important role as they discriminate between self and infectious non-self. These receptors recognize unique conserved molecular patterns, such as lipopolysaccharides and peptidoglycans, found only in bacterial and fungal pathogens. The regulation of the recognition is facilitated and controlled mainly by activating and inhibitory CLIP domain serine protease. Intra-cellular signal transduction is highly conserved and comprises protein interactions similar to the NFKB signal pathway found in vertebrates. The protein modifications and interactions will eventually lead to the release of transcription factors, which are translocated to the nucleus and drive the expression of immune response

genes. Depending on the pathway used the response will either be the production of antimicrobial or antifungal peptides humoral response or activation of hemocytes (cellular response). However, to some degree, there is crosstalk between the two pathways.

Haemocytes

Crustacea have an open circulatory system with no equivalents to vertebrate red blood cells, but analogues of the white blood cells appear to exist. Crustacean haemocytes play an important role in the host immune responses including self/non-self recognition, phagocytosis, encapsulation, melanisation, cytotoxicity and cell-cell communication. The invertebrate blood cells collectively called as haemocytes or ceolomocytes were broadly classified in general, into progenitor cells, that are small, ranging from 4-10 µm in diameter with high nuclear cytoplasmic ratio; phagocytic cells, whose main function is phagocytosis of non-self material invading the host; homostatic cells, that participate in blood coagulation and wound sealing; Nutritive cells, that play an important role in nutrition and Pigmented cells, that are assumed to have respiratory pigments like haemocyanin. Percoll density gradient haemocyte population separation techniques followed by Soderhall and Smith (1983) in some decapod crustaceans paved the way for precise classification and characterization of crustacean haemocytes based on their morphological criteria into Granular, Semigranular and Hyaline cells. Similar classifications were made in urochordates, echinodermates, molluscs.

Martin and Graves (1985) studied fine structure and classification of penaeid shrimp haemocytes showing the morphology of hyaline cells with absence of any granules, although cytoplasmic inclusions of unknown origin was noticed. Similar findings were documented in palaemonid prawns by Tsing *et al.*, (1989). The hyaline cells of crustaceans were shown to have properties that readily attach and spread extensively on glass surfaces and phagocytose.

The semigranular cells contain a number of small granules, which upon activation lyse rapidly and release their contents *in vitro*. They concluded that the semigranular cell is responsible for recognizing and responding to foreign molecules and particles by de-granulation and subsequently attaching and spreading on the foreign surface in crustacea. Further the de-granulation response of semigranular cells could be induced by microbial lipopolysaccharides and β-1,3-glucan. Besides this, the encapsulation reaction was also found to be engaged by the semigranular cells.

The granular cell is filled with fairly large granules and its main function seems to be a repository for the prophenoloxidase activating system in freshwater crayfish,. The granular cell can be triggered to undergo exocytosis and release of the proPO system by two endogenous proteins, namely, the 76 kDa factor and the β-1, 3-glucan binding protein. Hose *et al.,* (1987) studied the shrimp haemocytes cytologically to show granular and semigranular cells to contain granules of proPO and peroxinectin. Division of circulatory haemocytes in *Penaeus paelensis, Macrobrachium rosenbergii, M. acanthurus* was observed. Thus, the new haemocytes need to be compensatory and proportionally produced and it is commonly believed that haemocytes are released continuously, although at varying rates, from a specialized haematopoietic tissue identified in several crustaceans like penaeid shrimp, *Sycionia ingentis* and lobster, *Homarus americanus*. In many crustacea, the sheet like haematopoietic tissue is situated on and covers the dorsal and dorso-ventral sides of the stomach and is surrounded by connective tissue.

Most invertebrate groups have a variety of fixed haemocytes that may play some role in the humoral and cellular defense mechanisms. Those include reticulum cells, podocytes,

pore cells, and sinus lining cells of gastropod molluscs and nephrocytes of crustaceans. Crustacean nephrocytes have been shown to be involved in the sequestration of soluble and small particulate materials from the blood. Following injection with bacteria, the gill nephrocytes become filled with debris and there was a marked increase in the number of pinocytotic vesicles.

Phagocytosis

Phagocytosis is the most common of the cellular immune mechanisms and together with natural humoral factors, undoubtedly forms the first line of defence, once the physico-chemical barriers have been breached and hemolymph coagulation and blood cell clotting have been circumvented. Crustaceans have been shown to be capable of overcoming infection by the clearance of foreign materials from the hemolymph. This was thought due to phagocytosis by the circulatory cells, followed by accumulation of the haemocytes in the capillary and lacunar networks, where a system of fixed phagocytes further participate in the degradation of infective particles. Regarding the process of phagocytosis to remove a wide range of bacteria, virus, parasite and fungi, chemotaxis, attachment, ingestion and killing stages can be identified in invertebrates. Several studies have been done on this phenomenon in different crustacea. Phagocytosis by separated haemocyte populations *in vitro* has been examined in the freshwater crayfish, in which the hyaline and the semigranular cells were phagocytic, where as in case of crabs it is restricted to the hyaline cells. In shrimp it is accompanied by the granulocytes, primarily the small granular haemocytes.

Using non-separated haemocytes in crustaceans, phagocytic rates ranging from 1-2% to 28% have been recorded. Soderhall *et al.,* (1986) reported phagocytosis from the isolated haemocytes of *Carcinus maenas.* In a detailed study of phagocytosis in the freshwater crayfish, *Parachaeraps bicarinatus,* it was found that *in vitro* phagocytosis of erythrocytes by haemocytes of the cray fish, required specific opsonins. These opsonins appeared to be haemagglutinins, which enhanced adhesion of erythrocytes to haemocytes.. A variety of vaccines from other gram-negative bacteria or Lipopolysaccharide endotoxins also increased resistance to Pseudomonas infection. The immunity was ascribed to a change in activity of phagocytic cells.

Efficient uptake of bacteria was dependant upon factor(s) present in the haemolymph. Smith and Soderhall (1983b) treated haemocyte monolayer with β-1, 3-glucan, a trigger of proPO system activation, to show five to seven times higher degree of phagocytosis than untreated control monolayer. This particular finding shows evidence for the involvement of proPO system in phagocytosis. Tyson and Jenkin (1973) have shown that removal of bacteria form the circulation of the freshwater crayfish *P. bicarinatus* follows an exponential curve and is dependant on the presence of opsonic factors in the haemolymph. Later on Soderhall *et al.,* (1986) using isolated hyaline cells from *C. maenas,* showed that the phagocytic rate increased three times by opsonic factors present in the haemocyte lysate. Opsonic factors in crustaceans are thought to be either the products of proPO activation or agglutinins, specifically lectins. These opsonic factors were only produced if the proPO system in the haemocyte lysate induced to its active form. Phenoloxidase as such appeared to lack opsonic properties. The release of proPO granules is thought to be the initial event in recognition of the non-self particle and activation of phagocytes.

The phagocytic defence reactions of the shore crab, *C. maenas* were studied following injections of the bacteria, *Bacillus cereus* and *Morexella* sp., by histological and ultrastructural examination of the gills, heart and hepatopancreas. The *in vitro* phagocytosis of giant

freshwater prawn, *M. rosenbergii* were studied following administration of Lipopolysaccharide (LPS) showed all the three types of haemocytes are able to phagocytose foreign particles.

During phagocytosis, particles or microorganisms are internalized into the cell, which later forms a digestive vacuole called the phagosome. The elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome and the generation of Reactive Oxygen Intermediaries (ROIs) like O-2 subsequently releasing hydrogen peroxide (H2O2), hydroxyl radicals (OH-) and singlet oxygen (1O2) through the process of respiratory burst (Rodriguez and Moullac, 2000). Hydrogen peroxide can be converted to hypochlorous acid (HOCl-) via the myeloperoxidase (MPO)-H2O2-CL system, forming a potent antibacterial system. Bell and Smith (1993), showing the O-2 generation from hyaline cells using phorbol myristate acetate (PMA) as elicitor, gave the first evidence of ROIs production in crustacean haemocytes.

Nodule Formation

Nodule formation has been reported in most of the invertibrates including crustaceans, when microbial invasion is far in excess of the phagocytic capabilities of the host. When the haemocoel/coelom is invaded by numbers of microorganisms in excess of those that can be effectively cleared by phagocytosis along, nodule or cell clump formation occurs in many invertebrates, thus parasites being entrapped in masses of phagocytic or nonphagocytic blood cells which may or may not, depending on the animal involved, become melanised and effectively kill the parasites. The bacteria were found adhering outside to the granular haemocytes, which then aggregate to form cell clumps that subsequently enlarge by the addition of haemocytes, forming concentric flattened layers around a central, compact, often necrotic and sometimes melanised core.

Encapsulation

Haemocytes encapsulations by free circulating cells are stimulated to adhere to substratum and to each other, when a large parasite is encountered. Larger entities are encapsulated by haemocytes that attach and spread over them and eventually build up to several layers of cells. Thus sealed off from the circulation.

In addition to phagocytosis and nodule formation, invertebrate haemocytes are capable of immobilizing or killing the parasites, such as cestodes, trematodes, nematodes, parasitoides, fungi and large protozoans that are too large to be ingested by a single blood cell by surrounding them with multicellular sheaths. Person *et al.,* (1987b) have demonstrated in crayfish *Astacus leptodactylus* that, *in vitro* encapsulation was carried out by semigranular cells on a variety of foreign particles tested, regardless of their origin and surface charge and granular cells on fungal spores. Similarly Azad *et al.,* (1995) have studied that the most of the crustaceans semi-granular haemocytes are the first to react to a foreign particles or organism by encapsulation. However, the hyaline cells could not be noticed to be involved in the defense reaction. It has been suggested that the components produced in the process of activation of the prophenoloxidase system are involved in the encapsulation response in arthropods. Several groups have found that substances, which elicit proPO activation in arthropods, also enhance phagocytosis in *vitro* (Smith and Soderhal, 1983a; Ratcliffe *et al.,* 1984; Leonard *et al.,* 1985) and that a haemocyte lysate supernatant in which the proPO system is activated, has encapsulation promoting activity *in vivo.* An encapsulation-promoting factor has been purified from haemocyte lysae of crayfish *Pacifastacus leniusculus* and found to be a 76 KD protein, acts as a binding factors in the

encapsulation process, released from degranulation of haemocytes upon activation by non-self materials such as proPO elicitors in the haemolymph.

Cytotoxicity Reactions

Cytotoxicity is the mechanism by which some cells of the haemocytes specialized to kill the target organisms by their toxic properties (Azad *et al.,* 1995). Yamazaki *et al.,* (1984,1985) recorded first cytotoxic activity in the early stages of an invertebrate life cycle relates to aplysianin E, which lyses murine and human tumour cells.

Haemocytes from the crayfish *Parachaeraps biraniatus* have been found to be cytotoxic for various tumour cell lines. In another study Soderhall *et al.,* (1985) reported crayfish *Astacus astacus* haemocytes displaying cytotoxic capacity towards all tested mammalian tumour and non-tumour cell lines. Separating and isolating the haemocyte populations of crayfish, Soderhall *et al.,* (1985) demonstrated that the semigranular and granular cells retained their cytotoxic capacity *in vivo.*

Superoxide anion production

Many oxygen compounds are toxic to living cells. In order to protect itself from toxic oxygen compounds formed in the oxygen metabolism, every cell must contains systems that deal with the undesired and potentially dangerous product's formed. The most important reactive oxygen species (ROS) can be said to be the superoxide ion radical O-2, hydrogen peroxide H_2O_2 , and the hydroxyl radical OH.

The reactive properties of oxygen compounds are not simply harmful; if directed at right target, they can also be used in the destruction of foreign material. It has long been accepted that a so-called oxidative burst with raised oxygen consumption occurs in reaction towards foreign material, in human as well as in plant. The membrane-bound enzyme complex, NADPH oxidase, will assemble after binding of the cell to foreign material and reduce molecular oxygen of superoxide. Superoxide production *in vitro* has been reported in hyaline cells of the shore crab *C. Maenas.* In, Drosophila, elevated superoxide levels during melanotic encapsulation of parasites have been reported and in the pond snail *Lymnaea stagnalis,* haemocytes have been reported to mediate cytotoxicity through production to reactive oxygen intermediates.

The superoxide anion O_2- can easily form in reactions where molecular oxygen is present, and is scavenged by the enzyme Superoxide dismutase (SOD) to form hydrogen peroxide and O_2.

Superoxide dismutase : $2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Catalase : $2\ H_2O_2 . \rightarrow 2\ H_2O + O_2$

Peroxidase : $H_2O_2 + A\ H_2 \rightarrow 2\ H_2O + A.$

Prophenoloxidase (proPO) System

One of the hallmarks of the crustacean immune system is the prophenoloxidase (proPO) enzyme cascade. Despite the primitive phylogenetic nature of crustaceans, they possess the complex and effective mechanism like proPO system for eliminating pathogens. Recent *in vitro* researches have shown that the phenoloxidase (PO) activating system and associated factors of proPo system are important mediators in crustacean immunity.

Prophenolxidase system acts as a major recognition and defence pathway in crustaceans and insects. The melanisation reaction, which is a common response to parasite entry in vertebrates, especially arthropods, is due to the activity of an oxidoreductase, phenoloxidase (PO). The enzyme is a part of a complex system of proteinases, pattern

recognition proteins and proteinase inhibitors constituting the so-called prophenoloxidase (proPO) system. The activation of proPO system results in the production of various proteins, including PO, which participate in melanisation around the parasite, coagulation, opsonisation of foreign materials and direct microbial killing. Furthermore, the proPO system has been proposed as invertebrate counterpart of the vertebrate complement system since it can be activated by β-1, 3-glucan and has got cascade reaction involving proteinases.

PO is a bifunctional copper containing terminal enzyme of the whole system, which catalyses both the O-hydroxylation of monophenols and the oxidation of phenols to quinones, thus converting tyrosine to dihydroxyphenylalanine (DOPA), as well as DOPA to DOPA-quinone, followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment. A 30 kD PO has been purified and characterized in *Penaeus setiferus* by affinity chromatography. Further, a purified a 76 kD glycoprotein proenzyme, proPO, from the blood cells of the crayfish. However, the conversion of inactive proPO into active PO needs a serine proteinase, named the prophenoloxidase-activating enzyme (ppA), which has been isolated and purified from several arthropods, viz., *Bombyx mori* and crayfish.

As early as 1977, Unestam and Soderhall showed that PO activity could be strongly activated from its inactive from, proPO to active PO by β-1, 3-glucans from fungal cell walls. Ochiai and Ashida (1988) purified a β-1, 3-glucan-recognition protein that when coupled with ligand activates the proPO system in silkworm plasma. Similar observations were recorded in purified β-1, 3-glucan binding proteins of crayfish, *Pacifastacus leniusculus*. In both crustaceans and insects, the proPO system can be specifically activated by β-1, 3-glucan, which are surface components of fungal hyphae. Johansson and Soderhall (1985) concluded that in crayfish, *Pacifastacus leniusculus,* lipopolysaccharide (LPS) serves as an indicator of Gram-negative bacteria and β-1, 3-glucan as an indicator of fungi, that elicits both the exocytotic release of the proPO system from the semigranular cells and the subsequent biochemical activation of this system. It was also shown that microbial cell wall-derived LPS could also initiate the cascade in the crayfish, *Astacus astacus*, in *Procambarus clarkii,* induced by LPS, glycolipids, zymogen A and trypsin, by trypsin in *Carcinus maenas* and in *Pacifastacus leniusculus*. Aspan and Soderhall (1991) concluded that crayfish proPO could be converted to active form, the terminal enzyme in the proPO activating cascade by an apparent proteolytic cleavage, not only by a commercial proteinase, but also an endogenous serine type proteinase. The evidence that the proPO system generates non-self recognition factors in arthropods is confined to two studies with the crustaceans, *Astacus astacus* and *Carcinus maenas*. Smith and Soderhall (1983b) have suggested the attaching proteins of the proPO cascade are strong non-self signals for the haemocytes, causing them to degranulate and release previously cell-bound recognition factors into the haemolymph, where they are free to trigger activation of adjacent haemocytes.

Humoral Defence Factors

Hydrogen peroxide is scavenged by catalase enzyme to form water and oxygen and by peroxidase in the presence of reducing agent. In human neutrophils, myeloperoxidase is a heam containing protein present during phagocytosis, will form hypochlorous acid (HOCl-) from hydrogen peroxide and chloride ions. HOCl will then destroy the ingested microbes. Eosinophilic peroxidase is a related protein involved in defence against larger parasites. Two peroxidase of this family have been found in Drosophila, where one of these, peroxidasin, has some peculiar properties characteristic of extra cellular matrix protein: The peroxidase domain is combined with six leucin-rich regions, four Ig loops, a thrombospondin/per collagen homology and an amphipatic α-helix. Another haem dependant peroxidase family is the cytochrome c-like peroxidase. Glutathione peroxidase

and catalase are other type of enzymes that scavenge hydrogen peroxidase. Invertebrates, although lack immunoglobulins, have a range of factors that mediate agglutinating, lytic and antimicrobial activities against various biological agents, These factors may be naturally occurring and/or formed after pathogenic stimulation, but generally do not show the amnestic properties of the immunoglobulin as in case of vertebrates.

Lectins/Agglutinins/Haemagglutinins

Lectins/agglutinins are in general multivalent, or at least bivalent, carbohydrate-specific-binding ubiquitous proteins or glycoproteins usually without catalytic activity having various biological applications including cell sorting, cellular and humoral immune recognition mechanisms, immunomodulation and ability to agglutinate erythrocytes (haemagglutinin), bacteria and other normal and malignant cells. Ratanapo and Chulavatnatol (1992) reported the purification of monodin, a sialic acid- specific lectin from *P. monodon* was found to induce the agglutination of *Vibrio Vulnificus,* a major infective bacterium of the prawn. Haemolymph of the crab, *Scylla serrata* contains a lectin specific for N-glycolyl-neuraminic acid and was able to agglutinate when mammalian erythrocytes was used as a pathogen model. Bachere *et al.,* (1995) purified a lectin similar to monodin of *P. monodon* in the semi-granular haemocytes from *P. japonicus* using monoclonal antibodies.

Limulin, a sialic acid-specific lectin from the horseshoe crab, *Limulus polyphemus* (Chelicerata), reported to possess cytolytic-activity indicating an additional role for lectin invertebrates. Dyrynda *et al.,*(1995) reported cytotoxic activity in zoanthid, echinoderm , and eggs of opisthobranch molluscs and tunicate larvae.

In vitro studies have shown that invertebrate agglutinins facilitate phagocytosis by opsonisation of the non-self particles (McKay and Jenkin, 1970b). Arason (1996) has emphasized the possible role of lectins as non-self recognition molecules in vertebrate and invertebrate immunity. Results of Vazquez *et al.,* (1997) suggested that there is an active participation of sialic acid-specific lectin from the freshwater prawn *Macrobrachium rosenbergii* haemocytes in the recognition of non-self cells for active phagocytosis.

Haemolysins

Investigations on the lytic systems of invertebrates have concentrated chiefly on their ability to lyse vertebrate erythrocytes *in vitro.* Naturally occurring haemolysins have been found in a number of invertebrates, including molluscs and crustaceans.

Lysozyme-like activity

Primary immune response in crustaceans is non-specific cellular immunity. Haemocytes play a crucial role in this immune response because of their participation in phagocytosis, encapsulation, nodule formation, and cytotoxic mediation. Phagocytosis is the most common cellular defence reaction and in combination with humoral components, it consists the first line of defense against parasites or other intruders that evade the physicochemical barrier of the cuticle. Franchini and Ottaviani (1990) have shown that phagocytes from the Cray fish, *Procambarus clarki,* produce lysosomal enzymes, which efficiently degrade and remove foreign material. Haemocyte phagocytosis in a number of animal species has only been proved indirectly by the detection of phagocytosis related lysosomal enzymes such as α-naphthyl acetate esterase, β-glucuronidase, and acid phosphatase.

Among the known antimicrobial peptides secreted by insects cell lines, lysozymes hydrolysed the β-1, 4 glycosidic bond between N-acetyl muramic acid and N-acetyl

glucosamine in the peptidoglycan layer of the bacteria cell envelop. In Gram-positive organisms such as *Micrococcus luteus* , the peptidoglycan layer is found at the surface of the cell and varies considerably among species . Among Gram-positive organisms, the cell wall of *M. luteus* is more susceptible to degradation by lysozyme than that of other bacteria with which it has been compared. A lysozyme enzyme was purified and its properties were documented in an insect *Hyalophora cecropia*. Brewers yeast is a source of nucleic acids and polysaccharides including glucan; β-1, 3-glucans have been recognized to effectively enhance immune function of many aquaculture species including tiger shrimp *P. monodon* and rainbow trout.

Antimicrobial Factors

Antimicrobial factors that contain peptides, proteinases, defensins, cecropins and other antibacterial compounds appear to be ubiquitous and multipotent components of the innate immune defense arsenal used by both prokaryotic and eukaryotic organisms. They may either bring about the killing/ lysis of bacteria (bactericidal or bacteriolytic factors) or may act as a general disinfectant in inhibiting the growth (bacteriostatic). Initially crayfish serum and haemolymph when tested against marine and terrestrial bacteria for the presence of bacteriocins, no bacterial killing was noticed. Later Nakamura *et al.,* (1988) purified and studied the structure of tachyplesin, a class of antimicrobial peptide from the haemocytes of the horseshoe crab *Tachypleus tridentatus* that inhibits the growth of both Gram-positive and Gram-negative bacteria at low concentrations and formed a complex with bacterial lipopolysaccharide. An antimicrobial chitin-binding protein from horseshoe crab has been purified and christened tachycitin that has got growth inhibitory action on both Gram-positive and Gram-negative bacteria The penaeid shrimps possess penaeidins, peptides that combine with a proline rich amino terminal domain and a carboxyl domain containing six cysteines engaged in three disulfide bridges.

Decapod crustaceans have the capability of rapidly clearing invading bacteria from their haemolymph. It has been reported that the Shore crab, Lobsters, and penaeid shrimps removing in excess of 75% of bacterial cells within 10 min. to 1 hr. after injection. Both humoral & cellular responses are thought to be involved in this clearance reaction. Crustaceans possess a wide variety of non-cellular factors that are both naturally occurring, inducible, bioactive molecules e.g. agglutinin, killing factors, Lysins, precipitins, and clotting agents . Some antibacterial factors have been found in Lobster plasma, such as bacteriocidin, which were perceived as being effective against a variety of bacteria in lobsters.

Other plasmic proteins

Apart from haemocyanin, the respiratory protein which is quantitatively the most important of the circulating protein in crustaceans. Other similar kinds of immunopotential proteins are also reported. A α2- macroglobulin (α2-M)-like protein has been identified in the shrimp using Western-blot assay in crayfish and purified by the shrimp-specific monoclonal antibody (Mab) (41B12). Shrimp α-2M-like protein appears to be dimmer of 340kD. In addition to the plasma, this protein is localized in the membrane of all haemocytes types and in large vesicular inclusions of the hyaline and semi-granular cells.

Pattern Recognition Receptors

Several pattern recognition receptors (PRRs) have been identified in crustaceans, most of them related to β-glucan recognition proteins. In the freshwater crayfish *Pacifastacus leniusculus* a lipopolysaccharide- and β-1,3-glucan binding protein (LBGP) has been isolated and characterized. LGBP has binding activity for LPS and β-1,3 glucans but not for peptidoglycans of Gram –positive bacteria. Experiments using a polyclonal antibody to

block LBPG indicated that this protein plays a role in the activation of the proPO system. In other penaeid shrimp a BGP and a LBGP have been reported. The BGP from *Penaus monodon* is not up regulated, whereas the LBGP from *Penaeus stylirostris* is up regulated after bacterial infection. Peptidoglycan recognition proteins and Toll receptors have not yet been found. However, We have recently isolated a partial sequence encoding a TIR domain in *Penaeus monodon* . The presence of this TIR domain suggests that a Toll receptor or intra cellular signal transduction molecules such as MyD88 are present. Besides the three major PRR groups other receptors like αα2-microglobulin, C-type lectins of penaeids and calcium independent lectins have been reported.

Regulation of Immune Responses

Modulation and amplification of extra cellular cascades occurs mainly by serine protease. These protease are active in a large number of biochemical processes and those that perform an immune function by cleaving a specific ligand that interacts with a receptor are difficult to identify. A cell adhesion protein, a mass like protein, form *Pacifastacus leniusculus* shows homology with serine protease, especially to the *Drosophila* Masquerade and may be such a a candidate serine protease involved in immune responses. After binding of this masquerade-like protein to micro organisms, a proteolytic enzyme cleaves this protein. The intact form of the masquerade-like protein has been extensively characterized and contains seven putative disulphide knotted motifs and is implicated in the immune response to a bacterial infection.

Effector mechanisms

In the crustacean cellular response three groups of cells can be recognized based on morphological differences: the first are hyaline cells, which phagocytes invading organisms. The second group is called the semigranualr group, which have a much broader function, encapsulation, (limited) pahgocytosis, storage and release of proteins of the proPO system, and cytotoxicity. The latter two functions are also found in the third group, the granular cells. The first antimicrobial peptide characterized in crustaceans showed a high identity with bactenecin-7. The next group of antimicrobial peptides identified was the penaeidin family from the Pacific white shrimp *Litopenaeus vannamei.* Although this family shows similarities to the insect praline-rich antimicrobial peptides, they do not have a strong activity against gram negative bacteria. Callinectin, isolated form the hemocytes of the blue crab *Callinectes sapidus,* demonstrated activity against *E. coli* I and possessed a praline rich domain. The peptide does not show significant homology with any known peptide. Crustin, a new family of antimicrobial peptides, also show no homology with other known antibacterial peptides, but does display sequence identity with proteinase inhibitory proteins.

The prophenoloxidase (proPO) cascade-activating system is implicated in the immediate defense of shrimp against a variety of stimulatory conditions. This system has been extensively studied. Phenoloxidase(PO; EC 1.14.18.1) is present in the hemolymph as an inactive pro-enzyme, proPO. Common chemical components of bacteria and fungi, such as β-1,3 glucans, are reacting with β-glucan binding protein (BGBP) and this complex induces degranulation and the activation of proPO system. This protein cascade is widely expressed and highly conserved in crustaceans. Proteins of the proPO system thus occupy a prominent position in non-self recognition, hemocyte communication and the production of melanin. Upon activation and degranulation of haemocytes, the inactive proPO is converted into the active phenoloxidase(PO) by prophenoloxidase activating enzyme(ppA). This quinines, and after several intermediate steps the formation of melanin. During this formation antimicrobial factors are formed. Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. The melanization

process is observed in response to foreign intruders in the haemocoel and during wound healing. An important factor that is associated with the proPO system is peroxinectin, which ws recently cloned for *P. monodon.* Peroxinectin displays cell- adhesion and peroxidase activity. Crayfish peroxinectin is synthesized in the hemocytes, stored in thesecretory granules in an inactive form, released in response to stimuli and activated outside the cells. Transmebrane receptors of the integrin family on the hemocytes play an important role in the cell adhesion function of peroxinectin. The cell adhesion can lead to attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading micro-organisms,

Conclusion

As a result of the molecular sequence information currently available on the expressed genes in insects, experiments have been performed adopting standardized challenge protocols either with bacteria or fungi to identify potential immune response genes. The problem with these experiments is that about 50%of the genes that are modulated after the pathogen challenge have an unknown function. Nevertheless these experiments have led to the identification of a large number of proteins involved in the immune response, notably those of the Toll and Imd pathway. Some of the modulation observed might be due to wounding alone in the case of a bacterial challenge where the bacteria are introduced by pricking the flies with a septic needle. This issue needs to be resolved. The fungal challenge is a more natural challenge as spores are simply coated onto the flies and this procedure does not require rupture of the cuticle. The fungus has the capability to penetrate the cuticle by itself. Given the relative close relationship between insects and crustaceans it is anticipated that the major immune signaling components and the full suit of effectors can be identified using the information from insects. However, this only applies for anti-bacterial and anti-fungal responses, but not for the immune response to pathogenic viruses. As far as we can ascertain little attention has been paid to the immune response to viruses, neither in insects not in crustaceans. This could be due to the lack of appropriate viral challenge models. However, currently much research effort is devoted to the white spot syndrome virus(WSSV). This virus has become a pandemic within a relative short time span, and is particularly virulent in *Penaeus monodon.* The genome of the WSSV has been completely sequenced and turned out to be the largest animal viral genome of 292,967 base pairs with184 open reading frames. To investigate the aetiology of the virulence and the immune response to a viral infection in crustaceans, adopting a similar approach as used in flies, namely expression profiling using micro arrays, may provide clues which pathways, signaling cascades and effector mechanisms are involved.

PATHWAY DATABASE AND CONCEPTS MODELING IN FISH DISEASES

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Pathway Database

The pathway database is a foundation for understanding the mechanism of cellular signaling. In order to be useful to the research community, this information must be synthesized and integrated into understandable paradigms of cellular communication. Here, we introduce an integrated system, called "Pathways Database System," with a set of software tools for modeling, storing, analyzing, visualizing, and querying biological pathways data at different levels of genetic, molecular, biochemical and organism detail.

Research will focus on functional studies of attributing functions to genes, their regulatory elements, and other DNA sequences. To facilitate the use of genomic information in such studies, a new modeling perspective is needed to examine and study genome sequences in the context of many kinds of biological information. To facilitate the use of genomic information in such studies, a new modeling perspective is needed to examine and study genome sequences in the context of many kinds of biological information. Pathways are the logical format for modeling and presenting such information in a manner that is familiar to biological researchers.

The conventional perspective for managing, analyzing, viewing and querying genomic information is in the context of DNA sequence. In this perspective, DNA sequences are annotated with the identity and location of genes, transcriptional motifs and other regulatory elements, repetitive DNA elements, and chromosome segments that have been conserved among various species during evolution. This perspective is appropriate for studying questions of genome organization and evolution, and for identifying mutated genes that are responsible for phenotypic variation including human diseases. However, DNA sequence does not reflect the context in which most genes act, that is, functionally related genes are usually not physically clustered in DNA, but instead are distributed among distant sites. The protein products of these genes assemble at appropriate cellular locations to coordinate their biological functions. Thus an alternative to DNA sequence for studying genomic information is biological pathways. Pathways are the sequential and cumulative action of genetically distinct but functionally related molecules. Each reaction in each pathway begins with specific substrates, uses various combinations of molecules as cofactors, activators and inhibitors, and ends with products that are chemically modified substrates. Individual steps in every pathway involve at least one genetically unique gene product which catalyzes the reaction. Thus pathways are an appropriate format for representing the functional role of most genes in the genome.

Types of Pathway

The three general classes of biological pathways are (1) **metabolic and biochemical**, (2) **transcription, regulation and protein synthesis**, and (3) **signal transduction**.

Metabolic and biochemical Pathway:

Metabolic pathways are responsible for carrying out the chemical reactions that provide basic biological functions such as DNA, RNA and protein synthesis and degradation, energy metabolism, fatty acid synthesis, and many others.

Transcription, regulation and protein synthesis Pathway

Transcription and protein synthesis are responsible for converting genetic information into proteins (gene products).

Signal transduction Pathway

Signal transduction pathways are responsible for coordinating metabolic processes with transcription and protein synthesis.

Each of these three kinds of pathways has distinct attributes, to be kept and managed in the pathways database.

Related Pathway Sources

List of Metabolic Pathway Sites

Name	**Web site address**
BioPath	http://www.molecular-networks.com/databases/biopath
AraCyc	http://www.arabidopsis.org/biocyc/index.jsp
EMP-Enzymes and Metabolic Pathways Database	http://www.empproject.com/
ExPASy Biochemical Pathways	http://www.expasy.org/tools/pathways/
IPath	http://escience.invitrogen.com/ipath/
KEGG	http://www.genome.jp/kegg/
Millipore Pathways	http://www.millipore.com/pathways/pw/pathways
MPB - Metabolic Pathways of Biochemistry	http://www.gwu.edu/staticfile/GW/Global/404.html
PathArt	http://www.jubilantbiosys.com/pathart.html
PathCase	http://nashua.cwru.edu/PathwaysWeb/
PathDB	http://www.ncgr.org/pathdb/
Ingenuity Pathways Knowledge Base	http://www.ingenuity.com/
PATIKA	http://www.patika.org/
PATRIC	http://patric.vbi.vt.edu/
PC - Pathway Commons	http://www.pathwaycommons.org/pc/
PDS	http://nashua.case.edu/pathwaysweb/
PlantCyc	http://www.plantcyc.org/
ProcessDB	http://www.pathguide.org/
Protein Lounge	**http://www.proteinlounge.com/**
RGD- Rat Genome Database pathway resource	http://rgd.mcw.edu/wg/pathway
RiceCyc	http://www.gramene.org/pathway/ricecyc.html
WikiPathways	http://wikipathways.org/index.php/WikiPathways
SMPDB	http://umbbd.msi.umn.edu/
Signaling Pathways	
CellML Repository	http://models.cellml.org/
CPDB	http://cpdb.molgen.mpg.de/
DOQCS	http://doqcs.ncbs.res.in/
CST	http://www.cellsignal.com/
JWS Online	http://jjj.biochem.sun.ac.za/

iPath	http://escience.invitrogen.com/ipath/
MetaCore	http://www.genego.com/metacore.php
Millipore Pathways	http://www.millipore.com/pathways/pw/pathways
PathArt	http://www.jubilantbiosys.com/pathart.html
PathDB	http://www.ncgr.org/pathdb/
Ingenuity Pathways Knowledge Base	http://www.ingenuity.com/
PDS	http://nashua.case.edu/pathwaysweb/
PID	http://pid.nci.nih.gov/
Protein Lounge	**http://www.proteinlounge.com/**
pSTIING	http://pstiing.licr.org/
RGD	http://rgd.mcw.edu/wg/pathway
ROSPath	http://rospath.ewha.ac.kr/
SMPDB	http://www.smpdb.ca/
SIGMA-ALDRICH	http://www.sigmaaldrich.com/life-science/cell-biology/neuroscience.html
Signaling Gateway	http://www.signaling-gateway.org/
SigPath	http://icb.med.cornell.edu/crt/SigPath/index.xml
STKE	http://stke.sciencemag.org/
STCDB	http://www.techfak.uni-bielefeld.de/~mchen/STCDB/
TRANSPATH	http://www.biobase-international.com/pages/index.php?id=39
TRRD	http://wwwmgs.bionet.nsc.ru/mgs/gnw/trrd/
WikiPathways	http://wikipathways.org/index.php/WikiPathways

The novel features of the Pathways Database System include the following:

- Genomic information integrated with other biological data and presented from a pathway, rather than the DNA sequence, perspective
- Design for biologists who are possibly unfamiliar with genomics, but whose research is essential for annotating gene and genome sequences with biological functions
- Database design, implementation and graphical tools which enable users to visualize pathways data in multiple abstraction levels, and to pose ad-hoc and predetermined queries
- An implementation that allows for web (XML)-based dissemination of query outputs (i.e., pathways data) to researchers, giving them control on the use of pathways data

At the computational level, Pathways Database System allows users to visualize pathways in multiple abstraction levels, and to pose a wide range of queries using a graphical user interface. By different abstraction levels, we refer to the representation of pathways at different levels of biological function. At one level, for example, all of the individual steps in methylation can be illustrated, while, at another level, the collections of steps are labeled methylation. Together this is an easy and intuitive way to query complex sets of genomic, genetic and biological information.

Generally pathways are categorized in to several groups according to their function or organism wise. Functionally they are divided in to 22 sub groups and organism wise they are divided into 15 sub groups.

Functionally	Organism wise
Adhesion	Algae
Apoptosis	Amphibians
Cell Cycle	Bacteria, *C.elegans*
Cell Signaling	Cyanobacteria
Cell Survival	Drosophila
Chemokines	Fungi
Cytokine	General
Degradation	Infectious Particles
Developmental Biology	Mammals
Disease	Plants
G-Protein	Plasmodium
Genetic Processing	Protozoa
Growth Factor	Viruses
Hormone	Yeast
Immunology	
Kinase	
Metabolic	
Motility	
Neuroscience	
Stress Activated	
Transport	
Vitamins	

In biology, signal means a cell converts one kind of signal or stimulus into another. Most processes of signal transduction involve ordered sequences of biochemical reactions inside the cell, which are carried out by enzymes and activated by second messengers, resulting in a signal transduction pathway.

Aeromonas hydrophila causes disease in fish known as "Hemorrhagic Septicemia", "Motile Aeromonas Septicemia", "Ulcer Disease", or "Red-Sore Disease". The many synonyms of this disease relate to the le-sions caused by this bacterium which includes lesions of septicemia when the bacteria or bacterial toxins are present within numerous organs within the fish, and ulcers of the fish's skin. When infected with *A. hydrophila*, fish develop ulcers, tail rot, fin rot, and hemorrhagic septicaemia. Hemorrhagic septicaemia causes lesions that lead to scale shedding, hemorrhages in the gills and anal area, ulcers, exophthalmia, and abdominal swelling.

A.hydrophila is a ubiquitous gram-negative, motile, rod-shaped' bacterium which can be commonly isolated from fresh water ponds and is also a normal inhabitant of the gastrointestinal tract of fish. The disease primarily affects freshwater fish such as catfish and bass, and many species of tropical or ornamental fish. Fish infected with *A. hydrophila* may have many different clinical signs. These range from sudden death in otherwise healthy fish to in appetence, swimming abnormalities, pale gills, bloat and skin ulcerations. The skin ulcers may occur at any site on the fish and often they are surrounded by a bright red rim of tissue. The pathogenicity of *Aeromonas* sps. is mediated by a number of extracellular proteins such as **aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins and enterotoxins**. However the pathogenic mechanisms of *Aeromonas* sps. are remain unknown. The recently proposed type III secretion system (**TTSS**) mediated pathogenic mechanism has been proven to play a pivotal role in *Aeromonas* pathogenesis. The TTSS is specialized protein

secretion machinery that export virulence factors delivered directly to host cells. These factors subvert normal host cell functions in ways that are beneficial to invading bacteria. In contrast to the general secretory pathway, type III secretion system is triggered when a pathogen comes in contact with host cells. **ADP-ribosylation** toxin is one of the effector molecules secreted by several pathogenic bacteria and translocated through TTSS and delivered into the host cytoplasm leads to interruption of NF-κB pathway, cytoskeletal damage and apoptosis. This toxin has been characterized in *A. hydrophila* (human diarrhoeal isolate), *A. salmonicida* (fish pathogen) and *A. jandaei* GV17, a pathogenic strain which can cause disease both in human and fish.

Sequence analysis and 3D modeling of some toxins/proteins of *Aeromonas hydrophila*

Example: ADP-ribosylating toxin (2GWL)

Hemolysin (1PRE)

Aeromonas salmonicida subsp. salmonicida A449 : Aerolysin: (aerA gene)

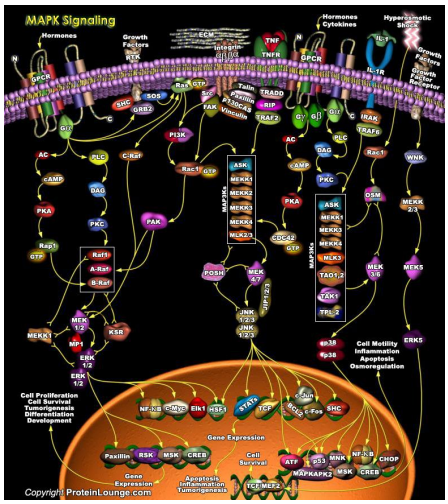
Some viral proteins

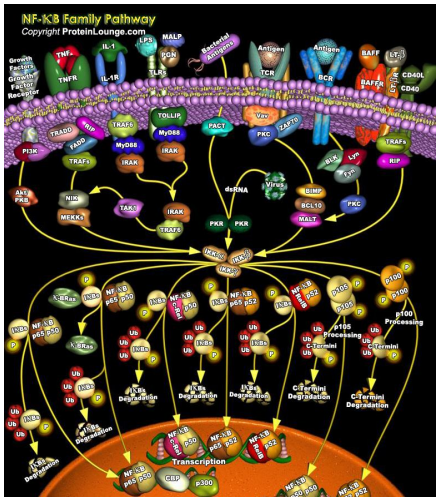
IPNV (Infectious pancreatic necrosis virus)

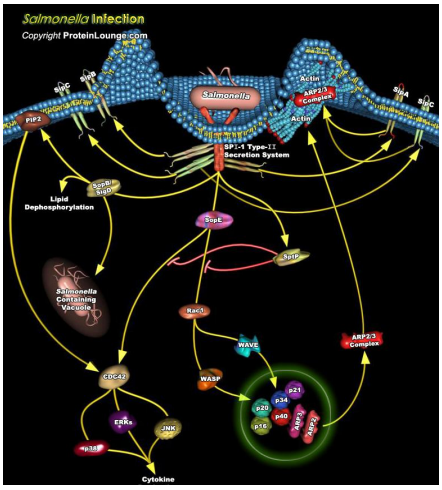
Proteins of IPNV

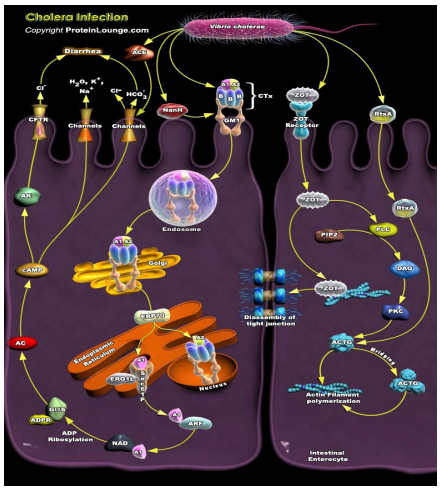
1. Viral Protein 1 (VP1)
2. Polyprotein (NP_047196): 972 aa

NCBI (BLAST): http://www.rcsb.org/pdb/home/home.do









APPLICATION OF SOFTWARES IN MOLECULAR MARKER DATA ANALYSIS

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Introduction

The development of DNA technology over the last few decades has led to Generation of huge amount of data from plant and animal genome. The main sources of markers are mitochondrial DNA (mt DNA), minisatellites, micro satellites and anonymous nuclear sequences assayed using highly specific PCR primers or by using the RAPD technique. A major contribution comes from molecular markers used for analyzing genetic variations. There has been the infusion of computers, softwares and online databases into the daily activities of many molecular geneticists. The identification of abundant, highly variable loci, detectable by a wide variety of techniques has led to a revolution in the way researcher view and analyses levels of variation. We will here discuss about current computer programs in this area to characterize the DNA profiles and data analysis for intra and inter population structure, phylogenetic inference and parentage determination.

In fish breeding, genetic markers can be used to investigate the genetic diversity present in the population to be used as brood stock. The breeding population can also be tested for Hardy-Weinberg equilibrium. The bands yielded from both dominant and codominant molecular markers such as Allozymes, RAPD, AFLP, RFLP, microsatellite etc. are converted into numerical data matrix to use in different genetic data analysis software. Plenty of software and computing materials are available in Internet and standalone Personal Computers. These are easily available and most of them are free to download from Internet. Here few selected programs are discussed, which are widely used globally.

Programmes

Various programmes are available now in the Internet. Some of these programmes are used for codominant and some are for dominant markers.

1. Genetic Data Analysis (GDA) version 1.1

The authors of this programme are Lewis and Zaykin (2000). GDA uses a data file format known as NEXUS. NEXUS files are plain text files, which can be viewed by any programme that is capable of displaying standard ASCII text files. These data files can be created by any word processor.

Analysis consists of a table of descriptive statistics, tests for Hardy- Weinberg and pairwise disequilibrium, linkage disequilibrium coefficients, estimation of F-statistics, bootstrapping over loci, genetic distances between populations and a dendrogram based on genetic distances.

Descriptive statistics include mean sample sizes over all loci, mean number of alleles per locus, the proportion of polymorphic loci, the mean number of alleles per polymorphic locus, the expected heterozygosity, the observed heterozygosity and an estimate of the fixation index (f_{is}). Tests can be performed for any departure from Hardy-Weinberg equilibrium at individual loci through an exact test and linkage disequilibrium will also be tested for all possible pairs of loci. Bootstrapping options include outputting a list of

replicate estimate of *f* (=f_{is}), F (=F_{it}) and Theta (=F_{st}). Jackknifing across populations to obtain variances of F-statistics estimates for individual lociis also available. The genetic distance measures will be clustered using the UPGMA (Unweighted Pair Group Method using Arithmetic averaging) and a tree depicting these phenetic relationships among populations will be drawn.

2. GENEPOP version 3.4

GENEPOP is another population genetic software package authored by Raymond and Rousset (1995). This software runs on any Window processors including Pentiums II and III without any need for extended memory. It is not protected and available free of charge. Haploid or diploid codominant data of micro satellite markers can be analysed with this software.

This software estimates, I). exact tests for Hardy-Weinberg equilibrium, for population differentiation and for genotyping disequilibrium among pairs of loci, ii) F-statistics and other correlations, allele frequencies etc and iii) analysis of isolation by distance.

GENEPOP provides P-values for Hardy-Weinberg equilibrium, linkage disequilibrium and population differentiation by Exact tests, either by complete enumeration or by Markov chain methods. The U-test for heterozygosity excess or deficiency is also available. Global Hardy-Weinberg test across loci or across populations is another option for GENEPOP. *F*-statistics or *Rho*-statistics can be calculated for all populations or all pairs of populations. The matrix for F_{st} values can be used for isolation by distance by the ISOLADE programme using rank correlation coefficient.

GENEPOP allows the transformation (ecumenicism) of the data input file toward other formats required by other programmes, which is unique for this software.

3. POPGENE (version 1.30) (Yeh and Boyle,1996)

POPGENE is a user-friendly **Microsoft®** Window-based computer package for the analysis of genetic variation among and within natural populations. The current version is designed specifically for the analysis of co-dominant and dominant markers using haploid and diploid data. It performs most types of data analysis encountered in population genetics and related fields. It can be used to compute summary statistics (*e.g.*, allele frequency, gene diversity, genetic distance, *F*-statistics, multilocus structure, *etc.*) for (1) single-locus, single populations; (2) single-locus, multiple populations; (3) multilocus, single populations and (4) multilocus, multiple populations. This latest version also includes modules for quantitative traits. The software programme opens in two windows namely data display window and dialog boxes, it is menu driven and very easy to run by selecting the menus from the task bar. The popgene have provision for dominant and co-dominant marker levels, Each have Haploid or Diploid data analyses provision, each again have provision for 3 population levels like single population, groups or multiple population, at last estimation of single locus or multi locus genetic parameter can be assigned.

4. Arlequin

Laurent Excoffier of the Computational and Population Genetics Lab of the Institute of Zoology, University of Bern, Switzerland (laurent.excoffier@zoo.unibe.ch), Stephan Schneider, and David Roessli have released **Arlequin** version 2.001, a program for population genetics analysis. It does many kind of analyses for different kinds of molecular

data of RFLP, AFLPs, minisatellites sequences, microsatellites, allozymes etc. It estimates gene frequencies, testing of linkage disequilibrium, and analysis of diversity between populations. It enables us to compute variety of genetic distance measures including Jukes and Cantor, the Kimura 2-parameter distance, and the Tamura-Nei distance, each of these with or without correction for gamma-distributed rates of evolution. It can also compute a Minimum Spanning Tree network. Arlequin has its interactive "front end" written in Java, and requires the Java Runtime Environment. The software and a PDF documentation file can be downloaded from http://lgb.unige.ch/arlequin/.

5. TFPGA (Tools for Population Genetic Analyses)

This is a Windows based programme for the analysis of allozyme and molecular population genetic data. Mark P. Miller developed this programme in 1997 from Northern Arizona University. This can analyse both haploid and diploid data and also dominant and co dominant. This software needs around 1 MB of hard disk space and any Pentium processor can do it.

The programme can estimate allele frequencies for diploid dominant markers by either as the square root of the frequency of the recessive genotype or by Taylor expansion. The analysis menus are descriptive statistics, F-statistics, Genetic Distance, Hardy-Weinberg, UPGMA, Exact Tests and Mantel Test.

Assignment Test

More information regarding population structure and gene flow can be available now with assignment test using highly variable microsatellite and RAPD marker. This test was developed originally by Paetkau et. al. (1995) for population differentiation. It was based mainly on frequency statistics using the likelihood of each genotype in each of the population. Individuals are correctly assigned to the population with the highest likelihood. The available softwares are as follows:

1. GENECLASS 1.0.02 (Cornuet et.al. 1999; http://www.ensam.infra.fr/URLB).

This programme involves both the frequency model and the Bayesian model with 'leave one out' option. The simulation - exclusion approach proposed by Cornuet *et al.* (1999) computes a probability that the individual belongs to a population by simulating 10000 genotypes and a p= 0.001. A threshold P-value is fixed according to the required certainty of exclusion. Each individual was assigned to the population in which it had the highest probability of occurring. When an allele in the individual to be assigned is absent from the population sample, the estimate of the corresponding allelic frequency is equal to zero, leading to a likelihood of zero and hence eliminating de facto this population. However, the allele in question may be rare in the population so that it was not represented in the sample (Cornuet *et al*. 1999). If the individual probability is lower than the decided threshold in a population, it considers that the individual does not originate from the population. Thus an individual was considered as correctly assigned to a population when it was excluded from all of the non-origin population (e.g. P< 0.001), but not from the true population of origin.

2. **Structure** (version 2) (Pritchard and Wen 2002; http://pritchard.bsd.uchicago.edu)

It is a model based clustering method using genotype data. The genotype data includes microsatellites, SNPs and RFLPs. The programme is divided into two steps. First step involves the estimation of number of population (K) and second step involves the assigning of individuals to each of the population with highest percentage of membership (q).

3. **Assignment Calculator** (Brzustowski 1998) http://www.biology.ualberta.ca/jbrzusto/Doh.php

This programme is an interactive one, therefore to run this software one has to log on to the website and use it. This calculates the assignment probabilities of the individuals to the most probable populations. That is, if individuals whose source population is unknown and we wish to find out the population in which their genotype are most likely, then this porgramme will help us. The input data can be directly imported from a Popgene file.

Genetic Distance- Similarity

Nei (1987) described the definition of genetic distance as " the extent of gene differences between populations or species that is measured by some numerical quantity". Various genetic distance measures have been used now for analysis of molecular marker data for the purpose of genetic diversity analysis. The commonly used measures of genetic distance using binary data are,(i) Nei and Li (1979), (ii) Jaccard's (1908) coefficient, and (iii) Social and Michener's (1958) simple matching coefficient.

Genetic similarity (GS) between individuals *i* and *j* is estimated according to the formula given by Nei and Li (1979):

$GS_{ij} = 2N_{ij} / (N_i + N_j)$

where N_{ij} is the number of bands common in individuals *i* and *j* and N_i and N_j are the total number of bands in individuals *i* and *j*, respectively, with regard to all assay units. Thus, GS reflects the proportion of bands shared between two individuals, and ranges from 0 (no common bands) to 1 (all bands identical).

Genetic dissimilarity (GD) is calculated as:

GD = 1 - GS.

Jaccard's coefficient (GD) takes into consideration only matches between bands-alleles that are present and ignores pairs in which a band or allele is absent in both individuals, where as Sokal and Michener's GD, a Euclidean measure of distance which takes into account of mismatches and matches, and gives equal weight to both in estimating genetic distance.

Phylogenetic tree construction:

1) Genetic distance based
2) Genetic similarity based.

In case of co-dominant data like microsatellites, various allelic combination (genotypes) the genotypes can be determined by recording the presence (1) or absence (0) of these bands only. Two assumptions were made for the analysis of the RAPD data: I) markers from different loci did not migrate to the same position on the gel, II) each band was assumed to represent the dominant genotype at the locus, whereas lack of the same band in another individual was assumed to correspond to the alternative homozygous recessive genotype in the Hardy- Weinberg equilibrium (Lynch and Milligan, 1994).

This matrix data can be used to construct a UPGMA dendrogram using SAS (v. 8.0 or latest) or Quantity One (BIORAD) or Tree Programs from NTSYS-pc, v. 1.6 (Rohlf, 1992) or any one of the these software that has option for phylogenetic tree construction.

The major features of some of the programs

Feature	TFPGA	ARLEQUIN	GENEPOP	POPGENE	GDA
Diversity					
Observed heterozygosity	√	√		√	√
Expected heterozygosity	√	√		√	√
No. of alleles per locus		√		√	√
Proportion of polymorphic loci	√	√		√	√
Hardy-Weinberg equilibrium	√	√		√	√
Population structure					
F-statistics		√	√	√	√
AMOVA		√			√
Homogeneity	√		√		
Migration		√	√	√	
Linkage equilibrium					
Two locus	√	√	√		√
Multilocus					√
Genetic distance					
Nei's	√	√		√	√
Rogers'	√				
Pairwise FST	√	√			√
Clustering					
UPGMA	√			√	√
Neigbour-joining					√
Neutrality test		√		√	

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MOLECULAR ASPECTS OF BACTERIAL GENOME STUDIES

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Introduction

Genome sequence information is very essential in understanding pathogenesis, virulence, immunogenicity, therapeutic targets and fish pathogens. In addition this information throws light into fish and pathogen interaction thus can help in finding preventive measures for fish shellfish diseases in aquaculture. Till date the number of completed genome sequence is available in the International Nucleotide Sequence Database Collaboration .A wide range of pathogen detection methods are followed including culturing methods, nucleic acid methods, immunological methods, microscopy, spectroscopy and bioluminescence with varying degree of cost, sensitivity and specificity. Traditional methods of microbial detection are slow and time taking in comparision to molecular methods of detection. In general, molecular methods combine higher discriminatory power and higher reproducibility than phenotypic tests. These advantages are a result of their ability to detect minor genome differences and the higher stability of molecular targets compared with that of phenotypic profiles for some species (Cano-Gomez et al., 2009). Bacterial genome study devoted to the molecular principles of the physiological processes involved in the life cycle of prokaryotic This includes gene expression and regulation, genetic transfer, the synthesis of macromolecules, sub-cellular organization, cell to cell communication, and molecular aspects of pathogenicity and virulence. Genome study primarily involved in the interactions between the various cell systems of microorganisms including the interrelationship of DNA, RNA and protein biosynthesis and the manner in which these interactions are regulated. Mainly bacteria are used for study of molecular aspects because of their relative simplicity, ease of manipulation and growth in vitro, and importance in medicine, and these tiny microorganisms were instrumental in the development of molecular biology. The complete genome sequence for a large number of bacterial species is now available. Molecular microbiology techniques are currently being used in the development of new genetically engineered vaccines, in bioremediation, biotechnology, food microbiology and environmental microbiology.

Table-1 Major fish pathogens and its virulence

Pathogenic Bacteria	Distribution	Disease affected part of Fish	Disease	Virulence factor
A. hydrophilla	Freshwater, marine water and brackish water	Wild fresh water fishes as well as in marine fishes	haemorrhagic septicemia (red sore disease), infectious dropsy, rubella, ulceration, exophthalmia, tail rot, fin rot, scale protrusion	enterotoxins, cytotoxin, haemolysin, endotoxin, aer-A and hly-A
P. aeruginosa	Fresh water, Brackish water	Fresh water fished	Epizootic Ulcerative Syndrome, Large hemorrhage cutaneous ulcers, epidermal degeneration and necrosis followed sloughing of scales	Lipopolysaccharide virulence factors (protease, hemolysins, exotoxin A, exoenzymes
F. columnare	Freshwater, and brackish water	Freshwater fishes	Gill and Skin disease	Gill necrosis and Columnare Disease
F. psychrophilum	Freshwater, and brackish water	Trout and salmon	Gill, tail rot and peduncle disease	Collagenase gene
V. parahaemolyticus	ubiquitous in estuarine, costal and pelagic waters and in marine sediments and fresh water	Shrimp, fresh water fishes, Marine water fishes	Vibriosis	Enterotoxin, TDH gene, Ctx A gene, extracellular products (ECP)
A.salmonicida	Freshwater, marine water and brackish water	Freshwater fishes	Furunculosis	extracellular products (ECP), lipopolysaccharide (LPS)

Y. ruckeri	Freshwater, marine water and brackish water	Freshwater fishes	Enteric red mouth disease	YhlA gene,YhlB gene, hemolysins
V.alginolyticus	ubiquitous in estuarine, costal and pelagic waters and in marine sediments and fresh water	Shrimp, fresh water fishes, Marine water fishes	Vibriosis	Enterotoxin, TDH gene
S. aureus	estuarine, costal, fresh water	Freshwater Fish	Eye infection (Eye ball disease),	Enterotoxin,

TYPES OF MOLECULAR TECHNIQUE

Many bacteria have become model organisms for molecular studies. Molecular techniques have had a direct influence on the clinical practice of medical microbiology. In many cases where traditional phenotypic methods of microbial identification and typing are insufficient or time-consuming, molecular techniques can provide rapid and accurate data, potentially improving clinical outcomes. Specific examples include:

- 16s rRNA sequencing to provide bacterial identifications
- Pulsed Field Gel Electrophoresis (PFGE) for strain typing of epidemiologically related organisms.
- Denaturing Gradient Gel Electrophoresis (DGGE), permitting the rapid detection of pathogen
- Direct detection of pathogenic genes related to resistance mechanisms, such as aerolysin gene and TDH gene in *Aeromonas hydrophilla* and *Vibrio parahaemolyticus* respectively
- RAPD (Randomoly Amplified Polymorphic DNA) and RFLP (Restriction Fragement Length Polymorphism) profile provideat species level identification of bacterial genome
- Isothermal amplification
- Fluorescence In situ hybridisation
- Multilocus Sequence Analysis (MLSA) is a robust and portable molecular technique that compares the nucleotide sequence of several housekeeping genes for genotypic characterization of bacterial groups
- Whole-genome fingerprinting methods have been used in several studies to refine the complex bacterial taxonomy and to identify pathogenic strains
- DNA microarray analysis

Study of 16s r RNA

The use of ssu r RNA as a phylogenetic tool was pioneered in the early 1970s by Carl Woese at the university of Illioni and the method is now widely used the classification of organisms traditionally has been based on similarities in their morphological developmental and nutritional characteristics. 16S and 23S rRNA are currently considered the most useful molecules for the determination of prokaryotic phylogeny. Acceptance of 16S rRNA as an

appropriate tool for the reconstruction of evolutionary history and phylogenetic relationships (Amann et al., 1995). The ssu r RNA sequence evolved very slowly with time. Therefore 16s r RNA have proven the most useful for establishing distant relationships because of their high information content conservative nature, and universal distribution. Although conserved in sequence overall, the 16S rRNA actually exhibit great variation in some regions. These differences in 16S rRNA sequence provide the basis for the design of nucleic acid probes of various specificities, ranging from probes targeting all living organisms to group-specific and species- specific probes. Terminal restriction fragment (TRF) analysis of 16s rRNA gene is an increasingly popular method for rapid comparison of microbial communities. Polymorphism in repeated 16S rRNA genes had been initially suggested by the observation of nucleotide variations from 1 to 5% between pairs of 16S rRNA sequences from one bacterial strain or different bacterial strains of the same species deposited in GenBank. Ribotyping or ribosomal RNA Restriction Fragment Length Polymorphism analysis (rRNA-RFLP) is based on the detection of sequence differences within or flanking the 16S, 5.8S and 23S ribosomal RNA genes (Cano-Gomez et al., 2009)

Study of Pulsed-Field Gel Electrophoresis (PFGE)

A new methodology for genetic diversity analysis based on DNA restriction using an endonuclease with a high cutting frequency (much like PFGE) has become available (Gonzalez-Escalona et al., 2007). DNA sequencing of highly variable genes will become the method of choice for molecular epidemiologists in the future; currently the "gold standard" technique used in this discipline is pulsed-field gel electrophoresis (PFGE). PFGE is essentially the comparison of large genomic DNA fragments after digestion with a restriction enzyme. Since the bacterial chromosome is typically a circular molecule, this digestion yields several linear molecules of DNA. The basic concept of interpretation of this experiment is the following: if one is comparing two strains that are clonal (i.e. the same strain), the sites at which the restriction enzymes act on the DNA and the length between these sites would be identical. Therefore, after digestion of the DNA and electrophoresis through an agarose gel, if the DNA banding patterns between any two isolates is identical, then these isolates are considered the same strain. Conversely, if two isolates are not the same strain, then the sites at which the restriction enzymes act on the DNA and the length between these sites would be different; thus their DNA banding patterns will be different.

Study of Denaturant Gradient gel Electrophoresis (DGGE)

It is an electrophoresis method that separates genes of the same size that differ in base sequence. When a double stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to melt, difference in melting properties are to a larger degree controlled by difference in base sequence. DGGE analysis yields a detailed picture of the number of phylotypes. Vibrio-specific 16S rRNA-directed primers and a competitive PCR protocol were adopted for separation and quantification of Vibrio populations using denaturant gradient gel electrophoresis (DGGE). Putative identification based on migration in the DGGE gel was verified by parallel cloning and sequencing of PCR products, and representative clones were also characterized by DGGE (Eiler et al., 2006).

Study of Multilocus sequence Analysis (MLSA)

Multilocus sequence typing (MLST) is a technique in molecular biology for the typing of multiple loci. The procedure characterizes isolates of bacterial species using the DNA sequences of internal fragments of multiple (usually seven) housekeeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each

housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type. MLST directly measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles. The principle of MLST is simple: the technique involves PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes (generally seven) depending on the degree of discrimination desired. The analysis of a single locus has potentially limited resolution power for closely related Vibrio species as discussed above. Therefore, extensive efforts have recently been made to develop a Multilocus Sequence Analysis (MLSA) approach for Vibrio species delineation (Sawabe et al., 2007). In general, MLSA is a robust and portable molecular technique that compares the nucleotide sequence of several housekeeping genes for genotypic characterization of bacterial groups (Gevers et al., 2005). In epidemiology, a variation of this technique known as Multilocus Sequence Typing (MLST) (Maiden et al., 1998) is used to characterize strains within named species by comparing allelic mismatches of a number of housekeeping genes (usually 7). The method is a refinement of the earlier Multilocus Enzyme Electrophoresis (MLEE) which evaluated genetic variation of metabolic enzymes based on their migration in starch gels during electrophoresis. MLEE was taken to a genomic platform with MLSA and MLST and the techniques are now considered standard typing tools for bacterial taxonomy and epidemiology studies (Maiden, 2006; Cano-Gomez et al., 2009). Thompson et al. (2005) explored the usefulness of three genes for identification of vibrios by MLSA; the RNA polymerase alpha subunit gene (rpoA), the uridylate kinase gene (pyrH), and the recA recombination and DNA repair protein gene (recA). The results were compared with the resolution power obtained by using the 16S rRNA gene. The genus Vibrio was found to be both heterogeneous and polyphyletic, and Vibrio species and strains showed high gene sequence variation for these loci (19% for rpoA, and 27% for the recA and pyrH genes). More specifically, V. harveyi-related species were found to have at least 3.5, 3, and 5% sequence variation for rpoA, recA, and pyrH, respectively.

Study of RAPD Randomly Amplified Polymorphic and Restriction Fragment Length Polymorphisms (RFLP)

Molecular polymorphisms can be identified at random and used to discriminate the phylogenetic relatedness of microorganisms. Using this clue a renowned molecular technique was developed. The technique is Randomly Amplified Polymorphic DNA assay. Actually this is a PCR reaction but it amplify segment of DNA, which are essentially unknown to the researcher (random). Often, PCR is used to amplify a known sequence of DNA that want to amplify, then design and makes primers which will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA. This makes it a tool of great power and general applicability. RAPD-PCR has been used to characterize population and strains of animals, plants, fungi and bacteria (Fani et al., 1993). RAPD-PCR is extensively employed in molecular ecology, population genetics, microbiology, Selection and breeding etc. Several authors have reported in the application of RAPD technique in microorganisms (Babalola et al., 2002). It uses random primers (William et al., 1990) and can be applied to any species without requiring any information about the nucleotide sequence (Babalola, 2003). Nowrouzian et al., 2001 designed a RAPD typing method for the identification of *E. coli* strains in the normal human intestinal microflora. The band pattern generated in the analysis represents genome characterization of particular bacterial strain (Welsh and Mc Cleland, 1990). In addition, the method has the potential for analyzing phylogenetic relation ships among closely related species (Williams et al., 1990) and can distinguish between strains within a species. RAPD is based on random amplification of genomic DNA fragments using a single primer of

arbitrary nucleotide sequence. Size separation of the resulting fragments allows differentiation between genetically distinct clonal lineages. The technique is fast, simple and inexpensive, although it suffers from poor reproducibility due to the randomness of the sites targeted by the primers (Cano-Gomez et al., 2009). Restriction enzymes recognize specific short sequences of DNA and cleave the DNA at that site. Single nucleotide changes can result in the gain or loss of a restriction site, thus altering the number of fragments produced following digestion of DNA. These restriction fragment length polymorphisms (RFLP) can be visualized following gel electrophoresis of the digested DNA to separate the fragments according to size. Restriction enzyme digestion of total genomic DNA preparations may produce fragment polymorphisms from different samples. However, detecting these differences within a mixture of many fragments of varying size usually requires the use of probes to identify the Polymorphic fragment (Cunningham et al., 2002). The 16S rDNA genotypes among the family Vibrionaceae were determined using PCR/RFLP analysis. Five tetrameric restriction enzymes (HhaI, DdeI, RsaI, Sau3AI and MspI) were used for RFLP analysis and adequate numbers of informative bands were obtained from each enzyme. Twenty-seven genotypes were obtained from 49 type and reference strains including 35 species (Urakawa et al., 1997).

Isothermal amplification

Within last twenty years many techniques have been developed that allow amplification of nucleic acid at isothermal condition. These techniques include Loop mediated amplification(LAMP),nucleic acid sequence based amplification(NASBA), Rolling circle amplification(RCA) and Strand displacement Amplification(SDA).These techniques may use several set of primers without thermal cycler. One of the major advantages of these processes is that genomic contamination will not affect the result as there is no thermal denaturation process as seen in PCR. Diseased fish showed fluorescent signals with NASBA assays for the detection of *Vibrio cholera*. A LAMP assay targeting the *invA* gene of *Salmonella* was developed by Wang et al. amplification time of approximately 60 minutes and run at 65°C. The detection limit of the LAMP assay was 100 fg of DNA per reaction, whereas a PCR approach gave a detection limit of 1 pg of DNA per reaction tube.

Fluoresence In Situ Hybridisation

Fluoresence in situ hybridisation is a technique for probe based identification of nucleic acids without amplification. In this technique rRNA molecules are targeted. Fluorescent labelled probes will generate specific signals when in contact with target sequence,which can be detected under Fluoresence microscopy. FISH technique can be used for the detection of pathogenic microorganisms using known probes.

STUDY OF MARKER GENE

There is an immense number of different bacterial species in the world. Although most of them are completely harmless for humans, a few dozen bacterial species can cause serious diseases. Established etiologic agents such as *Vibrio cholerae* (cholera), *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Salmonella typhimurium (diarrhea)*, *Shigella flexneri* (shigellosis) and *Legionella pneumophila* (Legionnaires' disease) elicit various diseases in humans. Pathogenic bacteria posses a specific gene which is associated with their virulence properties. A virulence gene, whose presence or activity in an organism's genome is responsible for the pathogenicity of an infective agent. The arsenal of virulence genes determines the kind of disease that a pathogenic bacterium can cause. *A. hydrophilla* possess hly A (Singh et al., 2009) gene and *V. parahaemolyticus* possess TDH gene (Caburlotto et al., 2008) which contribute pathogenecity to these bacteria. These two genes act as identifying markers gene and can be detected by Polymerase Chain Reaction. Duchaud *et al*. (2007)

found the homologue of *V. anguillarum* haemolysin VAH5 in the genome of *F. psychrophilum*, as well as the homologue of the pore-forming toxin thiol-activated cytolysin family (TACY). Such toxins appear to be involved in the pathogenicity of *F. psychrophilum*. Wiens *et al*. (2008) reported several virulence-related factors found in the genome *R salmoninarum*, including the haem acquisition systems, capsular synthesis, secretion systems, major soluble antigen (MSA), proteases and haemolysins. The carA gene which encodes carbomoyl phosphate synthase in *Pseudomonas* sp was used to distinguish between different strains (Ercolini et al.,2007). The hsp60 or groEL gene that encodes a heat shock protein in bacteria contains a fragment that helps in determining phylogentic relationships among bacteria. The *rpsU-dnaG- rpoD* region is another locus has been used to differentiate between different bacteria.

Study of Whole-genome analyses

DNA–DNA hybridization (DDH) evaluates the DNA similarity between two bacterial strains and has been regarded as the gold standard technique for bacterial species delineation. The method is also used for the definitive assignment of a strain with ambiguous properties to the correct taxonomic unit (Wayne et al., 1987). Although DDH is required for proposal of new bacterial species, it is not suitable for routine identification of isolates since the technique is complex, restricted to a few laboratories and non cumulative, requiring the inclusion of reference strains in each identification (Gevers et al., 2005). Recent advances have however taken the technique to a microarray platform and with this approach an open database of hybridization profiles can be used if standard genome chips for bacteria are available. Gomez-Gil and coworkers (2004) made use of DDH to precisely identify 39 presumptive *V. harveyi*-related isolates as *V. harveyi*, *V. campbellii* and *V. rotiferianus*, and demonstrated that these three closely related species share N65% DNA–DNA similarity. Due to the labour intensive protocols and the cost involved in performing DDH analysis, several studies have evaluated the potential for using whole-genome fingerprinting techniques as alternative methods for species classification. These methods are also used in epidemiological studies for identification of constraint groups of bacteria, such as strains from the Harveyi clade. So far, three wholegenome fingerprinting techniques have been used for analysis: Amplified Fragment Length Polymorphism (AFLP), Repetitive Extragenic Palindromic Elements PCR (REP-PCR) and Random Amplified Polymorphic DNA (RAPD). AFLP is based on size separation patterns of fragments amplified with two primer sets after initial restriction cutting of genomic DNA. In general, each bacterial species has a specific AFLP pattern. Since the grouping corresponds well to that obtained by DDH, the technique can be used as an alternative bacterial identification tool (Janssen et al., 1996). For vibrios, DDH similarities can be predicted from AFLP similarities, with band pairwise similarities of around 70% corresponding to DDH similarities of ~80 to 100% (Thompson and Swings, 2006). REP-PCR uses PCR primers to amplify highly conserved DNA sequences present in multiple copies within the genome. After resolving the amplified fragments in a gel matrix, a REP-PCR fingerprint is created and used to differentiate bacterial isolates at the species and strain level. Finally, RAPD is based on random amplification of genomic DNA fragments using a single primer of arbitrary nucleotide sequence. Size separation of the resulting fragments allows differentiation between genetically distinct clonal lineages. The technique is fast, simple and inexpensive, although it suffers from poor reproducibility due to the randomness of the sites targeted by the primers. Whole-genome fingerprinting methods have been used in several studies to refine the complex *V. harveyi* taxonomy and to identify strains associated with diseases in marine animals. Epidemiological studies have used whole-genome fingerprinting methods to associate specific banding patterns to particular pathogenic strains or clones (Wassenaar, 2003). RAPD fingerprinting can be used to discriminate between virulent and avirulent *V. harveyi* strains in several host species (Alavandi et al., 2006). For use in taxonomical classification, RAPD should be complemented

with a more accurate and reproducible identification tool (Gürtler and Mayal, 2001). Despite the usefulness of whole-genome fingerprinting methods for strain identification and characterization, the techniques have their limitations (Cano-Gomez et al., 2009).

Microarray Detection

In addition to southern blot analysis, gel electrophoresis, probe based analysis microarrays have been used to analyse PCR results. In DNA microarray technology, fixes probe based oligonucleotide in specific location on glass or substrate. Following hybridization of the target sequence to probes anchored on chip's surface fluorescence detection can be used to monitor binding events. Microarrays may be an effective way of distinguishing between nonspecific and target product formation and therefore this detection strategy may allow the use of more primers in a multiplex PCR assay than would normally be possible. Amplification methods have been used in combination with microarray technology E.coli O157:H7.Wilson et al.,were able to detect 18 pathogenic bacteria, virus ,prokaryotes and eukaryotes using PCR along microarray analysis containing 50,000 probes and specificity upto 10fg of DNA.

Conclusion

In the 40 years since the advent of molecular biology, developments in DNA technology have revolutionised biological research. Numerous molecular methods have been developed that are rapid sensitive and can identify fish pathogens from few nucleotide bases. The study of fish disease is no exception and great advances have been made in this field, particularly in the last 10 years. The time has now come to reap the benefits of work that has provided the means to improve the diagnosis and control of fish disease. These new techniques have yet to be fully incorporated in fish health legislation. Progress in techniques aids epidemiological studies as well as identifying causes of disease outbreaks or the presence of pathogens. Molecular methods have slowly established a place in the diagnosis of disease in aquaculture. The time has now come for their wide-scale adoption and application. Microfabrication, microfluidics, and nanoparticle conjugation will play a vital role in future systems.Further funding is required to enable high-quality validation and trial of these methods as we move into the next century of fish health research and development.

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NORTHERN BLOTTING :
A GOLD STANDARD IN GENE EXPRESSION ANALYSIS

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Introduction

Northern blotting is a molecular technique used in molecular biology for the detection of RNA molecules. RNA is broadly classified into three types *viz;* tRNA (transfer RNA), rRNA (ribosomal RNA) and mRNA (messenger RNA). Northern blotting classically deals with the detection of RNA which is the product of DNA transcription, and the raw material for the translation (protein synthesis). It provides information about the size, abundance and the presence or absence of desired RNA that helps to analyse the level of gene expression at different stages of development and during abnormality or diseased condition of a cell .

History

Northern blotting was developed in 1977 by Alwin *et al* at Standford university. It receives its name due to its similarities with Southern blotting which was developed by Edwin M. Southern in 1975. The only difference between the two techniques is that Northern blotting targets RNA instead of DNA .

Procedure

Northern blotting basically involves four steps:-

i) RNA separation by gel electrophoresis
ii) RNA blotting on a solid matrix
iii) Hybridization with a specific labelled probe
iv) Detection of the probe

Prior to RNA separation through electrophoresis, RNA isolation should be carried out from a definite source as per the need of the researcher. It is a very critical step as RNA is highly prone to degradation (due to the presence of RNase enzymes). Therefore, in order to get intact and high quality RNA, care should be taken that all reagents used in the RNA isolation process are RNase free. After getting the total RNA from the tissues or cells , the mRNA should be purified. Purification of the mRNA can be done with the help of oligo (dT) attached column chromatography. As the mRNA from eukaryotic source possess poly A-tail , it would bind to the oligo (dT) present in column.

i) RNA separation by gel electrophoresis

Secondary structure interferes in the migration rate / speed of the RNA molecule which creates difficulties in analysing the length of RNA molecule by measuring the migration distance. Therefore, denaturation of the RNA is required. Formaldehyde is generally used as denaturing but, glyoxal / DMSO can also be used.

Generally 1 - 1.5 % of agarose gel is used to separate RNA fragments. RNA/mRNA has to be loaded in one well or lane, in other well a ladder which will help to decipher the size of the RNA molecule. The buffer used in gel electrophoresis is generally 1X TBE. Polyacrylamide gel can be used in Northern blotting if the size of the RNA molecule is very small. An electric current has to be passed through the gel to facilitate the movement of the RNA molecule away from the negative electrode (cathode) towards the positive end (anode). After electrophoresis, the size of the RNA fragment can be measured according to the distance covered by them. The gel has to be stained with EtBr (ethidium bromide) and

then with the help of a gel documenting system (or exposing them to the UV – light) the RNA band can be visualized and analysed.

ii) Blotting

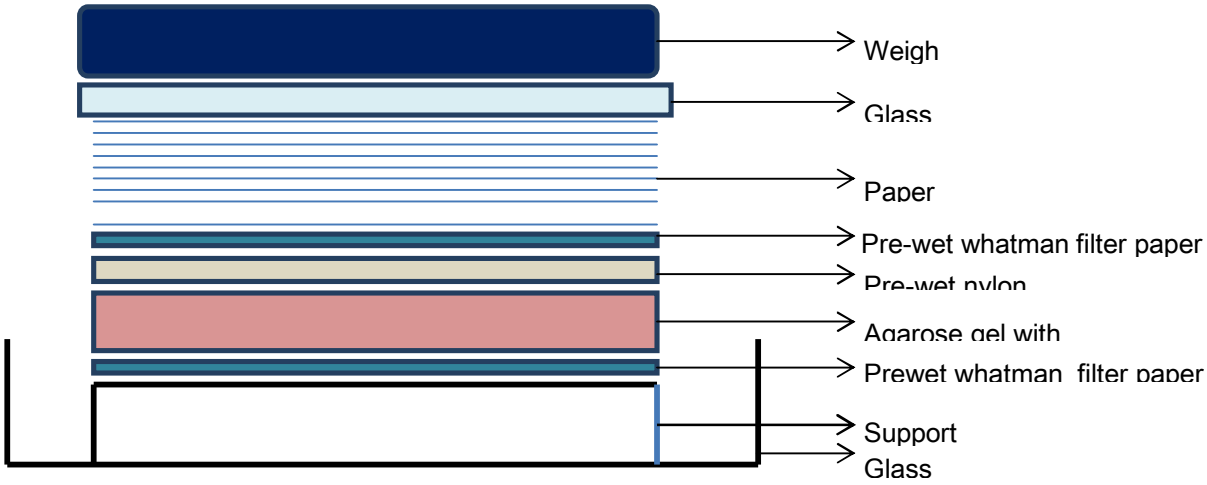
This process involves the transfer of separated RNA molecules from the agarose gel to a suitable solid matrix . Two different kinds of blotting technique are used

i) Capillary transfer
ii) Electroblotting

Capillary transfer

The RNA molecules are transferred to a solid matrix like filter paper, nitrocellulose paper /filter or nylon membrane passively with the help of capillary force. The binding ability of the nitrocellulose membrane varies according to the size of RNA and also requires washing at high temperature, which makes the membrane brittle. Nylon membrane is durable than the nitrocellulose membrane and nucleic acids bind irreversibly to it. It also allows the nucleic acid to get immobilised on it in low ionic strength buffer. Generally two types of nylon membranes are available: - unmodified (or neutral) nylon and charged - modified (positively charged) nylon .The positively charged nylon membrane has greater ability to bind nucleic acid . Capillary transfer can be done in two different ways.

Upward capillary blotting: It is very simple and economic. It requires overnight transfer of nucleic acids from gel to membrane where the buffer flows in an upward direction. Here, the filter paper placed under the gel has to be fully saturated or otherwise some of the RNA can move from the gel in a downward direction counter the flow of the buffer.



Upward capillary transfer

Downward capillary blotting: This technique is better than the above one, as it does not cause flattening of the agarose gel and provides faster transfer of nucleic acid from the gel to the membrane. It has the advantage as the blotting of RNA takes very less time e.g. RNA molecules up to 8 kb in size can be transferred within one hour at either neutral or alkaline pH. First the gel is placed on a sponge which is previously soaked in a buffer or salt solution. The nitrocellulose paper is placed on the gel slabs and above that many layers of papers are placed. The solution passes through the gel and nitrocellulose filter to the paper layers. This process takes about 12 --16 hours.

Electroblotting

Electroblotting involves the use of electric current to transfer the RNA molecules or fragments from the gel to a solid matrix. Mostly the filter paper is used as a solid matrix. In this process, the filter paper is saturated with the transfer buffer. The gel is then sandwiched between the wet blotting paper and wet sponge. It is rolled with a glass rod and inserted into a blotting chamber. Transfer of RNA from gel to uncharged nylon membrane is carried out at a neutral pH using 20X SSC (saline sodium citrate). Transfer of RNA to the positively charged nylon membranes is carried out at alkaline pH with the help of NaOH and HCl. This partially hydrolyses the RNA and increase the speed and efficiency of transfer of large RNAs.

This requires baking under vacuum for 2 hours , by heating in a microwave oven for 2-3 minutes or by exposing the nylon membrane to UV – radiation at 254/312 nm. The later one generates more intense signals in hybridization than baking.
The blotting process is followed by the hybridization process which enables the detection of the RNA molecule .

iii) Hybridization of RNA molecule

Probes used in Northern Blotting are basically composed of nucleic acid which has partially or total sequence complementarity to the target / desired RNA fragment. Bond forms between RNA and the probe where it finds the complementary sequence .Therefore, the design of the probe is to be done according to the sequence of the target RNA which is to be detected . After getting the complementary nucleotide sequence, the probe should be labelled .There are two basic techniques used for the labelling the probe: radioactive labelling and the chemical labelling. Radioactive labelling is done with a radioactive element usually 32P. Chemical labelling is carried out using an enzyme that catalyses the breakdown of a chemiluminescent substrate, and emits light that can be detected with the help of a detector. The chemical labelling can be done in various ways. The nucleotide probe may be attached to the enzyme or to the ligand or the antibody – labelled nucleotide probe may be attached to the enzyme.

The nitrocellulose membrane containing the bound RNA molecule is hybridized with the labelled probe by incubating the membrane in a probe containing solution; this is usually done overnight in a rotating oven to facilitate the probe to bind to the complementary regions of the target RNA. This step is followed by washing step in order to remove all the excess and un-hybridized labelled probes.

iv) Detection of RNA molecule

The detection process depends on the type of probe used in hybridization . If the probe is labelled with a radioactive substance, then the membrane is detected using an autoradiography or a phosphoimager. In case of a chemically labelled probe, the membrane is incubated with suitable reagent or substrate that results in the emission of detectable light which is captured by the detector. In both cases dark bands on the X – ray film indicates the RNA bands. The X-ray film obtained can then be further analysed with a computer densitometric software , that would quantitate the amount of target RNA present on the blotting membrane.

Applications

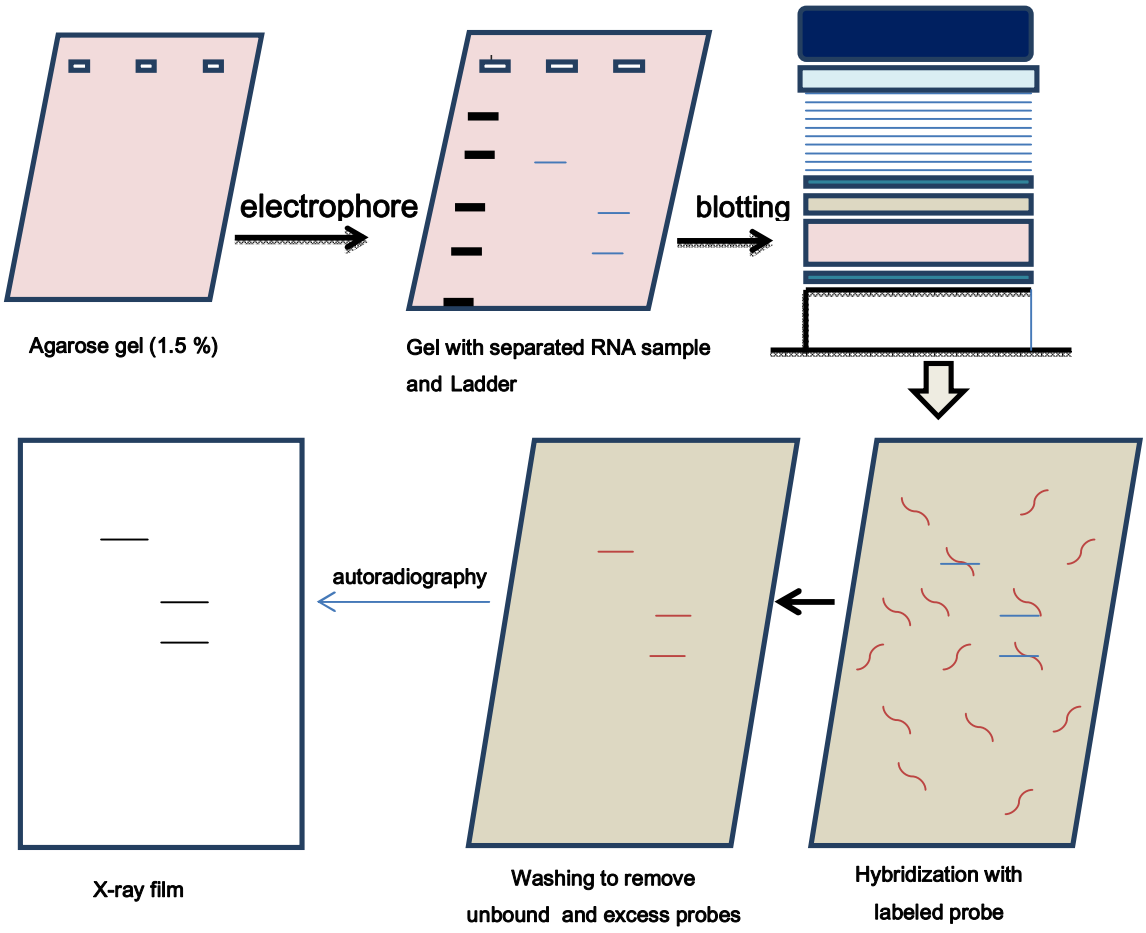
Northern blotting is used to analyse the pattern of gene expression in different tissues, organs and also in different developmental stages of the cell . It helps to analyse the gene transcript level of the cells under stress condition. It helps to study up-regulation and down-regulation of certain gene under different environmental/ external conditions. Therefore, many experiments involve Northern blotting *viz.* to identify different ligand /inducers that increase the expression of immune response related gene, to analyze genetic

and immunological characterization of multixenobiotic resistance in some fishes, identification of different receptors in fishes which hold importance in describing different molecular pathways.

Advantages and disadvantages

Advantage: Northern blotting can efficiently detect small changes in gene expression, it is highly specific and there is less chance of getting false positive result. Because of its simplicity, this technique is still considered as a gold standard in RNA analysis

Disadvantage: Processing of samples are difficult, as they are prone to degradation by RNase enzymes, and all steps requires highly sterilised RNase free condition. Furthermore, the chemical used in this technique like formaldehyde, EtBr, DEPC and UV -light have harmful effect. It is likely to be a little less sensitive than other improved and advanced techniques.



Schematic diagram of Northern

OUTER MEMBRANE PROTEIN: A POTENTIAL CANDIDATE VACCINE FOR AQUACULTURE

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Membranes are highly viscous yet plastic structures. The plasma membrane forms close compartments around protoplasm thus separating one cell from another. The plasma membrane acts as a selective barrier thus maintaining a balanced composition between inside and outside of the cell. A plasma membrane contains about equal masses of lipid and protein. A protein that resides in a membrane is called transmembrane protein. There are two types of membrane associated proteins, peripheral membrane protein and integral membrane protein. Peripheral membrane protein can dissociate from lipid bilayer by either treatment with polar reagents such as extreme pH or high salt concentration. Once dissociated from the membrane, peripheral membrane protein are soluble in aqueous buffer. However integral membrane protein can only be released by reagents that disrupt hydrophobic interactions.

About 50% of the outer membrane mass consist of proteins either in the form of integral membrane protein or as lipoprotein anchored to the membrane. Integral membrane protein account for 20%-30% of total protein. Two major types of integral protein are seen α-helix or β-barrel. α-helix are predominantly found in the cell membrane of eukaryotic cells while β-barrel are located in the outer membrane of Gram-negative bacteria and in the outer membrane of mitochondria and chloroplast (Cavilier,2000 and Gray 1999).The β-barrel protein play an important role in nutrient uptake, trafficking of solutes and ions into and out of the cell, pathogenesis, protein secretion and adherence to the host cell. The outer membrane protein(OMP) are β-barrel protein.

Bacterial Membrane

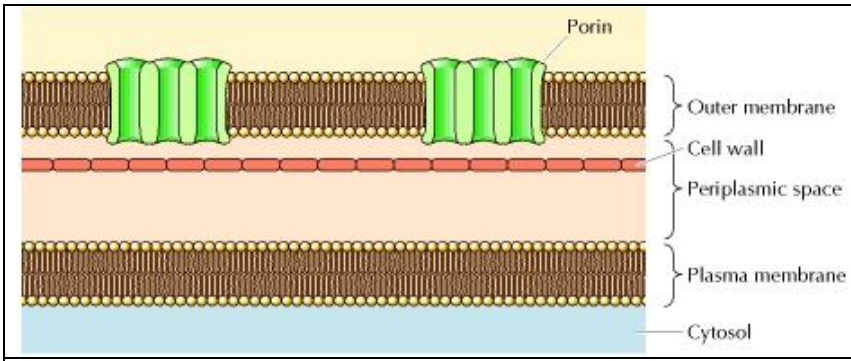


Fig1: Bacterial Outer Membrane

From: Structure of the Plasma Membrane The Cell: A Molecular Approach. 2nd edition.Cooper GM.Sunderland (MA): Sinauer Associates; 2000.

The gram negative bacteria are usually bounded by two membranes .The inner one (IM) called the plasma membrane is a trilamellar structure of protein and lipid bilayer. The outer membrane (OM) also is a trilamelar structure consisting of proteins, including porins, receptors and asymmetric distribution of lipids. The outer portion of the outer membrane is composed of lipopolysaccharide (LPS) projecting outside and the inner leaflet contains

phospholipids and lipoproteins. The LPS of a Gram negative bacteria consists of three different sectors

1. Lipid-A
2. The core polysaccharide comprising the inner and outer cores
3. The O-specific polysaccharide chains projecting outward

The inner membrane and outer membrane are separated by a gel-like layer known as the periplasm. The periplasm contains a thin layer of peptidoglycan which is connected to the OM and IM through protein-protein interactions. The OM contain unique trimeric channel forming proteins known as Porins. Porins allow the passage of small molecules <600 daltons to pass through and enter the periplasmic space. In *Pseudomonas aeruginosa* there are three lipoproteins, OprI, OprL and OprF that connect the OM with the PG layer. In wild- type Salmonella spp there are specific domains in the envelope that promote interactions between the outer membrane protein (Omp) and the peptidoglycan layer (PG) and also interactions between the Omp and the inner membrane protein (IMP) involving the PG; (Deatherage *et al.,* 2009).

Bacterial Outer Membrane Proteins

The outer membrane of Gram negative bacteria, contain transmembrane β-barrel protein known as OMP. Typically OMPs consist of an even number of β strands ranging from 8-24 in antiparallel fashion(Ricci *et al.,*2012 and Fairman *et al.,* 2011). While most OMP share a common β-barrel structure they are functionally very diverse. Based on their function, OMPs can fall into one of these six categories: nonspecific porins, substrate specific channels, translocons for substrate export, auto transporters, enzymes, and structural OMPs. (Nikaido 1994). When these proteins were classified into protein families based on their sequence homology and function, several families were found to be common in OMVs derived from several species of Gram-negative bacteria:

(1) The abundant OMPs, Porins (Omps, PorA, PorB and OprF), which were found in most OMVs;

(2) The murein hydrolases (Met and SLT), which are responsible for the hydrolysis of certain cell wall glycopeptides, peptidoglycans in particular;

(3) The multidrug efflux pumps (Mtr, Mex, and TolC), which are known to release toxic compounds (Kobayashi *et al.,* 2000);

(4) The ABC transporters (LamB and FadL);

(5) The protease/chaperone proteins (DegQ/SurA)

(6) The motility proteins related to fimbriae/pili (FliC/PilQ) were found in OMVs from different strains.

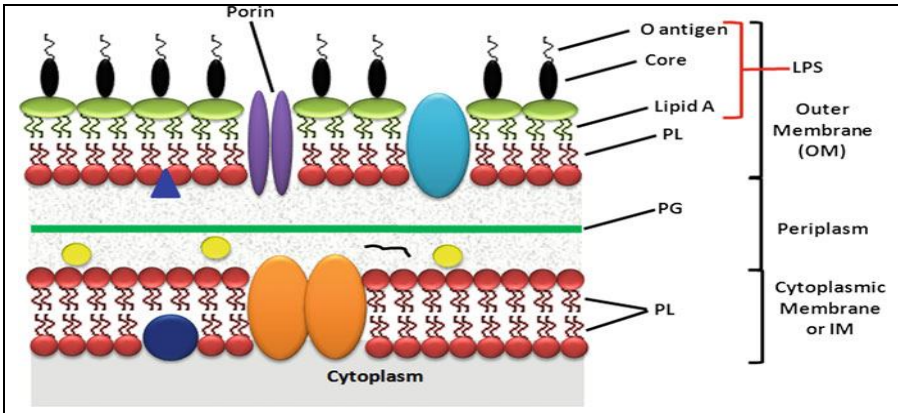


Fig2: A detailed diagrammatic presentation of Gram negative Bacterial OM

Biogenesis of OMP

The field of OMP biogenensis research has seen many changes in the recent years. Bacterial OMP biogenesis begins in the cytoplasm with cleavable N-terminal target sequence that guides them to the inner membrane. The OMPs are translocated across the membrane via the Sec-translocation system into the periplasm (Danese *et al.*,1998).The released OMPs are then transported across the periplasmic space via either SurA pathway or Skp/DegP pathway.(Volokhina *et al.,* 2011 and Eppens *et al.,* 1997).

All prokaryotic proteins are synthesized in the cytosol but they require a chaperone to help them in folding and a translocation system to carry them to the final destination. In Gram negative bacteria one of the least understood methods of membrane protein assembly is that of outer membrane protein (OMPs) which are β-barrels. A significant breakthrough came, however with the identification of a protein complex known as β-barrel assembly machinery (BAM) complex that catalyzes OMP folding and its insertion into the phospholipid bilayer. Absence of the BAM complex results in misfolded OMPs aggregation in the periplasm and eventual cell death (Tommassen,2010 and Knowles 2009). The OMP substrates are recognized by the BAM complex via their C-terminal targeting signal that has a consensus sequence of X-Z-X-Z-X-Z-Phe/Trp where X is hydrophobic and Z is any amino acid. The exact consensus sequence varies from one bacterial species to another and the BAM complex can only recognize OMP from same species(Robert 2006).The first discovered component of BAM complex is BamA, which is associated with four lipoproteins: BamB, BamC, BamD and BamE.

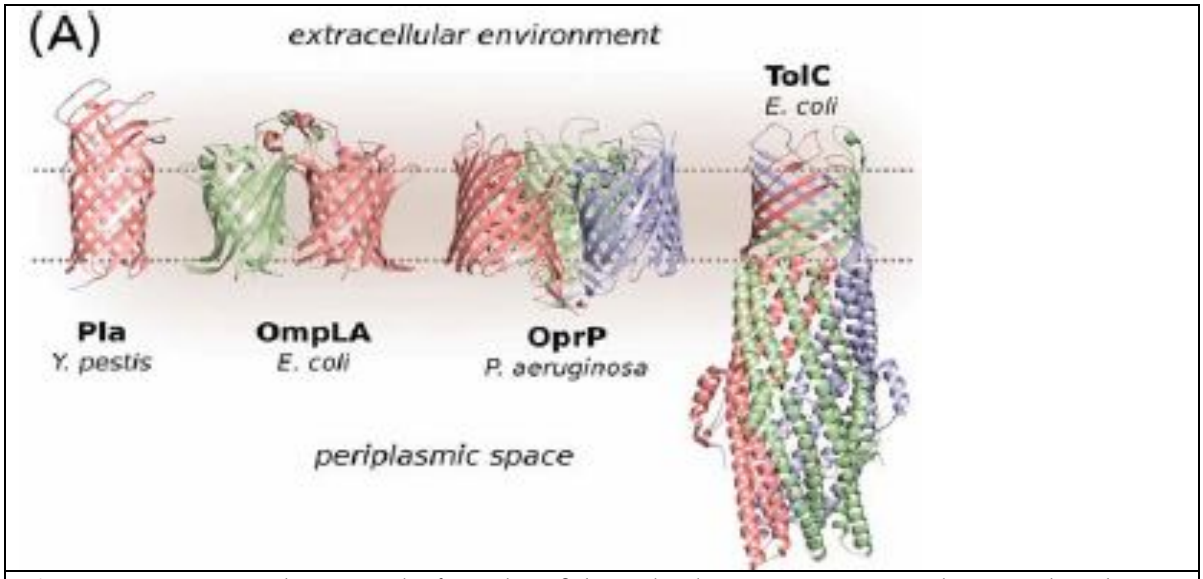


Fig 3: OMP are predominantly found as β-barrel. Pla is a monomer where each subunits forms its own β-barrel, While OmpLA from *E.coli* and OprP from *Pseudomonas aeruginosa* are oligomers and in TolC are oligomers with multiple subunits.

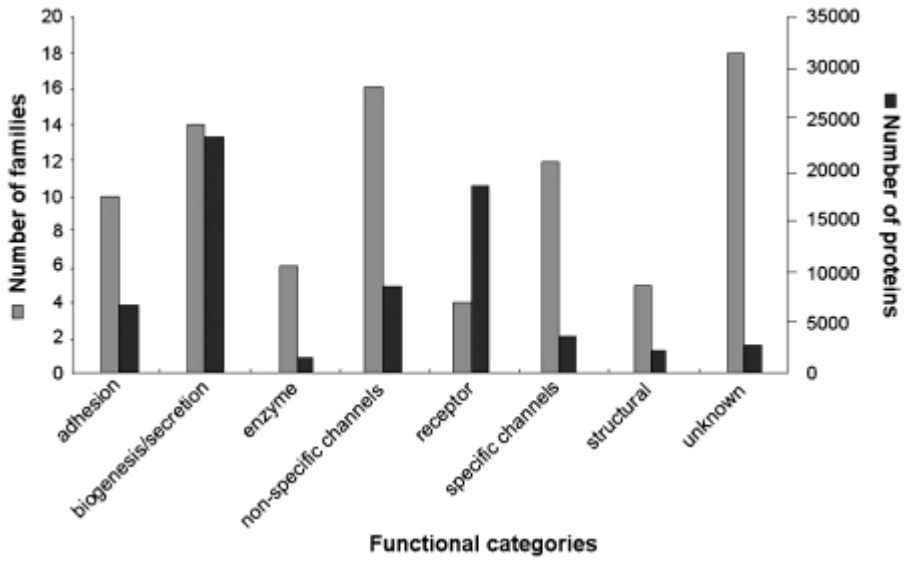
Function of Bacterial Outer Membrane Protein (OMP)

- OMPs of microorganisms function as a dynamic barrier between the cell and its surroundings.
- These structural OMPs include proteins involved in peptidoglycan formation, OMP assembly, as well as usher proteins that transport and polymerize pili subunits required at the bacterium's exterior.
- They form porins for active and passive transport of solute and ions eg TonB receptor complex

- Enzymatic OMPs discovered to date have functions that include proteases and phospholipases
- They help in adherence to host cell thus have pathogenic properties
- They are important elements of antimicrobial resistance in microorganisms
- protein–protein interactions involving the PG are primarily responsible for giving the required strength and stability to the bacterial envelope or rather the surface structures of bacteria.
- Translocons include OMPs that are involved in the export of proteins, drugs, and other molecules, with examples including TolC as well as members of the two-partner secretion system.
- Autotransporters cover the category of OMPs that have a transporter domain embedded in the outer membrane that secretes a passenger domain found on the same polypeptide; this secreted protein is usually a virulence factor, and thus, autotransporters are commonly found in pathogenic strains of Gram-negative bacteria.

(Desvaux 2004)

OMPdb, in its first version, contains 69 354 β-barrel outer membrane proteins, originating from 2712 Gram-negative bacterial species and strains, which are classified into 85 families.



A demonstration of the total numbers of families and protein entries in OMPdb that belong to each of the eight functional categories were created. There are still a lot of families with β-barrel proteins whose function remains unclear, whereas specific and non-specific channels, together with biogenesis/secretion proteins seem to play a vital role in the bacterial life. Receptors are under-represented in terms of the number of families, but they constitute the second largest category of β-barrel OMPs in absolute numbers of proteins. In general, the largest proportion of outer membrane proteins in Gram-negative bacteria serve as receptors or play a role in biogenesis/secretion. A smaller amount comprises of non-specific channels and adhesion proteins. The category of proteins with unknown function, though it contains a lot of families, has a relatively small number of protein members, which

may be due to the progress in whole genome annotation that is being made regarding Gram-negative bacteria.
(From: Nucleic Acids Res. 2011 January; 39(Database issue): D324–D331.)

Given below in the table are few OMP which are of importance in fish disesase

Omp family	**Structure**	**Function**	**Bacteria**
OmpA	N-terminal ß-barrel domain formed by 8 beta strands long flexible loops at the external ends that anchors the protein to the outer membrane	required for bacterial conjugation and for maintenance of outer membrane stability	OmpA is largely expressed in the outer membrane of *Escherichia coli*
OmpX	consist of 8 transmembrane beta sheets and 4 cell surface-exposed mobile loops	Required for invasion and adhesion to host cells	The Ail protein of *Yersinia enterocolitica*
OprD	monomeric 18-stranded ß-barrel.	facilitates the diffusion of basic amino acids as well as some structurally analogous beta-lactam antibiotics	porins in Pseudomonas species
OprF	N-terminal ß-barrel domain formed by 8 beta strands long flexible loops at the external ends that anchors the protein to the outer membrane	maintenance of outer membrane stability	*Pseudomonas aeruginosa*.
OmpW	8-stranded ß-barrel.	OmpW is also the Receptor for colicin S4	*Salmonella typhimurium, Escherichia coli* and *Vibrio cholerae*
MipA/OmpV	ß-barrel.	MipA is considered to be a structural protein mediating the assembly of MltA to PBP1B into a complex. OmpV, is the Major Outer Membrane Protein (MOMP) of *Vibrio cholerae*	*Vibrio cholerae*
AT-2 (Autotransporter)	ß-barrel with 12 transmembrane beta-strands, comprised by 4 strands from each subunit	attachment to the surfaces of host cells and protect bacteria against complement and the bactericidal activities of defensins.	All pathogenic bacteria
OMR	22-stranded antiparallel ß-barrel traverses the outer membrane with the	outer membrane proteins act as receptors for organic iron chelators (siderophores) and vitamins necessary for bacterial	Pathogenic bacteria, *Flavobacterium collumnare*

	adjacent beta-strands being connected by large extracellular loops and periplasmic turns	life	
FUP(Fimbrial Usher Protein) OmpC	ß-barrel proteins and form channels consisting of 24 beta strands	proteins work in conjunction with a periplasmic chaperone in order to assemble and secrete more than 30 different surface molecules in a broad range of Gram-negative bacteria	*Edwardsiella tarda,*
GBP-1(General Bacterial porin) Omp32, OmpF, Phosphoporin, OmpK36	6-stranded ß-barrel is a common structural feature	facilitating the diffusion of small hydrophilic molecules through the outer membrane in a non-specific manner	*Aeromonas*

Virulence and Vaccination

The outer membrane (OM) of Gram-negative bacteria is in direct contact with the external environment, and contains proteins, lipoproteins, phospholipids, and lipopolysaccharides (LPSs). OM proteins (OMPs) are often directly involved in the virulence of pathogenic bacteria. *Aeromonas hydrophila* is a major fish pathogen causing haemorrhagic septicaemia, tail and fin rots, red sores, ulceration and ultimately cell death. Recent research have shown that virulent strains have following virulent factors haemolysin (hlyA), pro-tease (oligopeptidase A), outer-membrane protein (OMP), multidrug-resistance protein and histone-like protein (HU-2) which are absent in avirulent strains of *A. hydrophila*. Several workers have studied the immunogenic properties of OMP of *Aeromonas hydrophila* in different fish models. Results of Vazquez-Juareza *et al.,*. (2005) also indicated that encoding major outer membrane proteins (OMP) of *A.veronii* into DNA vaccines afforded some protection in spotted sand bass (*Paralabrax maculatofasciatus*) against a homologous challenge. Rahman and Kawai (2000) showed that OMPs of *A. hydrophila* has immunogenic properties in gold fish. In a similar study the immunogenic properties of OmpTS protein from *A.hydrophila* was studied in Indian Major Carps by Khushiramani *et al.,*(2007).

Enteric redmouth (ERM) is an acute or chronic generalized bacterial septicaemia in fish caused by *Yersinia ruckeri* mostly affecting the salmons. SDS-PAGE analysis of OMP of *Yersinia ruckeri* showed five types of OMP in the range of 36.5-40.5 kDa.(Davies 1991). Though vaccines have been developed against ERM but due to vast intraspecific variance there is no complete prevention against all the pathogenic strains. Based on OMP profiling and biotyping six clonal groups were proposed by Davies.

E. tarda produces the disease commonly known as 'fish gangrene', 'emphysematous putrefactive disease of catfi sh' or 'red disease of eels', hereafter known in this text as *Edwardsiella* septicaemia (ES), and *E. ictaluri* causes 'enteric septicaemia of catfish' (ESC). Fimbrial proteins OMPs and Lipopolysaccharides are recognized as important antigens of *E. tarda.* The major drawback of *Edwardsiella* bacterins is short span of protection and lack of broad strain protection. The lack of protection may be overcome by a 37kDa OMP which

produces effective protection against different serotypes of *E. tarda* (Kawai *et al.,*2004).Fish immunized with OMP have shown higher survival rate compared to normal fish.

Vibriosis occurs in cultured and in wild marine fish in salt or brackish water, particularly in shallow waters during late summer. The characteristic clinical signs of vibriosis include red spots on the ventral and lateral areas of the fish and swollen and dark skin lesions that ulcerate, releasing a blood exudates. The outer membrane protein-OmpK has been considered as a vaccine candidate for the prevention of infections due to *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in fish. Interestingly, the polyclonal antibody raised against the recombinant OmpK from V. harveyi strain EcGs020802 recognized the OmpK homologues from other strains of Vibrio species by immunoblotting (LiN *et al.,*2010).

There are three primary bacterial species within the genus *Flavobacterium* that cause signifi cant diseases to hatchery-reared and wild populations of freshwater fish. Columnaris disease may cause both external pathology and systemic infections. *F. Columnare* can be isolated readily from gills, body surface lesions and from internal organs (e.g. kidney, spleen and liver) of many of the fish. Similarly 62 OMPs have been identified from tthe 2D analysis of SDS profile of *F. Columnare* by Dumpala *et al.,.,*2010.

Conclusion

Essentially OMP plays a vital role in trafficking of molecules across the lipid bilayer, thus can be of special importance in drugs metabolic pathway. Furthermore some OMP play a direct role in virulence extending the importance of studying bacterial OMPs and their biogenesis to medical interest**.** Several pathogenic bacterial genomes and fish genomes have been fully sequenced. So more knowledge has to be gathered from the genome database regarding specific drug targets. New drug development focuses at a suitable drug target. In this aspect BAM complex could serve as suitable target because it is very essential for survival of Gram negative bacteria and its functionally redundant. A detailed study of the protein family in the OMP database will throw light into the various pathogenic pathways in different bacteria affecting the host. Several researchers have studied and found the immunogenic properties of OMP. Thus OMPs can be used as a potential candidate for vaccine development for a broader spectrum of diseases

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A NEW IRON BASED COMPOUND INDUCES CELL DEATH IN TUMOR CELLS THROUGH CASPASE DEPENDENT PATHWAY

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Abstract

Transition metal complexes have recently been in focus for its potential as anti-tumor drugs. Due to its reactivity with oxidant molecules, iron based compounds possess the capability of inducing cell death that is hitherto less investigated. An iron(salen)Cl derivative has been synthesized that remains active in monomer form. The efficacy of this compound as an anti-tumor agent was investigated in human and mouse leukemia cell lines. The results showed that Fe(salen)Cl induced cell death in specifically in tumor cells and not in normal primary cells. Mouse and human T cell leukemia cell lines, EL4 and Jurkat cells were found to be very susceptible to Fe(salen)Cl and underwent apoptosis, but normal mouse spleen cells and human peripheral blood mononuclear cells (PBMC) remained largely resistant to Fe(salen)Cl. Fe(salen)Cl treated tumor cells showed significantly higher expression level of cytochrome c that might have triggered the cascade of reactions leading to apoptosis in cancer cells. For the first time we demonstrated that Fe(salen)Cl specifically targeted the tumor cells, leaving the normal cells least affected, indicating an excellent anti-tumor potential for this compound. Further, the study emphasizes that Fe(salen)-induced cell death in tumor cells is though caspase dependent cytochrome c mediated apoptosis pathway.

Introduction

Cancer still possesses a major threat to mankind despite intensive research focused on study in cancer. Amongst the chemotherapeutic drugs for cancer, cisplatin is mostly being used albeit it has serious side effects [1]. Investigation in this area is centered on finding out a suitable drug that will specifically target the cancer cells leaving the normal cells unaffected or least affected. In this effort, transition metal complexes are of intense research interest because of their structural diversity [2-4]. Amongst the metallo-drugs, Fe(porphyrin) [5] and Fe(salen) [6] compounds have been shown to have cytotoxic effect *in vitro* in cancer cells, but their effect on normal cells remain largely undefined. Fe^{III}(porphyrin) complexes are widely used to mimic the role of cytochrome P-450 class of enzymes that are responsible for the oxidation of various organic substances [7]. In this context we have synthesized Fe^{III}(salen) complexes (Scheme 1) to mimic the activities of Fe^{III}(porphyrin) complexes. A nice similarity exists between Fe^{III}(porphyrin) and Fe^{III}(salen) complexes [8, 9]. Mandal and his colleagues [10] have synthesized a series of Fe^{III}(salen) derivatives and studied their biochemical effects on DNA.

In the present study, we have synthesized Fe^{III}(salen) complex and have studied its anti-cancer activities in cancerous tumor cells and normal primary cells in an effort to study its potential as anti-cancer drug. From single crystal X-ray structural analysis it is found that Fe^{III}(salen) complexes could exist as monomeric (1A) or dimeric (1B) fashions depending on the solvent used for crystallization. Although single crystal data obtained from CH_3CN medium suggest that the compound is indeed dimeric in nature in solid phase, the solution phase electrochemical studies indicate that the compound is monomer in solution (Scheme 1). The interaction of Fe^{III}(salen) with live cell is very scarcely reported in literature [11]. Mn(salen) [10, 12, 13] and Fe^{III}(salen) [14] have been reported to induce cell death in breast cancer cell line *in vitro*, suggesting that salen compounds may have anti-tumor properties,

although the mechanism by which salen compounds induce cell death is yet to be elucidated. Nevertheless, the effect of salen compounds on the viability of primary cells is largely unknown. Oxidative stress exerted by redox active metals like iron may be responsible for DNA/RNA damages *in vitro* [15-17]. In the present study, we show for the first time that Fe(salen)Cl specifically kills the cancerous cells and spares the primary normal cells. Further, the study emphasizes that Fe(salen)-induced cell death in cancerous cells is possibly due to caspase dependent cytochrome c mediated apoptosis.

Materials and Methods

Synthesis of Fe(salen)Cl

Fe(salen)Cl has been synthesized by following the procedure reported earlier [18]. Fe^{III} chloride and salen ligand were mixed in (1:1) mole ratio in absolute methanol ($25cm^3$). The resulting mixture was heated to reflux under a dinitrogen atmosphere for 2h. The concentrated solution was kept in the refrigerator overnight. The precipitate which formed on cooling was filtered and washed thoroughly with ice-cold water followed by diethyl ether. The product was recrystallized from acetone.

Cytotoxicity assay

The cytotoxicity of Fe^{III}(salen) was evaluated by using Trypan blue dye exclusion method as described earlier [20]. About 1 million cells in IMDM media were seeded in a 24 well culture plate (Nunc). Fe^{III}(salen) was dissolved in DMSO and diluted further in IMDM culture media and was added to triplicate wells in a concentration of 0μM (control), 0.625μM, 1.25μM, 2.5μM, 5μM and 10μM. Control wells were added with equivalent amount of diluted DMSO. After 18 h of incubation cells were taken out from each well and were subjected to death assay by trypan blue dye exclusion method. IC_{50} value for the Fe(salen) metallo-complex was determined by counting the percentage of live and dead cells under the microscope.

Flow cytometry

For a better understanding of the pathway, by which Fe(salen) induced cell death in cancer cells, we studied apoptosis by staining the cells with Annexin V-PE and 7AAD kit (BD Biosciences) according the manufacturer's protocol with a little modification [21, 22]. The cells were centrifuged at 1500 rpm for 5 minutes and the pellet was washed with PBS (Phosphate buffer saline) and then resuspended in binding buffer. Annexin V-PE and 7AAD were added to the cells and were kept for 5 min at room temperature. After the incubation the cells were analyzed by flow cytometer (FACS Calibur, BD Biosciences).

Western blot analyses

Cytochrome c plays an important role to trigger the cascade of reactions leading to the induction of apoptotic pathway. The enhanced apoptosis in Fe(salen)Cl treated EL4 and Jurkat cells encourages us to investigate the expression status of cytochrome c. Western blot analyses were done on Fe(salen)Cl treated Jurkat cell lysate. In brief, Jurkat and EL4 cells were cultured in presence (2.5 μM) and in absence of Fe(salen)Cl for 18h. After the incubation, cells were harvested and washed in PBS and were re-suspended in lysis buffer containing protease inhibitors. The lysate proteins were separated by SDS-PAGE and were transferred on PVDF membrane (Millipore) and were blocked in 5% non-fat milk in PBS. The membrane was probed with rabbit anti-cytochrome c (Imgenex) and were detected with goat anti-rabbit antibody conjugated to peroxidase (Jackson).

Results:

Cytotoxic effects of Fe(salen)Cl on tumor and normal cells

The effectiveness of an ideal anti-cancer candidate should selectively target the cancer cells and not the normal cells. Thus the ability of Fe(salen)Cl to induce cell death was investigated in cancer cell lines and was compared with its effect on normal cells. EL4 (mouse T cell lymphoma) and Jurkat (human acute T cell leukemia) cells were studied to investigate the efficacy of Fe(salen)Cl in inducing cell death. Mouse splenocytes and normal human peripheral blood mononuclear cells (PBMCs) were used as primary cells to monitor the effects of Fe(salen)Cl on normal cells. Cells were cultured in presence and in absence of Fe(salen)Cl and were incubated at different concentrations of Fe(salen)Cl. After 18 h of incubation, live and dead cells were counted by trypan blue dye exclusion method. Our data indicated that Fe(salen)Cl induced cell death in EL4 in a dose dependent manner and the LC_{50} for EL4 was about 1.25 μM. Normal mouse splenocytyes were more resistant to Fe(salen)Cl compared to its cancerous counterparts and less than 10% cells were found to be dead at a dose as high as 10 μM, at which more than 95% EL4 cells were found to be dead. Similarly the result showed that Jurkat cells were susceptible to Fe(salen)Cl even at a concentration less than 1.25 μM and more than 20% of the cells were found dead. The LC_{50} of Jurkat cells was found to be around 2.0 μM. Interestingly, normal human PBMCs were significantly resistant to Fe(salen)Cl in comparison to Jurkat (Figure 2) and only about 3-4% cells were found to be dead at a dose of 10 μM, whereas at this dose more than 95% Jurkat cells were found dead. The data possess a significant finding that Fe(salen)Cl was potent to induce cell death specifically to cancer cells both in mouse and human, whereas it has little effect on normal cells. This indicated that Fe(salen)Cl specifically targets the cancer cells and not the normal ones.

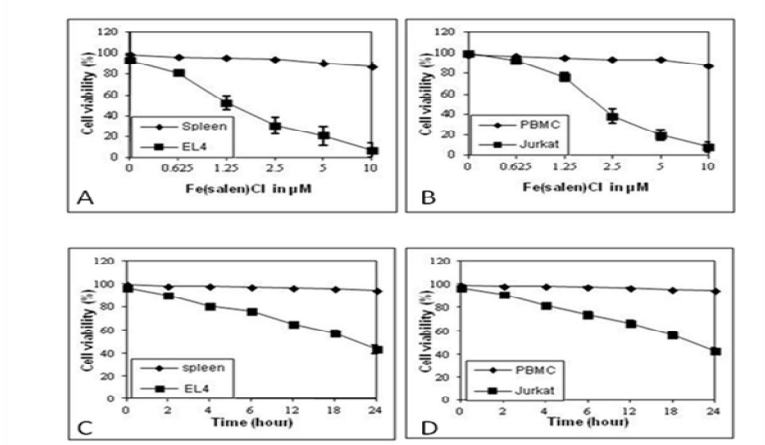

Fig. 1. Fe(salen)Cl induces cell death in EL4 and Jurkat cells but not in mouse splenocytes and human PBMCs.

Dose (upper panels, A and B) and time kinetics (lower panels, C and D) of Fe(salen)Cl on cell survival in mouse splenocyte and EL4 cells and in human PBMC and Jurkat cells. Normal mouse splenocytes, human PBMC, EL4 and Jurkat cells were cultured in a 24-well plate in presence of different concentrations of Fe(salen)Cl as indicated and were cultured for 24 h (A and B). For time kinetics, cells were incubated with 2.5 μM of Fe(salen)Cl for 0, 2, 4, 6, 12, 18 and 24 h, respectively (C and D). Fe(salen)Cl efficiently induced cell death in EL4 (A and C) and Jurkat cells (B and D) but did not affect normal mouse splenocytes and normal human PBMCs.

Fe(salen)Cl induced of apoptosis in tumor cells but not in normal cells

Induction of 'programmed cell death' or apoptosis in susceptible cells is a salient feature of most anti-cancer agents. Thus, the induction of death by Fe(salen)Cl in tumor cells was investigated to find out whether this death is due to apoptosis (Figure 3). Combination of Annexin V-PE and 7AAD was used to detect any apoptosis in EL4 and Jurkat cells by Fe(salen)Cl. The cells were incubated with different doses of Fe(salen)Cl for 18h and were stained with Annexin V-PE and 7AAD. As the figure 3 suggested both EL4 and Jurkat were undergoing apoptosis after treatment with Fe(salen)Cl. Interestingly, the induction of apoptosis was found to be dose-dependent and with the increase in the dose of Fe(salen)Cl, the number of apoptotic or dead cells increases.

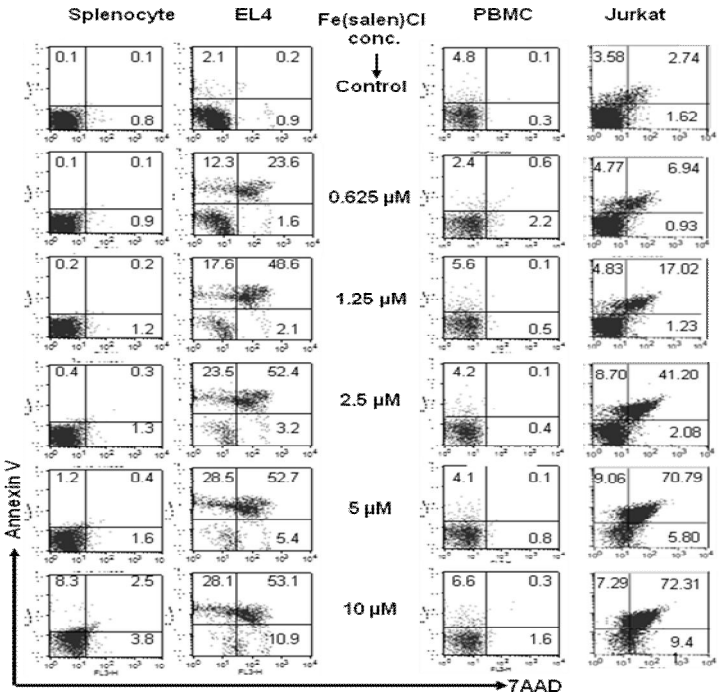

Fig. 2. Fe(salen)Cl induces apoptosis in EL4 and Jurkat cells.

Flow cytometric analysis of apoptosis in mouse splenocytes and EL4 (left panles); and human PBMC and Jurkat cells (right panels) treated with Fe(salen). Cells were cultured for 24 h in presence of different concentrations of Fe(salen)Cl as indicated. Cells were harvested, washed in PBS and were stained with Annexin V and 7AAD. Data were analyzed by Flow cytometry. Numbers in the quadrants are percentage of positive cells in the respective quadrant and show that with the increasing dose of Fe(salen)Cl the number of Annexin V and 7AAD positive cells increase.

Expression level of cytochrome c was induced in tumor cells

Cytochrome c plays an important role in the induction of apoptosis and it triggers a cascade of reaction that leads to apoptosis. Any change in the cytochrome level in a cell could be an indicator of its apoptotic status (Figure 4). Once it is found that the cells are undergoing apoptosis in presence of Fe(salen)Cl, we studied the expression level of cytochrome c level Fe(salen)Cl treated cells and interestingly it is found that the level of cytochrome c are indeed significantly increased in treated cells as compared to untreated cells. It suggests that Fe(salen)Cl was capable of inducing the expression of cytochrome c level that in turn initiates the apoptotic pathway.

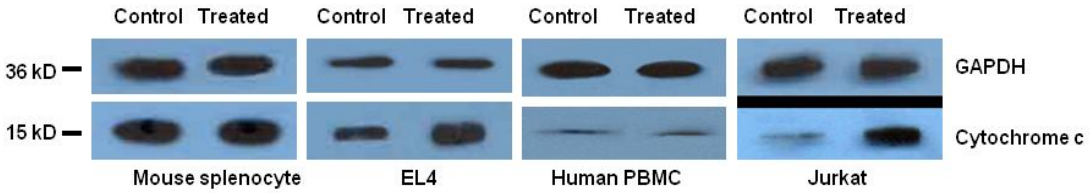

Fig. 3. Fe(salen)Cl upregulates cytochrome c expression in EL4 and Jurkat cells but not in mouse splenocytes and human PBMCs. Cells were cultured for 24 h in presence of Fe(salen)Cl (2.5 µM) and the cell lysates were subjected to Western blot analysis using anti-cytochrome c antibody (lower panel). Anti-GAPDH antibody was used as loading control.

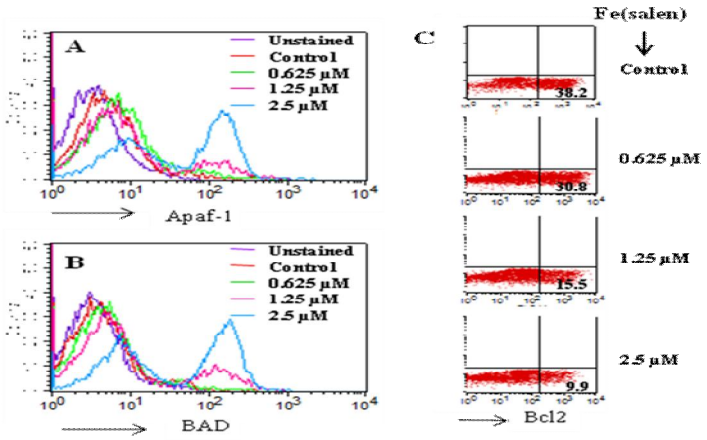

Fig. 4. Fe(salen)Cl upregulates expression of Apaf-1 (A) and BAD (B) and downregulates Bcl2 (C) in Jurkat cells. Jurkat cells were cultured for 24 h in presence of different concentrations of Fe(salen)Cl as indicated and were subjected to flow cytometric analysis. Apaf-1 (A) and BAD (B) expression in Jurkat cells were upregulated when cells were incubated with Fe(salen)Cl at 1.25 µM and 2.5 µM. Bcl2 (C) expression was downregulated in a dose dependent manner with the increasing doses of Fe(salen)Cl.

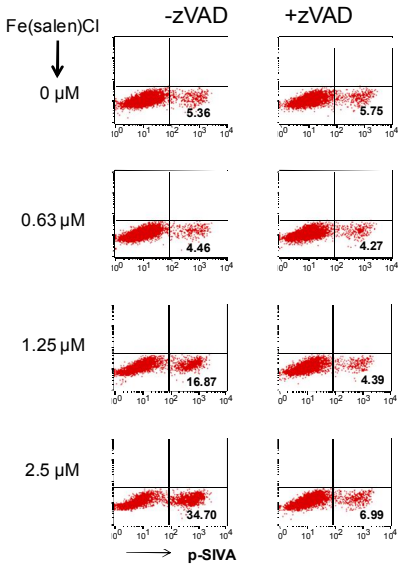

Fig. 5. zVAD rescues Jurkat cells from Fe(salen)-induced apoptosis. Jurkat cells were cultured in a 24-well plate in absence or in presence of zVAD along with different concentration of Fe(salen)Cl as indicated and were cultured for 24 h. Cells were harvested and were stained with p-SIVA-IANBD for flow cytometric analysis of apoptosis.

Discussion

One of the main methods of modern cancer treatment is chemotherapy [23]. The majority of drugs used for the treatment of cancer today are cytotoxic drugs that work by interfering in some way with the damaging effects on DNA [24]. A major challenge is to design new drugs that will be more selective for cancer cells, and thus have lesser side effects. With the advancement in the field of inorganic chemistry, the role of transition metal complexes as therapeutic compounds is becoming increasingly important [25]. These complexes offer a great diversity in their action; they not only have anti-cancer properties but also have anti-inflammatory [26], anti-infective [27] and anti-diabetic [28] effects.

Recent studies showed that metallo-salen compounds may have the potential to induce cell death. It has been reported that Mn(salen) [12] and Ru(salen) [29] was able to kill the cells by damaging DNA. These studies inspired us to find out the potential of Fe(salen)Cl to induce cell death. Our results show that Fe(salen) was indeed able to induce cell death in dose dependent manner in both mouse and human T cell leukemia cell lines (EL4 and Jurkat, respectively). The LC_{50} of Fe(salen) in EL4 cells is around 1.25 μM, whereas in Jurkat around 2 μM. At a dose of 10 μM of Fe(salen), the cell death percentage reaches above 95% in both EL4 and Jurkat cells. Very interestingly, Fe(salen)Cl hardly affect the cell viability in normal mouse splenocytes and human PBMCs, even at a dose of 10 μM. This data is very important considering the fact that an ideal anti-tumor drug should selectively target the cancerous tumor cells and not the normal cells.

Albeit, the Fe(salen) complexes have been reported to induce cell death *in vitro*, it was not well-defined, how this compound elicit cell death. In the present study, we show that Fe(salen)Cl actually induces programmed cell death or apoptosis in EL4 and in Jurkat cells indicating that Fe(salen) could be a strong candidate for anti-cancer drug. Ansari et al. [6] have reported that Fe(salen)Cl compound was able to induce cytotoxic effects in breast cancer (MCF7) and colon cancer (CCL228) cell lines. MCF10, a non-malignant breast epithelial cell line have shown somewhat resistance against Fe(salen) as compared to MCF7 [12].

The pro-apoptotic nature of Fe(salen) and its selective specificity against cancer cells, led us to investigate the pathway by which the apoptosis is triggered. Studies on this metallo drug demonstrated that it was also triggering the intrinsic pathway of apoptosis by permeabilization of the outer mitochondrial membrane and the release of proteins from the mitochondrial intermembrane space [30]. Among these factors, there are several proteins that initiate or regulate caspase activation, including cytochrome c. The expression level of cytochrome c showed a remarkable increase in Fe(salen)Cl treated EL4 and Jurkat cells in comparison to untreated controls. The higher intensity band of cytochrome c in treated cancerous cell lines is the hallmark of apoptosis. The data indicated that Fe(salen)Cl triggered apoptosis in treated cells via mitochondrial pathway.

Our results showed that the Fe(salen)Cl metallo compound indeed has cytotoxic effects in cancer cells via induction of apoptosis and our data suggest that the salen compound may

induce apoptosis by mitochondrial pathway by releasing cytochrome c to cytoplasm which in turn results in caspase activation leading to cell death.

Tumor cell produces a large amount of hydrogen peroxide compared to normal cell [31]. The exact cause of this hydrogen peroxide generation is unclear. It is well known that the combination of Fe(II)/Fe(III) and hydrogen peroxide produces free $OH^{\bullet}$ radicals via the Fenton or Haber-Weiss process [32]. Iron is primarily responsible for helping the Fenton reaction and to produce hydroxyl radical ($OH^{\bullet}$) inside the cell and this hydroxyl radical ($OH^{\bullet}$) cleaves the DNA by damaging both bases and deoxyriboses [33]. It explains the toxic nature of the free $OH^{\bullet}$ radicals generated inside the cell and ultimately causes apoptotic cell death. Production of $OH^{\bullet}$ radicals from various Fe(II) chelates has been shown by Yamazaki and Piette [34] by using the spin trapping technique and it has been concluded that the overall yield of the $OH^{\bullet}$ radicals depends on the ancillary ligand functionalities.

In conclusion, we have demonstrated that treatment of EL4 and Jurkat cells with Fe(salen)Cl is capable of inducing apoptosis via a cytochrome c-mediated intrinsic pathway indicating that Fe(salen)Cl seems to be effective in arresting tumor cell growth *in vitro*. We have also demonstrated for the first time the specificity of the drug, Fe(salen)Cl, towards the tumor cell line. However, further studies are warranted to establish both the cancer preventive and therapeutic efficacy of Fe(salen)Cl.

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REAL-TIME PCR : BASIC PRINCIPLE AND APPLICATION IN FISH IMMUNOLOGY

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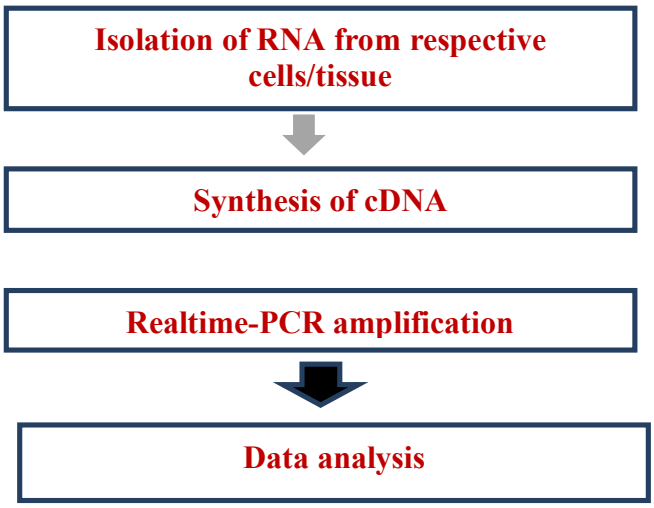
Introduction

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) or Kinetic polymerase chain reaction (KPCR) is a laboratory technique based on PCR which is used to amplify and simultaneously quantify a targeted DNA molecule. It was first introduced in 1992 by Higuchi and his co-workers [1, 2] . This technique can be opted for its precise quantification of specific nucleic acid molecule present in a complex mixture even at lower concentration. This methodology reveals its excellent feature when there is an amplification of targeted DNA molecule using FRET technology (fluorescence resonance energy transfer). It produces a clear image of how quickly the amplified target reaches a threshold detection point which correlates with the quantity of starting material present. It can be applied to traditional or conventional PCR applications as well as new-fangled applications that would have been less effective in comparison to the conventional PCR. With its ability in quantifying and collect data in the exponential growth phase, the command of PCR has been expanded to various applications such as: genotyping [3-5], quantifying viral load in patients [6], and assessing gene copy number in cancer tissue [7-9]. However the most common and unique use for this technology has been studied in quantization of gene expression levels by blending it with a procedure called reverse transcription [10-14]. By combining the two technologies (called real -time RT-PCR), one can measure RNA transcripts level in a quantitative fashion. There are many different variations of using real time PCR assay like 5'exonuclease activity, Taq Man probe as well as the most significant and universal method SYBR Green which has extensively been adopted in many laboratories.

Fig.1.Generalized scheme of Real Time PCR



<u>Real Time PCR measures the amplicon during the exponential phase</u>

The amplicon or the product accumulation is measured "real time" by the use of various fluorescent labeled substrates thereby making the process expedient as compared to the conventional PCR. Some of the techniques and their principles are precisely described below.Three general methods are used for the quantitative assays.

(a) ***SYBR Green I technique:*** SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

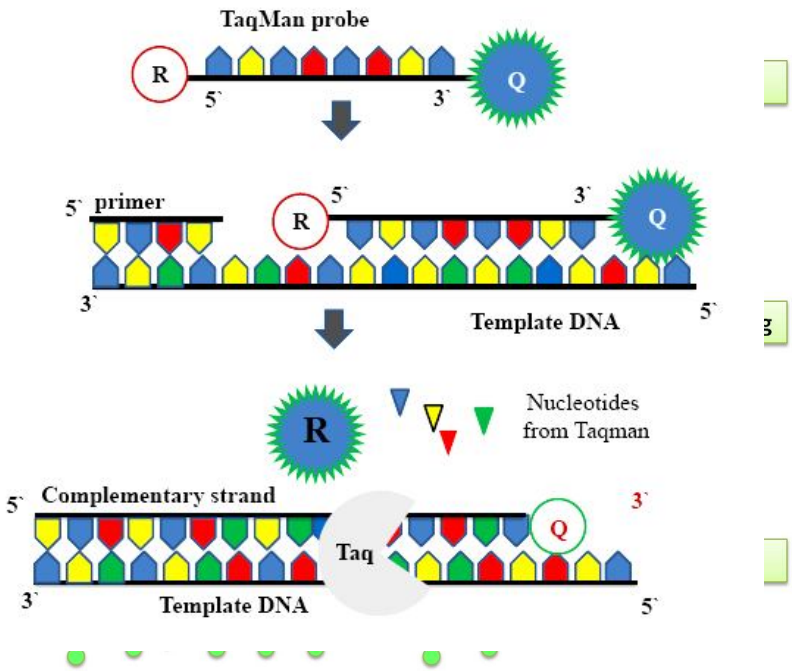


Fig.2. Schematic representation of SYBR green I technique: The fluorescence of this dye significantly increases when it binds to the minor groove of the dsDNA

(b) ***Hydrolysis probe technique (TaqMan probes and Molecular beacons):*** The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the

Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

(c) ***Hybridization probes technique:*** In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

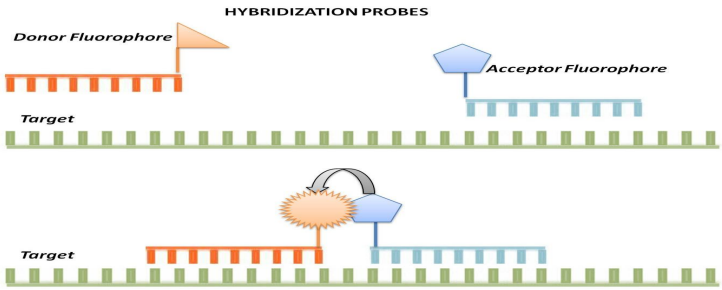


Fig.5. Hybridization probe Assay

Strategic designing of real time PCR

The Real Time PCR method includes a multiple step protocol hence requires:

- ✓ High quality RNA purification, hence it is recommended to purchase the kits from commercial suppliers thereby minimising the time consumed in preparing the reagents and validate them.
- ✓ Optimum conversion of RNA to cDNA by reverse transcriptase enzyme and additional buffers standardized by professional suppliers.
- ✓ Sensitive quantification methods for accurate determination of PCR products "real time".

The detailed explanation of a Real Time PCR methodology is given below:

Step 1: Converting RNA into cDNA

The most important and general method to start working in RT-PCR is its checking the purity and integrity of RNA. The RNA is highly sensitive and less stable than DNA due to its ubiquitous nature and constancy of RNases compared with DNases. Therefore proper maintenance must be carried out as RNA is chemically less stable than DNA and is easily degraded in solutions. Thus, it is important to stabilize the RNA preparations as quickly as possible. On a usual practice in laboratories, RNA is stabilized by adding RNase inhibitor diethylpyrocabonate (DEPC) and is stored as -70˚C. Besides this, DNA contamination might be a significant problem, thus major strategically approaches are taken to treat the samples with RNase free-DNase which are supplied by various manufactures. There are various methods of isolating total RNA depending on the protocol used which is tissue specific. While, selecting an RNA isolation protocol one must understand what to use total RNA or mRNA as the target material in RT-PCR assay. However, it is important to use total RNA as the purification is simpler and provides sufficient amount and quality for the RT-PCR assay.

There are various manufacturers who supply day to day kits in use which are easy to work for RNA isolation with automated standardized procedures for an easy differentiation with other experiments (Ambion, Stratagene,Qiagen and Invitrogen).The very first step in the RT-PCR reaction is to selectively convert the RNA molecules encoding the proteins containing genes into cDNA. The RNA that encodes the protein or carries message to synthesize proteins is mRNA. The messenger RNA that is extracted from cell or tissues must be in a purified form. To convert RNA into cDNA, it follows the reverse transcription process which in an experimental point of view has many variations. Basically, the purified form of RNA is converted to cDNA by RNA dependent DNA polymerase enzyme known as reverse transcriptase. A short DNA molecule strand called oligonucleotide primer is hybridized to complementary mRNA, which extends the primer by the activity of reverse transcriptase enzyme and produce a complementary DNA strand (cDNA). The designing of sequence of a primer or probe can be in such a fashion that it can bind to the target DNA molecule or to the mRNA with efficacy. The two most common strategies include the use of an oligo (dT) primer that can hybridize to the poly (A) tail at the 3' end of almost all the eukaryotic mRNA or the random hexamer oligonucleotide that can easily hybridize to any region of mRNA strand thereby produce nonspecific strands of complementary cDNA which are lowly expressed transcripts. Although oligo (dT) is the most preferred method in converting RNA into cDNA but random hexamers priming method are also of importance when converting bacterial mRNA into cDNA.

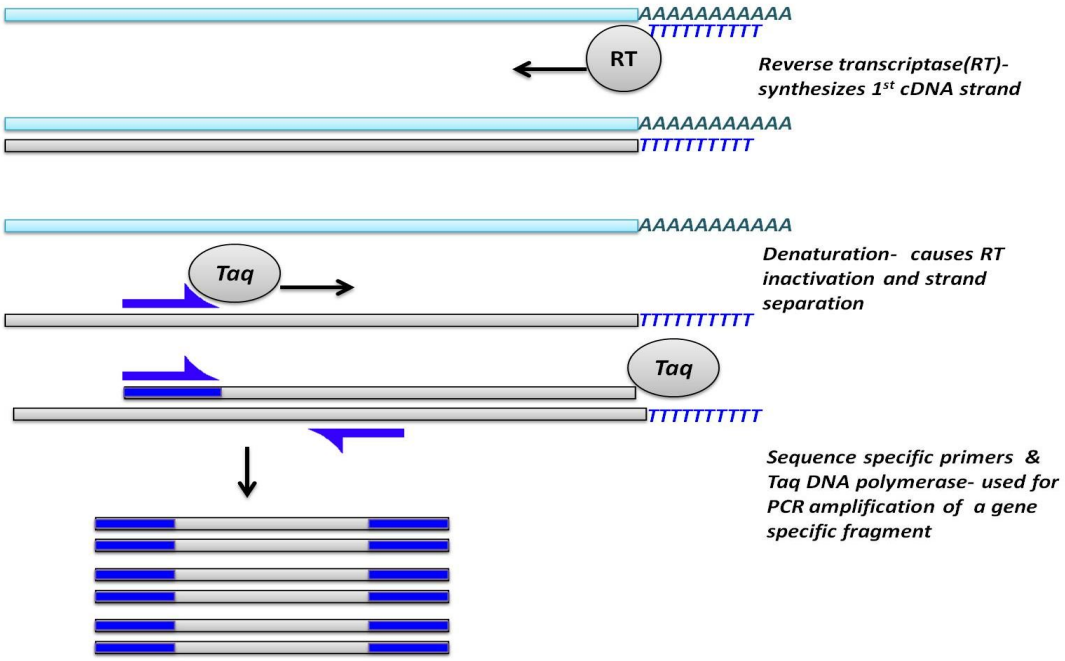


Fig.6. A comprehensive model of cDNA synthesis and PCR amplification of target sequence using reverse transcriptase and *Taq* DNA polymerase respectively.

Step 2- Standardizing the PCR Reaction for accurate "real time" analysis

After subsequent generation of cDNA template, it is amplified into double stranded DNA by using definite set of primers that are required for a particular PCR reaction.
Several factors that are to be considered preceding the PCR reactions include:

- ✓ The use of particular thermal cycler

- If regular → the reaction amplification specificity is confirmed by gel electrophoresis.
- If real time → the product amplification and accumulation is measured by the use of specific fluorescent labeled dyes (Please refer to page no.2-4) and by melting point analysis.

✓ The optimization of PCR reaction.

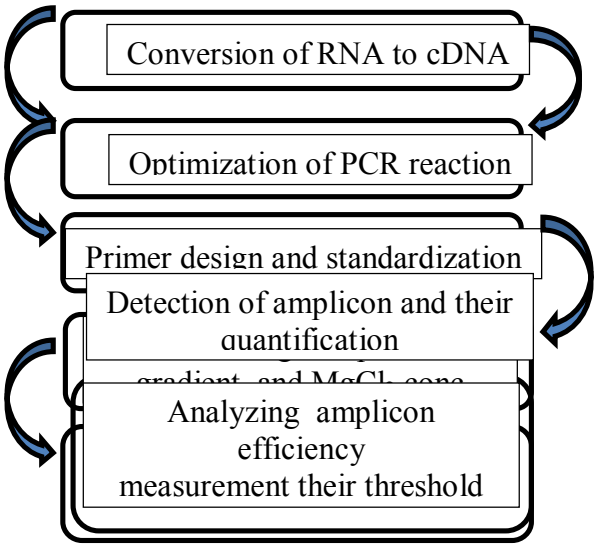


Fig.7. Schematic Depiction of the Real Time PCR reaction

A comprehensive explanation of all the factors determining the reactions are as follows

Primer designing : normalizing the concentrations and the annealing temperatures

Optimizing the design of the primers used in Real Time PCR is the most pertinent factor determining the efficiency of amplification of preferred sequences. As discussed earlier a successful RT-PCR reaction basically depends upon the amplification of a short product (ideally 100-200 bp) specific to the particular mRNA. Effective designing requires sequence information on the genomic DNA and direct alignment with the cDNA coding sequence. This information can be retrieved via a number of public websites viz., NCBI (*http://www.ncbi.nlm.nih.gov/*) and EMBL (*http://www.ebi.ac.uk/embl/*). The precise designing of primers is further aided by various softwares (e.g., Vector NTI, Mac Vector and DNA star) which include several factors like

✓ Elimination of primer dimmers due to complementary sequence b/w primers
✓ Matched annealing temperatures b/w the primers
✓ Calculation of precise difference b/w the annealing temperatures of primer pairs and the PCR product

A number of other factors are also summarized below

I. Primers should bestride the intron or flank a large exon.
II. The binding sites of the primers should not have extensive secondary structures and so as the 3' sites of the primers.
III. The primers should not notably complement each other or themselves.

IV. The primer pairs must have an approximately equal GC content and should have similar annealing temperatures

Annealing Temperature

To normalize the annealing temperatures of primer pairs

- A temperature gradient analysis should be done.
- Another optional method is to analyze the melting temperatures (T_m) of the primer pairs which reveal the extent of annealing under incrementally increasing temperatures thereby selection of an ideal temperature.

Primer concentration

Gradually increasing concentration of primers enhances the PCR reaction but too high concentrations adversely results in formation of non-specific products like primer-dimers. In general the optimal concentrations should be b/w 0.05 and 0.9 µM but sometimes they may vary for different primer pairs. Ideally the primer concentrations that give the lowest "Cycle Threshold" (C_T) values are considered as lower CT values corresponds to more efficient product formation.

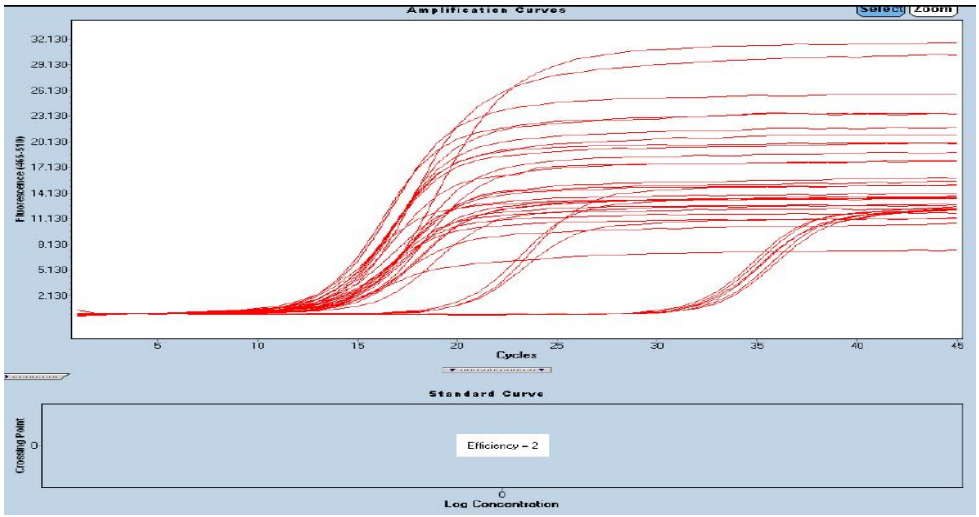


Fig.8. Cycle Threshold and optimization of primer concentration, an example.

Other reaction components such as magnesium concentration and template purity

Other than primer concentration and design, factors like the $MgCl_2$ concentrations, template purity and concentration also significantly determines the efficiency of a RT-PCR reaction. Magnesium chloride concentration impacts ranges from no product formation if too low to multitude of non-specific products if too high. This considerably results in significant increase of PCR artifacts. The normal concentration of $MgCl_2$ should be 1 to 5 mM which are mostly aided by buffers provided in commercially available kits. Template purity and concentration can be of considerable importance in determining the efficiency of PCR product formation due to presence of certain PCR inhibitors during the preparation of the template. Especially blood samples and some other environmental samples contain inhibitors like humic acids and heavy metals. Therefore it is advised to use low concentration of template at the starting point which dilutes these contaminants thereby increasing the effectiveness of product formation.

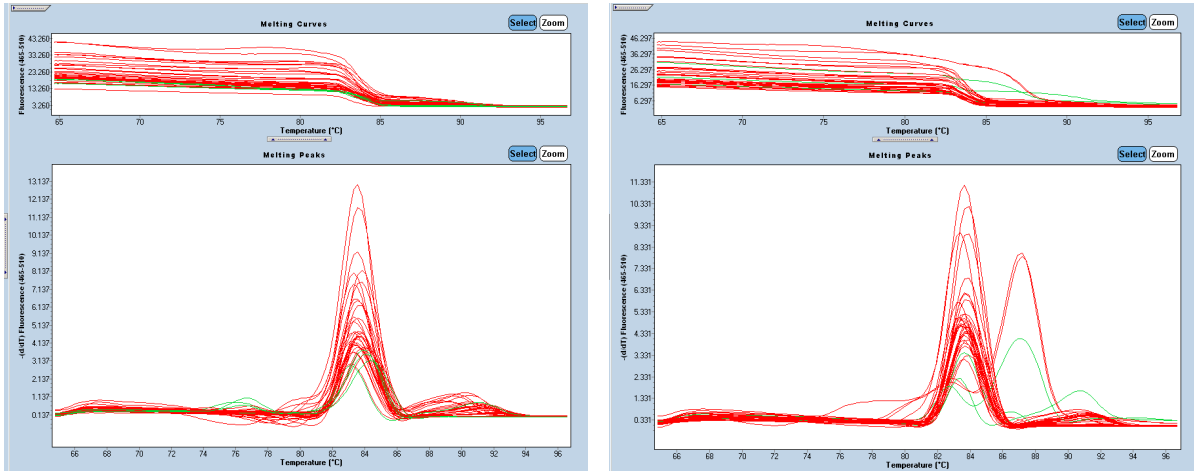
The amplicon detection in real time thermal cycler

Real-time PCR thermal cyclers combine PCR product generation and recognition into one integrated format that allows for the subsequent analysis of the captured data (Fig. 10.3.7). To accomplish these two tasks, real-time PCR machines incorporate traditional PCR

thermal cycler technology with integrate fluorimeters and detectors that provide the ability to both excite a fluorochrome and detect the emitted light. There are several suppliers of such equipment and these can range in cost depending upon the features. For many laboratories, this represents a substantial investment but can result in savings, as the analysis of PCR products can be conducted without running agarose gels, representing a savings in both time and money when processing a large number of samples. Because real-time PCR reactions are quantified at each cycle by measuring fluorescence, most real time thermal cyclers need to be calibrated for the particular tube or micro titer plate and micro titer plate seal used, and for the PCR reaction volume. Therefore, it is important to know which real-time thermal cycler will be used for the experiment before setting up the reactions.

Melting curve analysis to verify amplicon specificity

Preceding the analysis of results obtained, one should recertify the specificity of the PCR amplification under actual real-time PCR conditions. This can be done via melting curve analysis feature in real-time thermal cyclers. This is a constructive practice that ensures a quality control step. The analysis is principally used to distinguish target amplicons from PCR artifacts such as primer-dimers or misprimed products. As conferred previously the melting curve analysis is also useful for optimizing primer- annealing temperatures



(A) (B)

Fig.9. Melting curve analysis performed on Roche Light cycler 480 II. (A) It depicts the single peak melting curve representing a single species of DNA molecule in the reaction. (B)It portrays the double peaks as a consequence of fragment denaturing at lower temperatures due to primer-dimer formation.

The utility of melting point analysis derives from the observation that the temperature at which a DNA duplex will denature is dependent upon length and nucleotide composition. Fluorescence measurements are made while slowly increasing the temperature of the reaction products (for example, from 60◦ to 95◦C). At the low temperature, the amplicons are all double stranded and thus bind the SYBR Green dye, producing a strong fluorescence signal. As the temperature increases, the PCR products are denatured, resulting in a decrease in fluorescence. The fluorescence is measured continuously and when the T_m of a particular dsDNA product is reached, there will be a rapid decrease in the fluorescence over a short temperature range. This is detected by the instrument and plotted as either total fluorescence or as the first negative differential of the fluorescence signal with respect to temperature. The advantage of the second method is that the plot will appear as one or more peaks centered on

different temperatures (Fig.9). These peaks represent the points at which the maximum rate of change in fluorescence is detected. Different amplicons can generate different peaks centered on different temperatures and, fortunately, PCR artifacts typically have lower melting temperatures than the target amplicon. By programming the instrument to detect fluorescence at the appropriate temperature, above the melting temperature of the misprimed or primer-dimer products but below the melting temperature of the target product, one can more accurately measure fluorescence of the target amplicon and not include the fluorescence from artifactual products. To conduct a melting point analysis, consult the manufacturer's manual of your real-time PCR thermal cycler for the details as to how to enter this feature into your PCR program. Generally it is a simple menu command.

Step 3 - Real time RT-PCR result analysis and quantification

Prior to design the experiment and setting up the work the most crucial point to set an eye on several quality control steps, including negative and positive controls. These will make certain that Real Time RT-PCR data being formed is of high purity and less prone to error results. Additionally, for quality control reasons, each sample is run in triplicate for better quality results and minimizing error. A dilution series is also required for each sample: serial dilutions or working dilutions establish amplification efficiencies and to generate standard curves.

A. Experimental design and quality control

Real Time RT-PCR includes the following controls to follow up the experiment:-
Negative control (at least two)

- ✓ No-template control
- ✓ No-reverse transcriptase control

Positive control (helps to ensure all reagents are working properly)

- ✓ DNA (a source of quantified DNA containing the target sequence).
- ✓ RNA (a sample of quantified RNA that contains the RNA target).

B. Setting the threshold value for detection of the amplicon:

Threshold value or Ct value is the point at which it detects the level of fluorescence signal which is sufficiently above the background noise and assumed to be as the reliable signal. The cycle at which threshold is met or exceeded is called the cycle threshold. Basically adequate settings must be done to avoid premature detection and unreliable data.
To set a threshold value, one must first identify the baseline value. Baseline values are generally determined from the fluorescence versus the cycle number. Typically, threshold values are set at three standard deviations above the mean baseline value. It is understood that the threshold be set to allow the detection of the product at the exponential phase of cycle. The point at which fluorescence detects the product and crosses the point is known as the C_T value.
In general, Ct value is measured by comparing the differences between the two samples. The efficiency of amplification is considered to be 100% when the amplified product is doubled at each cycle.

$$2^{(C_{T1}-C_{T2})} = \text{fold difference in the amount of starting target}$$

Where, C_{T1} refers to the C_T value for sample one
C_{T2} refers to the C_T value for sample two

Thus, difference of 4 C_T equals to 16-fold difference in starting amount of target sequence.

C. Amplicon quantifiaction

Before conducting a real time RT-PCR setup, one must understand what should be taken before quantifying the amplicons using either relative quantification or absolute quantification.

Absolute Quantification:

Absolute quantification results in measure of the amount of target sequence in the sample in copy number or concentration. It gives the exact value of the target sample while generating a standard calibration curve to plot the values of standard that has already been standardized, taking it as an endogenous control in terms of copy number or concentration.

Relative quantification

Relative quantification allows providing the relative value expressed as a ratio of amount of initial target sequence between the experimental treatments and control that are normalized relative to some internal standard expression of housekeeping gene (constitutively expressed). It requires internal standard sample for variability between samples. Typically these are housekeeping genes such as β actin , cyclophilin, or glyceraldhehyde-3-phosphate dehydrogenase (GAPDH).Wisely, housekeeping genes must be used as they are invariant in expression according to the treatment. However, there is an advantage of using relative quantification as there is no requirement of calibration curve analysis. If amplification efficiencies of the target and control sequences are identical, then one simply compares relative expression values for the target gene in different samples expressed as a ratio. The ratio is then normalized using the housekeeping gene's expression in the same samples.

Basic protocol

Removal of genomic DNA from RNA preparations

1. Combine the following into an RNase and DNase-free 0.5-ml microcentrifuge tube

Component	Quantity
Total RNA	1-5 µg
10X buffer	1µl
DNase I	1µl
DEPC-treated wate	to10 µl final volume

2. Incubate the reaction at 37˚C for 30 min.
3. Add 1µl of 25 mM EDTA and incubate at 65˚C for 10 min.

Prepare the **First strand cDNA synthesis** mix adding each component in the following order:

Component	**Quantity**
Total RNA	0.1-5µg
Oligo (dT) Primer	1µl
Random hexamer	1µl
DEPC-treated water	to 12µl total volume

1. Mix gently and centrifuge briefly and incubate at 65˚C for 5min. Chill on ice, spin down and place the vial back on ice.
2. Add the following components in the indicated order

Component	**Quantity**
5X reaction buffer	4µl
Ribolock RNase inhibitor (20U/µl)	1µl
10mM dNTP mix	2µl
Revert Aid M-MuLV reverse transcriptase (200u/µl)	1µl
Total volume	20µl

3. For incubation, oligo (dT) or gene specific primed synthesis incubate for 60 min at 42˚C. For random hexamer primed synthesis, incubate for 5min at 25˚C followed by 60min at 42˚C.
4. Terminate the reaction by heating at 70˚C for 5min.

Real Time PCR amplification and analysis

- Preparation of master mix : components
  2 X reaction buffer
  50 µM forward primer
  50 µM reverse primer
  RNase free water

1. Assemble or arrange the components mentioned above in ice container.
2. Prepare a serial dilution or working dilutions of a cDNA sequence that has to be amplified. The dilution should be typically four to five fold dilutions.
3. Prepare a master mix in a 1.5 ml micro centrifuge type for the sample PCR to avoid the number of PCR tubes.
4. Mix the master mix thoroughly, centrifuge briefly to collect disturbed contents, and dispense equal aliquots into each well of real-time PCR plate.
5. Add the serially diluted template to each well as needed (be sure to include a negative control that does not receive any template).
6. Set up and program the PCR machine as it will take quite time to warm the machine according to the manufacturer's instructions.

7. Place the real-time PCR plate in the thermal cycler and start the program.
8. After PCR, run melting curve analysis
9. Check the product in gel

Trouble shooting

1. **Poor Quality of the amplicon product in low quantity**

Poor RNA quality may contribute to low yield in RT-PCR. Running the RNA preparation on a denaturing agarose gel containing formaldehyde can reveal the integrity of the RNA. No or low product yields in RT-PCR may be due to inefficient cDNA synthesis or low amplification of cDNA. This may require setting up different reactions where varying amounts of RNA are added to the cDNA synthesis reaction. Additionally, variable amounts of cDNA can be added to the PCR reaction.

2. **Band formation by negative RT reaction control**

This indicates contamination of the RNA due to-

- ✓ Reagents used in RT reaction.
- ✓ Presence of genomic DNA in RNA preparation.

To avoid this it is best to repeat the reaction using new reagents (except RNA Prep). If the problem disappears, one can proceed. But if this persists digesting the RNA with RNase free Dnase and re-isolating the RNA is recommended.

3. **Band production by negative PCR control**

This indicates the amplicon contamination of the reagents. It is advised to repeat the reaction with new reagents (apart from *Taq*). If the problem is solve, one may carry on. But if the problem continues the *Taq* is retested in a secured new tube of enzyme. Avoiding the use of block pipette tips is advisable to prevent the contamination of amplicon.

4. **Extensive differences b/w duplicate and tri-plicate samples**

If there is huge variability in the duplicate and tri-plicate samples it indicate the pipetting error and problem in preparation of the master mix. It is suggested to check and mix the master mix appropriately and to calibrate the pipette so that equal amount of water is dispensed reproducibly.

5. **Reduced amplification efficiency**

If the PCR amplification reaches an early plateau stage compared to other PCR reactions, this might be result in poor amplification efficiency (< 90%).If this is the case, the primers are redesigned and synthesized yet again. Careful optimization of the primer pairs by the use of temperature gradient analysis and melting curve study is otherwise recommended.

6. **Melting curve reveals primer-dimers**

A double peak in melting curve analysis is basically due to formation of primer-dimers. This is due to low template concentration and it is advised to increase the concentration optimally.

Advantages of real time PCR

1. It does not allow non-specific amplification.
2. No-post PCR processing of products (high throughput, low contamination risk).
3. Ultra-rapid cycling (as fast as 25 min).
4. Wider dynamic range of up to 10^10 fold.
5. Detects or quantify the value present at a very low range of concentration.
6. Easily accessible, specific, sensitive and reproducible.

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RNA ANTIBODIES

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Introduction

Antibodies are pretty amazing not only do they protect us and serve diagnostic functions in the clinic, they are also indispensable tools for biological research. They are used for protein separation, for detecting proteins and protein interactions, for imaging in cells and as chaperones for protein crystallization. Recent genomic studies have brought to light the existence of an array of noncoding RNAs. RNA antibodies would certainly be welcome tools to study the biology of these molecules, but the reality is that very few antibodies to RNA exist. Methods to generate synthetic antibodies to RNA should open up several new applications for studying RNA biology. The reason why, as structural biologist Anthony Kossiakoff of the University of Chicago explains, is that "the process that you would generally use to make hybridoma-based antibodies just don't work at all."

Present Context

The concept of RNA antibodies is quite new to the field of aquaculture but its application has great avenues to disease diagnosis and drug delivery in aquatic organisms.Nucleic acid reactive antibodies are greater intrest in clinical medicine, cell biology and molecular biology. They are used to serve as marker in certain auto immune disease. The traditional process to make antibodies is to inject an animal with the antigen toelicit an immune response, but foreign RNAs apparently just get chewed up by nucleases. To circumvent this challenge, Kossiakoff and Shohei Koide, also a structural biologist, and Joseph Piccirilli, an RNA chemist, developed a synthetic phage display-based approach to select specific antigen binding fragments (Fabs) that bind to RNAs. As a target RNA antigen, the researcher selected the ΔC209 P4-P6 domain of the *Tetrahymena* group I intron, which folds into a well defined three-dimensional structure. Using the phage display method, the researchers selected seven Fabs, expressed them as soluble proteins, and identified two that strongly bound to the target RNA structure. Because ΔC209 P4-P6 requires magnesium to achieve its folded structure, and the Fabs did not bind when magnesium was absent, the researchers concluded that the Fabs were recognizing the tertiary RNA structure, not just a linear epitope. The recognition was also extremely specific.

Structure and Functions of RNA antibodies

Antigen binding fragments

The fragment antigen-binding (Fabs) is a region on an antibody that binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain. These domains shape the paratope — the antigen-binding site — at the amino terminal end of the monomer. The two variable domains bind the epitope on their specific antigens.

In an experimental setting, Fc and Fab fragments can be generated in the laboratory. The enzyme papain can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment. The enzyme pepsin cleaves below hinge region, so a F(ab')2 fragment and an Fc fragment is formed. The variable regions of the heavy and light chains

can be fused together to form a single-chain variable fragment (scFv), which is only half the size of the Fab fragment, yet retains the original specificity of the parent immunoglobulin.

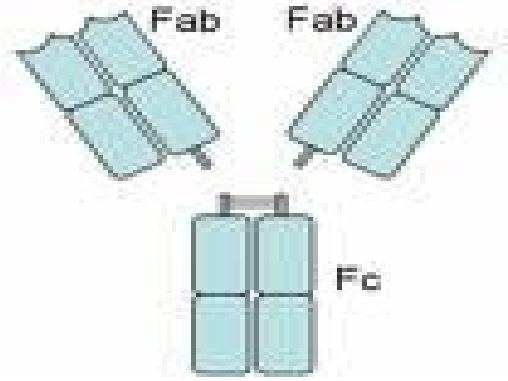

Fig. 1: An antibody three fragments: two Fab fragments and one Fc fragment

Phage display- based approach

This technique is used for producing antibody-like molecules. Gene segments encoding the antigen-binding variable of V domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bateriophage. A collection of recombinant phage, each displaying a different antigen-binding domain on its surface, is known as a phage display library. In much the same way that antibodies specific for a particular antigen can be isolated from a complex mixture by affinity chromatography, phage expressing antigen-binding domains specific for a particular antigen can be isolated by selecting the phage in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody.

The genes encoding the antigen binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining then to parts of immunoglobulin genes that encode the invariant parts of the an antibody. When these reconstructed antibody genes are introduced into a suitable host cell line, the transfected cells can secrete antibodies with all the desirable characteristics of monoclonal antibodies of the suitable host cell line. Phage display can be applied for epitope mapping of monoclonal and polyclonal antibodies, generating immonogens, identifying peptide ligands (drug), defining substrate sequences, defining protein-protein interaction and isolation of high affinity antibodies.

Kossiakoff and Koide have been interested in developing the use of chaperones, such as antibodies, to improve protein crystallization. They reasoned that charperons could also aid the crystallization of RNAs. Kossiakoff explains the rationale: "RNAs have two issues. One is that they are the second is that (because) the backbone is charged and there are just four bases, you have a situation where you don't have the specificity for lattice contacts." The researchers crystallized ΔC209 P4-P6 in complex with one of their Fabs and demonstrated an improvement in resolution (1.95A) over previous structure. (2.25).

1. Chaperones

In molecular biology, chaperones are proteins that assist the non-covalent folding/unfolding and the assembly/disassembly of other macromolecular structures, but

do not occur in these structures when the latter are performing their normal biological functions. The common perception that chaperones are primarily concerned with protein folding is incorrect. The first protein to be called a chaperone assists the assembly of nucleosomes from folded histones and DNA and such assembly chaperones, especially in the nucleus (Richardson, 2006; Alekseev, 2009), are concerned with the assembly of folded subunits into oligomeric structures (Ellis, 2006).

Chaperones do not necessarily convey steric information required for proteins to fold: thus statements of the form `chaperones fold proteins` can be misleading. One major function of chaperones is to prevent both newly synthesised polypeptide chains and assembled subunits from aggregating into nonfunctional structures. It is for this reason that many chaperones, but by no means all, are also heat shock proteins because the tendency to aggregate increases as proteins are denatured by stress. New functions for chaperones continue to be discovered, such as assistance in protein degradation, bacterial adhesin activity, and in responding to diseases linked to protein aggregation

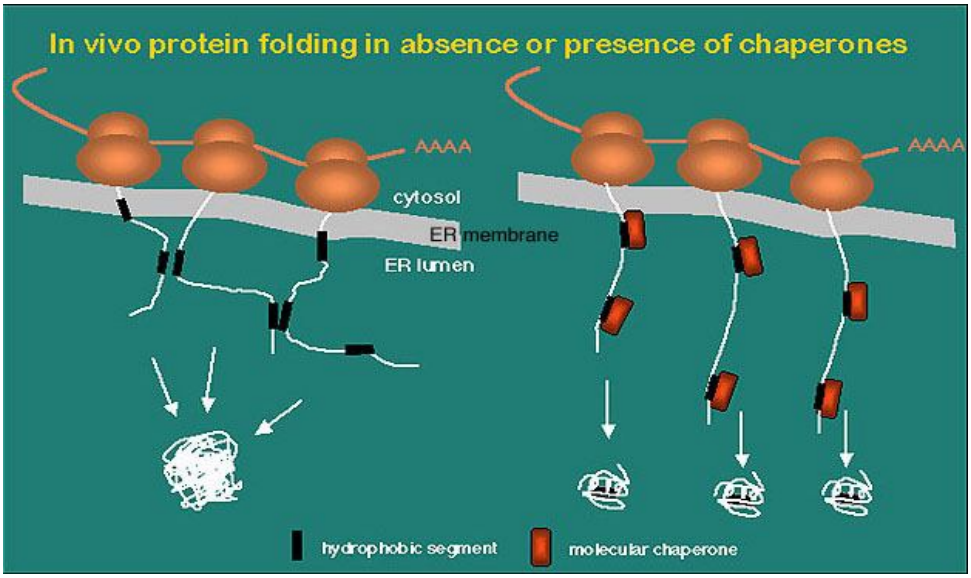

Fig.2: In the absence of molecular chaperones (left) exposed hydrophobic segments of nascent polypeptides associate and lead to aggregation. Molecular chaperones (right) interact with hydrophobic segments in regulated cycles of binding and release, preventing aggregation and allowing productive folding. The chaperone does not actually direct folding pathways but rather prevents non-productive aggregation reactions.

Kossiakoff believes that such an approach could be used for crystallizing even larger RNA structure. He explains: "The [strategy of the] structural RNA community has been to divide and conquer"; you chop thing up in sable pieces and obtain structures for the fragments, then piece them together "So if you think of a big RNA molecule you [could} have two different chaperones binding at different parts of the molecule.' Such that the entire RNA could be crystallized in one piece. RNA crystallography is just one potential application; Kossiakoff acknowledges this work is just the tip of the iceberg for antibodies to RNA. He suggests using antibody Fabs for immunopreccipitating RNA protein complexes, recognizing conformational switches, localizing RNAs in cells and using them as inhibitors. "Anything that you

can do with a protein antibody interaction you can do with an RNA antibody", he says.

Applications

Nucleic acid reactive antibodies are found to be wide range of applications in the detection, estimation and isolation of specific nucleic acids and nucleic acid components. They are used as reagent to locate modifications or conformational variations in nucleic acid polymers even when they occur in combinations with other macro molecule like proteins.They have good potential as inhibitors of nucleic acid functions.Their used as anticancer agents has been proposed(Senapathy and Jacob, 1979).

Conclusions

RNA antibodies are going to play an vital role in the field of Nucliec acid immunology. This is certainly being a welcome tool to study and identification of partner proteins with that of the research on potential powerful approach in biology.

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siRNA IN REVERSE GENETICS

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RNA interference (RNAi) is a mechanism where the expression of a particular gene is interfered by the presence of small ds RNA (double stranded RNA) that shares homologous sequence with that gene. This is an exceptionally powerful and important tool in reverse genetics, as it is very specific to the target, simple to design and easy to deliver. Inside the cells, RNAs perform two broad functions. Single-stranded messenger RNAs (mRNAs) are vital intermediaries in gene expression that transmit information between DNA and protein. Ribosomal and transfer RNAs have structural, catalytic and information-decoding roles in the process of protein synthesis.

In 1998, Andrew Fire, Craig Mello and colleagues discovered the fact that expression of a gene in nematode worms can be knocked down or silenced by double-stranded RNA molecules and they announced their discovery as RNA interference (RNAi). Since then, RNAi became an extremely useful experimental tool to understand the function of a gene. Later, it was observed that, it's an evolutionarily ancient method of genome defense in many organisms. Double-stranded RNA was originally found to silence genes by targeting mRNA intermediaries for destruction. But at least two more mechanisms have been uncovered: blocking transcription (the synthesis of mRNAs from DNA) and inhibiting translation (the synthesis of proteins from mRNAs). Surely, this is an interesting story and possibly not the end!

Mechanism of RNA-interference

In plants, it was observed that short RNAs (~ 20 to 25 nt) down-regulate some genes, where there sequences match correctly. These short RNAs were characterized later as siRNAs (silencing RNAs), and were much smaller than mRNA and rRNA which were routinely being observed in the cell. Biochemical characterization revealed that these siRNAs are 21–23-nt long dsRNA duplexes with 5′-phosphate and 3′-hydroxyl groups, and have 2–3-nt symmetrical overhangs at 3′ end. This chemical structure shows that it is the product of RNASE III-like enzymatic cleavage pattern, which led to the identification of the highly conserved dicer family of RNase III enzymes as the mediators of the dsRNA cleavage. Biochemical and genetic evidence have shown that in the cytoplasm, dicer cleaves long dsRNA to produce siRNAs. These siRNAs are incorporated into a multi-protein RNA-inducing silencing complex (RISC). However, to enter RISC, siRNAs must be 5′ phosphorylated, and siRNAs that lack a 5′phosphate are rapidly phosphorylated by an endogenous kinase 26. The duplex siRNA is unwounded : the sense (non-guide) strand of the siRNA duplex is cleaved by argonaute 2 (Ago2), and the antisense strand
(guide) RISC to its homologous target mRNA. The target mRNA is then cleaved by Ago2 at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, which is 10 nucleotide apart from the 5′ end of the siRNA.

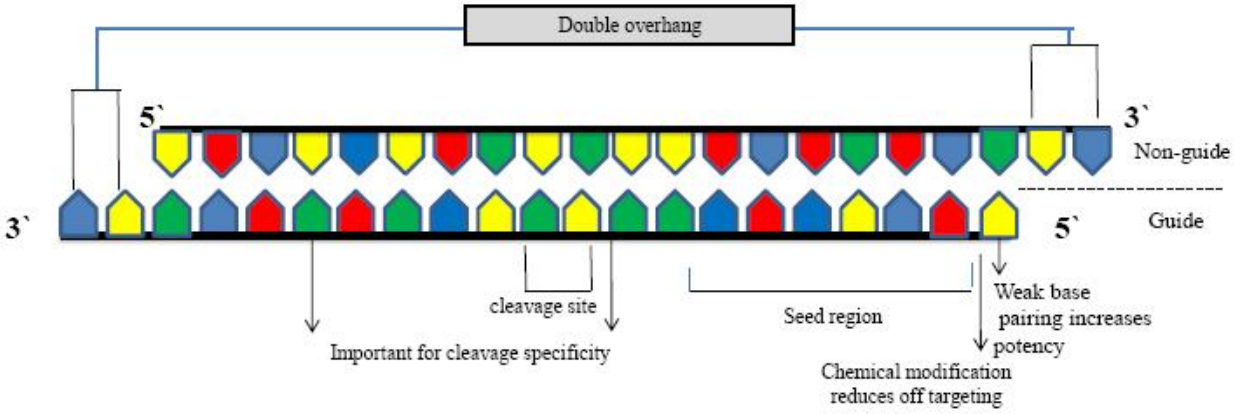
Fig.1 : Structure of a siRNA

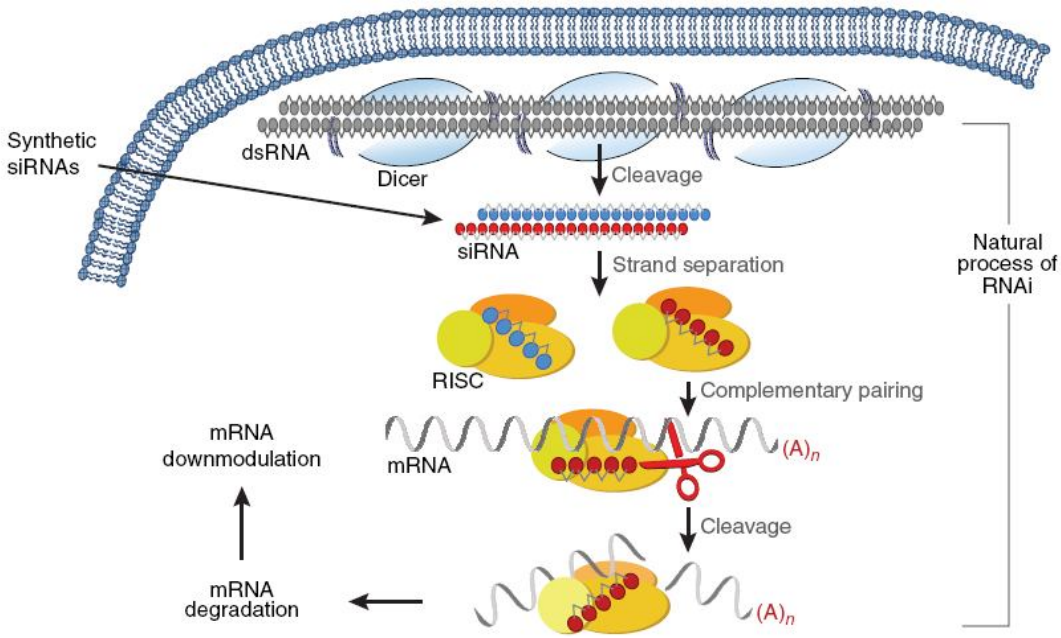
Critical nucleotide positions in siRNA, showing mRNA recognition, mRNA cleavage and cleavage specificity (Adapted from Nature Chemical Biology, vol 2 : 2006

Fig: 2 Cellular mechanism of RNA-interference

Long double-stranded RNA (dsRNA) is cleaved by dicer to synthesize small interfering RNA (siRNA). Then siRNA enters into the RISC and strands separation takes place. RISC with anti-sense siRNA binds to complementary mRNA sequence and is then cleaved by argonaute 2 (Ago-2)(Adapted from Nature Chemical Biology, Vol 2 : 2006)

Source of siRNA oligos





Suppliers of RNA synthesis reagents are Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, USA), Pierce Chemical (part of Perbio Science, Rockford, IL , USA), Glen Research (Sterling, VA, USA), Chem Genes (Ashland, MA, USA), and Cruachem (Glasgow, UK). In India siRNA are synthesized by Invitrogen, SIGMA, IDT and Thermo Scientific etc.

Delivery of siRNAs in to the cell

To deliver siRNAs inside the cells, electroporation is a very efficient way for floating cells. For electroporation, 1-10 µl of a 250 picomol/µl siRNA solution are added to pre-chilled 0.4 cm electrode gap cuvettes (Bio-Rad, Hercules, CA). Cells (1.5 $x10^7$) are re-suspended in cold, serum-free RPMI media, added to the cuvettes, mixed, and pulsed once at 250-450 mV (the voltage has to be optimized for cell type and the individual electroporator), 960 uF, 200 ohms with a Gene Pulser electroporator (BioRad, Hercules, CA). Electroporated cells are then plated in cell culture plate. Cell viability immediately after electroporation is typically around 40-60%, or even less. siRNA can also be delivered by transfection with lipofectamine (Invitrogen), and this is particularly important for those cell types which are adherent in nature.

Tools to analyze functional efficacy of RNAi

Efficacy of RNAi can be assessed by analyzing the expression / function of a particular gene . Generally, three basic techniques are used for gene function analysis.

- Western blot with specific antibody to the target protein
- Northern-blot with radio-labeled or cold labeled probe to the target gene
- Semi-quantitative reverse transcriptase (RT)-PCR of the expressed target gene

siRNA in functional genomics

To date, several reverse-genetic approaches like antisense and gene targeting by homologous recombination and RNAi are in use for the validation of gene function. The major advantage of RNAi is that it can be applied to study the function of many genes of different cell types. This technology entirely depends upon the nucleotide sequence of a gene, and with the exponential increase of the gene bank database it became a virtual reality to study any kind of gene function by RNAi. Majority of the important application of RNAi was obtained from the experiment in *C. elegans.* This is because *C. elegans* is a highly genetically tractable system, a large bank of mutant worm lines was established using traditional genetic techniques, and those mutant worm lines can function as a reference point for large-scale RNAi screens.

Although many studies on functional-genomics with RNAi were carried out using dsRNA injection, but the most promising approach for large-scale RNAi studies was the development of feeding RNAi-libraries. Several groups have used RNAi libraries that express dsRNA in *E. coli* to screen for genes that are involved in various traits, including abnormal anatomy and motility, altered sex ratios, sterility, longevity and fat-regulatory genes. Several reports described the use of RNAi technique for functional genomic analysis in mammals but in fish some sporadic reports are available only in zebrafish.

Comparative efficacy of antibodies and RNAi

Antibodies	RNAi
o Antagonism or agonism of target	o Antagonism only
o Extracellular targets	o All targets, including 'non-drugable targets'
o Slow optimization	o Rapid optimization
o Relatively difficult, and takes much time to produce	o Easy to synthesize and very fast

Applications of RNAi technology

- Besides being an area of intense, upfront basic research, the RNAi process holds the key to future technological applications. This technology is proving to be useful to analyze quickly the functions of a number of genes in a wide variety of organisms.
- In many infectious diseases, development of drug resistance is a major obstacle. However, as RNAi targets the multiple regions of the viral genome it may overcome this obstacle. Similarly, targeting viruses with multiple interfering RNAs reduces the chances of a virus escaping RNAi repression through spontaneous mutation.
- RNAi is a fundamental cellular mechanism for silencing gene expression that can be harnessed for the development of new drugs. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that are difficult to modulate selectively with traditional pharmaceutical approaches involving small molecules or proteins.
- To date, siRNAs have been shown to inhibit infection by human immunodeficiency virus, poliovirus, and hepatitis C virus in cultured cell lines, and respiratory syncytial virus causing severe respiratory disease in neonates and infants
- Identification of some biochemical signaling cascades, embryonic development and other basic cellular process have been studied using RNAi technology (e.g. *D. melanogaster*)
- In plant, toxin producing genes may be silenced using this technique.
- siRNA treatment has also been shown to reduce the expression of the BCR-ABL oncoprotein in leukemia and lymphoma cell lines, leading to apoptosis in these cells. With respect to future medical applications, siRNA based therapy seems to have a great potential to combat carcinomas, myeloma, and cancer caused by overexpression of an oncoprotein or generation of an oncoprotein by chromosomal translocation and point mutations.
- Use of siRNA has been reported in the specific silencing of White spot syndrome virus (WSSV) infection in shrimp.

Conclusion

A new era of reverse genetics has been started with the application of RNAi technology. In a very short period, remarkable progress has been made in advancing RNAi as therapeutics. RNAi offers many potential advantages over the gene-knockout techniques by providing the machinery of gene switch off and on. The siRNA approaches for gene silencing holds great therapeutic promise, as siRNAs, are naturally used by cells to regulate gene expression, and are therefore nontoxic and highly effective. Although many technical challenges are associated with the therapeutic application of siRNAs, such as synthesis, delivery and specificity, it has numerous advantages over other gene-silencing approaches. RNAi is target specific, because if there is mismatch of one nucleotide within the 19- to 20-mer siRNAs, it effectively stops functioning in disrupting and degrading the target mRNA. However, this gene specificity of siRNAs needs to be confirmed on a genome-wide scale, and further research into the fundamental mechanisms of RNAi could unveil new dimensions of siRNA-mediated gene silencing that will have profound implications for understanding gene regulation, functional genomics and therapeutic applications.

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SMALL INTERFERING RNA: POSSIBILITIES IN FISH HEALTH

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Introduction

The idea of gene silencing by siRNA(short interfering RNA) is a still developing field in biology. As promising these studies are both had to deal with a severe drawback of systematic siRNA applications. The challenge is to increase nuclease resistance and improve cellular uptake while maintaining gene silencing processes. The preclinical examples support the notions that local admistrations of siRNA could be more favorable for clinical development at the moment than systematic aproaches. siRNA is thought to be a part of nuclic acid based immune systems.

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 nucleotides in length, that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome; the complexity of these pathways is only now being elucidated.

siRNAs were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England, as part of post-transcriptional gene silencing (PTGS) in plants (1. Hamilton and Baulcombe, 1999). Shortly thereafter, in 2001, synthetic siRNAs were shown to be able to induce RNAi in mammalian cells by Thomas Tuschl (2. Elbashir *et al.*, 2001). This discovery led to a surge in interest in harnessing RNAi for biomedical research and drug development.

Why RNAi ?

RNA interference (RNAi) represents an evolutionarily conserved cellular defense for controlling the expression of foreign genes in most eukaryotes including humans. RNAi is triggered by double-stranded RNA (dsRNA) and causes sequence-specific mRNA degradation of single-stranded target RNAs homologous in response to dsRNA. The mediators of mRNA degradation are small interfering RNA duplexes (siRNAs), which are produced from long dsRNA by enzymatic cleavage in the cell. siRNAs are approximately twenty-one nucleotides in nucleotide 3'-overhangs. Chemically synthesized siRNAs have become powerful reagents for genome-wide analysis of mammalian gene function in cultured somatic cells. Beyond their value for validation of gene function, siRNAs also hold great potential as gene-specific therapeutic agents.

RNAi is a system within living cells that helps to control which genes are active and how active they are. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to specific other RNAs and either increase or decrease their activity, for example by preventing a messenger RNA from producing a protein. RNA

interference has an important role in defending cells against parasitic genes – viruses and transposons – but also in directing development as well as gene expression in general.

The RNAi pathway is found in many eukaryotes including animals and is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short fragments of ~20 nucleotides. One of the two strands of each fragment, known as the *guide strand*, is then incorporated into the RNA-induced silencing complex (RISC). The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand base pairs with a complementary sequence of a messenger RNA molecule and induces cleavage by Argonaute, the catalytic component of the RISC complex. This process is known to spread systemically throughout the organism despite initially limited molar concentrations of siRNA.

The selective and robust effect of RNAi on gene expression makes it a valuable research tool, both in cell culture and in living organisms because synthetic dsRNA introduced into cells can induce suppression of specific genes of interest. RNAi may also be used for large-scale screens that systematically shut down each gene in the cell, which can help identify the components necessary for a particular cellular process or an event such as cell division. Exploitation of the pathway is also a promising tool in biotechnology and medicine.

History & Discovery

The discovery of RNAi was preceded first by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants, (3. Ecker and Davis, 1986) and more directly by reports of unexpected outcomes in experiments performed by plant scientists in the U.S. and The Netherlands in the early 1990s (4. Napoli *et al.,* 1990) in an attempt to alter flower colors in petunias, researchers introduced additional copies of a gene encoding chalcone synthase, a key enzyme for flower pigmentation into petunia plants of normally pink or violet flower color. The overexpressed gene was expected to result in darker flowers, but instead produced less pigmented, fully or partially white flowers, indicating that the activity of chalcone synthase had been substantially decreased; in fact, both the endogenous genes and the transgenes were downregulated in the white flowers. Soon after, a related event termed *quelling* was noted in the fungus *Neurospora crassa*, (Romano and Macino, 1992) although it was not immediately recognized as related. Further investigation of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation. This phenomenon was called *co-suppression of gene expression*, but the molecular mechanism remained unknown (Mol and Krol, 1991).

Not long after, plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. While it was known that plants expressing virus-specific proteins showed enhanced tolerance or resistance to viral infection, it was not expected that plants carrying only short, non-coding regions of viral RNA sequences would show similar levels of protection. Researchers believed that viral RNA produced by transgenes could also inhibit viral replication (Covey *et al.,* 1997). The reverse experiment, in which short sequences of plant genes were introduced into viruses, showed that the targeted gene was suppressed in an infected plant. This phenomenon was labeled "virus-induced gene silencing" (VIGS), and the set of such phenomena were collectively called post transcriptional gene silencing (Ratcliff *et al.,* 1997).

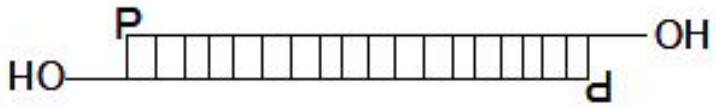
After these initial observations in plants, many laboratories around the world searched for the occurrence of this phenomenon in other organisms (Guo and Kemphues, 1995; Pat-Bhadra *et al.,* 1997). Craig C. Mello and Andrew Fire's 1998 *Nature* paper reported a

potent gene silencing effect after injecting double stranded RNA into *C. elegans* (Fire *et al.*, 1998). In investigating the regulation of muscle protein production, they observed that neither mRNA nor antisense RNA injections had an effect on protein production, but double-stranded RNA successfully silenced the targeted gene. As a result of this work, they coined the term *RNAi*. Fire and Mello's discovery was particularly notable because it represented the first identification of the causative agent for the phenomenon. Fire and Mello were awarded the Nobel Prize in Physiology or Medicine in 2006 for their work (Daneholt and Bertil, 2006).

Biotechnology

Structure of Small interfering RNA (siRNA)

SiRNAs have a well-defined structure: a short (usually 21-nt) double strand of RNA (dsRNA) with 2-nt 3' overhangs on either end:



Schematic representation of a siRNA molecule: a ~19-21basepair RNA core duplex that is followed by a 2 nucleotide 3' overhang on each strand. OH: 3' hydroxyl; P: 5' phosphate.

Each strand has a 5' phosphate group and a 3' hydroxyl (-OH) group. This structure is the result of processing by dicer, an enzyme that converts either long dsRNAs or small hairpin RNAs into siRNAs (Bernstein *et al.,* 2001). SiRNAs can also be exogenously (artificially) introduced into cells by various transfection methods to bring about the specific knockdown of a gene of interest. Essentially any gene of which the sequence is known can thus be targeted based on sequence complementarity with an appropriately tailored siRNA. This has made siRNAs an important tool for gene function and drug target validation studies in the post-genomic era.

Cellular mechanism of RNA interference

RNAi is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute. When the dsRNA is exogenous (coming from infection by a virus with an RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved to short fragments by the enzyme dicer. The initiating dsRNA can also be endogenous (originating in the cell), as in pre-microRNAs expressed from RNA-coding genes in the genome. The primary transcripts from such genes are first processed to form the characteristic stem-loop structure of pre-miRNA in the nucleus, then exported to the cytoplasm to be cleaved by dicer. Thus, the two dsRNA pathways, exogenous and endogenous, converge at the RISC complex (Bagasra and Prilliman, 2004).

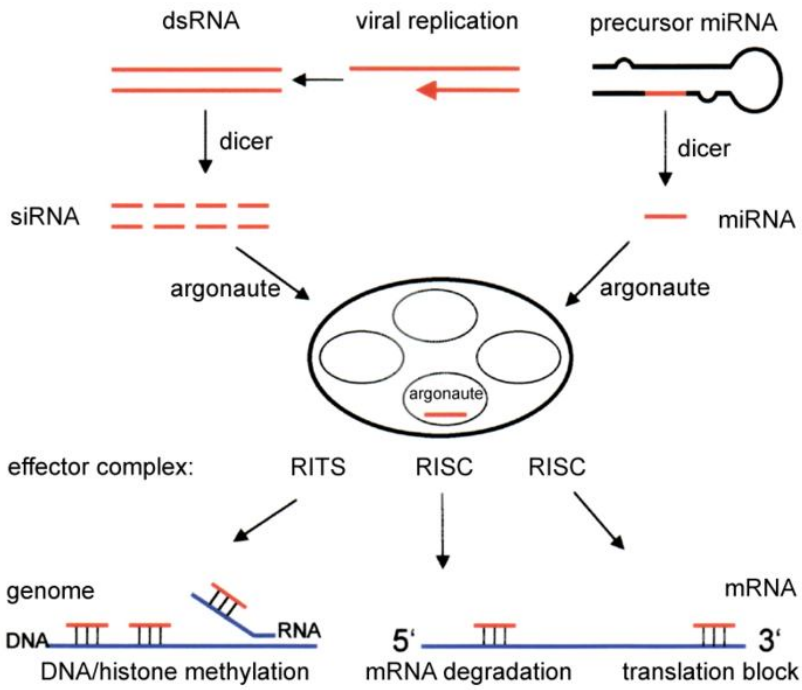


Fig.1: The enzyme dicer trims double stranded RNA, to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation.

dsRNA cleavage

Exogenous dsRNA initiates RNAi by activating the ribonuclease protein Dicer, (Bernstein *et al.,* 2001) which binds and cleaves double-stranded RNAs (dsRNA)s to produce double-stranded fragments of 21–25 base pairs with a few unpaired overhang bases on each end. Bioinformatics studies on the genomes of multiple organisms suggest this length maximizes target-gene specificity and minimizes non-specific effects (Qiu *et al.*, 2005). These short double-stranded fragments are called small interfering RNAs (siRNAs). These siRNAs are then separated into single strands and integrated into an active RISC complex. After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template (Ahlqist, 2002).

Exogenous dsRNA is detected and bound by an effector protein, known as RDE-4 in *C. elegans* and R2D2 in *Drosophila*, that stimulates dicer activity (Parker *et al.,* 2006). This protein only binds long dsRNAs, but the mechanism producing this length specificity is unknown (Parker *et al.,* 2006). These RNA-binding proteins then facilitate transfer of cleaved siRNAs to the RISC complex (Liu *et al.,* 2003).

This initiation pathway may be amplified by the cell through the synthesis of a population of 'secondary' siRNAs using the dicer-produced initiating or 'primary' siRNAs as templates.[15] These siRNAs are structurally distinct from dicer-produced siRNAs and appear to be produced by an RNA-dependent RNA polymerase (RdRP) (Pak and Fire, 2007; Sijen *et al.*, 2007).

RNAi induction using siRNAs or their biosynthetic precursors

Transfection of an exogenous siRNA can be problematic because the gene knockdown effect is only transient, particularly in rapidly dividing cells. One way of overcoming this challenge is to modify the siRNA in such a way as to allow it to be

expressed by an appropriate vector, e.g., a plasmid. This is done by the introduction of a loop between the two strands, thus producing a single transcript, which can be processed into a functional siRNA. Such transcription cassettes typically use an RNA polymerase III promoter (e.g., U6 or H1), which usually directs the transcription of small nuclear RNAs (snRNAs) (U6 is involved in gene splicing; H1 is the RNase component of human RNase P). It is assumed (although not known for certain) that the resulting siRNA transcript is then processed by Dicer.

RNA activation

It has recently been found that dsRNA can also activate gene expression, a mechanism that has been termed "small RNA-induced gene activation" or RNAa. It has been shown that dsRNAs targeting gene promoters induce potent transcriptional activation of associated genes. RNAa was demonstrated in human cells using synthetic dsRNAs, termed "small activating RNAs" (saRNAs). It is currently not known whether RNAa is conserved in other organisms.[4]

How Does it Work?

But how does the double stranded RNA cause gene suppression? Since the only RNA found in a cell should be single stranded, the presence of double stranded RNA signals is an abnormality. The cell has a specific enzyme (in Drosophila it is called Dicer) that recognizes the double stranded RNA and chops it up into small fragments between 21-25 base pairs in length. These short RNA fragments (called small interfering RNA, or siRNA) bind to the RNA-induced silencing complex (RISC). The RISC is activated when the siRNA unwinds and the activated complex binds to the corresponding mRNA using the antisense RNA. The RISC contains an enzyme to cleave the bound mRNA (called Slicer in Drosophila) and therefore cause gene suppression. Once the mRNA has been cleaved, it can no longer be translated into functional protein (Figure 2).

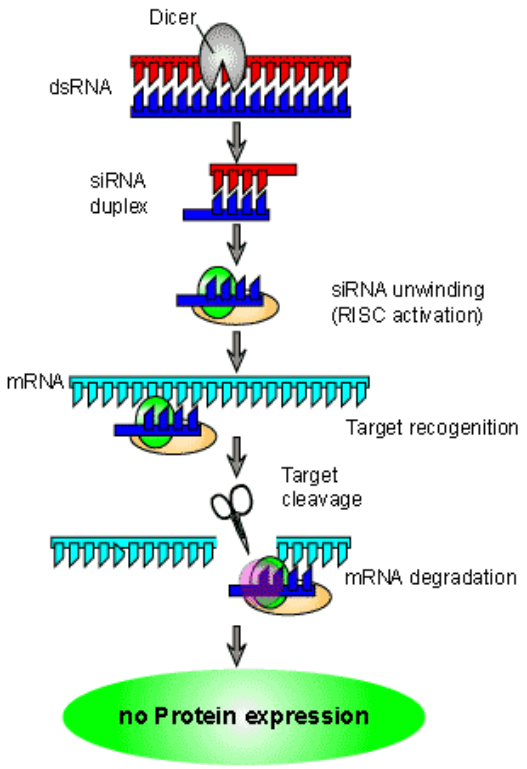


Fig. 2: Mechanism of action of RNAi. Double stranded RNA is introduced into a cell and gets chopped up by the enzyme dicer to form siRNA. siRNA then binds to the RISC complex and is unwound. The anitsense RNA complexed with RISC binds to its corresponding mRNA which is the cleaved by the enzyme slicer rendering it inactive.

Uses of siRNA

Knock out organism

The suppression of protein synthesis by introducing antisense RNA/siRNA into a cell is very useful. A gene encoding the antisense RNA can be introduced fairly easily into organisms by using a plasmid vector or using a gene gun that shoots microscopic tungsten pellets coated with the gene into the plant cells. Once the antisense RNA is introduced, it will specifically inhibit the synthesis of the target protein by binding to mRNA. This is a quick way to create a knockout organism to study gene function. Using antisense RNA as a tool in this way is an exciting prospect for many molecular biologists.

Pharmacokinetics

Antisense RNA is also being investigated for use in cancer therapy. Injecting aRNA that is complementary to the proto-oncogene BCL-2 may be useful for treating some B-cell lymphomas and leukemias. Antisense oligodeoxynucleotides (ODNs) are also being studied for human therapy. ODNs are similar to antisense RNA, but they are made synthetically and are deoxynucleotides (like those in DNA) rather than nucleotides. ODNs are being tested for their effectiveness against HIV-1, cytomegalovirus (a member of the herpesvirus group), asthma and certain cancers.

Food Engineering

Antisense RNA methods have also been used for commercial food production. You may have heard of the Flavr Savr tomato. This tomato was developed by Calgene Inc. of Davis, California in 1991 and was approved by the U.S. FDA in 1994. The tomato was the first whole food created by biotechnology that was evaluated by the FDA. One of the problems associated with tomato farming is that the fruit must be picked while still green in order to be shipped to market without being crushed. The enzyme that causes softening in tomatoes is polygalacturonase (PG). This enzyme breaks down pectin as the tomato ripens, leading to a softer fruit. Calgene suppressed the expression of the gene encoding PG by introducing a gene encoding the antisense strand of the mRNA. When the introduced gene was expressed, the antisense strand bound to the PG mRNA, suppressing the translation of the enzyme. The Flavr Savr tomatoes therefore had low PG levels and remained firmer when ripe. This meant the Flavr Savr tomatoes can ripen on the vine and then be shipped to market. Although the Flavr Savr tomatoes were approved for sale in the U.S., production problems and consumer wariness stopped the production of this fruit in 1997. RNA interference is a field that was stumbled upon by accident while trying to improve the colour of petunias, however its implications may be far reaching in the near future.

Challenges & Concerns

Because RNAi intersects with a number of other pathways, it is not surprising that on occasion nonspecific effects are triggered by the experimental introduction of an siRNA. When a mammalian cell encounters a double-stranded RNA such as an siRNA, it may

mistake it as a viral by-product and mount an immune response. Furthermore, because structurally related microRNAs modulate gene expression largely via incomplete complementarity base pair interactions with a target mRNA, the introduction of an siRNA may cause unintended off-targeting.

Innate immunity

Introduction of too much siRNA can result in nonspecific events due to activation of innate immune responses. Most evidence to date suggests that this is probably due to activation of the dsRNA sensor PKR, although retinoic acid inducible gene I (RIG-I) may also be involved. The induction of cytokines via toll-like receptor 7 (TLR7) has also been described. One promising method of reducing the nonspecific effects is to convert the siRNA into a microRNA. MicroRNAs occur naturally, and by harnessing this endogenous pathway it should be possible to achieve similar gene knockdown at comparatively low concentrations of resulting siRNAs. This should minimize nonspecific effects.

Off-targeting

Off-targeting is another challenge to the use of siRNAs as a gene knockdown tool. Here, genes with incomplete complementarity are inadvertently downregulated by the siRNA (effectively, the siRNA acts as an miRNA), leading to problems in data interpretation and potential toxicity. This, however, can be partly addressed by designing appropriate control experiments, and siRNA design algorithms are currently being developed to produce siRNAs free from off-targeting. Genome-wide expression analysis, e.g., by microarray technology, can then be used to verify this and further refine the algorithms. A 2006 paper from the laboratory of Dr. Khvorova implicates 6- or 7-basepair-long stretches from position 2 onward in the siRNA matching with 3'UTR regions in off-targeted genes (Birmingham *et al.*, 2006).

Possible therapeutic applications and challenges.

Given the ability to knock down essentially any gene of interest, RNAi via siRNAs has generated a great deal of interest in both basic (Alekseev *et al.*, 2009) and applied biology. There are an increasing number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. Because disease processes also depend on the activity of multiple genes, it is expected that in some situations turning off the activity of a gene with an siRNA could produce a therapeutic benefit.

However, applying RNAi via siRNAs to living animals, especially humans, poses many challenges. Experimentally, siRNAs show different effectiveness in different cell types in a manner as yet poorly understood: some cells respond well to siRNAs and show a robust knockdown, whereas others show no such knockdown (even despite efficient transfection).
Phase I results of the first two therapeutic RNAi trials (indicated for age-related macular degeneration, aka AMD) reported at the end of 2005 demonstrated that siRNAs are well tolerated and have suitable pharmacokinetic properties (Tansey, 2006). siRNAs and related RNAi induction methods therefore stand to become an important new class of drugs in the foreseeable future.

In 2008, a team of researchers from Texas Tech University and Harvard University announced the development of a siRNA-based treatment that may ultimately counteract the

Human Immunodeficiency Virus (HIV). Human cells infected with HIV, injected into rats, have been cured by the experimental treatment. Clinical trials on humans are expected to begin by 2010 (Swaminathan, 2008).

In 2008, a novel DNA-siRNA delivery system that could lead to more efficient and more disease-specific vaccines against infectious diseases was developed and by researchers at The University of Texas at Austin. Biomaterials based micron size particles carrying both the DNA vaccine and the siRNA to immune cells show potential to divert immune response in desirable directions (http://www.bme.utexas.edu/news/2008_roy_VDS.cfm).

Concluding remarks: siRNA & techno-economic Aquaculture

Recognizing that the value of an novel use of siRNA cannot be separated from its socio-technical context will also have enormous implication for the manner in aquaculture research and its objectives. Recently, a few groups have reported that viruses from the aquatic animals could be inhibited by exogenously synthetic long dsRNAs or siRNAs (Robalino *et al*., 2004, 2005; Tirasophon *et al*., 2005; Westenberg *et al*., 2005; Xie *et al*., 2005). They showed that long dsRNA molecules could induce a sequence-independent antiviral immunity in shrimp (Robalino *et al*., 2004, 2005), and viral replication could be effectively inhibited by long dsRNA molecules and siRNA molecules in shrimp or fish cells (Tirasophon *et al*., 2005; Xie *et al*., 2005). siRNA cannot act on non-specific sequence. However, it has been shown that non-specific siRNAs also reduce mortality in shrimp (Westenberg et al., 2005), and neither sequence-dependent nor sequence independent sequence independent siRNAs could induce the antiviral action (Robalino et al., 2005).

Yue *et al.* (2007) described specific silencing of five white spot syndrome virus (WSSV) genes in *Litopenaeus vannamei* in vivo with sequence-specific siRNAs. These genes included DNA polymerase (dnapol), ribonucleotide reductase small subunit (rr2), thymidine kinase and thymidylate kinase (tk-tmk), vp24 and vp28. At 6 days post-challenged, the relative survival rates of shrimp injected with siDNApol, siRR2, siTK-TMK, siVP24 and siVP28 (siRNAs for dnapol, rr2, tk-tmk, vp24 and vp28 genes) reached 50%, 50%, 66%, 33% and 33%, respectively. Specific siRNAs of the five WSSV genes could result in suppression of the target genes and a significant reduction in the viral proliferation. In negative controls, sequence-independent siRNA (mutant siRNA) could not inhibit expression of these five genes or viral replication. Consequently, injection of sequence-dependent siRNA could induce anti-WSSV activity in shrimp. These results suggest that siRNA can suppress WSSV efficiently in shrimp, and it may provide a potential approach to the therapy of aquaculture viral disease.

Effective time of RNAi in vivo is limited, and the delivery efficiency of dsRNA or siRNA is not difficult to control and detect (Robalino *et al.,* 2004;Westenberg *et al*., 2005). The half-life of synthetic RNA duplexes is very short. In some circumstances, these siRNAs will not stay long enough for complete elimination of virus replication in an infected cell. Further studies are necessary to construct high expression vectors and better transfection approaches of dsRNA/siRNA in shrimp in vivo. Therefore, it is necessary to study siRNA injection continuously into shrimp instead of single injection so that the siRNA may perform more effectively. Moreover, a permanent shrimp cell line would bring further advantages. Although there are a number of important issues to be resolved, these siRNAs may be a potential candidate for therapeutic measures against the current disease caused by WSSV.

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SPERMATOGONIAL STEM CELL RESEARCH: STRATEGIES AND PROSPECTS

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Stem cells are the parent cells for all tissues and organs of the body. They exist mainly to maintain and repair cells in the areas where they are found. Stem cells are found in the blood, bone marrow, skin, muscle, and organs like the brain, testis and liver. There are mainly two kinds of stem cells i.e. embryonic stem cells, and adult stem cells. Stem cells are unique cells within the mammalian body with the capacity for self replication and tissue regeneration. Stem cells have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiologic or experimental conditions, they can be induced to become cells with special functions such as the beating cells of the heart muscle or the insulin producing cells of the pancreas.

Properties of stem cell:
The classical definition of a stem cell requires that it possess two properties:

- ***Self-renewal*** - the ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
- ***Potency*** - the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotent - to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred to as stem cells.

Potency definitions:
Potency specifies the differentiation potential (the potential to differentiate into different cell types) of the stem cell.
Totipotent stem cells can differentiate into embryonic and extraembryonic cell types. Such cells can construct a complete, viable, organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent.
Pluripotent stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers.

- **Multipotent** stem cells can differentiate into a number of cells, but only those of a closely related family of cells.
- **Oligopotent** stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.
- **Unipotent** cells can produce only one cell type, their own,but have the property of self-renewal which distinguishes them from non-stem cells (e.g. muscle stem cells).

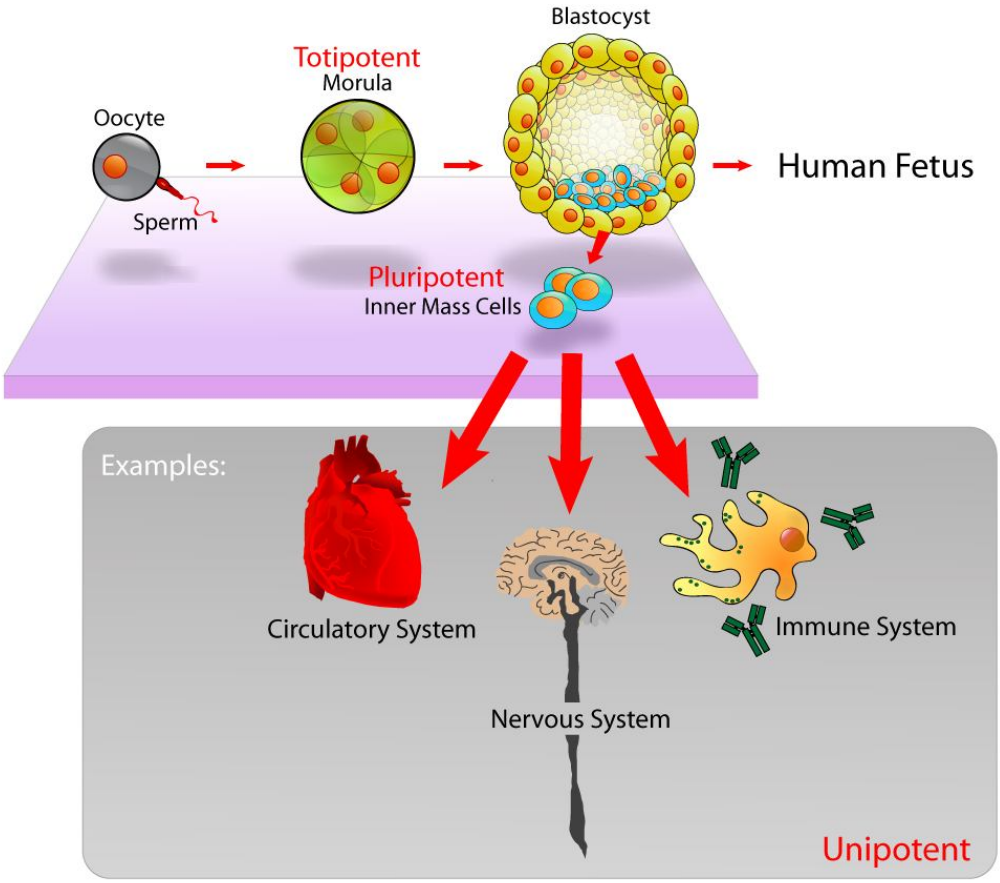


Fig. 1: Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta.

Spermatogonial stem cell:

A cell that is incapable of fertilizing an egg cell but can give rise to cells that develop into sperm is sperm stem cell. Sperm stem cells can be grown in laboratory culture, providing a ready source of these cells, as was first demonstrated in mice. These are also known as spermatogonial stem cell. The primitive germline cells are called primordial germ cells (PGCs), which are segregated from somatic cells, early in development and migrate to the embryonic female and male gonads, where they differentiate into oogonia or gonocytes, respectively. Oogonia in the female gonad directly enter meiotic prophase (arrested in meiosis I before birth) as oocyte, and hence, adult ovary lacks germline stem cells; whereas, in the male, gonocytes cease mitosis in the fetal seminiferous tubules (in the centre of the tubule) of the testes and resume mitotic activity after birth. Mammalian and fish PGCs proliferate during and after their migration, respectively. After birth gonocytes migrate to the basement membrane of the seminiferous tubules. At the onset of spermatogenesis, testicular gonocytes or prospermatogonia resume proliferation and become undifferentiated type-A spermatogonia, the male germ stem cells called spermatogonial stem cells (SSCs) that self-renew themselves to maintain the stem-cell pool or differentiate through meiosis into haploid spermatids that undergo through spermiogenesis to become motile and functional sperm. Spermatogonia are basically categorized in two types: undifferentiated

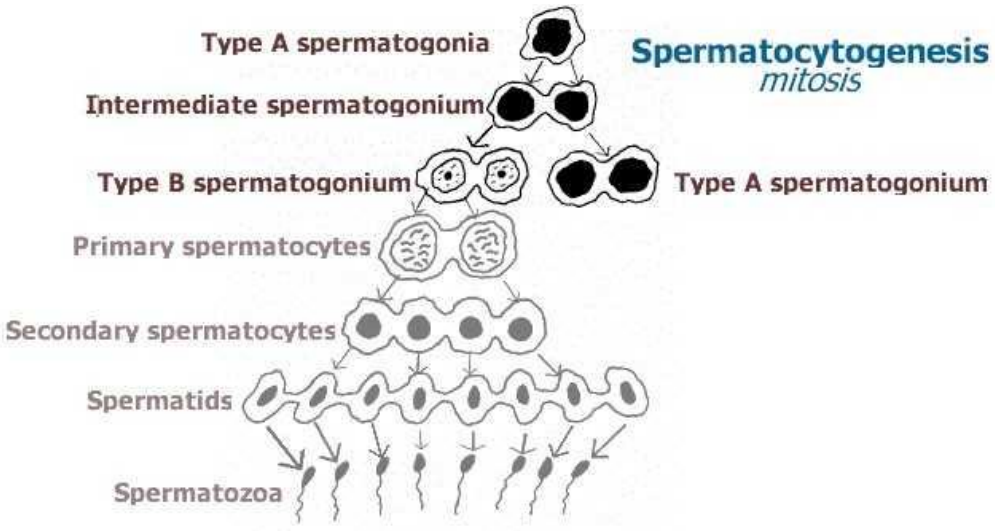
spermatogonial stem cells and differentiated spermatogonia committed to mitotic proliferation leading to meiosis. The former possesses an intrinsic ability to self-renew throughout the life-time of animals and continuously supply spermatozoa through the process of spermatogenesis.

Spermatogenesis:

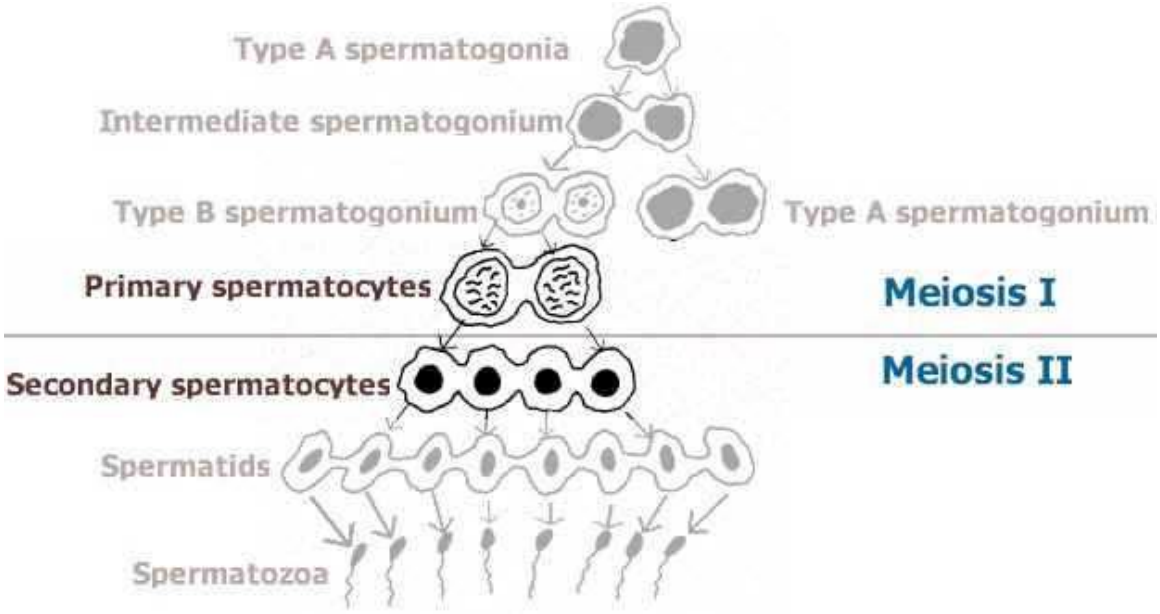
Spermatogenesis is the process by which undifferentiated germ cells within the testis divide and mature to produce millions of spermatozoa daily. Continual function of this process is a requirement for sustained male fertility.

Spermatogenesis is the process by which stem cells develop into mature spermatozoa. There are three phases: (i) Spermatocytogenesis (Mitosis), (ii) Meiosis, and (iii) Spermiogenesis.

(i) **Spermatocytogenesis** (also called Mitosis): Stem cells (Type A spermatogonia; singular = spermatogonium) divide mitotically to replace themselves and to produce cells that begin differentiation (Type B spermatogonia). Spermatogonia have spherical or oval nuclei, and rest on the basement membrane.

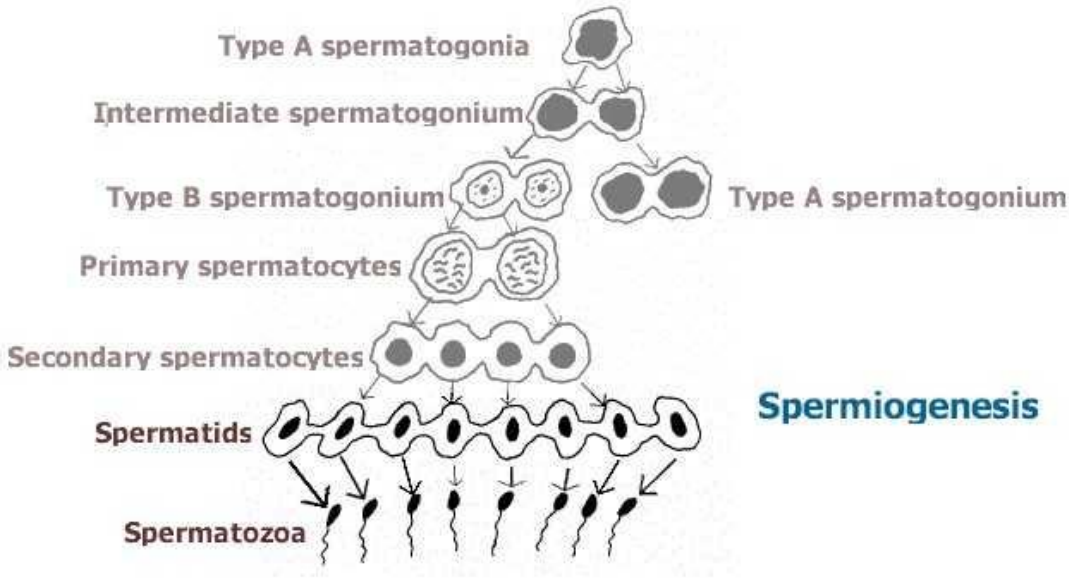


(ii) **Meiosis**: Cells in prophase of the first meiotic division are primary spermatocytes. They are characterized by highly condensed chromosomes giving the nucleus a coarse chromatin pattern and an intermediate position in the seminiferous epithelium. This is a long stage, so many primary spermatocytes can be seen. Primary spermatocytes go through the first meiotic division and become secondary spermatocytes. The cells quickly proceed through this stage and complete the second meiotic division. Because this stage is short there are few secondary spermatocytes to be seen in sections. You are not responsible for identifying secondary spermatocytes in lab. Meiosis is the process by which the diploid number of chromosomes present in spermatogonia (the stem cells) is reduced to the haploid number present in mature spermatozoa. The products of the second meiotic division are called spermatids. They are spherical cells with interphase nuclei, positioned high in the epithelium. Since spermatids go through a metamorphosis into spermatozoa, they occur in early through late stages. All of these progeny cells remain attached to each other by cytoplasmic bridges. The bridges remain until sperm are fully differentiated.



(iii) **Spermiogenesis:**

This is the metamorphosis of spherical spermatids into elongated spermatozoa. No further mitosis or meiosis occurs. During spermiogenesis, the acrosome forms, the flagellar apparatus forms, and most excess cytoplasm (the residual body) is separated and left in the Sertoli cell. Spermatozoa are released into the lumen of the seminiferous tubule. A small amount of excess cytoplasm (the cytoplasmic droplet) is shed later in the epididymis.



Spermiogenesis is a process of metamorphosis from a round cell with typical organelles to a highly specialized, elongated cell well adapted for traversing the male and female reproductive tracts and achieving fertilization of an egg. Spermatogenesis is cyclical to a varying extent depending on the species, and under endocrine control. Spermatogenesis is maintained and regulated by male steroid hormones such as testosterone, which is produced by the interstitial or Leydig cells found in the connective tissue of the testis. Interstitial cells, in turn, are stimulated by luteinizing hormone (LH) which is produced by

the pituitary gland. The male testis-regulating hormone was formerly known as interstital cell-stimulating hormone (ICSH), but it is now known to be identical to LH.

Impact of Spermatogonial stem cell propagation:

The SSCs biology should be an excellent model system for *in vitro* spermatogenesis to study underlying molecular mechanisms that regulate germ-cell development: mitotic proliferation and differentiation of stem cells, meiosis, and their regulation in a vertebrate. Once SSCs are formed, they continue to self-renew and produce spermatogonia that are committed to differentiate into spermatozoa, as controlled by their gene expression and environmental cues. The programming of self-renewal and differentiation of SSCs must be finely tuned so as to maintain normal spermatogenesis; however, the mechanisms governing the fate decision – self-renewal or differentiation – are largely unknown. By establishing culture system for fish SSCs, it will be possible to generate enriched stem cells that can be utilized to uncover mechanistic pathways and/or factors responsible for fate decision involving either self-renewal or differentiation. Understanding above mechanisms of germ-cell development is crucial to reproductive medicine, and animal/fish production. Hence, SSCs biology should be a novel tool to analyze spermatogenesis to study underlying molecular mechanisms that regulate germ-cell development: mitotic proliferation and differentiation of stem cells, meiosis, and their regulation in a vertebrate. This will also open the door to establish molecular markers based on distinguished gene expression patterns characteristic of spermatogonial stem-cell population that is poorly understood. The SSCs technology can also be used for cloning based biodiversity rescuing, germ-line mutagenesis and gene targeting, as an alternate to ES cells technology especially in fish species in which no ES cells for targeting could be made possible as yet despite several attempts.

Indian major/minor carps including domesticated comprises one of the major sources of Indian livelihood supplying protein rich diet. Moreover, freshwater aquaculture and animal husbandry together have been the industry to support economic growth in India. It is obvious that the availability of cultivable SSCs for animals and carps should be an asset to be used as potential carrier of delivering exogenous gene of interest for new transgenic technology in addition to knowledge to be gained from basic research. Thus, carp SSC technology will provide new possibilities in animal/aquaculture biotechnology in India.

OCCURRENCE AND IDENTIFICATION OF MARINE FISH PARASITES

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Parasitic fish diseases constitute one of the most important problems in the fisheries sector. Cymothoids (Cymothoidae, Crustacea, Isopoda,) are obligatory fish parasites, infest mostly commercially important fishes. They are protandric hermaphrodites and blood suckers, living on the skin, gill filaments, or in the mouth of the fishes. These parasites retard growth followed by emaciation. Pathological conditions resulting from parasitic diseases assume high magnitude of epidemics under crowded and other unnatural conditions among fish. The order Isopoda includes both terrestrial and aquatic species and represents the second largest order of crustaceans. Isopods are widely distributed in all types of habitats, from terrestrial to marine, fresh and ground waters, and some species are known to be parasitic. Ectoparasitic isopods of the family Cymothoidae inhabit freshwater, brackish and marine environments, utilizing various fish hosts. Bruce.N.L., Harrison-Nelson (1988) reported *Nerocila trichiura*, parasitic on flying fishes of the genera *Exocoetus and Cypselurus*. They resemble free-living isopods except for their hook-like legs. The stages normally found are the non-swimming, permanently attached mature females, often with a small male nearby.

Isopods have been accused of directly transmitting lymphocystis disease, but this is unlikely considering their feeding habits and behavior. As cymothoids penetrate the skin with their pereopods and mouthparts, and the tissue-inhabiting forms maintain a small opening to the outside, secondary infections occur. The wounds they cause may provide entry points for microbial diseases. Pathogenic microorganisms in the aquatic habitat pose problems to the economic important fishes due to their secondary invasion on the body of the fish, which got isopod parasitic infestation primarily (Ravichandran et al. 2001; Ravichandran and Ajith Kumar 2008). Parasitic crustaceans (Isopods and Copepods) often possess attachment organs that are employed to anchor into host tissues. Others may move freely on the host's surface causing widespread necrosis and disruption of the protective mucus covering of the skin at anchor points. The size and age of the host, host health status, the species of parasite, and developmental stages present, affect the severity of the disease. Severe wounding can result in host death due to osmotic imbalance or by providing entry points for other pathogens. Economic losses incurred other than direct mortality of farmed fish are generally due to reduced growth of infected fish, negative impacts on edible tissues and costs associated with treatments. Cultured cobia are susceptible to the sea lice *Caligus lalandei* (Chang and Wang, 2000), *C. epidemicus* (Ho et al., 2004) and in the Peng–hu region of Taiwan, Ho and Lin (2001) reported infestations by *Parapetalus occidentalis*. In the wild, cobia have been reported with *C. coryphaenae* (Causey, 1953), *Lernaeolophus sultanus* in conjunction with the barnacle *Conchoderma virgatum* (Dawson, 1969), *Tuxophorus caligodes*, *Euryphorus nordmanni*, *L. longiventris*, *L. hemiramphi*, and *C. haemulonis*, (Bunkley –Williams and Williams, 2006).

Nematodes or roundworms are commonly present even in healthy fish but when their abundance is large, roundworm infestations may cause death. Roundworms are generally cylindrical, smooth and long worms which distinguishes them from the segmented tapeworms. Similar to tapeworms however, nematodes can reduce reproductive performance and have a negative impact on

feed conversion efficiencies leading to reduced growth and overall performance of cultured fish. Economic loss can also occur due to the burrowing of worms into various tissues including musculature. Nematodes can cause hemorrhaging and the development of granulomatous tissue resulting in visible bumpy or nodular appearance that can downgrade the value of fillets. Bruce and Cannon (1989), describe *Iheringascaris inquires* from the stomach of cobia caught in Australian waters which may be synonymous with *Contracecum megacephalum* (Oschmarin, 1963) taken from the stomach of a cobia from the South China Sea. *Mabiarama prevesiculata* derived from the stomach of cobia has been described from specimens taken in Brazil whereas *Goezia pelagia* was isolated from fish in the Gulf of Mexico (Bun- kley –Williams and Williams, 2006). Arthur and Te (2006) also include *Anisakis* sp. and *Philometroides* sp. isolated from cobia in Vietnamese waters. To date no information is available on roundworm infestations in captive cobia but in other cultured species serious infections have been associated with serious tissue necrosis around the sites of intestinal intrusion. This may provide portals for bacterial and other infections.

Marine Nematode, & Crustacean Parasites in East coast of India

A. Marine Nematode Parasites on *Otolithus argenteus* along the Madras coastal water, Bay of Bengal:

Nematodes of the dracunculoid family Philometridae are frequent parasites in various body tissues and cavities of freshwater and marine fishes. *Philometra rajani* the gonadial nematode parasite was first described by Mukerjee R. P. 1963, from the ovaries of fishes *Polynemus polydactylus* and *Sciaena coiter*. Anniqiri (1961) recorded Philometrids from *Otolithus argenteus* and from his account it appears to be the same species described by Mukerjee R. P. 1963. From Japan, Yamaquti S (1961) recorded philometrids from the sciaenid fish *Sciaena schegeli.* While examining the sciaenid fish *Pennahia aneus* (Bloch) from Mandapam and Pamban (Palk Bay) many specimens infested with *Philometra rajani* were observed by Lal Mohan, 1970.

The present study reports the prevalence of philometrid nematode, *Philometra lateolabracis* infections in two species of Sciaenid fishes along the Madras coastal water, Bay of Bengal during December, 2008 to July, 2012 (Fig.1-4). Out of total 295 sciaenid fishes examined, *Otolithes ruber* and *Pennahia macrophthalmus* showed 70 % & 32.5% of parasitic prevalence on the subcutaneous region on caudal fins (Table1). The number of parasites per fish varied from 1 to 7 with an average of 3.18±1.64. The respective size of all the parasites varied from 5-16 mm with an average length of 11.18±4.62 mm. The minimum and maximum sizes and weight of Sciaenid fishes were caught from Kasimedu landing centre (Chennai) during October-December, 2012 varied from180-287mm and 120-245 gm respectively.

B. Marine Crustacean Parasites in East coast of India:

1. OCCURENCE OF PARASITIC COPEPODS, *Lernaeenicus sprattae* ON KING MACKEREL (*Scomberomorus commerson*), ALONG SOUTH-EAST OF INDIA:

This study was carried out to investigate the extent of parasitation of copepod pennellidae on King Mackerel (*Scomberomorus commerson*), along South-East coast of India during September 2009.This is the first report from this region and the infection is discussed in relation to biological parameters. Fifty to sixty numbers of parasites were recorded from belly region of King Mackerel. Parasitic copepod identified as *Lernaeenicus sprattae*, (Fig.A-C) belongs to family Pennellidae harms several fish species, which, feed voraciously and kill fry and fingerlings of the many marine fishes.

Lernaeenicus parasites would stunt the growth of fish through inhibiting reproduction, thus these type of parasitation would lead to severe economic loss in the commercial species of the marine fishes of India.

This is the first report on the occurrence of an infection by parasitic copepod; *Lernaeenicus sprattae* in the seer fish from the South-Eastern coast of India, Such mass infestation of pennellid copepod, on belly portion of seer fish is probably uncommon, but not accidental. Parasites in the belly portion are usually associated with atrophy and partly from damage associated with feeding and attachment leads to anaemic condition. This may lead to severe economic loss in the commercial species of the marine fishes of India particularly Seer fishes (Bragoni, *et al.,* 1984).

Table 1. Details on the number of parasites infected on Sciaenid fishes during 2008-2012

Species of fishes examined	Number of fishes examined	Number of fish infected	Prevalence (%)	Fish Size (mm)
Otolithes ruber	80	56	70	150-319
Johnius carutta	70	Nil	Nil	130-210
Nibea maculata	55	Nil	Nil	120-209
Pennahia macrophthalmus	40	13	32.5	130-311
Johnius dussumeri	50	Nil	Nil	110-212

2. Infestation of Yellow tip halfbeak fish, *Hemiramphus marginatus* (Lesueur 1821), a new host of *Nerocila exocoeti* (Crustacea, Isopoda, Cymothoidae).

Fifteen (22.5%) out of the 150 yellow tip half beak fish *Hemiramphus marginatus* examined specimens were infested with parasitic isopods. The parasites were found on the body surface. Based on morphological characteristics provided below, the isopods were determined as *Nerocila exocoeti* (Fig.5-7a&7b). Body surface of the infested fish was abraded due to the infestation.

Prevalence and infestation intensity: The prevalence of infestation of *Nerocila exocoeti* on *H. marginatus* was 22.5% (15 of 150) of the examined fishes. Ten ovigerous females of *N. exocoeti* were collected. Infestation intensity ranged from 1-2 per fish.

Hosts: The members of family Hemiramphidae are the specific hosts for *N. exocoeti*. Known host spectrum includes *Parexocoetus brachypterus, Cypselurus comatus* (Bruce and HarrisonNelson, 1988) *Exocoetus volitans* (Kanagasabapathy etal; 2011) and the range of host fishes is here extended and now includes *Hemiramphus marginatus* (present study). In this study, *N. exocoeti* is recorded for the first time from the *Hemiramphus marginatus*, together with India, which is a new geographic record. According to its rare occurrence, it is probable that *Hemiramphus marginatus* might be an occasional or accidental host for this species.

3. Co-infection of Yellowtip Halfbeak Fish (*Hemiramphus marginatus*) with Isopod and Copepod Parasites from the Coromandal Coast, India

Isopod parasite of the family Cymothoidae under order Beloniformes have been reported from about 350 fish species and over 80% of these occurrence are from tropical and subtropical seas, majority being from the Indo-Malaysian archipelago (Lester, 1995). Approximately 10,000 species of copepods have been described out of which 2,000 species are reported to be parasites of fish. Among the copepod parasites, those belonging to Pennellidae are widespread and gonochoristic in marine fishes.

Isopods and copepods have been reported parasitizing several fishes of Parangipettai coastal waters in India (Rajkumar et al., 2004). Double parasitism in Commerson's Anchovy (*Stolephorus commersonii*) is also reported from Parangipettai coastal waters (Rajkumar et al., 2006). Gopalakrishnan et al. (2010) reported double parasitism (isopod Cymothoidae and copepod Pennellidae) on the black-barred halfbeak fish, (*Hemiramphus far*) from southeast coast of India. In the present study an attempt was made to study the parasite infection in yellowtip halfbeak fish *Hemiramphus marginatus*, which occupy similar food niche in this ecological region (Fig. 8).

Parasite infestation was observed in 60% of fishes collected and the number of parasites per fish ranged from 1 to 3 with a mean of 1.73 ± 0.59. Double infection of an isopod (Cymothoidae) and a copepod (Pennellidae) was observed in yellowtip halfbeak fishes of size ranging from 265 to 310 mm and mean total length TL of 287.13 ± 14.15 mm.

The weight of the fish varied from 53-93g with average of 75.06 ± 11.88 g. The cymothoid isopod was identified as *Mothocya plagulophora* (Fig.9a&b) and both sexes of this species were found to co-habit as pairs in the gill region. Both the parasites *M. plagulophora* and *Lernaeenicus hemiramphi* (Fig.10) recorded in the present study were attached to the host fish.

Table. 2. Nature of isopod infection in clupeid fishes of Chennai coast.

Serial Number	Species of fishes examined	Number of fishes studied	No of fishes infected with *Joryama hilsae*	Number of fishes infected with *Joryma sawayah*
1	Pellona ditchela	130	23(17.7)*	42 (32.3)*
2	Thryssa mystax	30	Nil	Nil
3	Thryssa setirostris	50	Nil	Nil
4	Tenualosa ilisha	40	Nil	Nil

* Prevalence (%)

4. Occurrence of isopod parasites in clupeids, Indian Pellona fish, *Pellona ditchela* off Chennai coast, India

The study was carried out to investigate the incidence of isopod parasites in clupeids, with special emphasis on the Indian Pellona fish, *Pellona ditchela* . Two species of isopods, *Joryma hilsae* and *Joryma sawayah* were found infecting this fish. The isopods of the genus *Joryma* are typical Indo-Pacific genus, found infecting the gills of marine fishes. However, this is the first report of these two species in Indian pellona, *P. ditchela* along the Bay of Bengal off Chennai coast, India. The prevalence (%) of *J. hilsae* and *J. sawayah* in *P. ditchela* recorded during the present investigation were 17.7 and 32.3 respectively (Tab.2). Cymothoids are common isopod parasites of marine fishes and their infection can cause serious damages to the fishes.

Body colour of *J. hilsae* (Fig.11& 12) was pale tan with large dark stripes on lateral parts of pereonites 6 and 7 and pleonites 1 – 5. The dorsal posterior side of *J. hilsae* showed clear blackish bands (Tab.3). The average length and weight of *J. hilsae* was 22.8 ± 4.13 mm and 0.405 ± 0.196 g respectively. In *J. sawayah* (Fig. 13) body colour was whitish with a slight greenish tinge on the dorsal side. The mean length and weight of *J. sawayah* was 24.05 ± 2.37 mm and 0.4194 ± 0.104 g respectively.

Table.3. Comparisons species of Joryma Isopod collected from Lighthouse kuppam, Marina Beach, off Chennai Coast, India.

Species	Head	Pareonie Lanteral expansion	Pleonites	Pleotelson	Antenna	Mandibular palp	Maxilliped ecurved spines	Uropodsrami
J. hilsae	conspicuous dorsally, reaching margin of pereonite 1 expansion	Bilateral, Slightlybl obed	Not overlapped	Broadly rounded	9 articles	3 segmented	4	Equal
J. sawayah	Exposed dorsally, not reaching margin of perionites 1 expansion	Bilateral, distinctly bilobed	1,2,3 overlapped laterally	Triangular	8	Not segmented	7	Unequal

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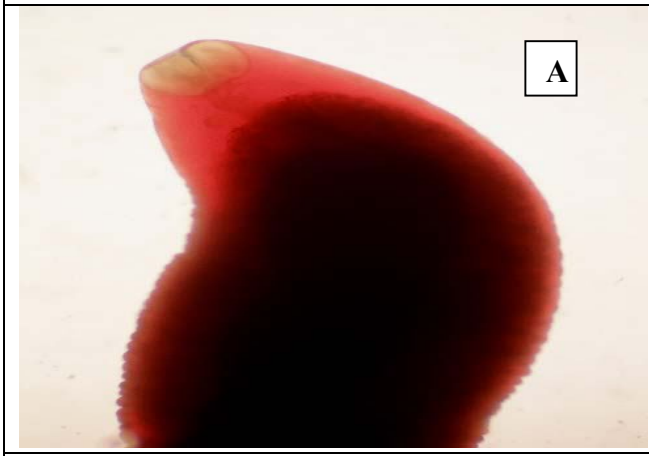
Fig.1. Grouper, *Otholithes ruber* infected with

Fig.2. Adult of *Philometra lateolabracis* isolated

Philometra lateolabracis **from sub-cutaneous nodules of caudal fins. Arrow points the attachment sites of the fishes from *O. ruber* from the Kasimedu Fisheries Harbour, Bay of Bengal off Chennai, TN.**

Fig.3. Gravid females and larvae of *Philometra lateolabracis* from *O.ruber.*



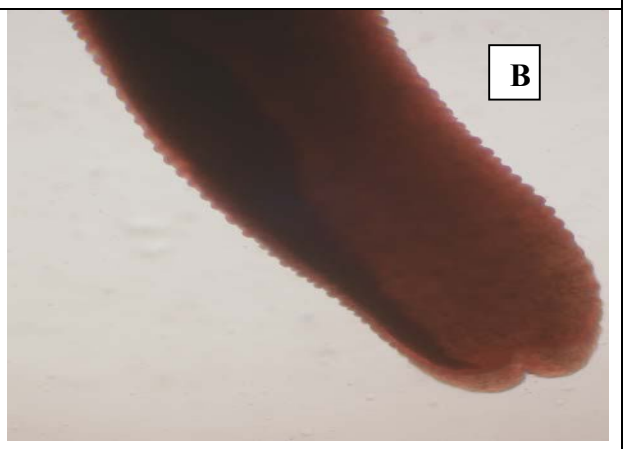


Fig.4. Anterior (A) and posterior (B) end of adult nematode, *Philometra lateolabracis* collected from *O.ruber* .

Fig.A : King Mackerel, *Scomberomorus commerson* collected from Kasimedu Fisheries Harbour

Fig.B : Parasitic copepods, *Lernaeenicus sprattae* attached massively on ventral portion of a King Mackerel, *Scomberomorus commerson.*

Fig.C: Group of parasitic copepods, *Lernaeenicus sprattae* collected from abdomen region of King Mackerel, *Scomberomorus commerson.*

Fig.5. *Nerocila exocoeti* attached to the head of Yellow tip halfbeak fish, *Hemiramphus marginatus* (lateral view)

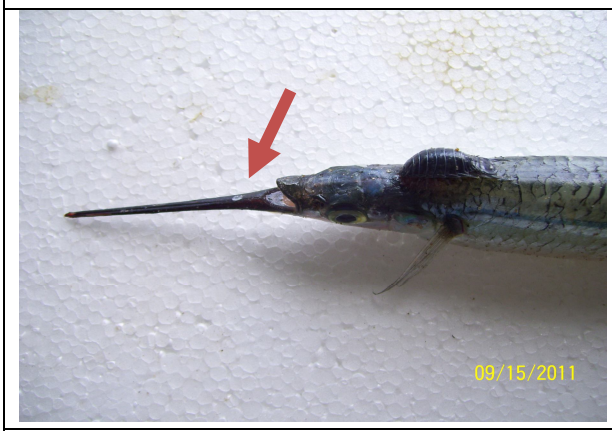


Fig.6. External Lesion due to the infestation of *Nerocila exocoeti*, the arrow points at the damaged skin patch on the head of *H. marginatus*

Fig. 11. Co-infection of *Joryma hilsae & Joryma sawayah* attached to the gill region of *P. ditchela*

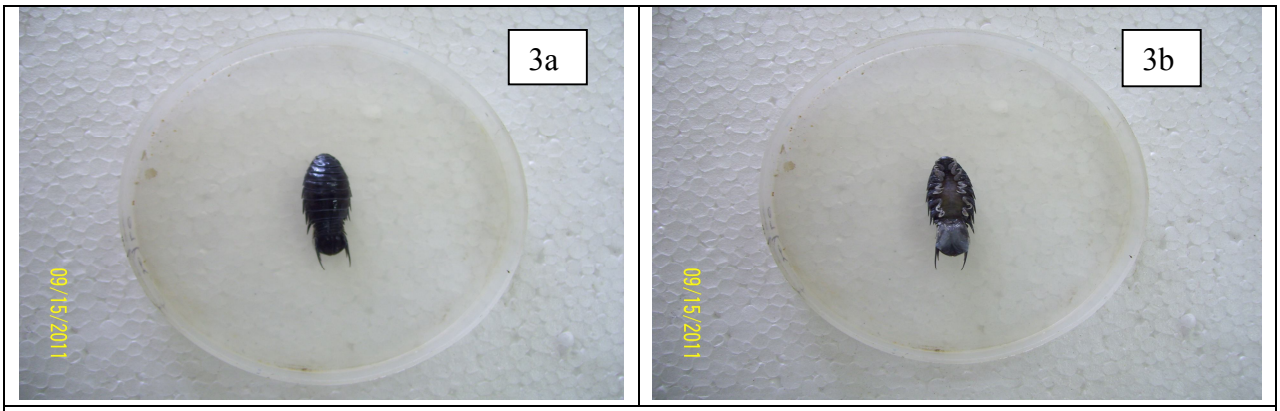


Fig. 7a&b. Dorsal and ventral view of the female of *Nerocila exocoeti* infested on *H. marginatus.*

Fig. 8. Parasitic Isopod, *Mothocya plagulophora* and Copepod *Lernaeenicus hemiramphi* attached to Yellow tip halfbeak fish (*Hemiramphus marginatus*)

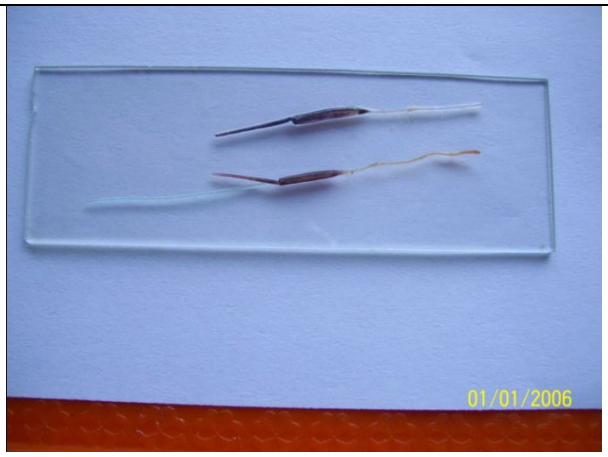


Fig.10. Parasitic Copepod *Lernaeenicus hemiramphi* attached to Yellow tip halfbeak fish (*Hemiramphus marginatus*).

Fig. 9 a& b. Gravid female parasitic isopods with embryos and larvae attached to brood pouch or marsupium isolated from Yellowtip halfbeak fish. (a. dorsal view & b.ventral view)

Fig.12. Gravid females of *J. hilsae* collected from *P. ditchela* (dorsal and ventral view)

Fig.13. Gravid females of *J. sawayah* collected from *P. ditchela* (dorsal and ventral view)

WESTERN BLOT HYBRIDIZATION: A MOLECULAR TECHNIQUE FOR DETECTION, CONFIRMATION AND CHARACTERIZATION OF UNKNOWN PROTEINS

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Introduction

Blots are the techniques for transferring DNA , RNA and proteins onto a carrier such as nitrocellulose membrane, PVDF etc. It is broadly categorized into three types : Southern blot, Northern blot and Western blot for DNA, RNA and protein respectively. The transfer - or "blotting" of electrophoretically separated proteins from the gel matrix to a membrane followed by subsequent antibody-based detection on the surface of the membrane is called Western - or Immunoblot. It enables detection of a specific target protein out of a complex protein mixture, e.g. tissue homogenate or cell extract, using highly selective and sensitive antibody-antigen interactions. It reveals both qualitative and semi-quantitative information about the protein of interest. It can also be used to detect changes in protein phosphorylation, lipid modification and gene expression studies. The method originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute, and the name Western blot was given to the technique by W.Neal Burnette**.**

SDS-Polyacrylamide Gel Electrophoresis SDS-PAGE)

The most common method of gel electrophoresis used for protein separation employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins based on their molecular weight. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the porosity and resolution of the gel. The greater the acrylamide concentration, the better the resolution of lower molecular weight proteins, and the lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Protein becomes negatively charged once it binds with SDS (an anionic detergent). Hence, proteins migrate on the gel towards the positively charged end of the electrophoretic apparatus according to their molecular weight. Therefore if proteins of known amounts are loaded on to the gel, the approximate molecular weight of your protein of interest can be found out. Following is a thumb rule guide for the separation of proteins based on their molecular weight.

Gel concentration (%)	Molecular weight of the separated proteins
15 %	**12-43 kDa**
10 %	**16-68 kDa**
7.5 %	**36-94 kDa**
5.0 %	**57-212 kDa**

Ingredients required for the preparing of gel

- Sodium dodecyl sulfate (SDS)
- Tris buffer (either glycine or tricine)
- Acrylamide and NN-bis-acrylamide

- TEMED: catalyst for polymerization (produces free radicals from APS)
- Ammonium persulfate (APS):source of free radicals for polymerization

Reagents for SDS-PAGE:

Sample Buffer (SDS reducing buffer)

(store at room temperature)

Deionized Water	3.8 mL
Tris-HCL(0.5M), pH 6.8	1.0 mL
Glycerol	0.8 mL
SDS (10% w/v)	1.6 mL
2-mercaptoethanol	0.4 mL
Bromophenol blue (1% w/v)	0.4 mL
	Total 8.0 mL

Dilute samples at least 1: 4 with sample buffer and heat at 95°C for 5 minutes

Electrode Running Buffer (5X), pH 8.3

Tris Base	9.0 g	(15 g/L)
Glycine	43.2 g	(72 g/L)
SDS	3.0 g	(5 g /L)
Water	(600 mL)	1000 mL

Store at 4 ^{0}C. If precipitation occurs, warm to room temperature before use. Dilute 60 mL 5X stock with 240 mL deionized water for one electrophoresis run.

Stacking gel: (4% acrylamide)

H_2O	3.075 ml
Tris-HCl (0.5 M), pH 6.8	1.250 ml
SDS (20% w/v)	0.025 ml
Acrylamide/bis-acrylamide (30% /0.8% w/v)	0.67 ml
Ammonium persulfate (APS) (10% w/v)	0.025 ml
TEMED	0.005 ml

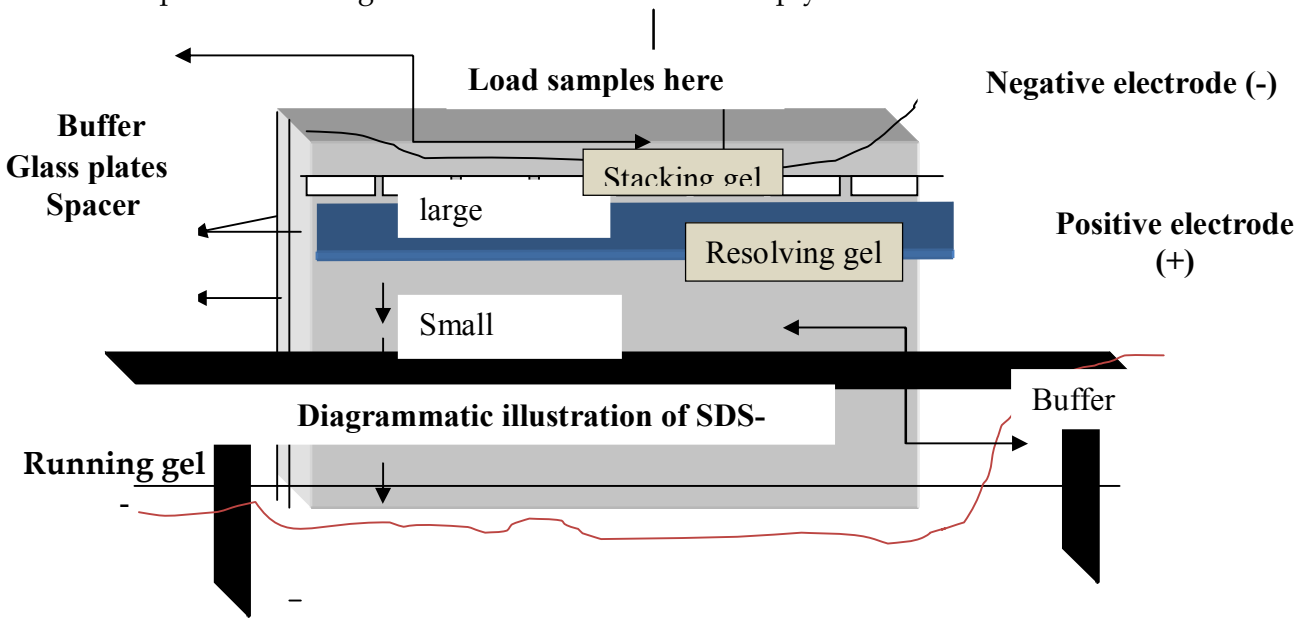
Resolving gel	**7 %**	**10 %**	**12 %**	**15 %**
H_2O	15.3 ml	12.3 ml	10.2 ml	7.2 ml
Tris-HCl (1.5 M), pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml
SDS (20% (w/v)	0.15 ml	0.15 ml	0.15 ml	0.15 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	6.9 ml	9.9 ml	12.0 ml	15.0 ml
10% (w/v) ammonium persulfate (APS)	0.15 ml	0.15 ml	0.15 ml	0.15 ml
TEMED	0.02 ml	0.02 ml	0.02 ml	0.02 ml

Casting of gel

- Pour running/resolving gel first. The percent of acrylamide may be adjusted for better resolution of proteins (5-15%)
  - Pour ethanol or distilled water on top of the gel for even polymerization.
  - Leave enough room to pour stacking gel, about one fourth of total gel.
  - After the running gel has polymerized, the gel is washed with distilled water to remove any debris on the gel to give a good interface between stacking and running gels. The stacking gel is poured. It contains Tris buffer at pH 6.8.
  - 5 % acrylamide is preferred for maximum porosity
  - Insert comb to form wells at an angle to prevent air bubbles.

Loading of samples

- Remove comb and wash wells out with running buffer.
- Best to use loading tips (Hamilton syringes also work) to load samples.
- Start at the bottom of well and work your way up the well.
- Glycerol in the loading buffer will keep sample in well.
- Optional- running buffer left in wells or wells empty.

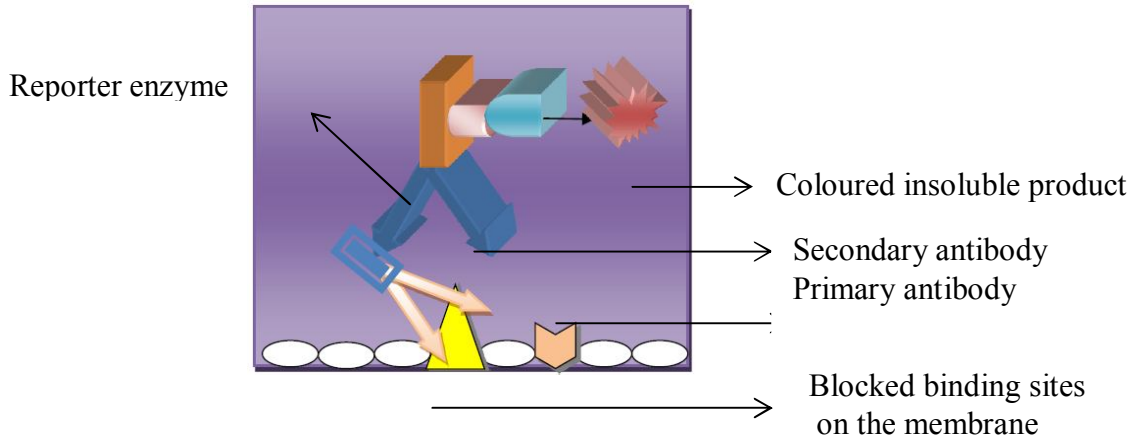


- After loading samples, added running buffer to upper reservoir and lower reservoir. Hint. Add upper reservoir first to detect leaks.
- Running buffer provides the ions to conduct the current through the gel.
- SDS make proteins negatively charged that attaches the proteins to the anode.
- Therefore in electrophoresis, the current must run from cathode (negatively charged, black) to the anode (positive charged, red).

Immunoblotting

In immunoblotting, proteins are transferred from electrophoresis gel to a membrane surface. The transferred proteins become immobilised on the surface of the membrane in a pattern that is an exact replica of the gel. Unoccupied protein binding sites on the membrane are blocked or saturated to prevent nonspecific binding of antibodies. This step is called "blocking"or "quenching".The blot is probed for the protein of interest with a specific primary antibody. The blot is probed again with a secondary antibody (conjugated with a

detectable enzyme) specific for primary antibody. The site of the protein of interest is thus tagged with an enzyme through the specificities of primary and secondary antibodies. The enzyme substrate that are converted into insoluble, detectable products are incubated with the blots. The products leave a colored trace at the site of the band or spot representing the protein of interest.



- Non fat dry milk, Triton X-100 and Tween 20 detergent are used to block the unoccupied sites in the membrane and are used as a carriers for the antibodies used to probe the membranes.

Blotting apparatus

Electro-transfer of proteins is done by directing an electric field across the thickness of the gel to drive proteins out of the gel onto the membrane. There are two types of apparatus for electro- transfer

1) Wet transfer
2) Semidry transfer devices

Membranes for immunoblotting

Membranes mostly used for immunoblotting are nitrocellulose and polyvinylidene fluoride (PVDF). Although, both bind proteins at 100 µg/cm^2, nitrocellulose is the best for initial stages of the experiments. PVDF can withstand the harsh chemicals of the sequenator, and can be used during proteins sequencing. It is more hydrophobic than nitrocellulose and will bind proteins more tightly and tolerate more SDS in the blotting system. Therefore, PVDF requires more stringent blocking conditions than nitrocellulose. Nylon is rarely used since it has two important drawbacks. In nylon membrane, complete blocking of unoccupied binding sites is difficult, and there is no well-developed protein-staining technique. Therefore, it suffers from high background (especially with detection by sensitive methods like chemiluminescence).

Buffers used for immunoblotting

Wet transfer

- Tris (25 mM)
- Glycine (192 mM)
- Methanol (20 % v/v)
- pH 8.3

Semidry transfer

- Tris (48 mM)
- Glycine (39 mM)
- Methanol (20%)
- pH 9.0

Antibodies used for immunodetection

Antibodies are proteins that bind specific epitopes of the proteins. Although, both monoclonal and polyclonal antibodies are used in western blotting, use of polyclonal antibodies are more frequent. Monoclonal antibodies are developed in mice, whereas polyclonal antibodies are in rabbit / goat / other animals. Monoclonal antibodies recognize single epitope, whereas polyclonal antibodies recognize multiple epitopes. So, it will be wise to select an antibody that recognizes and binds multiple epitopes in the protein of interest. Sometimes, when proteins get denatured, its epitope is changed and the antibody is not able to recognize it.

Protocol for immunoblotting

(A) Equipments and reagents

1. Electro-transfer apparatus: Wet transfer tank or semidry apparatus.
2. Filter papers, sponges and power supply.
3. Blotting membranes; nitrocellulose or PVDF.
4. Transfer buffer
5. TBS and TTBS
6. Primary antibody
7. Secondary antibody with conjugated enzyme
8. Substrate for the conjugated enzyme
9. Total protein stain

Detection of protein

Colorimetric detection

Colorimetric detection depends on incubation of the blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry or spectrophotometry.

Chemiluminescent detection

Chemiluminescent detection depends on incubation of the blot with a substrate that emits light when exposed to the reporter on the secondary antibody. The emitted light is either captured by photographic film, or by a CCD camera. The intensity of the image may be analyzed by densitometry. Various software may also be used for image and data analysis.

Radioactive detection

Radioactive labels require X-ray film, and eliminate the use of enzyme substrates. It is carried out by placing the film over the membrane. The dark regions reveals the protein bands of interest. In spite of its many advantages like detection of protein in very small quantity, the use of this technique is declining mainly because of health hazards.

Fluorescent detection

Fluorescent labeled probe is excited by light, and the light emission is detected by photosensor. CCD camera with emission filters captures a digital image of the blot, and helps in analyzing various data *viz.*, molecular weight and quantitation. Although,

fluorescence is considered as one of the best methods for quantification, but it is less sensitive than chemiluminescence

Western blotting: Conventional method and Chip nano method

Conventional method	Chip nano method
Blots are blocked for 1 hour in 5 % skim milk powder in TBS containing tween-20.	The blots are blocked for 1 hour in 5% skim milk powder in TBS containing tween-20.
The primary antibodies are added to the blots in TBS-Tween, with 2 % milk powder for 2 hours with gentle agitation.	The membrane is mounted in the CHIP and the same primary antibodies and concentrations that are used in the conventional Western are printed in an array, over the MHC. Each drop contained 100 pl, printed 0.15 mm apart, creating a line, 8 mm in length.
Excess primary antibody is removed by washing and then the HRP-linked secondary antibody is added for a further hour, followed by more extensive washing.	The CHIP is then used to wash the spots to remove non-specific binding and then a secondary antibody is printed in the same way as the primary antibody.
The interaction is detected using enhanced chemiluminescence reagents and film.	The interaction is detected using enhanced chemiluminescence reagents and film.
Less cost effective, and more time required	Most cost effective and less time required
Multiple individual blots are used for each anti-body	Multiple anti-bodies are used in one blot. It is an easier and quicker method to interpret and visualize

Critical points in western blotting

- Western blotting can detect immunogenic responses from infectious agents like bacteria and viruses
- Western Blotting utilizes not only antigens, but also antisera as a diagnostic tool. Antisera is widely used in the test for HIV presence.
- Compared to ELISA, Western blotting has higher specificity; the higher specificity, the more the method is independent of the specificity of antibodies.
- Among three common enzyme substrates, Fluorescent and Chemiluminescent create light detectable through X-ray or scanners. This ability enables high levels of sensitivity and quicker processing time
- A non-intended protein has a chance of reacting with the secondary anti-body, resulting in the labeling of an incorrect protein.
- Incidental phosphorylation or oxidation of proteins may result in multiple bands appearing after sample is processed.
- The appearance of bubbles may occur when transferring the sample from the gel/membrane sandwich and may also occur when incubating the sample with antibodies, resulting in a skewed band reading.
- If the transfer time is not sufficient when transferring proteins to the membrane, the larger proteins of higher molecular weight will not transfer properly, resulting in an incorrect or no band reading at all.
- Too much methanol in the transfer buffer decreases the transfer efficiency of proteins from the gel to the membrane; however methanol aids in protein binding to several different membranes, so a correct balance is required.
- Western Blotting is a very delicate process requiring the correct amounts of each component in order for successful identification of the presence of proteins. An imbalance in any step of the procedure may ruin the entire process.

WHITE TAIL DISEASE IN GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* (DE MAN): AN UPDATE

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The giant freshwater prawn *Macrobrachium rosenbergii* is an economically important and a moderately disease resistant farmed crustacean species cultured in Caribbean countries and South-East Asia including India. In India, the peak production of freshwater prawn recorded 12-fold increase from 35,573 tonnes in 1995 to 42,780 tonnes in 2005-06 because of its potential of fast growth, large size, high nutritional and export market value and disease tolerance. However, pathogenic diseases have become a threat to the prawn farming due to which the production rate has been decline to 6,570 and 13,525 in 2009 and 2012, respectively, in the near past.

Despite of the conviction that the giant freshwater prawn is free from the diseases, several epizootics have plagued the world's shrimp culture industries. Intensification of culture systems, poor hatchery management and polluted aqua system have opened route for the easy entry of the opportunistic pathogens. The most important diseases of cultured shrimp have had viral or bacterial etiologies, but the continuing outbreak of white tail disease (WTD) is a serious threat to the hatcheries and farms. The causative agent of WTD has been identified as *Macrobrachium rosenbergii* nodavirus (*Mr*NV) included in Nodaviridae family and it is the second most dreadful virus since the discovery of parvo-like virus some years ago. From the phylogeny and sequence analysis, it has been proposed that the virus belongs to the gammanodavirus, whereas betanodaviruses mainly affects the fish and alphanodavirus affecting insects. This virus-borne disease was first observed in Island of Guadeloupe in 1995 followed by West Indies, Taiwan, China, Thailand and most recently in Australia.

Clinical signs

The WTD affected prawns show lethargy, milky whitish coloration of tail region and abdominal muscle, degeneration of telson, uropods and 100% mortality within few days causing a great financial loss to the farmers. The discoloration starts from the tail and affects all part of the body. Mainly the post-larvae and early larvae are more prone to this disease, and it may be due to their poor immunity where as the adults are found to be the carrier of this virus. The histopathology of affected muscles revealed the presence of small basophilic inclusions in the cytoplasm and necrosis on muscles.

Virus and pathogenicity

Investigations by transmission electron microscopy of diseased post-larvae homogenates or by direct observation by negative staining showed the presence of two types of viral particles: small one *Mr*NV is an icosahedral, non-enveloped particle, 26–27 nm in diameter and has a genome with two fragments of single-stranded RNAs (ssRNA). RNA-1 encodes for an important viral enzyme RNA-dependent RNA polymerase (RdRp) of 3.2 kb and RNA-2 encodes capsid protein of 1.2 kb, respectively. RNA3, a subgenomic RNA of RNA1 was transcribed to B2 protein during virus replication that produces a non-structural protein being able to inhibit the cellular RNA interference. Till date, the role of B2 is confined to antiviral silencing using RNA*i* mechanism. The capsid protein of *Mr*NV consists of a single polypeptide of 43 kDa as confirmed on SDS-PAGE. On the other hand, XSV is also an icosahedral virus of 15 nm in diameter, genome consisting of a linear single-stranded

positive-sense RNA coding for a capsid protein. Because of the absence of RdRp gene, XSV is suggested to be a satellite virus that helps *Mr*NV in pathogenicity and recently it has been demonstrated that only the higher infectious dose of *Mr*NV is able to cause the disease, not XSV.

Diagnostic tools

For routine monitoring of health status of the animal and to restrain the disease outbreak, sensitive and easy diagnostic methods are highly essential for this important aquaculture species. Unlike earlier days, today a lot of diagnostic tools are available to confirm the presence of virus but the identification of natural carriers, mode and environmental condition of virus infection is still unknown. The sensitive viral genome as well as antigen based detection methods such as reverse-transcriptase polymerase chain reaction (RT-PCR), loop-mediated isothermal amplification (LAMP), double antibody sandwich enzyme-linked immunosorbent assay (DS-ELISA), triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), dot blot and *in situ* hybridization are widely used for screening of this disease.

Histopathology

The histopathology and electron microscopy studies revealed the myofibril degeneration of muscles along with necrobiotic myopathy. In all the diseased prawns some additional dense cytoplasmic inclusions were found. Eosinophilia or hyalinization, granulation and haemocyte infiltration were some of specific feature being reported in affected prawn confirming the presence of *Mr*NV. In the affected prawns some changes are observed in comparison to healthy ones like necrotic myopathy with single, rows, aggregations and sheets of hyperchromatic nuclei. The specific viral inclusions can be stained with special stains like pyronin methyl green which can distinguish viral inclusions from haemocyte nuclei by making the former green stained. Hence pyronin methyl green is usually recommended as diagnostic stain for *Mr*NV. The electron microscopic examination helped in identification of *M*rNV by observing the virus particles in cytoplasm in infected muscle cells. The histopathological sign of WTD is closely related to idiopathic muscle necrosis disease (IMN) and confused with that disease. But in case IMN the opaque muscle was found in abdominal segments 3-6 and it affects the PL of 19-21 days of age where as unlike IMN in WTD, whitish muscles were observed in abdominal segment 2-6 and the prawn were of age 28 days or more.

Genome-based diagnostic tools

Reverse-transcription polymerase chain reaction (RT-PCR)

The genome based diagnostic tools such as RT-PCR, real time PCR and *in situ* hybridization have been proved to be the most sensitive and rapid methods in comparison to the antigen-based diagnostics. The genome of *Mr*NV and XSV are linear RNAs that are more prone to degradation, hence they are reverse-transcribed to DNA using specific enzymes. Further, specific primer pairs were designed based on the gene sequences (capsid or RdRp for *Mr*NV) and XSV and used in PCR method. However, second step nested PCR can be carried out taking the product of first step, to detect the minimum viral copy number. Despite the high risk of mutation in the RNA-viruses, its genome seems to phylogenetically conserve till date and thus RT-PCR method has succeeded in detecting the virus in field level as well as in laboratory condition, with high sensitivity and accuracy with small amount of virus materials. Both the viruses can be detected with maximum detection limit up to 400 virus particles or 1fg of RNA. Moreover, it is a time-consuming process and can be completed within 7-9 hrs of sample collection. Other more sensitive processes like multiplex

PCR, nested PCR and single-tube nested PCR have also been developed for rapid detection of this disease.

Real-time RT-PCR

Real time PCR is an efficient diagnostic tool, that not only detect the presence of virus but also able to quantify the infected material. The method is similar to RT-PCR, that uses the primers and nucleic acid, and performed on technologically improved and expensive equipment. However, to understand the mechanism of virus distribution in different tissues and quantification of viral particle, the methodology is highly applicable.

LOOP-mediated Isothermal amplification (LAMP)

LAMP is also a convenient and rapid diagnostic method for *Mr*NV detection. It is a specific nucleic acid detection method used for recognition of different segments of DNA using specially designed primers. The advantage of LAMP over conventional PCR is, the reaction being carried out in a single temperature (under isothermal conditions) using a simple and inexpensive water bath. The results can be visually observed using magnesium pyrophosphate giving a whitish precipitation; hence it does not require an agarose gel and hazardous ethidium bromide. The amplification efficiency of the LAMP method is extremely high and minimizes the time loss in thermal changes. All these advantages make LAMP a suitable method for diagnosis in field level.

Dot-blot hybridization

Dot-blot analysis is a suitable and specific pathogen detection method used for *Mr*NV and XSV using DIG-labelled cloned parts of the virus as probes. The RNA of virus was hybridized taking different dilution of viral RNA and found that the successful detection limit for MrNV and XSV was 140 µg and 2.5 pg, respectively.

***In situ* hybridization**

This method is generally used to localize the virus using DIG-labelled *Mr*NV gene probes, which needs more technical skill. From the study, it is confirmed that *Mr*NV is confined to the striated muscle and connective tissue of the abdomen, cephalothorax and appendages and cytoplasm of the infected cells.

Antigen based diagnostic tools

Sandwich ELISA

In 2003, Romestand and Bonami developed a sandwich enzyme-linked immunosorbent assay, which was found to be a sensitive and inexpensive method. The rabbit polyclonal antibody was produced against the pure viral extract in Balb/C mice and used for the detection of viral antigens using ELISA method. An antibody coated plate was used to bind to the capsid protein antigen present in the virus, thus forming an antigen-antibody complex. This complex was again trapped by using a secondary biotinylated antibody. Although this is a sensitive and rapid method, but the unavailability of surplus amount of antibodies was found to be a major drawback for it.

Other ELISA methods like double antibody sandwich enzyme linked immunosorbent assay (DS-ELISA) and triple antibody s-ELISA (TAS-ELISA) were also developed for rapid diagnosis of the disease.

Transmission

This virus can transmit vertically as well as horizontally, but vertical way is the main route of transmission. The *Artemia nauplii* are considered as a most possible vector for vertical transmission. Insects like *Belostoma* sp., *Aesohna* sp., *Cybister* sp. and *Notonecta* sp. are screened as natural insect carriers and used for infectivity assays in insect cell line and reported to be susceptible to infection with *Mr*NV and XSV.

Conclusion

Among all the reasons attributed for declining production and productivity of giant freshwater prawn, WTD is seemed to be the most widely discussed. Recently it has also been observed in hatchery reared post-larvae of marine shrimp *Panaeus monodon* and *Panaeus indicus*. Hence, only through adoption of better management practices, timely disease diagnosis and quality seed production, impact of the disease can be minimized. Strict quarantine measure should be followed. Use of immunostimulants, drugs, recombinants proteins and vaccines could be of help to prevent the prawns from mortality.

FISH DISEASES IN OPEN WATER ECOSYSTEM: THEIR DIAGNOSIS AND CONTROL MEASURES

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Introduction

Inland open water fishery sector viz. rivers, reservoirs, lakes and wetlands, in the country has ben playing a major role in fulfilling protein requirement of the population besides supporting socioeconomic requirement of lakhs of people. Open water fisheries in India holds considerable potential for further growth, through species diversification and further horizontal and vertical expansion of culture sector. Inland fish production in India estimated to be around 2.83 million tones during 1999-2000 (Anon, 2001) is chiefly contributed by the capture fisheries from open water ecosystems. Present per capita availability of fish is still only about 9.5 kg per anum in the country as against world average of 12.1 kg. To fulfill this minimum requirement for the country's projected population, assuming 56% of the population as fish eaters, the present annual fish requirement has been computed to be 8 million tonnes and double of that by 2010 (Bhaumik and Paria, 2003; Sinha 2001). The above said scenario warrants a concerted effort to increase fish production from inland waters, where the resources are vast and diverse with estimated potential of 4.5 million tones. The last two decades have witnessed immense growth in the fish production from freshwater pond culture, because of emphasis given to popularization of scientific technology of seed production and composite fish culture (Bhaumik and Paria, 2003). Small reservoirs and floodplain wetlands in the country are thus increasingly been used for enhancing fish production during the last few years. Floodplain wetlands constitute a group of lentic water bodies associated with the floodplains of Indian rivers, known by different local names such as *beels, chaurs, mans, boars, hoars* and *pats*. They are usually cut-off river meanders (oxbow lakes), tectonic depressions, back swamps or sloughs, covering a total surface area of more than 200,000 ha spread over the eastern and north-eastern regions of the country. Floodplain wetlands in India support subsistence and commercial capture fisheries, practiced by the local fisher communities. However, to achieve this task of food productivity enhancement, fisheries in India must be regulated to grow in a sustainable manner. The ultimate goal is to achieve optimum fish production per culture unit in most aquaculture operations, whether it is a pond, raceway, reservoir or tank for culture of fish. New methods of fish culture viz. Cage culture, pen culture are being widely used to increase fish yield. All these practices influence the environmental and biological factors leading to disease occurrence in these culture systems (Das, 2003).

Health management in open water culture systems:

In particular, one of the most significant constraints for fisheries development is realized to be the threat of diseases. Among the infectious diseases, viral diseases, often known to cause rapid onset, high mortality leading to heavy damage to production. Outbreak of different bacterial, viral, fungal and parasitic diseases have been reported in fish and shellfish (Table-1). Maximum production is dependant upon many factors in the final analysis including water quality, environmental conditions and the incidence of infectious diseases. Occurrence of disease in a fish population depends on interactions of

variables defined for the host, the pathogen and the environment. Absence of pathogens would reduce the potential for adverse environment to influence disease outbreak. The dose or the number of pathogens, their route of entry to host and the duration of the exposure directly influence the severity of subsequent infection. Therefore, in order to confront the threat and transmission of infectious pathogens, to help for sustainable development, it is imperative to adopt holistic approaches utilizing biotechnological tools for health management in aquafarming. For sustainable aquafarming, the health management programme should focus on two main approaches like (i) Ecosystem management : environmental management with optimum water, soil quality maintenance, with proper stocking density, devoid of stress to animals and (ii) Disease diagnostics : development of rapid and sensitive immunological and molecular techniques for detection and characterization different fish and shellfish pathogens

There are several parameters used to assess the quality of water. The three main objectives of assessing water quality of culture system are, (i) To know the productivity status of the system (ii) to know the presence of microorganisms pathogenic to fish and prawn, in the culture system. (iii) to know degree of pollution/contamination of water with wastes and chances of transmission of such pathogens of public health significance to humans through fish/ prawn.

Principles health management in openwater culture system:

Even though the fish in their culture and natural environment are continually bathed in water suspension containing a variety of microorganisms, both pathogenic and non-pathogenic, they do not all become diseased. Several parameters associated with the fish are directly related to the occurrence of disease upon interaction with appropriate pathogen and environmental factors or conditions.

a) Host: It is understood that disease is a result of the complex interaction between the host, the pathogen and the environment (Snieszko, 1974). Host factors include those factors that are constantly present (constitutive) such as the fish species, genotypes, age, size, developmental stages, nutritional and reproductive statuses and innate defenses related to immune competence. Additional factors that affect host susceptibility to disease include adoptive factors which result from previous interactions with the pathogen or environment (Anderson, 1996). The ability of certain fishes to resist pathogens was demonstrated to have high heritability, although fish strains with resistance to one pathogen are not necessarily resistant to another (Coutant, 1998). Another factor considered important in disease outbreaks in fish population in openwaters is that disease outbreaks have been recognized as a major controlling factor in the abundance of both cultured and wild fish. Disease can directly influence performance, susceptibility to predation, success of reproduction and other critical factors required for survival and propagation of a species. These effects can be cumulative and have catastrophic consequences for wild fish population. Thus the fish with higher vigor, resistance and better ability to accommodate in environmental floctuations, survive in nature.

b) Pathogen : Aquatic environment severs as a platform both for maintenance, survival and transmission of such pathogens. Mere isolation of any pathogen not necessarily indicate health status of fish or prawn, however it can indicate the concentration or dose of such pathogens in aquatic environment. Isolation of pathogen from diseased fish, their isolation, re-induction of disease in fish and re-isolation of that pathogen, are the steps followed to conclude a pathogen responsible for disease.

c) The environment: The quality of the water in which fish are contained is very important to their livelihood. Adverse environmental parameters can have direct or indirect effects on fish. Poor water quality has been recognized as one of the main factors responsible for occurrence of disease problems in aquatic animals. Poor water quality has been recognized as one of the main factors responsible for occurrence of disease problems in aquatic animals. Incidence and severity of infectious diseases are very often dependant on the quality of the environment in which the animal lives. Therefore determination of the environmental factors that are important for occurrence of disease and the measurement and assessment of these environmental factors is critical to our understanding of disease (LaPatra, 1998). In other wards the quality of environment can indicate the health status of animals. This emphasizes the important role that environmental factors play in the pathogenesis and manifestation of disease outbreaks. Thus, the first and foremost important step in controlling infectious diseases is by maintaining the best quality environment possible in the culture unit. The details of various environmental factors and their interaction with pathogen and fish resulting in disease induction have been already described (Mishra, 2001).

Common fish diseases in open water culture systems:

Disease conditions in fish can be of two major types, i) Non-infectious diseases and ii) infectious diseases.

1.Non-infectious diseases : These include the direct effect of all environmental factors on the health of the fish, which cause stress to animals and may also lead to induction of infectious diseases.

The environmental parameters which need to be regularly monitored are

i) Dissolved Oxygen (DO): Main factors that affect DO are Temperature, salinity and atmospheric pressure. Water holds less oxygen at low atmospheric pressure. Other factors which affect DO in water include phytoplankton, bloom, organic load, respiration of aquatic organisms
ii) Ammonia : The total ammonia concentration in water consists of two forms (i) NH_3 - unionized ammonia and (ii) NH4 - ionized ammonium ion.NH_3 is more toxic to fish. Ammonia in water can originate from a) decomposition of organic matter b) industrial and domestic pollution c) excretion by aquatic organisms d) denitrification e) death & decomposition of phytoplankton.
iii) Nitrite : It is highly toxic to fish.It reacts with blood hemoglobin to formmetahemoglobin, which gives brick-red color to fish gills. The problem can be corrected by proper stocking, feeding & fertilization practices and addition of NaCl @ 250mg/l and biofiltration
iv) Suspended solids: The high tubidity due to suspended solids reduce penitraion of light in to culture ponds, reducing productivity.
v) pH: Optimum range is 6.0 – 9.0

Direct effect of environmental factors :

i) Temperature : A sudden rise or fall of temperature leads to stress. High temperature lead to fall in DO and cause respiratory distress and death.
ii) Sudden change in pH : due to acid rain, industrial pollution, cause death
iii) Gas bubble disease : When water becomes supersaturated with air (atmospheric nitrogen),small gas bubbles form within the tissues, under skin, eyes, fins, cause tissue damage, gill damage and death.
iv) Suspended solids : Cause irritation to gill and respiratory distress.

v) Endogenous toxins : These nitrogenous waste products from fish like Ammonia & nitrite cause chronic gill problems and death. High levels of carbon dioxide cause deposition of calcium salts in kidney and damage the tissues.
vi) Endogenous toxins : Effluents from industrial, agricultural and domestic wastes, heavy metals, petrochemicals, chlorine/ hydrogen sulphide gases, pesticides, algicides etc. Cause tissue damage and acute mortality.

2. Infectious bacterial diseases :

The majority of bacteria causing disease in fish are normally present in fish (surface or gut) or its culture environment and cause disease when there is stress to fish. Most Gram- negative bacteria belonging to *Aeromonas, Vibrio, Pseudomonas, Yersinia, Edwardsiella, Pasteurella, Cytophaga* cause disease in fish. There may invariably be haemorrhagic septicaemia with or without skin ulcers. Prevalence of different bacterial, fungal and paretic disease in cultured fishes in lakes and reservoirs and wetland have been elaborated by Das (2003). Mass mortality of common carp *Cyprinus carpio* and tilapia *Oreochromis mossambicus* in Kalyani wetland was traced to bacterial infection caused by Aeromonas sp. (Reddy etal., 1994). Occurrence of EUS in different open water culture systems have been reported (Das, 2003; Das and Das, 1995). Common diseases reported in cultured fishes have been given as under:

a) Bacterial skin disease : It is a systematic bacterial infection. There are red areas on body, depression with swollen eyes and abdomen. A wide variety of bacteria mainly belonging to Gram-negative rods are involved. Many pathogens are present only at skin lesions, especially Flexibacteria, Aeromonads, Vibrios etc. There may be necrotic lesions on fins (fin rot). The disease may become systematic, more severe form causing mortality.

b) Motile Aeromonas septicaemia (MAS): It is the most common bacterial disease of freshwater fish. *Aermonas* species which are ubiquitous in freshwater environment, are responsible for much of disease problems in fish, cause Aeromoniasis, haemorrhagic septicaemia. *Aeromonas hydrophila, A. sobria, A caviae, A veronii, A.veronii bv. sobria* are involved. There are red areas on body, skin ulcers, swollen body, abdomen and eyes.

c) Edwardsiellosis : Also called *Edwardsiella tarda* infection, Edwardsiella septcaemia. .There are deep ulcers and red areas on body of fish. Common carp, grass carp, gold fish, tilapia, are affected.

d) Vibriosis : It is mainly a disease of marine fishes. *Vibrio anguillaru, V. ordalii, V. damsela V. alginolyticus, V. vulnificus, V.fischeri, V. harveyi* etc. are involved.

e) Epizootic ulcerative syndrome : It is an important bacterial-fungal disease responsible high mortality in freshwater fishes. Fish species commonly affected are Catla catla, L. rohita, C.mrigala, C. carpio, Channa sp., Puntus sp., and G. chapra (Das and Das, 1993; 1995).The fishes become lethargic and float on the surface of the water, sometimes with the head projected out of water. Initially, the disease appears as red coloured lesions, haemorrhagic in nature. These red lesions spread and enlarge gradually becoming deeper and assuming the form of ulcers. Patches of fish tissues fall off, causing high mortality. Although the exact role of pathogens not known, it is suggested that the prime causative agent to be a fungus called *Aphanomyces* species along with seconday bacterial infection. A number of pathogenic bacteria have been isolated from affected fishes of which *A.hydrophila* has been very common. A number of viruses have also been isolated from affected fishes.

Table 1 : Common disease in fish, their clinical signs and possible causes

Clinical signs	Possible causes
Sudden death of fish without much preceeding symptoms	Low oxygen level (DO) Exogenous toxins Poisoning Per-acute bacterial disease
Gasping, crowding at inlets	Low DO Gill parasites/Gill disease Bacterial gill disease, gill mycosis Anaemia
Jumping, flashing, rubbing	Ectoparasites, toxins, irritants in water
Excess production of mucus from skin/ gills	Ectoparasites, toxins, irritants in water
White cotton-wool growth on skin	Funga l infection (Saprolegenia) Cytophaga infection
White spots on skin	Parasitic disease (Icthhyopthirius)
Swelling on skin	Parasitic cysts, tumours
Haemorrhages on scales, fins	Bacterial or viral infections
Skin lesions/ ulcers	Ectoparasites with secondary bacterial infections Systemic bacterial infection Nutritional imbalances Physical damages/Cannibalism
Fin rot	Cytophaga, Saprolegnia infection, Physical damage, Cannibalism, Nutritional imbalance

Fungal diseases of fish:

Only a small number of fungal species are pathogenic to fish. Mostly these are present in water and when the water quality is poor or fish are under stress or infected, they attack the fish causing skin lesions. The important fungal pathogen in freshwater fishes is *Saprolegnia*. It is usually a secondary invader of damaged or stressed fish and also infect fish eggs. The infection appears as raised white "cotton wool" appearance, may look coloured by trapped organic matter or sediment.

Parasitic Diseases :

Ectoparasites

i) Protozoa : Myxobolus spp., *Oodinium spp., Chilofonrlla spp., Trichodina* spp. and *Ichthyophthirius multifiliis. Thelohanellus catlae* and *Myxobolus bengalensis* cause "Gill spot disease". In this case gills are covered with whitish cysts, thereby reducing respiration, excessive secretion of mucus and gills become pale. The *ichthyopthirius,* a ciliate up to 1mm in dia, causes " white spot" or "Ich" in most freshwater fishes. *Trichodina* browse over gills and skin, damaging the host tissue and consuming the resulting dead tissues. Repeated chemical treatments are necessary to eliminate the parasites.

ii) Helminths diseases- Monogenian trematodes a)*Gyrodactylus* spp., commonly knows as skin flukes, attached to the body surface, fins and gills by a hook, 1mm in length, b) *Dactylogyrus* spp., known as gill flukes. Repeated chemical treatments are necessary to eliminate parasites produced from newly hatched eggs. Good husbandry, cleaning and sterilizing the facility, help to eliminate the eggs.

iii) Crustaceans : Most important are a) *Lernae* spp., adult up to 22mm long, attach to the surface of the skin. b) *Argulus* spp., the freshwater louse, large, flat, up to 1cm long, mostly found on head of the fish or behind the fins c) Ergasilus, which atck the gills, operculum and fins. Repeated treatments with organophosphorus is effective to destroy juvenile stages. Draining and drying will kill all stages, including eggs. Periodic removal of vegetation with attached eggs will help to reduce the parasite numbers.

Diseases of shrimp:

a) **Viral Diseases** : Shrimp, mostly *Penaeus monodon*, are affected with a number of viral and bacterial diseases, mostly in hatchery and intensive, semi-intensive culture system. Diseases are seen in juveniles in grow-out ponds but are often associated with poor water quality , poor pond bottom conditions and poor pond fertility. Although there are more than 17 different viral diseases in shrimp reported (Lightner, 1996; Lightner and Redman 1998), three have been reported in India, i.e, White spot syndrome virus (WSSV), Monodon Bacululovirus (MBV), Yellow head virus (YHV) and in giant freshwater prawn, *Macrobrachium rosenbergii*, prawn noda virus (MvNV) have been isolated and identified.

b) Bacterial diseases:
i) Bacterial necrosis : All ages of larvae are susceptible to this disease. There is progressive erosion of appendages with blackening (melinization). Larvae become weak and die, even up to 100%. Poor water quality with secondary bacterial infection with *Vibrio* spp. and *Aeromonas* spp. are responsible for this disease.

ii) Shell disease : This is a common bacterial disease of shrimp, usually with no mortality. There are black-brown spots present on gills, carapace (Cuticle or shell), abdomen and appendages. After each molt, the old disease is lost. If there is secondary infection, the underlying tissues get affected and lead to bacterial septicemia and death.
iii) Luminous vibriosis : Mostly shrimp larvae and post-larvae (PLs) are affected, resulting in mortality up to 100%. At night, affected larvae emit greenish luminescence, which can be seen in naked eyes. The luminous bacteria *Vibrio haeveyi* is responsible for this disease. Bacteria multiply in gut, spread to haemoceole, body cavity and cause death.

iv) Bacterial septicaemia : This occurs due to poor water quality, over crowding, wounding in larvae, PLs and juveniles. Ubiquitous bacteria which are in the culture environment and present in host as normal microflora are responsible for this disease. These opportunistic bacteria are *V. alginolyticus*, *V. parahaemolyticus*, other *Vibro* spp., *Aeromonas* spp, *Pseudomonas* spp. etc. Larvae may look red , stop eating, their mid-gut gland look opaque and finally resulting in death of larvae.

v) Filamentous bacterial Disease : It is caused due to *Leucothrix mucor*, a filamentous bacteria ubiquitous in the marine environment. The bacteria multiply in large numbers on larvae, PLs, there by affecting respiration, feeding, swimming, and moulting, and finally resulting in mortality. Disease outbreaks often occur in ponds with high organic load and low DO.

c) Fungal Disease: Larval mycosis is an important systemic fungal disease of larvae and post-larvae, causing serious mortalities in hatcheries throughout the world. A sudden onset

of mortalities in seen and within 24 hours the entire stock of larvae become dead. It is considered that *Phycomycete* fungi in the genera *Lagenidium, Sirolpidium* and *Haliphthoros* are responsible for this disease.

d) Protozoan disease: Protozoan fouling is another important disease of larvae, PLs and juveniles, in the form of epicommensal surface fouling, caused by peritrichus ciliates (*Zoothamnium*, *Epistylis* and *Verticella*). These protozoans multiply in large numbers on body surfaces causing obstruction of gill filaments, there by affecting respiration, feeding, swimming, and moulting, and finally resulting in mortality.

Methods used in disease diagnosis:
The common principles of fish disease diagnosis include-

1. Previous history of disease : A meaningful diagnosis is most likely where the fishes are under close supervision. A good history along with personal observation is important. Two important points need attention are i) previous history to know whether the previous disease problems have been seasonal or related to a particular group. It is also possible that certain fish stocks are more affected or the problems are with recent fish stocks ii) Nature of the problem, i.e. a rapid increase in mortality might suggest an infectious agent, while intermittent or low level mortality might be due to a variety of causes. Mass fish kills might be attributed to an oxygen deficit or a toxic agent.

2. Observation : In case of disease problems it is important to have on-site to examination of fish stock both in culture environment and at close quarters when captured. As suggested by Collins (1993) some of the points to watch are :

a) Swimming and shoaling : Sick fish are usually lethargic, lying apart from the shoal
b) Abnormal movement : Ataxia, spiral swimming and spasmodic bursts
c) Side swimming : May be due to cold-shock or physical trauma or problem in swim-bladder
d) Jumping : Excessive jumping may be due to ectoparasite irritation or environmental disturbance or stress or self-inflicted trauma.
e) Flashing : Fish rubbing on their surroundings due to ectoparasites or self-inflicted trauma.
f) Respiratory activity : Fish may become hyperventilating either lying near the surface or crowding near inflows due to gill disease, anaemia or low oxygen levels.
g) Colour : Sick fish usually appear either darker or paler than normal and may collect in slack-water areas.
h) Body surface abnormalities : Excess mucus, superficial lesions, accumulation of organic debris and algae on lesions.
i) Feed response : Fall in feed intake clue to health problem in fish.
j) Gross external lesions : Observe for abnormal, raised surfaces, proliferative lesions, localised swellings or ulcerative lesions on skin and erosions in fin and gills. Observe presence of cotton like growth of fungus, excessive mucus secretions due to bacterial colonization, haemorrhages, ectoparasites on skin and gills.

Live fish, tissue and blood samples may be sent to pathology laboratory for further pathological and microbiological studies and diagnosis of etiological agents involved.

3). Microbial isolation and identification

The first step in diagnosis is isolation and identification of causative agent involved. The basic steps for microbiological analysis are i) isolation and identification of microorganisms from samples/ tissues using a variety of cultural, selective and differential media, ii) study of their morphology structural characteristics, iii) metabolic properties to various substrates using a series of biochemical tests and iv) genetic characteristics.

4). Molecular diagnostic methods :

a) Polymerase chain reaction (PCR) & related techniques : PCR is a rapid and inexpensive way of preparing specific DNA segment by cyclic amplification, which can be used in characterizing the pathogen and various disease condition. Numerous modifications of the standard PCR procedure have been developed since its inception, which have effectively expanded the diagnostic capabilities of PCR. RT-PCR has been developed to amplify RNA targets. In the process, RNA targets are first converted to complementary DNA (cDNA), by RT and then amplified by PCR. This technique can be useful for detection of fish & shellfish RNA-viruses (Tang *et al*., 1997). Another modification is "Nested PCR", which is basically designed mainly to increase the sensitivity, uses two sets of primers. PCR and nested PCR have been applied by different researchers for detection of WSSV, MBV in shrimp and found to be more useful in screening of samples under field condition. A number of pathogens can simultaneously be detected using the process of PCR amplification called "Multiplex PCR". For diagnostics use, multiplex PCR can be set up to detect internal controls or to detect multiple pathogens from a single specimen (Tang et al., 1997). Another important development in PCR technology is "Real-time PCR". Real-time PCR can be accomplished with the fluorogenic 5´-nuclease assay called TaqMan and a spectrofluorometric thermal cycler. Such techniques are not only highly sensitive, requiring only few minutes, compared to days as required by other methods, but can also be applied in cases where other methods fail to detect the pathogen. Hence these techniques have wide scope of application in disease diagnosis and quarantine programme.

b) Nuclecic acid Probes: Nucleic acid probes are capable of identifying microorganisms at species and strain level. DNA probes facilitate the identification of pathogens that do not grow well or not easily cultured or cannot be cultured at all under laboratory conditions (Tang *et al*., 1997). The application of non-radioactive labelling methods has made gene probe technology readily available for diagnostic laboratories DIG-labelled probes have been applied to pathogen detection in several ways, in "Dot blot" hybridization" or "*in situ* hybridisation is used to detect viral and other genomic sequences with specific complementary DNA probes. *In situ* hybridisation methods have been developed for the shrimp pathogens- IHHNV, HPV, MBV, BP, WSSV group, YHV, TSV, HNP, *Agasoma* spp. (Lightner & Redman, 1998).

Treatment and control measures for fish disease:

Treatment of fish bacterial diseases : In open-waters, treatment is very difficult due to large water body, extensive way of culture practice, difficulty in assessing the condition of fish and large dose of chemicals/ antibacterials required to treat the disease and economy. However, treatment can be tried only in small culture areas, pond, lakes below 40 ha. Treatment of bacterial diseases includes:

1. Prophylactic treatment *:* During post-monsoon period, the disease prone water areas can be treated with lime, CaO at 50 kg/ha followed by application of bleaching powder at 0.5 ppm after one week.

2. At the initial stage of lesion formation, lime (CaO) is applied at 100 kg/ha followed by application of bleaching powder at 1 ppm after one week.

3. Gammaxene treatment can be done at 1 ppm.

4. Benzalkonium chloride : It is used as a disinfectant and treatment of external bacterial infections. Dose : Bath treatment 10mg/l for 5-10 minutes, For prolonged immersions, 0.5-1.0 mg/l

5. Antibiotics given via water : Oxytetracycline 20-120 mg/l, Oxolinic acid 10mg/l, Chloramphenicol 20-50 mg/l, Co-trimazine (80mg trimethoprim and 400mg sulphadiazine per ml) 1ml/100l, Neomycin 50mg/kg, Kanamycin 50-100mg/l

6. Antibiotics given via food : Oxytetracycline at 75mg/kg, Amoxycilin 80mg/kg, Furazolidone at 75mg/kg, Co-trimazine at 30mg/kg, Oxolinic acid 10mg/kg.

Treatment for fungal diseases:

The disease can be controlled by adopting good husbandry, removal of dead fish and use of topical fungicide such as malachite green. For treatment of fish eggs, use malachite green at 50mg/100l (2ppm) for 30-60 min, in flow thru. system. For bloodstock, 5-6g/100l (50 ppm) as dip treatment for 30 sec. For bath treatment 100-200mg/100l for 30-60min or 10mg/100l for prolonged (3-4 days) treatment.

Treatment for fish parasitic diseases:

i) Bathing in 2.5% solution of coomon salt (NaCl) for 3-5 minutes or 5.0% NaCl for 1.0min are found to be effective
ii) Formalin 250 ppm treatment is effective in cambating flukes.
iii) Application of lime at 100 kg/ha.
iv) Chloramine T : It is most useful against *Myxobacteria, Costia, Trichdina*, white spot, *Gyrodactylus* and other parasites. Dose : Bath treatment 50-200mg/100l
v) Copper sulphate : Good for ectoparasitic infections but now not used. Dose 0.1mg/l for long term bath treatment.
vi) Mixture of 0.25ppm Malachite green and 100ppm formalin is useful in combating protozoan parasites.
vii) Dichlorvos and Trichlorphan : Dichlorvos is effective for sea lice while Trichlorphan is effective against *Argulus* spp. , *Lernaea* spp., gill flukes and skin flukes(*Dactylogyrus* and *Gyrodactylus* spp.). Dose : Dichlorvos : Bath treatment at 1mg/l for up to 60 min. against sea lice. Trchlorphon : 0.1- 2mg/l (0.1-2 ppm) long term exposure, which may be repeated.
viii) Formalin (34-38% formaldehyde) : widely used for ectoparasite infection, at 160-250 ppm for 1 hour, i.e 20-25ml/100l for 30-60min. For prolonged treatment, 2ml/100l for 12-24 hours.
ix) Leteux-Meyer Mixer (Formalin-Malachite green Mix) : Add 3.68g malachite green to 1lit formalin. Use 2.5ml/100l for 1 hour or 1.5ml/100l as prolonged treatment, which is to be repeated 3 times at 3-4 days interval.
x) Potassium permanganate : For treatment of protozoal and monogenean parasites. Dose : For bath treatment use 500mg/100l for 1 hour, for dip 100g/100l for 30 sec. The dose may be increased or treatment is repeated, if organic load in water is high.
xi) Sodium chloride : Good for treatment of ectoparasites. Dose: For bath : 1.0-1.5kg/100l for 20 minutes, for dip: 2-3 kg/100l for few seconds. For prolonged treatment use 500g/100l.

Treatment of shrimp diseases:

For bacterial disease in larvae & PLs : Give Furazolidone at 10mg/l,
Nifurprinol, 1mg/l,
Oxytetracycline, 60-250mg/l.

For Filamentous bacterial disease : Cutrin-plus at 0.1-0.5mg/l,
Oxytetracycline at 100mg/l,
Nitrofurazone at 100- 500 mg/kg feed

For Shell disease in juveniles : Oxytetracycline at 1.5g/kg feed
Nifurpirinol, 100-500mg/kg feed
Nitrofurazone, 100-500mg/kg feed

For Fungal disease (larval mycosis): Malachite green at 6-10ug/l
Treflan (trifuralin) at 10-100ug/l as a continuous treatment.

Protozoan fouling : Formalin at 1.5-2.5ml/ 100l
Chloramine T at 5mg/l

Table 2. Commonly used chemicals & antimicrobials in water quality maintenance and fish culture for different disease condition.

Chemical or Drug	Application	Recommended Dose/ Concentration	Approval for use by USFDA
Benzalkonium chloride	Disinfectant, useful for external bacterial infection	0.1-05mg/l in water 5mg/l for 30min. bath	Approved for use as a disinfectant
Chloramine T	Antimicrobial,	0.5-2mg/l bath,	Not approved
Chlorine/ Calcium hypochlorite	Disinfectant, oxidizing agent, anti-microbial	0.5-10 ml/l depending on organic load & pathogens	Approved
Ozone	Disinfectant, Anti-microbial, anti-viral	0.5mg/l	Approved
Copper sulphate	Ectoparasitic, used in marine system	0.1mg/l in marine water	Approved
Formalin	Anti-bacterial, anti-viral and anti-parasitic	2.0-5.0ml/100l prolonged bath or 20ml/100l, bath for 30-60 min.	Approved
Potassium permanaganate	Anti-bacterial, parasiticide	2-4mg/l	Approved
Sod. Chloride	Antiparasitic, osmoregulator	0.5 – 3%	Approved
Acetic Acid	Parasiticide	500-1000mg/l as bath	Approved
Acriflavin	Antibacterial, disinfectant, parasiticidal	10mg/l for 1 hr or 2mg/l for prolonged	Not approved
Iodophores (Betadine, Wescodyne)	Antibacterial, antiviral, disinfectant	50-200 mg/l as static or flush treatment for 15 min. for fish eggs.	Approved as disinfectant for fish eggs only
Malachite green	Fungicide, parasiticide	0.15ml/l as bath, 1 hr 0.1 mg/l indefinite	Not approved

Sulpha drugs (Sulphamerazine , sulphadiazine, sulphisoxazole)	Antibacterial	200mg/kg fish /day in feed for 14 days	Approved for food fish
Romet-30 (Ormetoprim & Sulphadimethoxi ne)	Antibacterial	50mg/kg/day for 5 days in feed	Approved for food fish
Quatinary Ammon. CompoundsHya mine & Roccal)	Antibacterial, Disinfectant	1-2mg/l for I hr bath/ flush treatment	Approved as disinfectant
Nitrofurans (Nitrofurazone, Furacin, Furazolidone	Antibacterial	200mg/kg/day in feed 20-100mg/l bath 1 hr.	Not approved
& Furance	Antibacterial	0.25 – 1.0 mg/l bath for 1 hr	Approved for Aquarium fish only
Oxytetracycline (Terramycin)	Antibacterial	50mg/kg/day in feed for 10 days	Approved for food fish

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STEM CELLS FOR AQUACULTURE AND FISH HEALTH MANAGEMENT

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Introduction

Stem cells are defined as master or mother cells having self renewal and differentiation capabilities into various cell lineages. In other words these are the natural seeds of biological system which may be pluripotent or multipotent (Nandedkar and Narker, 2003). Stem cells are distinct and diverse types of undifferentiated primal cell population that can divide indefinitely. Higher animals are composed of many functional organs like brain, heart, kidney, liver, various glands and many more. They all originally come from a single totipotent cell. i.e., zygote. So this zygote has innumerable potential (or is the stem cell) to develop into all types of cells (up to certain stages of development) in a developing organism. They are uncommitted and remain as such till reception of appropriate signals to specialized cell types under both *in-vivo* and *in-vitro* system.

Stem cells have lots of applications in the field of basic and applied biology. In different animals these are the best model system for elucidation of genetic functions, differentiation, vertebrate developmental events and toxicological screening platforms. This can be best described by applications through modern genetic techniques like transgenesis, germ line chimera production, gene trapping, gene targeting and knock out mutant production etc. These technologies are fully or partially established in many animals like mice, primate, farm animals and fishes like zebrafish and medaka. In humans, the goal of stem cell technology is to direct cultured, undifferentiated cells into forming functional cells, organs and tissues for transplantation therapies in treating specific degenerative diseases like diabetes, Parkinson's, Alzheimer's, neural degeneration, Huntington's disease, brain injury, cardiomyopathies and other pharmacokinetic applications where adequate therapies do not exist (Draper et al., 2004 and Sameer et al., 2006). In other animals stem cells have potential to achieve enhanced growth, disease resistance, surrogate brood development and analysis of species specific gene functions. (Ma et al., 2001 and Takeuchi et al., 2001)

According to nature of stem cells can be classified as:

Totipotent: These cells have the potential to develop both embryonic and extra-embryonic parts *i.e.* to complete individual *e.g.* Zygote.

Pluripotent: These cells have the potential to differentiate into any cell of the body derived from three germ layers *i.e.* ectoderm, mesoderm and endoderm.

Multipotent-These cells have certain restriction due to their tissue specificity, can be differentiate into limited cell types but have got tremendous plasticity depending on source of origin.

Types of Stem Cells based upon source of derivation:

According to origin and differential potentialities stem cells are classified into (1) Embryonic stem (ES) cells, (2) Adult stem Cells and (3) Specialized stem cells.

(1) ES Cells

Embryonic stem cells are undifferentiated cell lines derived from early developing embryos of animals, retaining their full developmental potential and capacity to differentiate

(Evans and Kaufman, 1981). When introduced into host embryo, these ES cells can participate in normal development and contribute to several tissues of the host including cells of the germ line. Mammalian ES cells are derived from embryos in the blastocyst stage and the same niche is maintained to restrict their differentiation. ES cells are of paramount importance because it causes a substantial technological challenge to be used as a source of well defined differentiated cells, either for drug discovery or toxicology in the short term or regenerative medicine and therapeutic cloning in the long term. However several mammalian ES cells are the models to understand its biology and an initial study in drug discovery (Lovell, 2001). ES cells in other animals have equal importance in germ line chimera production, surrogate brood stock development for economically viable species and conservation of endangered species. (Chen et al., 2003).

This also provides a link between *in vitro* and *in vivo* manipulations of vertebrate genome and germ line transmission of transgenes. It has a unique opportunity to identify and to know the function of novel genes of particular interest by gene trapping and knock out technology. ES cells allow identification of genes involved in development via monitoring expression patterns of reporter constructs randomly inserted into the genome of ES cells. (Hong et al., 1996).

(2) Adult Stem Cells

Many organs of an adult body have stem cells that undergo continued renewal. These cells include bone marrow cells, epidermis (skin), hair follicles, intestinal villi etc. They exist in a dormant state and a specific niche maintaining their cellular homeostasis or repairing damaged tissue upon stimulation. Adult stem cells have the capacity to self renew and generate functionally differentiated cells that replenish lost cells in the organ throughout the life span of the organism. Culture of cord blood is also a source of stem cells, classified as adult stem cells. Their niches balance the dual process of self-renewal lineage commitment by enabling the niche-specific spatiotemporal interactions (Gilbert, 2006). According to origin they are again classified as epithelial derived, mesenchymal, neural, endothelial, mammary and hematopetic stem cells.

Their high potential in the treatment of blood cancers, Type-1 dibetes mellitus, spinal cord injury, muscle damage etc has increased further explorations (Hall and Watt, 1989). Adult stem cells have an advantage over embryonic stem cells with respect to ethical issues.

(3) Specialized Stem Cells

Certain cells of the body follow the properties intermediate between ES cells and adult stem cells. These include spermatogonial stem cells (SSC) from adult testis, oogonial stem cells (OSC) and primordial germ cells (PGCs) in the developing embryo and even in the adult gonads. SSCs are pluripotent and have differentiation potential similar to ES cells (Guan et al., 2006). But workers classified them as adult stem cells. PGCs are often termed as mother of all stem cells as they form germ cells in a developing animal capable of viable gamete production.

Mitotic division in stem cells

Stem cells show symmetric division and asymmetric division types of special mitotic divisions .In symmetric division two daughter cells are produce which are exact replica of mother cells. In asymmetric division the two daughter cells produce by mother cell have two different fates. One will be a stem cell and the other one progenitor cell. The progenitor cell again goes on dividing to producing terminally differentiated cells. Asymmetric division is of two types: environmental asymmetry and divisional asymmetry. In environmental asymmetry at beginning there fate of terminally differentiated daughter cells are same, but later the cell fate changes according to the molecular niche provided to them based upon the

environment cues. While in divisional asymmetry the fate of terminal daughter cells are predetermined.

Properties of stem cells under *in-vitro* condition

- Round, polygonal in shape having high colony forming ability.
- High nucleus cytoplasm ratio and have short doubling time.
- Generally immortal in nature (varies according to source of origin).
- Exhibit and maintain a stable complete (diploid), normal complement of chromosomes (except aneuploid embryonic carcinoma cells).
- Show high alkaline phosphatase activity.
- Can undergo de-differentiation, meta-differentiation and trans-differentiation.

(Meta- differentiation is the ability of stem cells to convert their differentiation pathways in between the germ layers).

- Express the transcription factors like Oct-4, Sox-2, Nanog, STAT3, Id, GATA they activate or inhibit, host of target genes and maintain stem cells to proliferate and remain in undifferentiated state.

Stem Cell Niche

Stem cells need a specific environment to be maintained in an undifferentiated state for a longer period. This is a dynamic multicellular structural unit consisting of stem cells, surrounding cells and some extra cellular substrates. All living cells need a continuous interaction with the environment (nearby cells) (paracrine signaling) and often with the cell itself (autocrine signaling). The ability of a cell to become stem cell is determined in large part by where it resides. Continuously proliferating stem cells are housed in compartments called stem cell niches (or regulatory microenvironments). In embryo there are particular places to become the niche (Gilbert., 2006). Understanding the biology of undifferentiated state is a major challenge. *In vitro* culture needs some specialized cytokines in addition to common

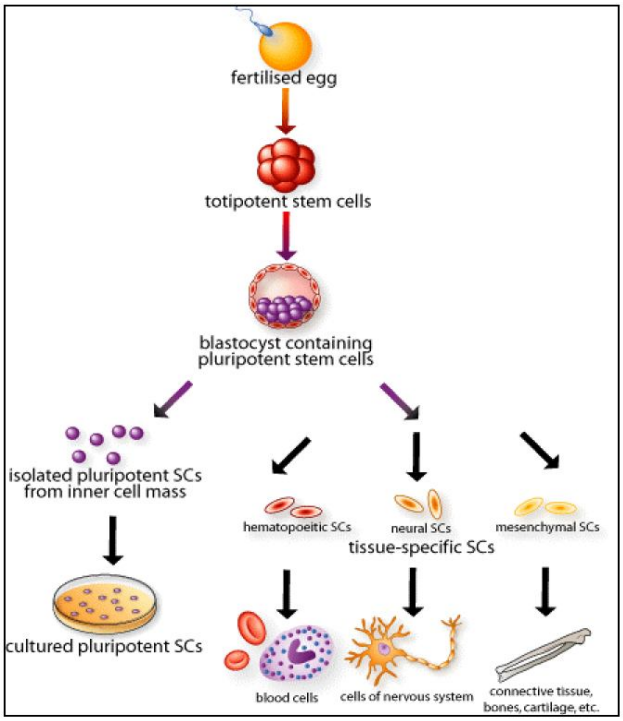

Figure 1: Schematic Diagram of Stem Cells and their origin

culture media. The general cytokines (protein or glycoprotein) include Leukemia inhibiting factor (LIF, cytokine of IL6 group), Bone morphogenic protein (BMP) and recently a heterocyclic compound, Scheme 1 (SC-1) are involved to maintain undifferentiated state (Chen et al., 2006). A schematic representation of derivation of stem cells is shown in Figure-1.

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mother of all stem cells as they form germ cells in a developing animal capable of viable gamete production.

2. Maintenance and Differentiation of Stem Cells

(a). *In Vitro* Culture and Preservation

In a sterile condition embryonic or adult stem cells are cultured in specially made culture plate supplemented with culture media and other growth factors. Now the culture plates containing cells (seeding) are maintained in an incubator with required temperature. The media must be changed periodically to refresh the *in vitro* condition. Stem cell culture often needs a feeder layer on which the original cells are plated. For long term preservation stem cells from culture with a freezing medium are packed in a cryovial and stored in liquid nitrogen at -196° C. Stem cells of different origin require different protocols with respect to cryopprotectants and cooling rate.

(b) Differentiation and Transdifferentiation

After establishment of a pluripotent stem cell line it is required to differentiate them for different applications. The signaling pathway that regulates the stem cell differentiation is yet to be elucidated completely. Specific differentiated cells are often required either for therapeutic application or drug testing. These cells can be differentiated into any type of lineages with specific signaling molecules. i.e., growth factors such as epidermal growth factor (EGF) for epithelial cells, nerve growth factor (NGF) for neural cells and so on. Some differentiated cells can be reprogrammed to behave as stem cells or some other differentiated called transdifferentiation Induced pluripotent cells are derived from fibroblast cell lines by transfection methods. More recently skin cells are dedifferentiated into pluripotent cells having embryonic stem cell properties.

3. Stem Cell Properties

Stem cells are characterized by their self renewal and undifferentiated properties. Stem cells may divide symmetrically (produce two same types of cell) or asymmetrically (one like parent type and other one is differentiated).They express certain cell surface receptors/markers such as SSEA-1(not in human), SSEA-3, TRA-1-60, TRA-1-81, SSEA-4, Thy 1 etc identified by immunocytochemistry. Some genes of developmental origin retain their expression in stem cells. They include oct-3/4, sox2, c-Myc, Nanog and Klf4 (confirmed by RT-PCR analysis). Specifically oct-4 and nanog restricts differentiation of stem cells. They remain in clumps (near about 400 cells) often interconnected with each other either by fibroblastic or neural out growth. They show strong alkaline phosphatase activity, high telomerase activity, upon injection to immunosupressed animal forms teratoma, show normal karyotype in every passage. Final characterization of stem cells in small animals requires germ line transmission and chimera formation.

4. Brief Status of Stem Cell Research

More than 2,000 scientists of international fame are involved to understand the biology of stem cells in different organisms, possible implications for human welfare (mainly to alleviate critical diseases) in cancer stem cell, tissue specific stem cells, technology development, clinical aspects and stem cells of different model organisms. Stem Cell research has been the focus of many biological sciences. This has been recognized by the Nobel Committee that conferred the 2007 Award to a stem cell research group, Sir Martin Evans, Mario R Capecchi, and Oliver Smithies for their discovery of "principles for introducing specific gene modifications in mice by the use of embryonic stem cells."

In aquaculture: ES cells in general possess a self-renewal capacity and developmental potential to differentiate into any cell type of an organism and can be regulated and maintained *in vitro* (Wobus & Keneth 2005). These cells provide a good *in vitro* model system to understand the molecular mechanism of pluripotency, conservation and basic biomedical research (Hong, Chen & Schartl 2000; Ma, Fan, Ganassin, Bols & Collodi 2001). The existence of ES cells has been reported in zebrafish, medaka, rainbow trout, sea perch, sea bream and

rohu (Wakamatsu, Pristyaznhyuk, Niwa, Ladygina, Kinoshita, Araki & Ozato 1994; Collodi, Kamei, Sharps, Weber & Barnes 1992; Chen, H-Q, Sha & Hong 2003; Dash, Routray, Dash, Guru, Swain & Sarangi 2008). ES cell cultures based on the original method established in mice (Evans & Kauffman 1981; Martin 1981 are maintained in a conditioned media (Pease & Williams 1990). Their characterization includes compact colony like morphology, normal karyotype, alkaline phosphatase activity, differential ability to form three germinal layers (Draper, Moore, Ruban, Gokhale & Andrews 2004), expression of specific cell surface markers (Henderson, Draper & Baillie 2002) and chimera production. However, species specific differences in their characterization patterns do exist. Fish is a powerful vertebrate model to understand the genetic analysis of developmental pathway and used for other applied sciences (Berghmans, Jette, Langenau, Hsu, Stewart, Look & Kanki 2005).

5. Future Challenges and Need for Stem Cell Research

The current advances in molecular biology, improvements in genetic engineering, post genomic capabilities and biotechnological advances need more collaborative work for elucidation and implication. Application of stem cell therapy and its vigorous research in basic science needs a cost effective approach. In different animals stem cell banks should be made industrially for potential use. For human ES cells there are constraints of ethical issues from certain parts of the world. However progressively this is solved by proper guidelines to obtain the embryo and other modern replacements like IPSCs and transdifferentiation. Proliferation of stem cells without differentiation, application in different fields and sophisticated research facilities are need of the time.

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SPECIFIC PATHOGEN FREE STOCK, BIOSECURITY AND THE ESSENTIAL PRINCIPLE OF HEATH MANAGEMENT IN SHRIMP (*LITOPENAEUS VANNAMEI) AQUACULTURE*

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Introduction

Around the turn of the century, some Asian countries began to culture Pacific white shrimp, *Litopenaeusvannamei*, a species native to Latin America. These white shrimp have a number of advantages like tolerance to a higher range of stocking densities, high growth rate, crowding tolerance, a wider range of salinities, lower temperatures, lower-protein food requirements and captive breeding which lead to its commercialization in America and Asia. Further, most important attribute of *L. vannamei* is successful captive breeding which enable to produce genetically superior and high health seed. Captured wild seeds were used in Latin America for extensive pond culture of *L. vannamei* until the late 1990s. It was introduced to Pacific islands in early 1970's and in 1996, it was introduced in China and subsequently in other coastal Asian counties from 2000-01. Introduction of *L.vannamei* in Indian aquaculture through SPF broodstocks opened up a new vista in coastal aquaculture of the country. However, worldwide shrimp farming activity is plagued by diseases and unsustainable practices which sometimes affect the coastal ecosystem and livelihood.

The ineffectiveness or belated enactment of regulations lead to the appearance of viral pathogens (including TSV, LOVV, REO III and BP), probably transferred through the importation of infected stocks of *L. vannamei*and now causing significant disease problems. Same is the story with the belated banning of its introduction in Malayasia, illegal importation of *L. vannamei in* Phillipines and Vietnam due to disease problems with the culture of *P. monodon* aggravated the situation.The SPF/ SPR lines of broodstock and culturing it under total biosecured system becomes the panacea for the aquaculture of shrimp industry. Indian government has shown great caution due to the potential risks involved, thus carried out a risk assessment and then finally allowed official import of certified SPF broodstock with such introductions, despite interest in the species due to its perceived tolerance to WSSV when SPF integrity is maintained with uncompromised farm biosecurity.

Present status of adoption and scope of up scaling:

World production of *L. vannamei* increased from about 150 thousand tonnes in 2000 to 2 million tonnes in 2008 increasing its share from a meager 10% to over 75% of total shrimp production. In India, shrimp aquaculture was synonymous with *P. monodon* culture and its growth rate of the sector was phenomenal till 1995. Since 1995, the sector is plagued by viral diseases, especially White Spot Syndrome Virus (WSSV). In 1997, Ministry of Agriculture established the Aquaculture Authority under the Environment Protection Act, 1986. In 2005, From 2000 onwards there was a gradual increase in production which reached a maximum of 1,40,000mt in 2006-07. But in 2007-08 and 2008-09, the production levels reduced drastically and reached the pre-1995 level of 75,000 mt.Introduction of *Litopenaeusvannamei*in 2009 led to the recovery of the sector with the total production levels reaching 2,20,000 mt in 2011-12.In the year 2012-2013, this pacific white shrimp vannamei production alone reached a level of 1, 47,516 tonnes from an area of 22,715 ha (Source: MPEDA)creating a demand for more than 1 billion seeds required for the purpose. The number & capacities of the hatcheries are to be increased to fulfill this requirement.

Genetic improvement program

Life Cycleand Reproductive Biology

L. vannamei has a complicated life cycle consisting of an offshore oceanic and inshore estuarine phase associated with spawning and juvenile growth respectively.In nature, they spawn in sea after successfulmating**.** When the eggs are released from the body they pass the sperm and get fertilized. *L. vannamei*weighing 30–45 g will spawn 100 000–250 000 eggs of approximately 0.22 mm in diameter. Floating eggs, 200-300 micron in diameter, drift with the sea currents and develop into nauplii. Hatching occurs about 16 hours after spawning and fertilization.The next larval stages (protozoea, mysis and early postlarvae respectively) remain planktonic for some time, eat phytoplankton and zooplankton, and are carried towards the shore by tidal currents.The larval stages, starting with the zoea are filter feeders. The postlarvae (PL) change their planktonic habit about 5 days after moulting into PL, move inshore and begin feeding on benthic detritus, decaying plant material, plankton, worms, bivalves and crustaceans. This species is one of the open thelycum species, and they differ from the closed thelycum species in the mating pattern. In open thelycum species, mating takes place when the female shell already has hardened. The spermatophore is visible as a coarse whitish mass of approximately 1 x 1 cm, glued to the female thelycum.In *L. vannamei* the color of the ovary changes from yellow to orange just before spawning, this can be used as a ripeness indicator.

Domestication and selection program:

Domestication and genetic selection programmes then provided more consistent supplies of high quality, disease free or resistant PL, which were cultured and reproduced. The selective breeding program in Hawaii and other places from 1989 resulted in the production of SPF and SPR lines, leading to the industry in the United States of America and Asia. Domestication and genetic selection programs for reproduction have paid a rich dividend in ensuring consistent supplies of high quality, disease free or resistant PL. Pacific White Shrimp Broodstock are selectively bred for good maturation performance, fast growth, resistance to diseases and high survival to the Hatcheries and generation after generation adding to the vigour. Now, the specific pathogen free (SPF) or resistant (SPR) lines with superior and high health seeds are available in Hawaii and other places.*L.vannamei*, selected for growth performance, has heritabilities of 0.15-0.3, meaning that the selected breeders produced offspring that grew 15-30% faster than the parents. With *L.vannamei* it was shown that domesticated stocks performed better then wild stocks. In fact, research work by some state and private companies has focusedefforts on the development of SPF strains that are also resistant to specificpathogens (SPF/SPR). These strains are typically resistant to only one pathogen,currently largely either TSV or IHHNV, but some work has indicated that strainswith multiple resistance to TSV and WSSV (at up to 25 percent survival tochallenge tests) may be possible. This is accomplished bychallenging sub-lots of shrimp families to a particular pathogen (or combination ofpathogens) and then selecting the most resistant families as broodstock for thenext generation. This negative correlation between growth and disease resistance (as it is found often like mean family survival to a TSV challenge) must therefore be taken into account. A US-based facility producing SPF and SPR *L.vannamei*has reportedly achieved a growth rate potential of 2g/week withfamilies of shrimp selected for resistance to TSV, with no negative correlationbetween growth and survival.

Other specific advantages of genetically improved variety include rapid growth rate, tolerance of high stockingdensity, tolerance of low salinities and temperatures, lower protein requirements(and therefore production costs), certain disease resistance (if SPR stocks areused), and high survival during larval rearing. SPR strains of shrimp, however, do not necessarily have to be SPF. Latin Americais now almost exclusively using pond-grown and (often) disease checked andquarantined SPR *L. vannamei*due to their better performance in maturation,hatcheries and grow-out ponds. At that stage depending only on the imported

bloodstocks may not be enough and economical. Therefore, India should have its own selective breeding program and multiplication centre to produce SPF seeds. Recently, Rajib Gandhi Centre for Aquaculture (RGCA) has tied up with M/s. Oceanic Institute, Hawaii, pioneers in the domestication of *L. vannamei* in the world for aquaculture at TASPARC (The Andhra Pradesh Shrimp Seed Production, Supply and Research Centre) facility of MPEDA at Vishakhapatnam in Andhra Pradesh. With the Germplasm (Postlarvae) provided by the collaborating institute a targeted high quality SPF broodstockof 4500nos can be supplied to the growing industry.

SPF and SPR Broodstock:

These **specific pathogen free (SPF), Specific pathogen resistant (SPR) or high health stock is one of the best way to recruit the stock in hatchery.** SPF broodstock are sourced by approved hatchery operators from approved international suppliers for seed production and supply to farmers. Hatcheries normally prefer wild broodstocks due to the belief that they produce more and healthier nauplii. In recent years, however, the high risk of introducing viral pathogens with wild broodstock has changed this preference. Domesticated stocks either genetically improved or suspected to be resistant or tolerant to specific pathogens may be used as broodstock. SPF stocks are generally maintained in highly biosecure facilities and their offspring (designated "high health" rather than SPF) are supplied to the industry. SPR shrimp are those that are not susceptible to infection by one or several specific pathogens, and Specific Pathogen Tolerant (SPT) shrimp are those that are intentionally bred to develop resistance to the disease caused by one or several specific pathogens.

The SPF stock of *L. vannamei* that is imported should be free of the following two sets of pathogens:

A. Pathogens exotic to India and likely to cause damage to *P. monodon*

1.Taura syndrome virus (TSV) 2. Yellowhead virus (YHV) 3.Hepatopancreaticparvo-like virus (HPV) 4.*Baculoviruspenaei*(BP) 5.Infectious myonecrosis virus (IMNV) 6.Necrotising hepatopancreatitisbacterium α-Proteobacterium (NHPB) 7. Necrotising hepatopancreatitis (NHP)

B.Pathogens reported from *L. vannamei* and which cause mortality or retarded growth /Pathogens not exotic to India and need to be avoided so that introduced *L. vannamei* performs well

1. White spot syndrome virus (WSSV) 2. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) 3.MonodonBaculovirus (MBV) 4. *P.vannamei*nodavirus (PvNV)

Emerging diseases:

EMS (early mortality syndrome):Asia's shrimp-farming industry has been heavily affected by early mortality syndrome or,more descriptively, acute hepatopancreatic necrosis syndrome (AHPNS) from 2009. Studies by the University of Arizona Aquaculture Pathology Laboratory identified the causative agent for AHPNS as a unique strain of *Vibrio parahaemolyticus* that can produce toxins responsible for the primary pathology in affected shrimp (Lighner group). Infected live shrimp and fresh shrimp tissues can transmit the disease to "clean" shrimp, but the agent is inactivated by freezing and thawing. Affected shrimp pose no human health concerns.AHPNS began to cause significant production losses in southern China in 2009, and by 2012 had spread to farms in Vietnam, Malaysia and Thailand. The disease has caused significant shortages of shrimp products for the global market, which in turn impacted the global price of shrimp.

AHPNS usually occurs within 45 days in shrimp ponds with newly stocked postlarvae of both black tiger shrimp, *Penaeusmonodon*, and Pacific white shrimp,

Litopenaeusvannamei.Shrimp with early AHPNS show pale to white coloration of the hepatopancreas, as well as atrophy that can reduce the size of the organ by 50% or more. In the terminal phase of the disease,black streaks or spots due to melanin deposition from hemocyte activity appear in the hepatopancreas.Mortality in affected shrimp ponds can approach 100%within a few days of disease occurrence.In the terminal phase of AHPNS, the hepatopancreas shows marked hemocytic infiltration and development of massive secondary bacterial colonization that occurs in association with the necrotic and sloughed epithelial cells. This unique pathology suggested that the primarylesions in the hepatopancreas are mediated by a toxin (Tran*et al*., 2013).

RMS (Running mortality syndrome):

RMS (Running mortality syndrome) is one of the emerging disease problem when the standing biomass is critical and /or because of some critical environmental factors (not known yet).If daily mortality remains low (<5) or subsides-no harvest & water exchange is required but informing to neighbors is mandatory. If shrimp size are small, it should not be abandoned rather the ponds should be disinfected.flag system should be followed, any dead shrimp should be removed, and neighboring ponds should be monitoredfor shrimp health. Emergency harvest prevents spread of the disease tand preserves the freshness and quality of the harvested shrimp.

Disease diagnostics:

The correct diagnosis is obviously a critical step in any control program. The available diagnostic methods that may be selected for diagnosis of the shrimp diseases or detection of their etiological agents are based on: Anamnesis, gross and clinical signs.

1) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, impression smears, haemolymph smears and wet-mounts of fecal strands as per the requirements.
2) Post mortem findings.
3) Histopathological and histochemistry findings.
4) Isolation and identification of the causative agents.
5) Bioassays of suspected or subclinical carriers using a highly susceptible host as an indicator.
6) Transmission or Scanning Electron Microscopy (TEM or SEM).
7) Antibody-based tests for pathogen detection using polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs).
8) Molecular methods.

Molecular Diagnostic methods

Nucleic acid based diagnosis is available for the important viral diseases affecting shrimps and fishes. Novel diagnostic tools having high specificity, sensitivity and speed emerged from very basic fact of uniqueness of genetic makeup or the genetic fingerprint of each organism or pathogen in the form of DNA or RNA. Use of these novel diagnostic tools is becoming the central part of disease prevention and management. These are also used for epidemiological studies for the better understanding of the pathogen. With the advent of DNA chips (DNA arrays), diagnosis of multiple pathogens is possible in one single step. This could involve preliminary screening with rapid (<30 min) immunodot-style test kits for WSSV , examination of faeces for HPV and MBV, and general microbial and morphological testing for other pathogens.

The polymerase chain reaction (PCR) enables amplification of the sequences of the nucleic acids of the pathogens represented at extremely low levels within extracted nucleic

acid pools (one copy of viral DNA per 70,000 cells), to levels detectable by gel electrophoresis and hybridization.

PCR screening at critical points would greatly reduce the risk of disease transfer from broodstock to larvae. Attention should also be placed on ensuring that reliable PCR results are obtained from the various PCR laboratories through intercalibration and/or harmonization operations.

Health assessment techniques:

The holistic quality assessment in hatchery is to be done at 3 levels

Level 1: Observation of animal and environment with examination based on gross features is to be performed on a routine basis. This should include: health examination of broodstock, sex determination, moult and ovarian development staging, removal of sick/moribund individuals, selection of nauplii by phototactic response, zoea/mysis stage feeding activity by observation of faecal strands, larval activity, postlarval health, activity and behaviour, stress. If a batch of nauplii shows poor phototactic and weak swimming behaviour, it is rejected without further examination.

Level 2: More detailed examination using light microscopy examination of larval and post larval quality looking in to various health-related features (cleanliness, feeding behaviour, digestion, etc.)and squash mounts, with without staining, and basic bacteriology of shrimp, feed and water is included to gain an understanding of the bacterial flora and to make a decision on whether the tank should be discarded or treated.

Level 3: Screening of broodstock, nauplli and postlarvae should be done by molecular techniques and immunodiagnostics (e.g. PCR). For *P. monodon* post larvae, WSSV is checked whereas for the *L. vannamei* WSSV, IHHNV along with other OIE listed virus should be screened.

Biosecurity principle and protocols

The role of biosecurity measures is to diminish the risks arising from the entry, establishment or spread of pathogens within the system to a manageable level. Biosecurity has been defined as "*...sets of practices that will reduce the probability of a pathogen introduction and its subsequent spread from one place to another...*" (Lotz, 1997).. Biosecurity protocols are advocated to be followed in all coastal aquaculture units to minimize the disease risk (Lightner, 2011)and become very important when viral /bacterial threat is imminent and moreover when specific pathogen free or specific pathogen resistant stocks are used (as in the case of *L. vannamei*).

The basic elements of a biosecurity programme in a shrimp hatchery include the physical, chemical and biological methods necessary to protect the hatchery from the consequences of all diseases that represent a high risk. Various levels and strategies for biosecurity may be employed depending on the hatchery facility, the diseases of concern and the level of perceived risk. The appropriate level of biosecurity to be applied will generally be a function of ease of implementation and cost, relative to the impact of the disease on the production operations (Fegan and Clifford, 2001). There are two common ways by which disease transmission occurs:

- **Vertical transmission** - from mother shrimp to the post larvae in hatchery systems
- **Horizontal transmission** - from one affected shrimp to the other in farming systems .

The components of biosecurity in an aquaculture facility comprises

i) **prevention**- protection of the cultured/managed organisms (especially pathogens) and the protection of humans and ecosystems from the adverse affects of the introduced culture system, and its targeted and non-targeted organisms, ii) **control**- control of the culture

system, the movement of organisms, risk related activities, and monitoring and recording of actions taken and iii) **contingency planning-** planning for all possible eventualities. The biosecurity issues in shrimp hatcheries may be either **internal** concerning the introduction and transfer of pathogens within the facility or **external** concerning the introduction and transfer of pathogens from outside sources to the facility or vice versa. In case of disease outbreak within the aquaculture facilities the options available are either **treatment-** by application of methods that reduce the effects of the diseases, **containment-** by restriction of the disease from spreading to other tanks/facilities, or **elimination-** of the diseases from the vicinity. Implementation of a biosecurity programme for a shrimp hatchery should include the following elements:

The physical separation or isolation of the different production facilities is a feature of good hatchery design and should be incorporated into the construction of new hatcheries. In existing hatcheries with no physical separation, effective isolation may also be achieved through the construction of barriers and implementation of process and product flow controls. Water distribution and discharge from the hatchery should also be compatible to all biosecurity protocols. The seawater to be disinfected with hypochlorite solution (20 ppm active ingredient for not less than 30 minutes) and inactivated. Vehicle tyre bath, Footbaths containing hypochlorite solution at >50 ppm active ingredient) and hands (bottles containing iodine-PVP (20 ppm and/or 70% alcohol) to be used upon entering and exiting the unit. All wastewater must be collected into another tank for chlorination (20 ppm for not less than 60 minutes) and dechlorination before release to the environment. All mortalities or infected animals must be incinerated or disposed of in another

Implementation of a biosecurity programme for a shrimp hatchery should include the following elements:

- Specific pathogen free (SPF) or high health (HH) shrimp stocks should be used.
- All the incoming stocks should be quarantined in the designated area.
- All incoming stock should be analyzed for diseases.
- All incoming water sources should be treated to eliminate pathogens.
- Equipment and materials should be sterilized and maintained clean.
- Personal hygiene measures including washing of hands and feet and clothing.
- Knowledge of the potential pathogenic diseases and the sources of risk and methods and techniques for their control and /or eradication
- Specific pathogen resistant (SPR) stocks to be used.
- Maintenance of optimum environmental conditions.
- Immune enhancers and probiotics to be used in place of antibiotics.

The following SOPs should be strictly followed to prevent the horizontal transmission of viruses in the culture ponds:

Elimination of viral particles from soil through drying and disinfection, Using quality water treated and filtered, Elimination and prevention of vectors entry, Prevention of viral pathogens in feed, Prevention of viral pathogens through birds and animals, Following strict sanitary protocols should be followed by all farm workers and disinfection of the feet and hands of the farm workers is mandatory before entering any pond, Disinfection of all the pond implements before use in any pond, Avoidance of fresh feed or farm made semi moist feed, Following proper disinfection protocol in case of disease outbreak or emergency harvest.

Preventing introduction, spread and increase resistance to a disease and eradicate it if it occurs (hatchery/farm) are the essential principle of biosecurity which comprises of proper engineering designing, broodstock screening or SPF/SPR broodstock and quarantine measures, monitoring health and protection of health, prioritizing and evaluating risks and critical control points, mitigating harmful diseases and other risks

through ideal biosecurity plans and programs including biosecurity check list and Financial dividends

Quarantine facility and protocols

SPF *L.vannamei* brood stock is to be imported into India only with a valid sanitary import permit through the designated port of entry and following strictly the guidelines for operation of aquatic quarantine facilities for the import of SPF *L. vannamei* .Before passing to the production system, the broodstock must be screened for pathogens (i.e. via dot blot, polymerase chain reaction (PCR), immunoblot etc.). Hatchery unit should comply with the set standards as propagated by CIBA and set by CAA. The quarantine facilities are essentially a closed holding area where shrimp are kept in individual tanks until the results of screening for viruses (and for bacteria, where applicable) are known. The broodstock quarantine unit should be physically isolated from the rest of the hatchery facilities. The quarantine unit should have the following characteristics:

- ✓ Adequate isolation from all of the rearing and production areas to avoid any possible cross contamination.
- ✓ Should be in an enclosed and covered building with no direct access to the outside.
- ✓ There should be means provided for disinfection of feet (footbaths containing hypochlorite solution at >50 ppm active ingredient) and hands (bottles containing iodine-PVP (20 ppm and/or 70% alcohol) to be used upon entering and exiting the unit.
- ✓ Entrance to the quarantine area should be restricted to the personnel assigned to work exclusively in this area.
- ✓ Quarantine unit staff should enter through a dressing room, where they remove their street clothes and take a shower before going to another dressing room to put on working clothes and boots. At the end of the working shift, the sequence should be reversed.
- ✓ An adequate number of plastic buckets should be available in the quarantine room to facilitate effective daily routine movement of shrimp in and out of the facility.
- ✓ The quarantine facility should have an independent supply of water and air with separate treatment and disinfection systems and a system for the treatment of effluents to prevent the potential escape of pathogens into the environment.
- ✓ The seawater to be used in the facility must enter a storage tank where it will be treated with hypochlorite solution (20 ppm active ingredient for not less than 30 minutes) before inactivating with sodium thiosulfate (1 ppm for every ppm of residual chlorine) and strong aeration.
- ✓ All wastewater must be collected into another tank for chlorination (20 ppm for not less than 60 minutes) and dechlorination before release to the environment.
- ✓ All mortalities or infected animals must be incinerated or disposed of in another approved manner.
- ✓ Used plastic containers and hoses must be washed and disinfected with hypochlorite solution (20 ppm) before reuse.
- ✓ All the implements used in the quarantine unit must be clearly marked and should remain in the quarantine area. Facilities for disinfection of all equipment at the end of each day should be available.

Shrimp Health Management:

Health management becomes very crucial especially as many viral and bacterial diseases are reported in this species. Seed being one of the major source of this virus and vertical transmission of the virus been established, the hatcheries have to adopt many precautionary principles. From hatching, it takes about 19-20 days to reach harvest at PL-10.

Care is taken to reduce bacterial/pathogen contamination of the larval facilities using a combination of periodic dry-outs and disinfections, inlet water settlement, filtration and/or chlorination, disinfection of nauplii, water exchange and the use of antibiotics or (preferably) probiotics. Biosecurity measures must be implemented for each phase, Water treatment, high quality feeds (both live and inert) and Rapid diagnostic methods for field use and expertise are required to use it. Live feeds should be proven free from pathogens, or treated suitably to kill them. Eggs and nauplius should be washed and cleaned using appropriate disinfectants to minimize "vertical" transmission of pathogens from broodstock to larvae. Only healthy nauplii should be selected.

The most successful strategies for controlling diseases in shrimp ponds are based on a combination of prevention by exclusion, and Better Management Practices that focus on creating a healthy, non-stressful environment for the shrimp. *L.*vannamei. SPF status of the broodstock and disease free seeds are to be procured and for this reason the biosecurity of the farm should be intact at all point of time. The gut content colour is a good indicator of the probable health status and corrective action to be taken. Black / brown / green gut content implies under feeding whereas a red or pink gut showed disease manifestation whereas a pale whitish gut showed gut infection. A normal gut will have a light or golden brown color. Adding lime to the water (100-200 kg CaO/ha) and spread lime on pond dikes if after rain shrimps distress immediately not feeding shrimp with crustaceans (crabs or shrimp) or by catch waste, following BMP not feed shrimp with crustaceans (crabs or shrimp) or by catch waste. Testing periodically for any disease prevalence like for WSSV and IHHNV during the culture is required to understand and act as per the infection presence.

In case of any sick or dead shrimps- gills, gut content of the shrimp, water quality and pond bottom condition can be checked, In case of wsd-informing neighbours; and if mortality is increasing over 2 days don't letting the water to change, in case. If >50% of the shrimp are not feeding, harvesting can be considered without draining the pond.

The other options to deal with the disease problems includes-

1. Immunoprophylaxis
2. Vaccination
3. Other novel therapeutic strategies (RNA interference approach)
4. Integrated eco based management

Immune mechanism and immunoprophylaxis:

Invertebrates, which lack adaptive immune systems, have developed defense systems that respond against antigens on the surface of potential pathogens. The defense mechanisms of crustaceans depend completely on the innate immune system that is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as lectins, antimicrobial, clotting, and pattern recognition proteins, which, in turn, activate cellular or humoral effector mechanisms to destroy invading pathogens. The main characteristics of the crustacean proteins that participate in immune defenseare elucidated by specific recognition of carbohydrate containing molecules, i.e. glycans, glycolipids, glycoproteins, peptidoglycans, or lipopolysaccharides from Gram-negative and Gram-positive bacteria, viruses, or fungi. The anti-viral defense system of lower vertebrates and invertebrates raise a challenge to our understanding the immunity which dare to continue & sustain these animals millions of years with all bacterial, viral and other challenges.

Eco-based culture practices:

The farm management in *L. vannamei*required more care because of stocking SPF seed and also stocking SPF seed alone don't give any guarantee of successful culture, so it depends upon how we are maintaining farm and Biosecurity measures in culture ponds. Production of shrimp with reduced water exchange in outdoor ponds is another promising method to minimize monetary losses and environmental degradation. Recent studies at CIBA showed that high-density nursery and grow-out of Pacific white shrimp are feasible, with excellent survival and yield.Even with high stocking of *L. vannamei* production and survival, weekly growth rate is at the rate of 1.5 to 2.0 g, depending on the stocking density. The increase of productivity in aquaculture has been accompanied by ecological impacts including emergence of a large variety of pathogens and bacterial resistance. These impacts are in part due to the indiscriminate use of chemotherapeutic agents as a result of management practices in production cycles.

Probiotic based farming system

Antibiotic and other chemotherapeutics agents and also pesticides were commonly used in fish farms either as a feed additives or immersion baths to achieve either prophylaxis or therapy, Nowadays, antimicrobial resistance is a growing public health threat and has been designated by the WHO as an emerging public health problem. Probiotic the natural beneficial bacteria are now well accepted and widely used in shrimp aquaculture.Antibiotics are used under the mistaken notion that they give better yield. Some hatcheries use banned antibiotics and other therapeutics causing environmental and health problems. Different probiotic strains have been shown in the literature, to be capable of modulating the immune system in several ways. These probiotics were widely used in culture practices of *L. vannamei* in India, but a proper mechanism for their screening and efficacy testing should be in place.

Biofloc and/orperiphyton based farming:

Biofloc is the conglomeration of heterotrophic bacteria, algae (dinoflagellates& diatoms), fungi, ciliates, flagellates, rotifers, nematodes, metazoans & detritus. Biofloc technology (BFT) is based on the concept of the retention of waste and its conversion to biofloc as a natural food within the culture system. This is a relatively new biotechnological means to maintain the water quality in aquaculture units, to reduce the water exchange and reutilize the feed and reduce the production cost. As the fish/shrimp ponds are rich in microbial community, the inorganic nitrogen added through the feed can be assimilated by these microorganism and converted in to microbial protein through an adjustment of C:N ratio. There is a need for constant aeration and agitation of the water column and addition of carbon sources as organic matter substrate to allow aerobic decomposition and maintain high levels of microbial floc in suspension in feed and/or fertilized ponds. In a typical brackishwater pond, 20–25% of fed protein is retained in the fish/shrimp, rest is wasted as ammonia and other metabolites, Organic N in feces and feed residue. Increased C:N ratio through carbon addition enhances conversion of toxic inorganic nitrogen species to microbial biomass available as food for culture animals.Microbial flocs produced in suspended growth bioreactors could offer the shrimp industry a novel alternative feed. CIBA have successfully demonstrated the biofloc and periphyton based nursery technology for this shrimp in tank system where a very high survival of 98-99 % was achieved in the treatments compared to 91-92 % in the conventional system. The grow-out culture was also demonstrated under biofloc based rearing in tank system with final weight of 22-23g in 110

days of culture and a production record of above 25 tons per ha. Now a daysbiofloc farming system become popular among the shrimp farmers because of its following advantages. The capital costs are most likely higher, but it will pay off in spades with the increased biomass yields with each harvest.

Integrated nursery system: There are some farmers practicing nursery system, first they stock seeds in small nursery tanks, they are speacially constructed near the farm, after acclimatizing the nursery seeds or juveniles were transferred to farms.

Adoption in BMPs in *L. vannamei* farming will have overall impact including Environment, Social, Reduced costs and improved Profits, Reduced risk to small-scale farmers, Increased co-operation and harmony among farmers, Better organized farmer groups, Reduced disease incidence, Reduced FCR and increased efficiency of resource use (feed, seed, energy, finance in particular), Reduced pollution , Reduced chemical and antibiotic use.

Need for Technology Improvements:

Though last three year of vannamei adoption went on smoothly and successfully, location specific fine-tuning is required. This species is found to be well suited to Indian conditions and being amenable for high stock densities, a higher productivity can be obtained. In this connection, reduction in the cost of production is the priority especially taking the indigenous culture and feed technology further. Following are some of the priority areas for research in *L. vannamei*.

- Multiplication centres of *L.vannamei* should be encouraged, but a mechanism to ensure Pathogen free status of the stocks imported/held/sold, should be put in place.
- Low larval survival rate in India compared to that obtained worldwide
- Robust disease surveillance during hatchery operations with special emphasis on problems like Zeoa2 syndrome
- Initiation for the establishment of Multiplication centers in India and related research on selective breeding program to avoid monopolistic situation of broodstock supply
- Continued development of SPR lines of *L. vannamei* for viruses including TSV, WSSV, IHHNV, BMNV and IMNV.
- Development of faster growing lines of SPF/SPR stocks.
- Effective disinfection procedures for eggs, nauplii and PL in hatcheries.
- Effective replacements (i.e. probiotics and immunostimulants) for antibiotics.
- Vaccination and other effective treatments for shrimp viruses.
- Replacement of non-ecofriendly and costly marine meals in shrimp feeds.
- Efficient water treatment and management systems for closed culture systems.

Conclusion:

Availability of captive broodstock; ability to conduct domestication and genetic selection work; availability of SPF and SPR lines; elimination of problems associated with wild broodstock and/or PL collection; and faster generation times are the advantageous attributed to *L. vannamei*growth. Implementation of a biosecurity measures by shrimp hatcheries will ensure restriction of the disease from spreading to other facilities or eliminationof the diseases from the vicinity of the aquaculture facility. Foolproof bio-security measures have to be put in place in hatcheries & farms and misconception like "SPF seed never get affected by virus" should be removed.However, keeping the requirements and future projection in mind, it is worthwhile to mention here that India should start a domestication program to produce a genetically improved*L. vannamei* through multiplication centre. SPF is very important for running a successful shrimp farm. The hatcheries should seriously consider adopting a risk assessment based approach using

HACCP guidelines. In the pretext of evolving culture practices and introduction and proliferation of farming of new species like *L. vannamei*, evolution of new culture practices like zero water exchange and/ or probiotic/ biofloc/periphyton based farming is to be encouraged.

SUGGESTED READING:

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Fig : SPF *L. vannamei* seeds, Farm biosecurity measures (bird fencing, crab

Fig : Innovations in culture

Fig: Biofloc based farming technology

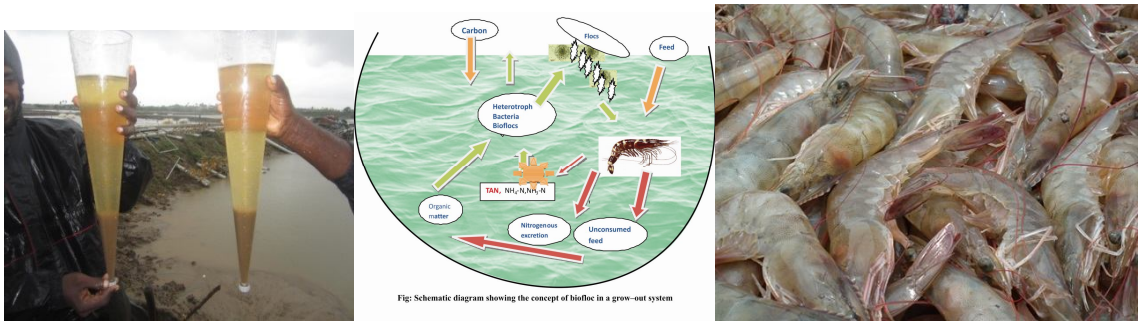
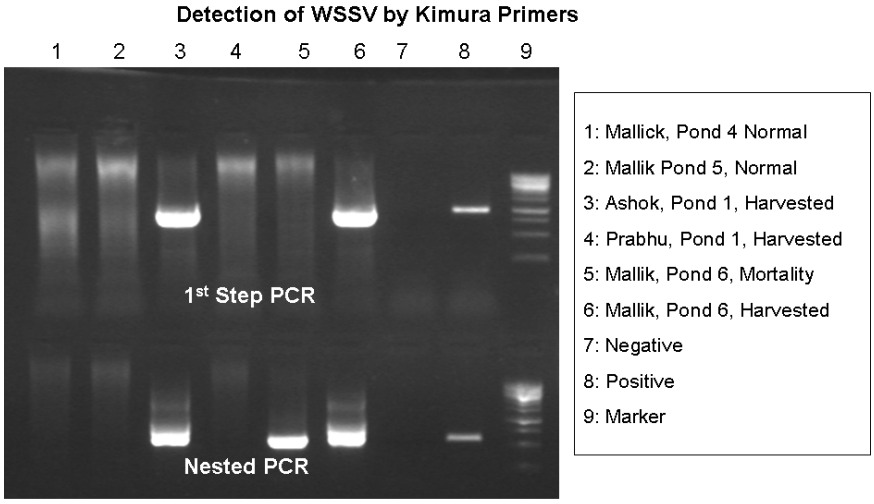
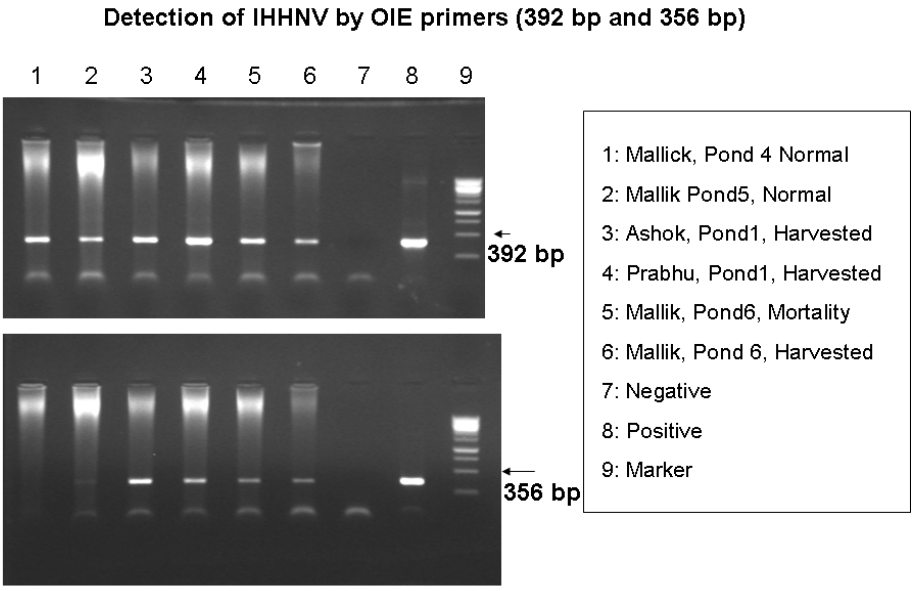


Fig: Diseases in *L. vannamei*

Fig. Molecular diagnostics of WSSV and IHHNV in farm reared L. vannamei





FUNCTIONALIZED CARBON NANOTUBES IN DRUG DESIGN AND DISCOVERY

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CONSPECTUS
Carbon nanotubes (CNTs) have been proposed and actively explored as multipurpose innovative carriers for drug delivery and diagnostic applications. Their versatile physicochemical features enable the covalent and noncovalent introduction of several pharmaceutically relevant entities and allow for rational design of novel candidate nonscale constructs for drug development. CNTs can be functionalized with different functional groups to carry simultaneously several moieties for targeting, imaging, and therapy. Among the most interesting examples of such multimodal CNT constructs described in this accounts is one carrying a fluorescein probe together with the antifungal drug amphotericin B or flurescien and the antitumor agent methotrexate. The biological action of the drug in these cases is retained or as in the case of amphotericin B constructs, enhanced, while CNTs are able to reduce the unwanted toxicity of the drug administered alone. Ammonium-functionalized CNTs can also be considered very promising vectors for gene encoding nucleic acids. Indeed, we have formed sable complexes between cationic CNTs and plasmid DNA and demonstrated the enhancement of the gene therapeutic capacity in comparison to DNA alone. On the other hand, CNTs conjugated with antigenic peptides can be developed as a new and effective system for synthetic vaccine applications. What makes CNTs quite unique is their ability to passively cross membranes of many different types of cells following a translocation mechanism that has been termed the *nanoneedle mechanism.* In that way, CNTs open innumerable possibilities for future drug discovery based on intracellular targets that have been hard to reach until today. CNTs excretion rates and accumulation in organs and any reactivity with the immune system will determine the CNTs safety profile and, consequently, any further pharmaceutical development. Caution is advised about the need for systematic data on the long term fate of these very interesting and versatile non-objects in correlation with the type of CNT material used. CNTs are gradually playing bigger and more important role in the emerging field of nonmedicine however, we need to guarantee that the great opportunities they offer will be translated into feasible and safe constructs to be included in drug discovery and development pipelines.

Introduction
The bioavailability and intrinsic toxicity of many potential low molecular weight drugs affect so much their pharmacological profile that their therapeutic efficacy is strongly compromised to the point that most of them have to be abandoned from late stage pharmaceutical development. This recurrent phenomenon in the drug development cycle has led to an almost critical situation plaguing the production pipelines of almost all pharmaceutical companies. This, in combination with the expiration of many patents protecting 'blockbuster drugs' is currently forcing the pharmaceutical industry to act creatively in rejuvenating their drug discovery programs and design more and better drug candidates for clinical development. One of the possibilities that can act as a potential source of new drug design strategies is the incorporation of nano technologies at an early stage in the drug development process. Moreover, the new drug conjugates are very expensive, and the search for new, more affordable constructs is highly desirable. The capacity of functionalized carbon nanotubes (*f* CNT) to act as carriers for the delivery of a wide range of

therapeutic agents, *f*-CNT conjugated to a drug can be also considered as a new entity with novel therapeutic or diagnostic properties.

Carbon Nanotube Functionalization Chemistry

CNT are tubular objects with a high aspect ratio and diameter in the nanoscale range. They can be classified by their structure into two main types: (i) single-walled carbon nanotubes (SWNT), which consist of a single layer of grapheme sheet seamlessly rolled into a cylindrical tube, and multiwalled carbon nanotubes (MWNT), which comprise multiple layers of concentric cylinders with the space of about 0.34 nm between the adjacent layers. From a chemical reactivity point of view, CNT can be differentiated in two zones: the *tips* and the *sidewalls* (**Scheme-1**).The tips are reminiscent of the structure of a fullerene hemisphere and are relatively reactive. The sidewalls can be approximately considered as curved graphite, the degree of curvature, of course, depending on the diameter of the tube. However, whatever the diameter, the reactivity of the sidewalls is considerably lower than that of the tips. Therefore, most reactions are expected to occur at the tips first and then, in some cases, at the sidewalls.

Since, the as produced CNT contain variable amounts of impurities, such as amorphous carbon and metallic nanoparticles. However, since the CNT tips do generally react under these conditions, the result is that the tubes open and the tips consist now of oxygenated functions, mainly carboxylic acids. Also, dangling bonds can react similarly, generating other functions at the sidewalls (**Scheme 2**). The carboxylic functions can, in turn, lead to further derivatization. After conversion to acid chlorides, reaction with amines can afford the corresponding amides (**Scheme 3**). Another opportunity is given by the reactivity of the sidewalls. Cycloadditions or radical reactions can be employed to covalently attach molecular appendages to the CNT sidewalls (**Scheme 4**).

Functionalized Carbon Nanotubes for Therapeutic and Diagnostic Application

If we assume that all the above described processes can be efficiently performed, we will have reached the goal of implementing three different functional units for potential targeting, diagnosis, and drug transport capabilities. All new functional groups, including amines and carboxylates, can be further modified with therapeutic agents to create CNT conjugates endowed with some kind of pharmacological activity. Nanotubes able to carry one or more therapeutic moieties with optical or other (e.g., magnetic) probes for imaging, and/or specific recognition signals for targeting, can offer multimodal options in the treatment of cancer and other types of complex diseases where activity is required only at specific sites in the body.

The possibility of developing CNT for biomedical applications became a reality after a series of powerful methodologies for their functionalization were described. Different approaches have been proposed to render the nanotubes soluble and compatible with physiological conditions. This is a key issue which is currently under careful and extensive examination from various laboratories.

The compounds shown in Table1 can be synthesized along the lines described above. The main features of compounds 1-9 are as follows:

1. The presence of hydrophilic appendages. All of the compounds bear an amino triethylene glycol chain for improving water solubility.
2. The presence of fluorescent probes as for compounds 3, 4, 5 and 7. Labeling can be performed, for instance, by condensing *f* CNT 1 or 2 with fluorescein isothiocyanae (FITC), giving 3 and 4, respectively. To improve water solubility, in some cases, e.g.,

4 it is possible to leave some of the amino groups unlabeled by playing with orthogonally protected functional groups.

3. The presence of amphotericin B as in the case of compound 5and 6. Amphotericin B as in the case of compounds a5 and 6. Amphotericin B is a potent commercially available antifungal agent.
4. The presence of the anticancer agent methotrexate as in the case of compound 7.
5. The presence of immunogenic peptides as in the case of compound 8 and 9.

Functionalized carbon Naontubes for Gene Delivery

Since *f* CNT 1 is cationic under physiological conditions, they were also found to efficiently complex and translocate DNA inside cells in the first studies exploring the capability of *f*CNT to deliver genes. We have produced the supramolecular complexes between *f* CNT 1 and plasmid DNA which express the βgalactosidase marker gene. The expression of the marker gene using *f* CNT 1 reached 5-10 times higher levels than the plasmid DNA delivered alone, which allows consideration of this novel approach for gene delivery as promising. the potential of gene therapeutics based on carbon nanotubes has been further explored for gene silencing applications. Complexes of single walled carbon nano tubes with siRNA strands modified with a hydrocarbon tail were used to target and specifically kill cancer cells. Further developments of this system are however necessary to validate the in vivo use of this methodology.

Functionalized Carbon Nanotubes for infectious Diseases

In another approach, carbon nanotubes could be funtionalized with antibiotics. Amphotericin B is an antimycotic agent used against particularly resistant fungal strains. It is, however, of limited use because it is highly toxic to mammalian cells, likely due to its low solubility in water and its tendency to aggregate and form pores in the cell membrane. The conjugation of amphotericin B to carbon nanotubes could modulate its properties in terms of toxicity and antimycotic efficiency. The first issue we addressed with *f*-CNT 5 was the cytotoxicity against mammalian cells. Amphotericin B is highly toxic at 10 μg/mL concentration, reaching 40% cell mortality, CNT conjugated AmB5 used at increasing concentrations (up to 40μg/mL, corresponding to a concentration of amphotericin B linked to the tubes of 10μg/mL), was not toxic. The cell penetration ability of *f*-CNT 5 bearing both amphotericin B and fluorescein was investigated. This latter component allows for the detection of *f* CNT 5 in the cells. The fluorescence was clearly evident inside the cell compartments. At the same time, the toxicity against fungi and yeasts was enhanced. Preliminary data (kindly provided by Prof. R. Gennaro and Dr. M. Benincasa, University of Trieste) Show that *f*-CNT 6 are also active on strains usually resistant to amphotericin B. The reason for this enhanced therapeutic effect is not totally clear. A more detailed study is currently underway to elucidate whether the mechanism of action of *f*CNT 6 is similar to that of amphotericin B itself or whether it differs in some aspects, then accounting also for the reduced toxicity to mammalian cells.

Functionalized Carbon Nanotubes for Oncology

f-CNT 7 contains a methotrexate molecule together with a fluorescein probe. Methotrexate is a well known and potent anti cancer agent, used also to cure autommune diseases. However, methotrexate suffers of low bioavailability and toxic side effects. Therefore, an increased bioavailability and a targeted delivery are highly desirable. *F* CNT 7 might offer the possibility for improving bioavailability and in the presence of a targeting unit, to address specifically cancer cells. Preliminary results hve shown that methotrexate conjugated to the nanotubes is as active as methotrexate alone

in a cell culture assay where jurkat cells were incubated up to 72h (unpublished data). The reason for lack of enhanced efficacy between the *f*CNT 7 and the nonconjugated drug could be attributed to the stable amide bond between methotrexate and the nanotubes. Indeed, the drug is probably released too slowly from the tubes into the cytoplasm for an efficient interactin with its receptor. It is certainly necessary to introduce a cleavable linker or a more enzymatically sensitive bond in the CNT 7 as demonstrated for conjugates based on dendrimers. However, *f*-CNT can be considered a promising construct, and we are currently improving its activity. Following an alternative approach, carbon nanotubes modified with a carborane moiety have been developed for cancer applications. Single walled CNT were functinalized with a substituted carborane cage for boron neutron capture therapy. The biodistribution study on different tissues showed that water soluble carborane nanotubes were concentrated more in tumor cells than in other organs when administered intravenously. These results were preliminary although also promising for future applications of carbon nanotube boron based agents for effective treatment of cancer.

Functionalized Carbon Nanotubes for Vaccination

Another class of carbon nanotube based therapeutic candidates consists of their constructs with synthetic peptide for immune system activation. The decoration of functionalized nanotubes with B and T cell peptide epitopes can generate a multivalent system able to induce a strong immune response. Peptide can be connected to the tubes using the chemoselective approach. This strategy is based on the preparation of functionalized carbon nanotubes with a maleimide group which readily reacts with peptides containing a cysteine residue at one end. The thiol group of cysteine selectively adds to the maleimide forming a stable covalent bond. The advantage of this method is that the peptide, obtained by solid phase synthesis, is fully deprotected and characterized before performing the ligation to the nanotubes. *f*-CNT 8 and 9 were prepared following this type of chemistry by linking a B cell epitope from a coat protein of foot and mouth disease virus (FMDV). The two conjugates differ in the amount of peptide around the nanotubes which was doubled for *f* CNT 9 using a lysine branch. The antigenic and immunogenic properties of these conjugates were measured. In particular, *f*- CNT8 and 9 elicited high antibody responses in comparison to the nonconjugated peptide. In addition, the generated antibodies had the capacity to neutralize the virus, thus demonstrating the potential of carbon nanotubes as components for synthetic vaccine development.

Conclusion

Besides the advances in novel synthetic chemistry that have led to the design and discovery of multiple small drug molecules, the product pipelines of pharmaceutical companies suffer from the lack of clinically viable product candidates. The incorporation of nanotechnology tools at early stages in the drug development process may offer new ways of rediscovering old drug molecules and also offer new discovery and design options for new molecules. Our laboratories have developed functionalized CNT with the objective of creating a completely new component for drug development. We described different strategies by which carbon nanotubes can be conjugated with many different small molecules or macromolecules that can act as therapeutic agents in order to achieve improved therapeutic efficacy. The current available examples in our laboratories include small molecule anticancer and antibiotic carbon nanotube conjugates as well as antigen presenting conjugates for the potential fabrication of vaccines and plasmid DNA complexes for construction of gene therapeutics. Although it may be premature at this stage to claim that carbon a nanotubes will be clinically

successful therapeutics, the results highlighted in this Account can certainly be considered promising. We anticipate carbon nanotubes to play a critical role as exemplary nanomaterials that can be clinically developed and constitute archetypal cases in the emerging field of nanomedicine.

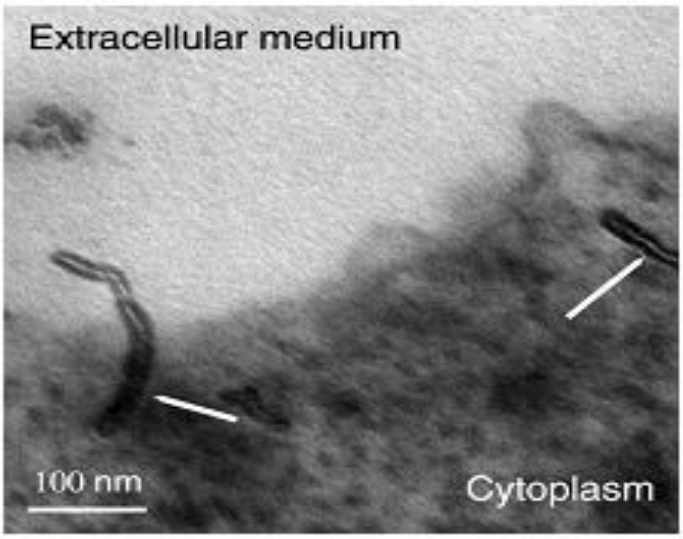
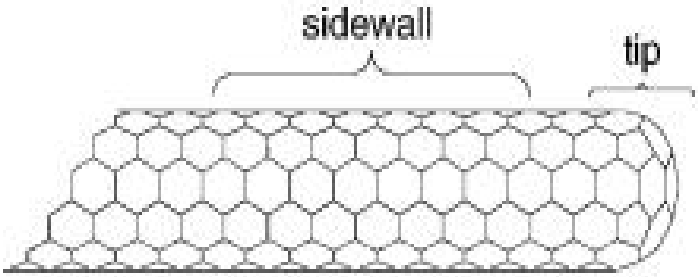
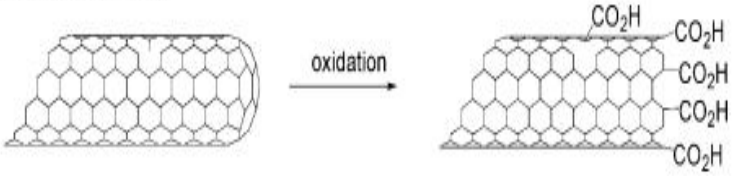


FIGURE 1. Ultrathin transverse section of a HeLa cell treated with ammonium-functionalized CNT **1**. Carbon nanotubes are crossing the cell membrane or visible into the cytoplasm (white arrows).

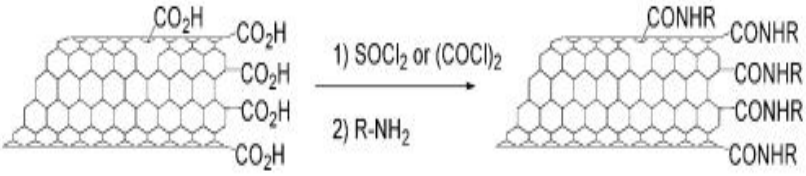
SCHEME 1. Molecular Structure of a Carbon Nanotube with Highlighted Tips and Sidewalls



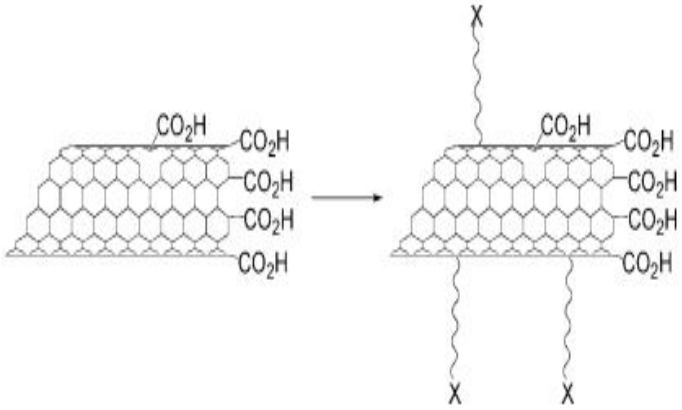
SCHEME 2. Oxidation of Carbon Nanotubes



SCHEME 3. Amidation Reaction of Oxidized Carbon Nanotubes



SCHEME 4. Functionalization of Carbon Nanotubes Using Addition Reactions (X = Functional Groups)



SCHEME 5. Insertion inside Carbon Nanotubes

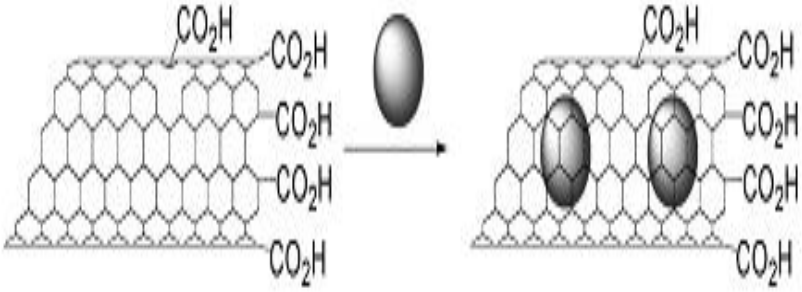


TABLE 1. Molecular Structures of the Carbon Nanotube Conjugated with Different Therapeutic Agents

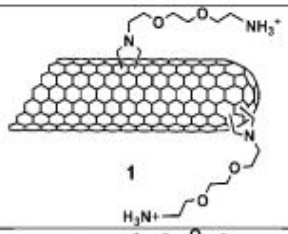
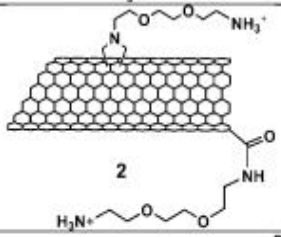
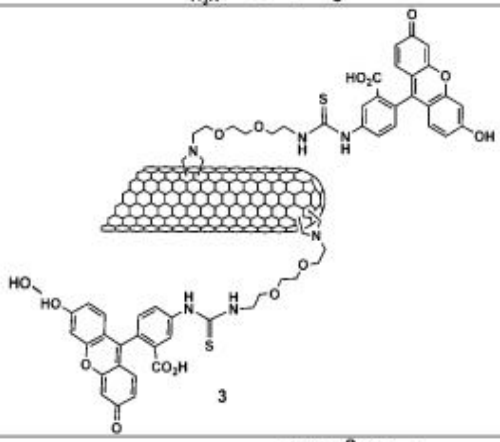
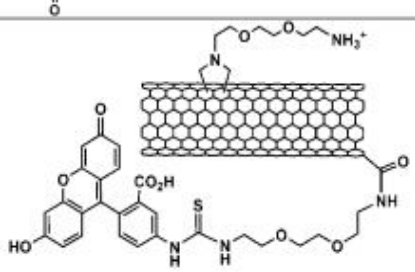
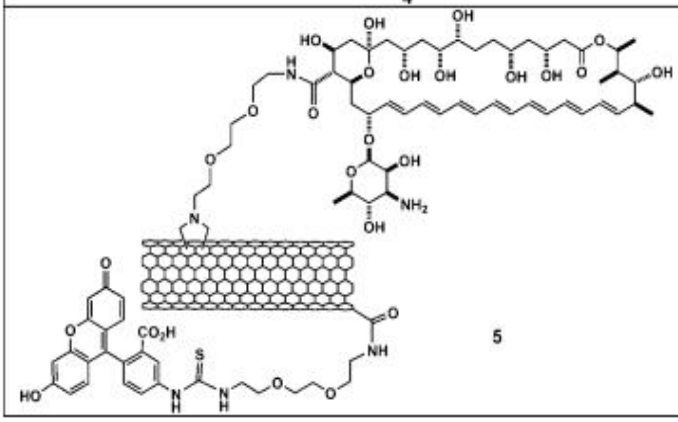
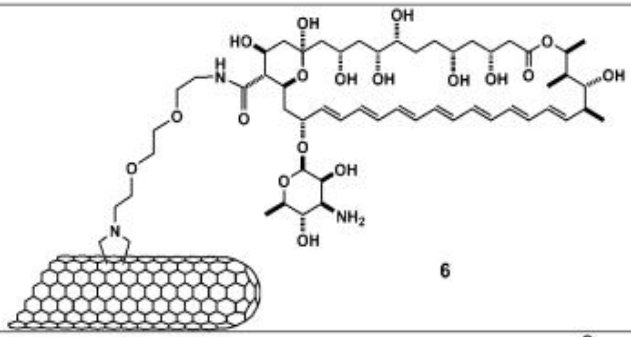
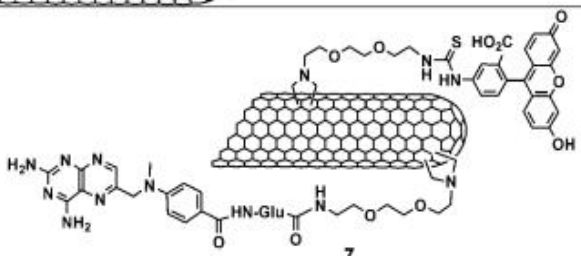
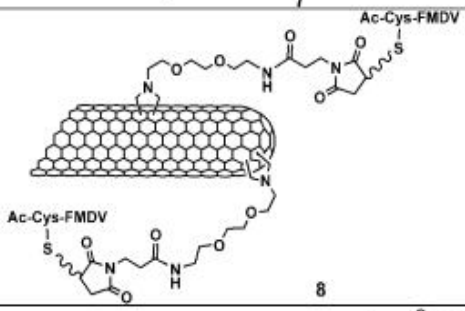
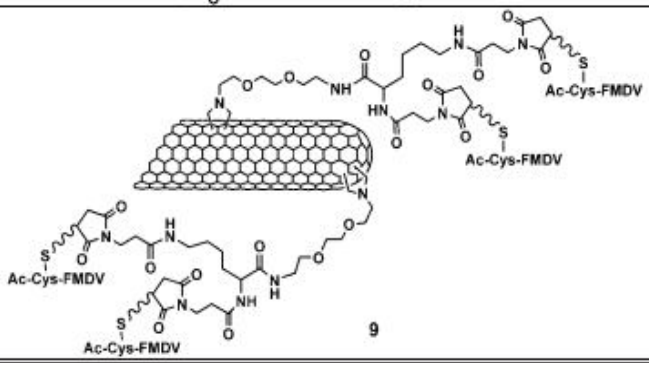
Compounds	Bioassays
 1	Cell internalization[24, 26, 27] Intracellular trafficking[24, 27] Cell viability[26] Plasmid DNA delivery[26, 32]
 2	Precursor for the preparation of CNT **4** and **5**
 3	Cell internalization[23-25] Intracellular trafficking[23-25] Cell viability[23-25]
 4	Cell internalization[24]
 5	Cell internalization[22, 24] Cell viability[22]

TABLE 1. Continued

Compounds	Bioassays
 6	Antibiotic delivery[22]
 7	Cell internalization[36] Cell viability[36] Anticancer delivery[36]
 8	Immunogenic activity[41, 42] (FMDV peptide corresponds to the 141-159 region of the viral envelope protein VP1 from foot-and-mouth disease virus)
 9	Immunogenic activity[42] (FMDV peptide corresponds to the 141-159 region of the viral envelope protein VP1 from foot-and-mouth disease virus)

MOLECULAR ASPECTS OF FUNGAL GENOME STUDIES

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INTRODUCTION

Under ideal conditions a healthy fish might be a carrier of numerous pathogens without showing any symptoms of disease. However under stressful conditions these symptoms are manifested. Under intensive aquaculture, stress increases hence there is ample chance of disease outbreak . Disease causes the largest economic losses in aquaculture and fungal infections are second only to bacterial disease in economic importance. (Ramaiah et al., 2006). Fungal infections are generally restricted to chronic, steady losses (Bruno et al., 1994). Fungal infestations of fish are widespread in freshwater and represent the most important pathogenic group affecting wild and cultured fish. Almost every freshwater fish is exposed to at least one species of fungus during its lifetime (Noga, 1993). *Aphanomyces invadans* is a peronosporomycete fungus associated with the serious fish disease, Epizootic Ulcerative syndrome (EUS). A survey of fish affected with EUS taken from outbreaks in countries from South and South-East Asia where mostly found to be members of Saprolegniaceae family including *Aphanomyces.* The mycelium from the strains produces an inflammatory response in healthy fish leading to myonecrosis with chronic epithelial reaction (Roberts *et al*.,1993).This disease has affected wild and farmed fish in Asia and Australlia over the last 30 years (Lilley *et al* 2003). *Dermocystidium marinum* is best known as a pathogen of oysterin eastern United states (Mackin et al., 1950). The most economically important of these genera, Saprolegnia, is a serious problem in aquaculture and numerous reports have implicated it as the etiological agent of fish mycosis (Molina et al., 1995). The *Saprolegniaceae*, in particular of the species *Saprolegnia parasitica*, is responsible for huge fungal infection, involving both living and dead fish, particularly in aquaculture facilities. It also infects moribund eggs by adhesion to and penetration of the egg membrane, and can spread from dead eggs to live eggs via positive chemo taxis.Gustafson and Rucker(1956) reported that Ichthyosporidium is a fungus, but it manifests itself internally. It primarily attacks the kidneys and liver, but it spreads everywhere else. The fish may become sluggish, lose balance and eventually show external cysts or sores. Exophiala salmonis and E.psychrophila; these fungal organisms have hyphae that are septated, irregular in width and branched (Robert, 1989). Both fungal diseases infected the many species of fish. Fish become darker and lethargic, with erratic and abnormal swimming behavior.Round yellow to white granulomas are present in visceral organs like liver, kidney and spleen with prominent enlargement of the posterior kidney(Verma 2008).

From fish pathologfist's viewpoint, there are mycoses that hinder the function of organs and kill the fish on mass scale and also there are mycoses depriving fish body of its natural strength. Fungal infections are common among many fish species and can prove fatal if not treated early. Scarfe et al. observed that aquaculture bio-security programs addressing aquatic animal pathogens and disease have become an important focus for the aquaculture industry. Disease outbreaks have threatened profitable and viable aquaculture operations throughout the world. Thus, information exchange between leading expert in different countries will have to increase for successful combating of infectious diseases (Ramaiah, 2006).

Karunasagar et al 2004, suggest that nearly 500 fungal species have been isolated from marine and estuarine environment of which a few are pathogenic to shrimp. Mostly larval stage of shrimp are affected commonly by *Lagenidium callinectes* and *Serolpidium* spp.Usually, fungal spores and mycelia can be observed in affected tissue, particularly in gill and appendages. Mycosis is problematic for larval stage in many hatcheries in India. Gopalan et al reported *Lagenidium marina* ande *Sirolpidium parasitica* infection in *P. monodon. Fusarium* spp. are opportunistic pathogens that may lead to high mortalities (90%).

Table 1: Species of Fungi reported as parasites of aquatic animals

Species	Host	Reference
Dermocystidium (*D.perce, D.pusula*)	Freshwater fish	Neish and Huge (1980)
Achlya	Freshwater fish and egg	Neish and Huge (1980
Aphanomyces (*A.astaci* , *A.piscida*)	Freshwater fish and egg	Wilson (1976)
Saprolegnia (*S. australis, S.delica*)	Freshwater fish and egg	Neish and Huge (1980)
Mucor	Softshell turtle	Jacobson (1980)
Fusarium (*F.solani, F.colmorum*)	Shrimps, fish	Macri et al (1984)
Aspergillus (*A.niger*)	tilapia	Olufemi and Roberts (1983)
Branchiomyces (*B demigrans.*)	Freshwater fishes	Wundsch (1929)
Lagenidium (*L. callinectes*)	Crab, Shrimp, egg and larvae of mangrove Crab	Couch (1942)

A thorough understanding of the basic molecular process, the nature of interaction with its hosts, and the identification of genes and proteins involved in these processes , could lead to novel control strategies that increase fish health, reduce disease losses and increase profit (Torto- Alalibo et al 2005). The DNA based composition analysis has been examined and is thought to be a promising potential taxonomic tool for infra-generic separation of *Oomycetes* (Green and Dick, 1992).

FUNGAL GENOME STUDY

Molecular markers have become enormously important in many areas of fungal biology, including strain typing, epidemiology, population genetics, fungal detection and identification genetic mapping, gene isolation, phylogenetics and evolutionary biology (Mc Dade and Cox 2001). These markers are based on minor difference that accumulates in the genome of members of a species as they diverge from one another over time. Markers can be present in the organism in a single copy or repated in multiple copies throughout the genome. Restriction fragment length polymorphisms and sequences of ribosomal DNA confirmed that A. invadans is distinct from all other species studied. A sequence from the internal transcribed spacer region ITS1, unique to *A. invadans*, was used to design primers for a PCR-based diagnostic test (Lilley et al., 2003). Restriction Fragment length polymorphism (RFLP) analysis and sequence of ribosomal DNA (r DNA) are used to compare with related taxa, with the aim of developing a specific PCR- based diagnostic test for *A. invadans.* Random amplification of Polymorphic DNA (RAPD) used to determine

whether strain difference could be identified between geographically diverse isolates. RAPD was previously been shown to be a sensitive method of distingushing **genotypes of the crayfish plague fungus, *Aphanomyces astaci* (Lilley et al 2003).**

Marker gene

Reliable methods for estimating genetic diversity within and between species are crucial for studying populations, classifying germplasm, breeding and identifying genes conferring particular traits. A comparision of the common DNA marker system has been presented elsewhere (Rafalski et al 1996). There are a number of "universal" sequences that can be used as markers in a range of different fungal genomes, with little or no prior knowledge of the genome. One widely used example is the r RNA gene, which contains both highly conserved and variable regions. Universal primers exist that can amplify regions of this gene from essentially any fungal species (White et al., 1990). Primes and probes can also be developed based on highly conserverd motifs found in fungal genomes.

Microsatellites

Microsatellites or simple sequence repeats (ssRs) are tandem array of short DNA sequences composed of 1-6 base pairs motifs. They are found ubiquitously in eukaryotic genomes, including fungi and are also found in some prokaryotic genomes. Microsatellite have been found in coding and non-coding regions and are co- dominantly inherited. They are often characterized by a high degree of length polymorphism; consequently the number tandem repeats at a given locus can vary from one individual to the next. Simple sequence repeats (SSRs) also known as microsatellites, comprise tandemly repeated genetic loci of 1-6 base pairs (bp) (Tautz and Renz 1984). SSRs are highly abundant, and exhibit extensive levels of polymorphisms in eukaryotic (Katti, Ranjekar, and Gupta 2001) and prokaryotic (Gur-Ari et al. 2000) genomes. They are found in protein-coding and non-coding regions (Katti, Ranjekar, and Gupta 2001; Toth, Gaspari, and Jurka 2000), with SSRs being more abundant in noncoding regions than in exons. Variation of SSRs is mainly due to slipped strand mispairing and subsequent resulting errors during DNA replication, repair and recombination (Tautz and Schlötterer 1994). Sequence polymorphisms in these loci arise by insertion or deletion mutations of one or more repeat units (Tautz and Renz 1984). SSR loci have high mutation rates ranging from 10-3 to 10-6 per generation (Xu, Peng, and Fang 2000). The mutation rate of SSRs generally increases with the increase of the repeat unit (Wierdl, Dominska, and Petes 1997). Fixation of de novo generated SSRs is determined by the interplay between the repeat type, the genomic position of a specific SSR, and the genetic/biochemical background of the cell (Toth, Gaspari, and Jurka 2000). It has been shown that different taxa exhibit different preferences for SSR types.

Internal Transcribed Spacer (ITS)

Advances in molecular technology show great potential for the rapid detection and identification of fungi for medical, scientific and commercial purposes. Numerous targets within the fungal genome have been evaluated, with much of the current work using sequence areas within the ribosomal DNA (rDNA) gene complex. This section of the genome includes the 18S, 5.8S and 28S genes, which code for ribosomal RNA (rRNA) and which have a relatively conserved nucleotide sequence among fungi. It also includes the variable DNA sequence areas of the intervening internal transcribed spacer (ITS) regions called ITS1 and ITS2. ITS (for internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Read from 5' to 3', this polycistronic rRNA precursor transcript contains the 5'

external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA and finally the 3'ETS. Although not translated into proteins, the ITS coding regions have a critical role in the development of functional rRNA, with sequence variations among species showing promise as signature regions for molecular assays. Multiple applications using the fungal ITS sequences are summarized here including those for culture identification, phylogenetic research, direct detection from clinical specimens or the environment, and molecular typing for epidemiological investigations. The breadth of applications shows that ITS regions have great potential as targets in molecular-based assays for the characterization and identification of fungi. (Iwen et al., 2002) The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner. Each repeat unit has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions. In some groups (mostly basidiomcyetes and some ascomycetous yeasts), each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vay among groups. Several restriction sites for EcoRI and BglII are conserved in the rDNA of fungi. Nearly all basidiomycetes we've studied share an EcoRI site within the 5.8S RNA gene along with a BglII site halfway into the LSU RNA sequence. Primers 5.8SR and LR7 include these restriction sites, which makes them convenient for cloning. The ITS region is now perhaps the most widely sequenced DNA region in fungi.[citation needed] It has typically been most useful for molecular systematics at the species level, and even within species(Chen et al ., 2001)

Isothermal systems do not require a thermalcycler to produce rapid temperature changes, but require only a simple platform such as heating blocks or water bath. Isothermal systems include rolling circle amplification (RCA) and loop-mediated isothermal amplification (LAMP).

Padlock probe technology and Rolling circle Mechanism

Detection and characterization of Single Nucleotide Polymorphism (SNPs) is becoming increasingly popular for pathogen identification, which had become a challenge for RT-PCR. Padlock probes (PLPs) are long oligonucleotides (about 100 bp) carrying a non-target-complementary segment flanked by the target complementary regions at their 5'and 3'ends,which recognize adjacent sequences on the target DNA(Nilsson et al. 1994). Briefly, various padlock probes are designed to target organisms and ligatedto DNA extracted from environmental samples or cultures (Van Doorn et al. 2007).

This method for SNPs detection based on DNA ligase-mediated single nucleotide discrimination with consecutive signal amplification by H-RCA has been developed for various groups of pathogenic organisms, including fungi, bacteria, and viruses (Kong *et al*. 2008, Zhou *et al*.2008, Kaocharoen *et al*. 2008,Wang *et al*.2009,2010)

<u>RAPD Randomly Amplified Polymorphic and Restriction Fragment Length Polymorphisms (RFLP)</u>

The technically demanding method of random amplified polymorphic DNA (RAPD) has been applied to the study of crayfish plague fungus, A. astaci (Huang et al., 1994). RAPD uses a single primer in low-stringency polymerase chain reactions (Welsh and McClelland, 1990; Williams et al., 1990). Random binding of primers results in different sizes of fragments from samples with nonidentical DNA. Application of the RAPD technique grouped different isolates of the fungus and provides the means to carry out

epidemiological investigations (Lilley et al., 1997). The method has also been used to examine another Aphanomyces species that has resulted in serious losses in both farmed and wild fish in Asia (Lilley et al., 1997). Other fish pathogens have been studied using RAPD, but problems with reproducibility and risks of contamination render the method unsuitable as a stand-alone method of diagnosis (Cunningham 2002). A PCR to examine ribosomal DNA from *Saprolegnia* isolates obtains from many geographical locations were developed by Molina *et al.* (1995). They discovered that the use of endonuclease BstUI produced identical fingerprints from all strains of *S. parasitica* examined, and suggested that this could form the basis of a diagnostic test to be applied in the absence of antheridia and oogonia. Dieguez-Uribeondo *et al.* (1996) have also developed a method employing RAPD, using PCR with DNA from *S. diclina-parasitica* complex isolates. They showed that Spanish isolates of this fungus had a genetic similarity of 85-100%, compared with only a 20-45% similarity with other strains of *S. diclina-parasitica* complex. Whisler (1996) noted that *S. parasitica* isolates from the Colombia river basin, USA recognized by RAPD analysis, were representative of the vast majority of the isolates cultured from fungal lesions. The use of single and paired primers with PCR amplification permitted identification of pathogenic groups and their distinction from other species of the genus considered more saprophytic in character (Whisler, 1996). Bangyeekhun *et al.* (2003) characterized thirty-one isolates of *Saprolegnia species* from infected salmon and trout by using RAPD-PCR and found majority of the isolates (6 of 31) was genetically almost identical. Restriction enzymes recognise specific short sequences of DNA and cleave the DNA at that site. Single nucleotide changes can result in the gain or loss of a restriction site, thus altering the number of fragments produced following digestion of DNA. These restriction fragment length polymorphisms (RFLP) can be visualised following gel electrophoresis of the digested DNA to separate the fragments according to size. Restriction enzyme digestion of total genomic DNA preparations may produce fragment polymorphisms from different samples. However, detecting these differences within a mixture of many fragments of varying size usually requires the use of probes to identifythe polymorphic fragment. Lilley *et al.* (2003) studied inter-specific relationships between *A. invadans* isolates and other aquatic animal pathogenic *Saprolegniaceae* from EUS affected areas by RAPD and RFLP.

DNA Microarray analysis

Microarray technology is a technique used to identify unique DNA (RNA) sequences first introduced by Schaena *et al.*(1995). DNA probes either short oligos or large fragments of DNA are radiolabelled or a chemiluminescent reporter are used to detect specific DNA sequence. The sample DNA that hybridizes to a specific location on the microarray can be detected by fluorescent array detection and the data analyzed by computer programs. Often more practical is to use competitive hybridization in which the test sample competes for hybridization to the tethered oligonucleotide, on the chip, with a fluorescent labeled competitor oligonucleotide. A fluorescent microarray detector and computer program can then analyze the fluorescent array for the presence or absence of the species/strain specific DNA sequence. Compared with traditional nucleic acid hybridization with membranes, microarrays offer the additional advantages of high density, high sensitivity, rapid detection, lower cost, automation, and low background levels (Shalon *et al.*, 1996).For most of the pathogens genetic sequence are available in the Genbank ,so oligonucleotide probe complementary to different strains can be made and added to the same chip. Thus several fungal strains can be identified simultaneously. As a result, microarray-based technology is potentially well suited for identifying
fish pathogens in fish populations . Microarrays are already proving valuable for assaying gene expression. A large number of probes on an array can reveal which genes are expressed or are present in the sample. This type of array would be particularly useful in studies of

pathogens, where the presence of certain genes or gene products indicate whether the organism is pathogenic or not. With the advent of customized oligonucleotide probes they can be made to identify specific fungal strains using ITS or ribosomal RNA information.DNA microarray technology will be used in the future for fish diseases diagnosis especially during the asymptomatic period of diseases.

CONCLUSION

Molecular tools are increasingly relevant to fish diseases. The sequencing of the complete genomes of pathogens is allowing great advances in studying the biology, and improving diagnosis and control of pathogens Molecular methods involving gene sequencing are objective, yield results that are uninfluenced by growth conditions, are frequently more rapid than phenotypic approaches and are capable of discriminating between fungi that fail to produce distinctive morphological features. Recent molecular studies have shown that many 'single species' of fungi pathogenic to humans in fact contain several phylogenetically distinct entities. Therefore, molecular biology can be a routine tool in the search for improved methods of diagnosis and control of fish pathogens and the epidemiology of infectious fish diseases

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DNA BASED DIAGNOSTICS AND PATHOGEN DETECTION IN AQUACULTURE

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Occurrence of diseases imposes heavy loss to aquaculture production and trade affecting both the economic development and socioeconomic revenue of the sector in several countries in the world. Apparently healthy looking carrier fish creates disaster by spreading contagious diseases in the fish populations. At stress condition, only manifestation of the disease becomes evident. With the technological advancement and improvement in culture practices intensive aquaculture is in the state of realization. Under this system of aquaculture the risk of stress increases and significant portion of the stock may be infected. With increasing worldwide demand for fish food, there is an alarming need for research to identify solutions to fish health problems.

Various factors have been related to the apparent increased incidence of diseases. It is widely recognized that diseases are outcome of complex interactions between pathogen abundance, host susceptibility and more importantly unfavorable environmental factors. As in most of the situations occurrence of the disease may change depending upon the season of the year and environmental conditions (Plumb, 1999), it may be difficult to sample an infected fish from a population. Therefore, an effective response to disease outbreaks requires a comprehensive diagnostic plan that addresses each phase of the outbreak: surveillance, response and control. Difficulty in getting affected individuals or low level of prevalence constraints consistent identification of causative agents in a fish population. Further the chances of identifying pathogen from carrier fish is increased by testing larger numbers of fish depending on the number of accuracy desired, the population size and assumed frequency of occurrence. Therefore, a broad spectrum of sensitive and specific diagnostic assays is crucial in providing timely and accurate diagnosis for immediate response for disease control.

Conventionally, disease diagnosis is carried out by in-vitro cultivation followed by phenotype and serological typing of the pathogens or histological investigation (Bernardet *et al.*, 1990; Pazos *et al.*, 1996). Additionally accurate identification of phenotypically similar bacteria of the same genera poised a problem (Shewan and McMeekin, 1983). Further. Biochemical tests have been employed in such cases to correctly identify the pathogens, but these techniques have some disadvantages such as need for previous isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen.

The advent of molecular biology techniques paved the way for understanding the host pathogen interaction at molecular level. At present molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish diseases and the epidemiology of bacterial, viral and parasitic diseases. Utility of nucleic acid detection techniques for identification of hardly cultivable, non-cultivable, and even dead microorganisms and scope for improvement have been realized. Molecular diagnostic techniques can be used to solve the problems faced by traditional methods, typically represent highly sensitive and specific means of detecting the nucleic acids of pathogens. Molecular diagnostic tools have been widely adopted for aquatic pathogen surveillance due to the attribute like speed, accuracy and robustness. Typing of pathogens from carrier fish is possible through employing molecular diagnostic tools thereby preventing disease outbreaks. Thus antibiotic treatment can be reduced therefore; creation of antibiotic resistant bacteria may be eliminated. These techniques include polymerase chain reaction (PCR),

restriction enzyme digestion, probe hybridization, in situ hybridization, microarray, real time PCR and next generation sequencing.

A. **(i) Polymerse Chain Reaction (PCR)**

A portion of DNA is specifically defined by a pairs of two "primers" is at least a million fold amplified in in-vitro condition through employing thermostable DNA polymerases in PCR. Generally the size of the PCR product varies from 150 bp to 3000bp and detected by gel electrophoresis (McPherson *et al*., 1991). The sensitiveness of the techniques greatly depends on the primers. Therefore, the primers should be sufficiently long to allow a high annealing temperature and reduce the opportunity for nonspecific primer annealing, but primers that are too long may facilitate nonspecific annealing even to regions of DNA that are not perfectly complementary to the primer sequence. The cocktail of reaction includes template DNA, primers, polymerase enzyme to catalyze creation of new copies of DNA, and nucleotides to form the new copies. The PCR reaction is carried out in a thermocycler and during each round of the thermocycling reaction, the template DNA is denatured, primers anneal to their complementary regions and polymerase enzyme catalyses the addition of nucleotides to the end of each primer, thus creating new copies of the target region in each round. In comparison to eukaryotic rRNA prokaryotic rRNA contains highly conserved sequences. In combination of PCR technology and conserved nature of prokaryotic rRNA, it is now possible to identify bacteria that is very difficult or impossible to culture citis made

In the similar fashion specific mRNA is detected employing reverse transcriptase ploymerase chain reaction (RT-PCR). In comparison to northern blotting analysis and RNase protection assay RT-PCR can be used to quantify mRNA levels from much smaller samples enabling quantification of mRNA from a single cell. With the advent of novel chemistries and instrument platforms it is now possible to detect PCR products on a real time basis (real time RT-PCR or qPCR) (Lockey et al. 1998). Therefore, it has been chosen as the method of choice for measuring level of gene expression worldwide. RT-PCR is the perfect choice of techniques for use in the disease surveillance and monitoring of infections due to its specificity and sensitivity.

(ii) Multiplex PCR

Simultaneous detection of more than one pathogen will increase the efficiency at the same time reduce the cost of the PCR technology. With the improvement in the formulating of PCR condition and advances in the primer designing methods it s now possible to detect several pathogen in a single reaction there by countering one of the foremost opinion against use of these technique as routine screening procedures (Williams *et al*., 1999). Multiplex PCR has the capability of potentially reducing the time, cost and effort within the laboratory without compromising its usable quality. Since the time of its inception in the field of infectious diseases it has been successfully utilized for the identification of viruses, bacteria, fungi and parasites.

(iii) Nested PCR

When amplification of a shorter fragment is targeted with the help of a second set primers on the first stage PCR product it is referred as nested PCR. Nested PCR increases the sensitivity of the PCR and at the same time two PCR products were produced for confirmation purpose. This technique has been successfully employed to detect a number of fish pathogens including betanoda virus of shrimps and white tail disease of *Macrobrachium rosenbergii*. However, one disadvantage of the nested PCR is the increased risk of cross-contamination due to the opening of amplification tubes to add an additional set of primers.

(iv) LAMP-PCR

Loop-mediated isothermal amplification is a novel method of amplifying DNA under isothermic conditions, and has been used by a number of authors to detect bacterial, parasitic and viral fish pathogens. It is faster and more sensitive than conventional PCR, and capable of detecting as few as six copies of DNA in the reaction mixture. (Notomi et al. 2000). The complete LAMP procedure can be performed in 90min and as it is carried out under isothermal conditions, it can be performed without the use of a thermocycler, making it suitable as a field test if a small oven is available.

(v) Random Amplification of Polymorphic DNA (RAPD)

The basis of RAPD technique is differential PCR amplification of genomic DNA. It deduces DNA polymorphisms produced by ''rearrangements or deletions at or between oligonucleotide primer binding sites in the genome'' using short random oligonucleotide sequences (mostly ten bases long). As the approach requires no prior knowledge of the genome that is being analyzed, it can be employed across species using universal primers. This technology successfully employed for phenotype typing of several fungal and bacterial pathogens (Sadok et al., 2013; Ye et al., 2013). The disadvantages with RAPD are its reproducibility risks of contamination render the method unsuitable as a stand-alone method of diagnosis. In the other hand RAPD can be a useful technique as a first step in the development of specific primers or probes and has been used in such a way in the study of several fish pathogens.

(vi) Amplified Fragment Length Polymorphism (AFLP)

AFLP is rapid PCR based techniques which combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers. Similar to RFLPs, the molecular basis of AFLP polymorphisms includes indels between restriction sites and base substitutions at restriction sites and base substitutions at PCR primer binding sites like RAPDs and AFLP can be used for typing prokaryotes and eukaryotes. In comparison to RFLP, AFLP requires small amount of purified genomic DNA to be digested with two restriction enzymes, one with an average cutting frequency (like *Eco*RI) and a second one with a higher cutting frequency (like *Mse*I or *Taq*I). Double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation, which allows simultaneous restriction and ligation, while religated fragments are cleaved again. An aliquot is then subjected to two subsequent PCR amplifications under highly stringent conditions with adapter specific primers that have at their 3' ends an extension of one to three nucleotides running into the unknown chromosomal restriction fragment (Ajmone-Marsan *et al.*, 1997). Its major weakness includes the need for special equipment such as automated gene sequencers for electrophoretic analysis of fluorescent labels; traditional electrophoretic methods can also be employed but they require the use of radioactive labels or special staining techniques such as silver staining. On the other hand AFLP procedures based on one RE enzyme with a single adapter and analysis by agarose gel electrophoresis can be used for typing.

B. Restriction Enzyme Digestion

Restriction enzymes (RE) or restriction endonucleases or molecular scissors have been predominantly isolated from prokaryotes. Restriction enzymes cleave the DNA in a very specific manner and three types of restriction enzymes were seen. Out of three types of RE type-II are most commonly used for DNA analysis and genetic engineering, each have a unique nucleotide sequence at which it cuts a DNA molecule. A particular RE cuts DNA at a particular recognition sequence, which may be a four, six or eight base pair palindromic

sequence. Due to its specificity restriction endonucleases were commonly used to generate fingerprint of a DNA molecule by cutting it into discrete fragments, which can be separate on gel electrophoresis. This pattern of DNA fragments generates a "DNA fingerprint" and each DNA molecule has its own fingerprint. The location of these restriction enzyme recognition sites on the DNA molecule can be compiled to generate a restriction enzyme map (Grizzle *et al.*, 2002). These maps are very useful for identifying and characterizing a particular DNA molecule (antibiotic resistance containing plasmid) or a particular region in the DNA.

C. Restriction Fragment Length Polymorphism (RFLP)

RFLP is the most widely used hybridization-based molecular techniques. RFLP technique was first used in 1975 to identify DNA sequence polymorphisms for genetic mapping of a temperature-sensitive mutation of adeno-virus serotypes (Grodzicker *et al.*, 1974). RFLP is a technique in which organisms may be differentiated by analysis of fragment patterns derived from cleavage of their DNA. This is possible due to the variation of the recognition site of a particular restriction endonuclease between the organisms. For example, if two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with that restriction enzyme. The variation in fragment patterns generated by the RE can be used differentiate species (and even strains) from one another. However, it is labor intensive and time consuming as it requires large quantity of DNA. With the advent of PCR technology it is now possible to amplify very small amount of DNA usually within 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time.

D. In-situ Hybridization

Fluorescence in situ hybridization detects nucleic be acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. In case of clinical microbiology ribosomal DNA or RNA is used as probe and the target is usually whole cell. The probes used are generally around 20 nucleotides in length. The probes are incubated in the presence of cells under appropriate conditions to permit specific hybridization of probe to target nucleic acid. Cells types that contain ribosomes with complementary RNA sequences become labeled by the binding of the fluorescent probe in situ. These labeled cells can then be visualized by flow cytometric or fluorescence microscopy. However some of the disadvantages of these techniques are false positive due to autoflourescences, lack of penetration, low RNA content and photo bleaching etc.

E. DNA Microarray

DNA microarray technology is based on the principles of hybridization where a large number of target sequences (RNA/DNA) are hybridized in parallel to a large number of DNA sequences (probes), immobilized on a solid surface in an ordered array. This is originally developed for the mapping of genes, is being used to detect a wide variety of pathogens. There are a number of ways of using DNA microarrays for the detection of unique DNA (or RNA) sequences. One method is to fluorescently label all the DNA sequences in the test sample.

Due to several attributes such as high density, high sensitivity, rapid detection, lower cost, automation, and low background levels (Shalon *et al.*, 1996), DNA microarray technology is the preferred method of identification of aquatic pathogens in recent years.

Availability of genetic sequences of most of pathogens in the public databases further facilitated designing of oligonucleotide probes complementary to all pathogens and can be inserted into microarray so that variety of microbes could be detected by a single microarray chip. The microarray techniques does not require such sequence conservation, however, because all of the diverse gene sequences from different populations of the same functional group can be fabricated on arrays and used as probes to monitor contagious fish disease especially during the asymptomatic period of the diseases (Zhou and Dorothea, 2004). The sample DNA that hybridizes to a specific location on the microarray can be detected by fluorescent array detection and the data analyzed by computer programs. Specific patterns of fluorescence are detected by a microarray reader which allows the identification of specific gene sequences found only in the aquatic pathogen of interest. This technology has the potential to identify the presence of agents of interest at the serotype or subspecies level, or to differentiate agents that cause similar clinical signs. Although the initial set up cost is high once established, cost per unit of sample analyzed will be low. Additionally, analysis time is short making it suitable to be used in the future for fish diseases diagnosis especially during the asymptomatic period of diseases.

F. Next Generation Sequencing

Conventional phenotyping of pathogen is based on morphological and biochemical tests which require the organism to be isolated by growth in-vitro condition. Only a small fraction of all bacteria can be successfully cultured, while clinically significant organisms may be slow-growing, fastidious, inert, or unviable. With the introduction of sequencing technology 16S ribosomal RNA (rRNA) gene sequencing is a popular alternative to traditional methods and provides several advantages. DNA sequencing is more accurate over traditional cultured based identification system. At the same time it is economic, less time consuming and labor intensive. However, 16S rRNA gene sequencing using bulk PCR products cannot be applied to polymicrobial specimens: the presence of multiple templates results in superimposed Sanger reads that are generally uninterpretable. Over the last several years, the advance in sequencing technologies has delivered the next-generation sequencing machines, which employ massively parallel approaches to produce millions of short DNA sequence reads in a single run. Their high-throughput capacity of sequencing DNA is also rapidly changing the landscape of life sciences and providing many novel biological applications to explore previously unanswered questions. The power of next generation sequencing in clinical epidemiology is realized through metagenomics research circumventing inherent limitations of Sanger's sequencing. Deep sequencing through massive parallel sequencing provides individual sequencing data for millions of DNA molecules allowing each to be classified independently. Next-generation 16S rRNA gene sequencing methods have potential for immediate utility for characterizing mixed infections from specimens containing non-viable or unculturable clinically important fish pathogens.

Conclusion

With the advent of molecular biology techniques molecular diagnostic tools are progressively more applicable in fish diseases. Deducing the complete genome sequence information of clinically important organisms is allowing great advances in studying the biology, and improving diagnosis and control of pathogens. Employing new methods of analyzing variation of target DNA sequences and sequence information can improve specificity, sensitivity, and speed of diagnosis and offer means of examining the relationships between genotype and phenotype of various pathogens. Development of new techniques aids in surveillance and epidemiological studies as well as identifying causes of disease out breaks or the presence of pathogens in the carrier individuals. Thus, molecular

biology can be a routine tool in the search for improved methods of diagnosis and control of fish pathogens and the epidemiology of infectious fish diseases.

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MOLECULAR MODELLING AND VACCINE DEVELOPMENT

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Though there are many effective vaccines available for fish diseases, and vaccination has proved to be an important target combating them yet there are still many diseases for which vaccination are not available. There are many factors responsible for the effectiveness of a vaccine like generating an immunogenic antigen when subunit or gene based vaccines are used, to induce a right type of immune response when cell mediated immunity is required Secondly when a new vaccine has to be tested it incurs huge investment on its trial, and a lot of fish sacrificed during fish challenge trials and the end result is always not positive.

Contrasting to the wet lab methods, *in silico* methods incorporate the knowledge from high-resolution 3D protein structures to probe structure–function relationships (Hillisch, *et al.*,2004), identify and select therapeutically relevant targets (assess druggability) (Hillisch, *et al.*,2004, Blundell, *et al.*,2006), study the molecular basis of ligand: protein interactions (Blundell, *et al.*,2006), characterize binding pockets (Halgren ,2009), develop target-specific compound libraries (Orry, *et al.*,2006 and Schnur, 2008), identify hits by high-throughput docking (HTD) (Cavasotto,2007 and Cavasotto, 2008) and optimize lead compounds (Lundstrom ,2007) , so that a potential vaccine candidate can be developed. Structural information of biological macromolecules is readily available in the Protein Data Bank (PDB), http://www.pdb.org. The Structure Based Drug Design are used to optimize these leads which can act as vaccine. The protein structure is used for suitable target identification by virtual screening of the different fragments in the selection. Thus the structure of the protein plays a very important role in optimising a suitable vaccine molecule. Numerous database are easily available to provide insight into the structure and function of these proteins. Database like SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html), Protein Model Portal (http://proteinmodelportal.org) and Modbase (http://modbase. compbio. ucsf.edu) contain protein models. These servers provide models that serve as starting point for biologists in Structural Genomics.

Thus the process of *In Silico* Vaccine development can be divided into following steps

- Target identification through sequence structure homology recognition
- Identifying ligand binding models
- Identification of hits and lead
- De Novo design

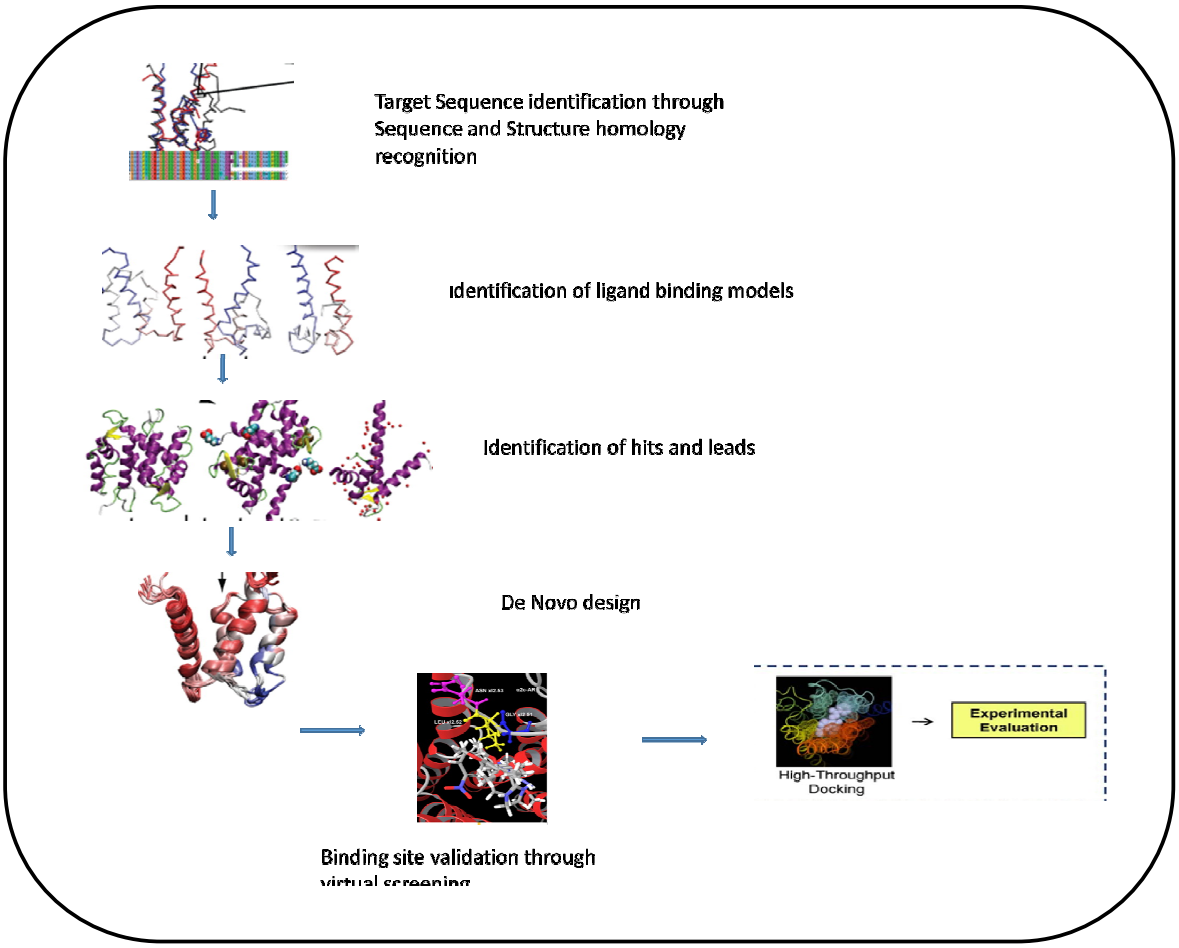


Fig1: Different stages of *In Silico* molecular modelling.

Target identification

Target identification is done through physical, genetic and mechanical approach. In the physical approach the diseased organism is directly studied. The disease manifestation at the organism level is the only direct approach for target identification. The mechanistic approach is to identify the target by comparing the intracellular pathways in vitro and in cell culture system. The last approach can be to compare the differential expression of genes and proteins in diseased and normal condition. This is a very challenging approach because for a particular disease condition several factors act in co-ordination and to identify a potential target amongst them is very difficult.

The structure of a protein gives information about the protein families and superfamilies which it belongs. Combined algorithms have been reported; for example, Gen THREADER uses the sequence comparison method to generate the sequence–structure alignment and then evaluates the alignment using threading potentials (Jones, 1999) . Once a homologue of known structure has been identified, it can be modelled using a variety of comparative (homology) modelling procedures, for example, those that use a fragment-assembly approach, such as Composer (Sutcliffe, *et al.*,1987) or SWISS-MODEL (Guex, 1999), or alternatively a restraint based approach, such as Modeller (Sali, and Blundell, 1993).These give good models if the sequence identity is >30% but the accuracy falls off sharply when it is lower, mainly because of the difficulties in obtaining good alignments, in predicting shifts of core residues and in building loops (Venclovas, *et al.*,2003).

Homology or Comparative Modelling

Comparative or homology modelling is a methodology to predict protein structure based on protein sequence. Sequence alignment plays an important role in homology modelling. Multiple sequence alignment methods simultaneously align several sequences to locate conserved regions, predict functional sites and protein function as well as aid phylogenetic analysis (Edgar, and Batzoglou, S. 2006). The process of understanding both the function and mechanism of action, termed target identification and validation, is of utmost importance before commencing any vaccine development program. With the increase in availability of 3D protein structures, several methods have been developed which supplement sequence information with structural information to probe biological function.

Identifying ligand binding regions

As structural genomics projects increase many 3D protein structures are produced from gene sequences. Thus it is becoming increasingly important to develop that will identify sites involved in productive intermolecular interactions that might give clues about functions and binding sites. There have been several attempts to predict functional or interaction sites computationally by finding different high energy conformations at active sites (Herzberg, and Moult, 1991) or through identifying clefts that occur at the active site (Laskowski, *et al.*,1996) . The most widely used method for identifying sequence conservation is evolutionary trace in which the residues that are conserved are highlighted in the structure.

Identification of hits and leads

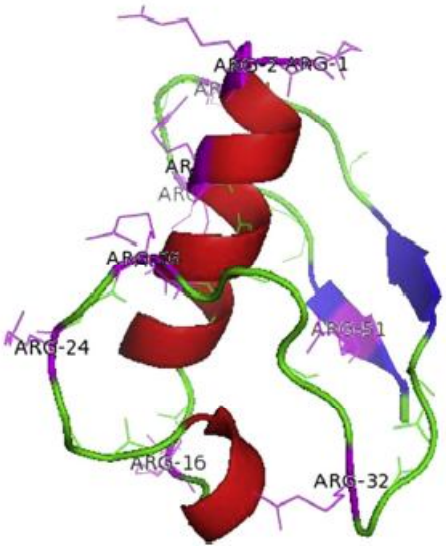
Hit identification is a very expensive and time consuming process. Rather than performing a large, costly high throughput screening a more focussed screening pattern is followed. Either virtual screening is done from already identified ligands or subset of samples are selected from the compound file. The first focus should be selecting a molecule that should bind to protein active site. Thus to predict the correct ligand conformation and orientation, in addition to any protein movement that is induced by the ligand, although for most applications of docking the protein is assumed to be rigid. As with virtual screening, there are several challenges in the correct orientation and scoring of designed molecules but, additionally, *de novo* design can often produce molecules that are difficult to synthesize. For these reasons, there has been a tendency to initiate *de novo* design from a moiety (or fragment) that is already exits and shows protein-protein interaction at the active site. As the designed compounds are based on active scaffold they are more close to real molecule and likely to bind to the receptor.

Application in fish immune system

Immunological predictions and simulations have been demonstrated highly useful in applied immunology in general, and in vaccinology in particular. It can be used as an efficient tool in epitope discovery for use in rational vaccine design, immuno-therapeutics and development of diagnogstic tools. A number of recent publications describe in great detail the values and benefits obtained by the use of immuno-informatics and predictions in applied immunology and vaccinology (Davies and Flower, 2007). Cytokines are signaling molecules that stimulate and regulate immune response. There is a direct correlation between cytokines and their receptor. Crystal structure of receptor ligand complex have been resolved in human IL-1/ILIR (Vigers *et al.*, 1997), TGFβ/TbR (Hart *et al.*, 2002). Huge information on mammalian cytokines crystal structure are available in the PDB but not much information is available on non-mammalian cytokines. Today the presence of IL-1 has been reported in all vertebrates except reptiles. Apart from IL-1β, several other IL-1 members have been discovered in fish. Analysis of multiple sequence alignments have shown that characteristic β trefoil structure is maintained across vertebrate IL-1 and that the

structurally important features are conserved. A number of IL-1R Toll-like receptors including IL-1RI, IL-1RII, IL-1RAcP, IL-18R and ST-2 have been recently discovered in Salmon, trout and Japanese pufferfish which wil help in the study of species specific ligand –receptor interaction. More ligands such as IL-55 are being discovered in human and mouse. Each species has evolved its own way of binding through ligand-receptor co-evolution. Modeling studies ,using human and murine structures as templates have been identified and based on these templates fish ligand- receptor structure can be modeled. Several peptides and fragments have been designed through virtual screening which are biologically active for human model can also be tested for other vertebrate species.

This first homology model of a fish IL-10 reveals a high degree of compatibility between the dimeric quaternary architectures of sbIL-10 and its mammalian counterparts. The phylogenetic analysis clusters sbIL-10 with other IL-10s, apart from IL-10-related molecules. Similar work was done to analyze properties and function of IL-10 by (Bikash *et al* ., 2010), they constructed a 3D model of IL-10 in the Indian major carp, the catla (*Catla catla*), which is a highly preferred fish species and the most commercially important one in the Indian subcontinent. The catla IL-10 model was constructed by comparative modeling using human IL-10 (2ILK) as the template, and a 5 ns molecular dynamics (MD) simulation was carried out to characterize its structural and dynamical features, which was validated by the SAVES, WHAT IF and MolProbity servers. Given below is the homology model of lenok cathelicidin. Cathelicidin are a family of endogenous antimicrobial peptide playing an important role in mammalian innate immune defense against invasive pathogenic bacteria(Zheng *et al.*,2013)



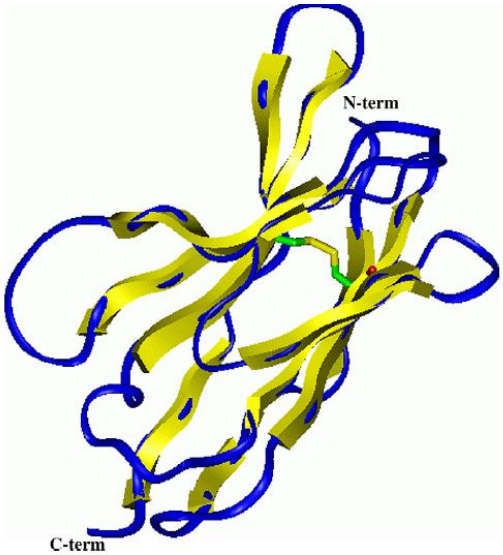


Fig 1: Molecular model of the lenok cathelicidin. Two stranded antiparallel β ribbons are flanked by the two α-helices on the left side and widespread coil construction.

Fig 2: homology model of sea bassCD8α.The backbone ribbon and secondary structure topology are shown, yellow arrows represent β-strands. Green and yellow stick indicate presence of a Cys-Cys bond.

Future Perspectives

Molecular Cloning and characterization has produced many molecules whose structural and functional information are also available in the database. Thus the focus should be to use all the available information to investigate the functioning of the teleost immune system, so that new molecules can be designed for vaccine development. Continued research on CD markers of teleost is needed. virtual screening of bioactive fragments and its structural design can definitely be used in generating new peptide fragments that are immunogenic and can act as potential vaccine candidate.

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EXPRESSED SEQUENCE TAGs (ESTs) and its implication in aquaculture

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Introduction:

Carps, the mainstay of Indian aquaculture, have received keen attention of the researchers in the country over last five decades and with the gradual decrease in marine catch, inland aquaculture has become more and more important for protein security, rural employment and livelihood. Augmenting production through genetic improvement of fish species has been accepted by the scientific and aquaculture community with the development of genomic tools and resources. Although a lot of literature is available on its culture, management, feeding and breeding aspect, unfortunately genomic resources such as EST database, molecular markers, genetic linkage maps or QTLs for marker assisted selection (MAS) are totally lacking in any Indian major carps including rohu. Even any future genetic manipulation (gene targeting, gene silencing or transgenic production) in these species requires more information about its genomic structure and gene expression and molecular biology of IMC largely remains unexplored so far. Therefore, genome-based technologies are not yet available for marker-assisted selection. ESTs will help us to relieve this limitation by capturing the vast majority of the transcriptome, allowing rapid gene discovery, identification, and annotation. The ESTs will also serve as the basis for the development of cDNA micro arrays that are fundamentally important for the analysis of genome expression in relation to function.

Rationale:

Expressed sequence tags (EST) provide a rapid and efficient method of gene discovery that has been widely applied in humans and others species. **ESTs are partial sequences (200-600 bp) of randomly selected complementary DNA (cDNA) clones isolated from a cDNA library**. Upon generation of short cDNA sequences, genes can be identified, annotated, and catalogued to establish orthologous relations with known genes from other organisms by comparison of similarities. EST has applications in discovery of new genes, mapping genomes and identifying coding regions in genomic sequences. EST analysis is a convenient way to examine the gene expression. Baseline expression levels of genes can be established by sequencing non-normalized cDNA libraries from specific tissues. As it represent transcripts of genes; therefore, their sequencing can also provide information on alternative splicing and differential polyadenylation. ESTs are important resources for gene mapping. ESTs can be directly mapped by Polymerase Chain Reaction (PCR) analysis for species in which radiation hybrid panels are available. For species without radiation hybrid panels, ESTs are a great resource for discovery of Single Nucleotide Polymorphism (SNP) as well as polymorphic markers by micro satellite tagging. Because many ESTs can be readily identified as orthologs of known genes by simple similarity searches, polymorphic microsatellites within the genes of known function would convert the micro satellites into type I markers and if found conserved in a wide spectrum of species will be useful for comparative gene mapping. Therefore, random sequencing of cDNA clones on one hand, provides a rapid and efficient means of developing EST markers associated with known genes, at the same time, it can also be used to develop markers for mapping economically important traits such as muscle built up, growth performance, resistance to diseases and reproduction etc. in fishes. Surveys of cDNA libraries have indicated that up to 8% of clones

may contain micro satellites and about 9% of catfish ESTs deposited to Genbank contain micro satellites, i.e. twice the rate of zebra fish, and seven times of the rate in mammals. Thus development and mapping of large number of gene associated micro satellites type I markers will greatly enhance the existing linkage maps, bringing application of genomics for the genetic improvement of fish brood through analysis of quantitative trait loci and marker assisted selection. However, despite their high utilities, type I markers are more difficult to develop, while non-gene sequences are free to mutate, causing higher level of polymorphism, sequences within protein coding regions generally show lower levels of polymorphism because of functional selection pressure. Lack of type I markers in many aquaculture species hinders major progress in genomics and genetic studies. And also from taking advantage of progress made in other species such as human, rat, zebra fish, and puffer fish.

Currently the number of fish related ESTs in the public databases is still small compared to mammalian and other species, which may be around 2% only of all ESTs in the dbEST. Almost 90% of it was generated from the two model species, Zebra fish (*Danio rerio*) and medaka (*Orizus latipes*) and recently from fugu, where resources from a large number of laboratories are contributing the sequence information. Out of nearly 38 million ESTs in databases at NCBI till date, 1.6 million are from fish species. Nearly 1.3 million are contributed from Zebra fish and medaka, and at best can provide ESTs through cross amplification for aquaculture species. But genomic model species are generally selected in view of suitability for experimental purposes and technical conveniences rather than ecological interest, evolutionary significance or any economic importance. Further, teleosts are the most divergent group among all the vertebrates, which hinders direct application of the sequence data from the model species to the economically preferred one like rohu. Obviously much effort needs to be directed towards the EST development in aquaculture species also. Among the aquaculture species EST has already been reported in Japanese flounder, rainbow trout, salmon, channel catfish, common carp and tilapia. Rainbow trout is most searched for ESTs contributing nearly 0.25 million entries, followed by Atlantic Salmon and channel cat fish but except a few (2), almost nothing is available for rohu. Targeted effort to explore ESTs from fish species of interest for Indian aquaculture is necessary; so that ESTs can be expanded covering more species using bioinformatics based tools.

International status:

Currently, the number of fish related ESTs in the public databases is still small compared with mammalian sequences. ESTs generated from teleosts account for only two percent of all ESTs in the dbESTs. Almost 90% of the teleost ESTs were generated from the model species like zebra fish, medaka and fugu. However, among the culture species EST has already been reported in Japanese flounder, *Paralichthys olivaceus;* rainbow truot, *Oncorhynchus mykiss;* salmon, *Salmo salar;* carp, *Cyprinus carpio* and catfish, *Ictalurus punctatus.* There was concerted effort to build up the EST database for most of the cultured species of economic importance.

1) Japanese flounder, (*Paralichthys olivaceus)*:

A cDNA library was constructed from Japanese flounder, leukocytes infected with Hirame rhabdovirus (HRV) to analyze the genes induced and expressed by virus infection .Out of 452 partial sequences representing 300 cDNA clones, three-quarters (217 clones, 72.3%) represented known genes in the public databases, whereas the remaining 83 (27.7%) of the clones did not show any significant homology with the sequences in the public databases.

Using expressed sequence tag (EST) strategy; from Japanese flounder, spleen cDNA library a total of 1010 ESTs were sequenced, out of which 61% were identified as being

homologous with known genes where as 39% appeared to be unknown and are likely to represent newly described genes. However, in the liver of Japanese flounder, the identified genes were classified with respect to a cellular role, which showed that 314 (58%) of the identified genes encoded proteins associated with primary liver functions such as transport, acute phase response, and blood clotting. Further, comparative analysis revealed that 26 unique genes had homologous to known genes highly expressed in mammalian liver systems.

2) Rainbow truot,(*Oncorhynchus mykiss):*
Over 45,000 ESTs were obtained by sequencing clones from a single normalized library constructed using mRNA from six tissues. The production of this sequence data and creation of a rainbow trout Gene Index eliminating redundancy and providing annotation for these sequences will facilitate research in this species.
Recently ESTs in rainbow trout were produced by normalization and subtraction, and 96,472 ESTs were submitted representing 42.5% of the overall sequence knowledge in this species. All these EST sequences have been clustered into a total of 52,930 clusters of putative transcripts groups, including 24,616 singletons which represent 2nd highest among the teleost after zebra fish.

3) Salmon, (*Salmo salar):*
The construction and quality analysis of six cDNA libraries from the liver, ovary, testis, brain, spleen and muscle tissues of adult Atlantic salmon has been reported. Subsequently, the 5'-terminal DNA sequences of 1152 cDNA clones, composed of 96 clones from each of the abundant and rare mRNA populations in the six tissues, were determined. Bioinformatics analysis revealed that 510 (50%) of the salmon expressed sequence tags (ESTs) of sufficient length showed significant homology to previously identified genes from salmonid and other species, while 517 (50%) of salmon ESTs were unidentified or novel. After accounting for multi-EST redundancy, the 510 identified ESTs provided DNA sequence markers for 178 salmon genes, which are listed in terms of tissue of origin and mRNA abundance class. A list of genes expressed in the liver of Atlantic salmon was compiled using the expressed sequence tag (EST) strategy. 733 ESTs, derived from 170 abundant and 563 rare mRNA encoding liver cDNA clones, were determined. Bioinformatic analysis revealed that 390 (53%) of the salmon liver ESTs could be ascribed to the transcriptional products of 93 identified genes including 7 previously described in the Atlantic salmon. The identified Atlantic salmon genes were classified with respect to cellular role which showed that 33 (36%) of the identified genes encoded proteins associated with primary liver functions such as transport, acute phase response, and blood clotting reported 80,388 ESTs from 23 Atlantic salmon (Salmo salar) cDNA libraries (61,819 ESTs), 6 rainbow trout (*Oncorhynchus mykiss*) cDNA libraries (14,544 ESTs), 2 chinook salmon (*Oncorhynchus tshawytscha*) cDNA libraries (1317 ESTs), 2 sockeye salmon (*Oncorhynchus nerka*) cDNA libraries (1243 ESTs), and 2 lake whitefish (*Coregonus clupeaformis*) cDNA libraries (1465 ESTs). The majority of these are 3' sequences, allowing discrimination between paralogs arising from recent genome duplication in the salmonid lineage. Sequence assembly reveals 28,710 different S. salar, 8981 O. mykiss, 1085 O. tshawytscha, 520 O. nerka, and 1176 C. clupeaformis putative transcripts. Higher- and lower molecular weight fractions of libraries were shown to contain distinct gene sets, and higher rates of gene discovery were associated with higher-molecular weight libraries. 2974 unique sequences, consisting of 779 contigs and 2195 singlets, were generated for Atlantic salmon from two cDNA libraries constructed from the gills and the intestine. Nearly 50% of the sequences were assigned putative functions because they showed similarity to known genes, mostly from other species, in

one or more of the databases used. The Swiss-Prot database returned significant hits for 1005 sequences. These could be assigned predicted gene products, and 967 were annotated using Gene Ontology (GO) terms for molecular function, biological process and/or cellular component, employing an annotation transfer procedure.

4) Common carp (*Cyprinus carpio*):

It has been reported that out of 200 single pass ESTs from common carp head kidney, 129 matched with known genes where as 71(35.5%) did not show any significant homology with sequences in public database. In another experiment, it was found that out of 188 EST clones from common carp head kidney, 127 clones (67.6 %) were completely unknown and are likely to represent newly described genes, whereas 61clones (32.4%) were matching to the sequences in the database. The putative genes contain ribosomal proteins, cytochrome oxidase, immunoglobulin light chain, FKBP, CXC chemokine receptor type 4, complement factor B/C2-A3, peptidylprolyl isomerase , cyclophilin S1, heat shock-70 kDa protein-4, stress-activated protein kinase-3. It has been reported a total of 530 expressed genes from the head kidney cells treated by immunostimulants.These included BPI/LBP, C-type lectins, fucolectins, CC-chemokine, CXC-chemokine, CD18, cyclophilin,FcgammaR, G-CSFR, HSP 70, Ig heavy and light chains, NITR, integrin beta2-alpha,Mx, interleukin-1beta, beta thymosin, lysozyme G & C, MHC class II associated invariant chain 1 and 2, granulin, CAAT binding protein and tumour necrosis factor-alpha induced protein.

5) Catfish (*Ictalurus punctatus*):

As part of transcriptome analysis in catfish to develop molecular reagents for comparative functional genomics, reported analysis of 1201 brain cDNA clones. Of the 1201 clones, 595 clones (49.5%) were identified as known genes and 606 clones (50.5%) as unknown genes. The 595 clones of known gene products represented transcripts of 251 genes. These known genes were categorized into 15 groups according to their biological functions. 606 clone of unknown genes showed no homology to any known genes in the GenBank databases and indicated that a great number of novel genes exist in teleost brain.

EST analysis using a cDNA library made from the head kidney in channel catfish analysed 2228 EST clones. Orthologues were established for 1495 (67.1%) clones representing 748 genes, of which 545 (36.5%) clones were singletons. The remaining 733 (32.9%) clones represented unknown gene clones, for which the number of genes was not determined. It has been analyzed that 1204 spleen cDNA clones in channel catfish of which, 665 clones (55.2%) were identified as orthologs of known genes from other organisms and 539 clones (44.8%) as unknown gene clones.147 novel genes were identified, and annotations were made to 118 of them. In addition, 389 novel EST clusters were identified. It has been analyzed that 1909-expressed sequence tags (ESTs) derived from a skin library. Of the 1909 ESTs, 1376 (72.1%) representing 496 unique genes had homologies with other organisms while 478 (25.0%) ESTs had no significant homologies and were designated as unknown. The remaining 55 (2.9%) EST clones were eliminated because of their low quality or short sequences. Of the 496 unique genes, 327 (65.9%) genes were singletons while 169 (34.1%) genes represented by two or more ESTs. A total of 1007 (52.8%) ESTs representing 235 unique genes matched previously reported channel catfish ESTs while 847 (44.4%) ESTs representing 261 unique genes were newly identified.

6) Tilapia *(Oreochromis sp)*:

A list of transcripts expressed in the hypothalamus of adult tilapia, *Oreochromis mossambicus*, was compiled using the expressed sequence tag (EST) strategy. Of 161 clones, 130 clones were unique genes and 31 clones were found to be redundant. Of 130 unique genes, 32.3% (42/130 ESTs) were identified as known genes and 67.7% (88/130 ESTs) as unknown genes. The functional categorization of the known genes was analyzed. Bioinformatic analysis revealed that 62 of 88 unknown genes (62/130 ESTs, 47.7%) showed a significant homology to neither nucleotide nor translated peptide sequences in the public database and these genes might be particularly expressed in the tilapia hypothalamus.

Therefore, the noticeable observation in all the above studies in aquaculture species is that a significant percentage of ESTs represented unknown or unidentified genes at a time when almost all the genes of human, mouse, zebrafish and other model species are already available in the genbank database.

Development of proper molecular markers is pivotal to structural genomics. Linkage maps have been constructed in several important aquaculture species, including Atlantic salmon. Rainbow trout, tilapia, catfish, eastern and Pacific oyster and shrimps. However most markers mapped in these aquaculture species were developed from anonymous genomic sequences (type II markers), prohibiting comparative genomic analysis. Catfish expressed sequence tags ESTs were rich resources for the identification of gene tagged micro satellites, which when mapped directly showed location of genes within the linkage map. Micro satellites and AFLP markers have been used efficiently in construction of the framework linkage map in catfish, but genomic locations of these types II markers cannot be compared to other species. Screening through cDNA libraries is an effective method for identification of polymorphic micro satellites within genes. The genomic resources of channel cat fish expressed sequence tags (ESTs) have been utilized to identify simple sequence repeats (SSRs) or microsatellite. A bioinformatic analysis of 43,033 ESTs identified 4855 ESTs containing micro satellites. A comparison of allelic variation between micro satellites markers developed from expressed sequence tags (EST) data and anonymous markers identified from repeat enriched libraries constructed from genomic DNA for rainbow trout (*Oncorhynchus myskiss*) has been performed, with respect to polymorphism information content (PIC), number of alleles, repeat number, locus duplication within the genome and ability to amplify in other salmonid species. Higher PIC was detected in dinucleotide micro satellites derived from ESTs than anonymous markers (72.7% vs.54.0%). In contrast dinucleotide repeat numbers were higher for anonymous micro satellites than for EST derived micro satellites (27.4% vs. 18.1%). A higher rate of cross-species amplification was observed for EST micro satellites.The occurrence of highly polymorphic micro satellites in the untranslated region s of expressed sequence tags (ESTs) is a potentially useful source of gene-associated polymorphisms, which has thus far not been utilized for genome screens in natural populations. It has been considered that the ESTs associated micro satellites as the best candidate loci affected by divergent selection, and hence, they serve as promising genes associated with adaptive divergence in Atlantic salmon. It has been also demonstrated that EST-linked micro satellite genome scans provide an efficient strategy for discovering functional polymorphisms, especially in non-model organisms.

Although, in most cases ESTs are made by large scale single-pass sequencing of cDNA clones randomly picked from libraries, the integrity of the data and the pace at which novel sequences can be identified depends to a great extent on the cDNA libraries that are used. Because altogether, in a typical cell the mRNAs of the prevalent and intermediate frequency classes comprised as much as 50-65% of the total mRNA mass,

but represent no more than 1000-2000 different mRNAs redundant identification of mRNAs of these two frequency classes is destined to become overwhelming relatively early in any such random gene discovery programmes, thus seriously compromising their cost effectiveness. For this reason the use of normalized libraries, in which the frequency of all classes is within a narrow range, has been shown to be beneficial for large-scale sequencing. Therefore most of the ESTs now days are made from normalized cDNA libraries. Commonly used normalization and subtraction procedures tend to select for shorter, truncated and internally primed inserts, seriously affecting library quality and thus led to the development of alternative procedure like oligonucleotide fingerprinting (OFP). Besides, a lot of improvement has occurred in normalization procedure itself, which was quite lengthy and complex and recently made easier and simple and used in oyster EST library preparation.

National status:

The Indian scenario in the above context is not encouraging and no concerted effort has so far been made except the genetic up gradation of rohu through selective breeding although India was quite ahead in cloning and producing transgenic fish for growth hormone gene for several fish**.** Genomic library was constructed for *Labeo rohita* and *Catla catla* for identification of structure and sequence data of functional genes by Dr Aparna Dixit and her colleagues at School of Biosciences, JNU under DBT project. A couple of transcribed repeat sequences identified in Indian major carps while attempting to develop **EST markers** of arbitrary genes and submitted (Acc #. AJ012657; AJ130723). DNA fingerprints were obtained in three species of commercially important freshwater fishes using Bkm and M13 multilocus probes. Two highly reiterated, tandemly repeated DNA and AT rich MboI DNA fragments in mrigal and *Clarias batrachus* have been cloned and characterized. Nucleotide sequence of growth hormone and its receptor mRNA from *Labeo rohita* were submitted by K.C. Majumdar in EMBL (Acc #. AY691177, AF418921, AF 416490, AF134200). A tissue specific cDNa library using polyA+RNA from pituitary glands of the Indian catfish *Heteropneustes fossilis* and a cDNA clone encoding growth hormone (GH) was isolated and characterized. Mature GH protein was efficiently expressed in bacterial and zebra fish systems using appropriate expression vectors. A modified Rapid Amplification of cDNA Ends (RACE) strategy for cloning highly conserved cDNA sequences. Using this modified method, the growth hormone (GH) encoding cDNA sequences of *Labeo rohita*, *Cirrhinus mrigala* and *Catla catla* were cloned, characterized and over expressed in *Escherichia coli*. Molecular cloning of growth hormone-encoding cDNA of *Labeo rohita* has been characterized and expressed. Higher growth enhancement and food conversion efficiency of **transgenic rohu** has also been reported.

ORNAMENTAL FISH BREEDING AND RELATED DISEASES

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Introduction

The ornamental fish is a promising sector within aquaculture which envisaged to be full of opportunities in terms of the growth and for generating income and employment to the large number of the skilled educated unemployed across the country side. At present a fraction of the domestic and international potential is harvested. But, in the recent times, the sector has shown a fasters growth upon concentrated efforts of the farmers and entrepreneurs to take up the ornamental fish as means of their business and livelihoods. A large number of the stakeholders' i.e fishers, farmers, breeders, traders, vendors, transporters and exporters are involved in the sector. The whole business of the ornamental fish is based on the supply of the fishes from two primary sources *i.e.* wild collection and captive breeding. There is a wide apprehension on the environmental impact of the wild collection and it has a damaging effect on the threatened fish biodiversity in the country. Therefore, the captive breeding is the foundation of the sustainable development of the enterprises.

Ornamental fish species for commercial production

Important groups of egg-layers are barbs, rasboras, goldfish, tetras, danios, bettas and gouramis and the major livebearers are guppies, platies, mollies and swordtails. Barbs are the most important group and most species of the group are known to have originated from India, *viz.*, rosy barb (*Puntiusconchonius*), melon barb (*P. fasciatusfasciatus),* aruli barb (*P. arulius*). *etc*. Among the exotic barbs, tiger barb (*P. tetrazona*) and its aquarium developed strains like green tiger barb and red tiger barbs are fascinating in modern days. The major species of the group danios include zebra danio (*Brachidaniorerio*), pearl danio (*B. albalineatus),* giant danio (*Danioaequipinnatus*), turquoise danio (*D. devario*), and malabardanio (*D. malabaricus*. Among rasboras, the slender rasbora (*Rasboradaniconius*), glowlightrasbora (*R. pauciperforata*), harlequin rasbora (*R. heteromorpha*) and scissors tail (*R. trilineata*) are important ones. The gold fish, *Carassiusauratus*, is the most commonly available fish, preferred by the hobbyists because of its attractive colouration ranging from pure gold to red, orange, black and albino. Some of the common varieties of gold fish available are comet, lion head, oranda (a modification of lion head), fringe tail, veil tail, fantail, shubunkin, telescopic eye, etc. These fishes grow up to 20 cm in length, but start breeding when they are only 6 cm long. Many aquarium strains of ornamental koi, *Cyprinuscarpio* (var. koi) are easily bread by available hormone injections. Its origin is from East Asia but strains like orenji-ogon, kin matsuba, kohaku, and shiro-bekoo are being cultured worldwide.

The tetras are small fishes of 3-8 cm long; majority of which has originated from South America. The most common species of the group are black widow tetra (*Gymnocorymbusternetzi*), serpae tetra (*Hyphessobryconsarpae*), lemon tetra (*H.*

pulchripinnis), flame tetra (*H. flammeus*), neon tetra (*Paracheriodoninnesi*), cardinal tetra (*P. axelrodi*), black neon tetra (*Hyphessobryconherbertaxelrodi*) and pretty tetra (*Hemigrammuspulcher*). The species, *Bettasplendens*commonly called as Siamese fighting fish occurs in varied colours like green, red, blue, albino and sometimes with a combination of two or three shades. The attractive colour and hardiness of the species are the characters of the species for its wide adoption by the hobbyists. The males are brightly coloured with beautifully spread-over fins. They show aggressive behaviour, only when other males are present. Angel fish, *Pterophyllumaltum*and *P. scalare* are important candidate species widely preferred for aquaria, with different developed varieties such as black, veil tail, gold and black marble, platinum, gold pearl, pink ghost and albino. The filamentous lower fins and their compressed body shape with their elegant movements are pleasant to look at. Among gouramies, three spot gourami (*Trichogastertrichopterus*), pearl gourami, (*T. leen*), moon light gourami (*T. microlepis*), snake skin gourami (*T. pectorails*), dwarf gourami (*Colisalalia*), honey gourami (*C. sota*), striped gourami (*C. fasciata)*, noble gourami (*Ctenopsnobilis*) and kissing gourami (*Holostomatemmincki*) are the important species. The bettas and gouramies are most popular among nest-builders, characterized by possession of accessory respiratory organs, thus making the species hardier. Among catfishes the exotic variety of sucker mouth catfish (*Hypostomusmultiradiatus*) is very popular, being originated from South and Central America it is available in every aquarist.

Essential aspects of the ornamental fish breeding technology

The breeding technology involves both science and art with high degree of skill for successful operations. The technology gets refined at the entrepreneurs level with experience and expertise as fines skills are learned by doing. The research institutes have committed to give attentions to develop these technologies. Below are few essential aspects of the ornamental fish breeding as tips to the entrepreneurs interested in the breeding.

Sexing the fish

Determining the sex of a fish is an important aspect. Most fish can be classified as sexually dimorphic or sexually isomorphic. In sexually dimorphic species, the sexes can be easily distinguished by size, shape, colour and fin pattern. Like all other animals of animal kingdom males are more colorful, larger, and have more elaborate finnage. Among the more brilliant outstanding of sexual dimorphism can be found in cichlids, killifishes, barbs and livebearers. In sexually isomorphic species like angel and discus fish it is generally difficult to identify sexual differences. But an experienced aquarist can identify the sex by virtue of experience or working with that particular fish for a long time. Often, the only way to distinguish between the sexes is the shape of the genital papilla, which is only visible during spawning period. In some isomorphic species, the males are slightly larger and the females are slightly oval in the belly.

(ii) Selection of correct brooder

Once the sexes have been distinguished, a suitable pair or spawning group can be selected. There are several important traits to seek in choosing the brood fish. The

fish that shows good markings and colour that would produce attractive young should be selected. It is better to use mature, healthy fish for spawning because unhealthy fish, if they spawn, may produce unhealthy or deformed hatchlings. It is to be ensured that the pair is well compatible. Many species cannot be put together in a breeding tank and expected to get along and produce young. In many cichlids, pairs form only after a group has been raised together for months. In certain species like gouramies, during spawning process, if the male is ready and female is not, in that case male may kill the female. This may be due to incompatibility among the sexes. The crossing between the different strains or color forms should be avoided because the young are often unattractive. It should always be ensured that the pairs are from the same species as usually hybrids are sterile.

(iii) Conditioning the brooders

Before placing the parent fish together for spawning, they should be conditioned through best feeding strategies with a variety of live foods to get them in excellent matured condition for spawning. The live foods such as tubifex, blood worm, mosquito larvae, zooplanktons etc. which not only gives the good growth but also triggers the spawning process.

Breeding strategies and spawning environment

Some of the ornamental fish species readily spawn in the aquarium or cement tanks, the eggs or hatchlings often do not survive because of predatory nature of the parents. Sometimes the mortality occurs due to unfavorable polluted water conditions. It is always better to breed the fish in a separate spawning tank. The spawning tank may be fitted with a thermostat (if necessary), a sponge filter and good aeration. Depending on the spawning method, the spawning tank can be set up in a number of different ways based on the spawning behaviour of the fish.

(a) Oviparous (egg layers)

Most of the aquarium fish are egg-layers with external fertilisation. Egg-layers can be divided into five groups' *viz*., egg-scatterers, egg-depositors, egg-burriers, mouth-brooders, and nest-builders.

(i) Egg-scatterers: Zebra fish (*Zebra danio*) is considered as egg scatterers, lay non-adhesive eggs. The larger ones are grouped under the genus *Danio* and other smaller varieties are under genus *Brachidanio*. The important varieties of danios include the giant danio (*Danioaequipinnatus*), pearl danio (*Brachidanioalbalineatus*) and zebra danio (*Brachidaniorario*). Since the egg scatterers often eat their own eggs, the spawning tank has to be set in such a way that the eggs fall out of the reach of spawning parents. These species do not show parental care and eat their own eggs.

For breeding such fishes the male and female ratio should be maintained at 1:1 or 2:1. 40-50 l capacity tank is sufficient for spawning for most eggs scatterers. The female is introduced in the breeding tank one day earlier than the males. But it is very difficult to know whether the female has already laid the eggs because of smaller size of eggs and remain hidden behind the pebbles. Sometimes the brood fishes are kept in a mesh net from where the eggs drop in to the bottom. If the

temperature is favourable, the eggs require two-three days hatching time. As soon as the tiny hatchlings seen in the aquarium tanks the parents are to be removed. The hatchlings take 2-3 days to absorb their yellow yolk sac. Then they are fed with infusorians for 4 days followed by rotifers and smaller zooplanktons for a week, after which they can be provided powdered formulated feed along with zooplanktons.

The egg scatters laying adhesive eggs are mostly the Gold fishes, *Carassiusauratus.* The common gold fish varieties are common gold fish, fringe tail, lion head, oranda, comet, shubunkin, telescopic eye, veil tail, red cap etc. When secondary sexual characters appears (by observing the maturity condition), the male and female gold fish are selected and kept in glass tanks or ferro-cement tanks. Since gold fish eggs are sticky in nature, they require some surface to adhere their eggs. Various types of natural submerged aquatic plants like *Hydrilla* can be used after proper treatment for this purpose. Artificial nests can be prepared by making split plastic ropes, with one end tied or burnt to make it blunt. Even polythene strips have been found suitable for the purpose.

The female and male in the ratio of 1:2 are released into breeding tank during late evening hours. The male chases the female, presses its operculum against female's abdomen and fertilizes the eggs, while swimming beside her. Egg laying usually takes place within 6-12 hrs of releasing the broods. After spawning the nest is transferred to a different container, or alternatively, the parent fish transferred from the breeding tank. If this is not done, the parents are most likely to eat away the eggs to compensate the post-spawning loss of energy.

Generally, the female lays about 2000-3000 eggs. Healthy eggs are golden transparent in colour and the unfertilized eggs will remain opaque and continue to remain as such with arrested growth. These dead eggs become pale white and hair like aquatic fungus would grow on all the sides, giving it the appearance of a small powder-puff. Under ideal condition, within three days, the eggs hatch out with a hatching rate of 80-90% when the young larvae start to float, the nest materials are taken out from the tank. Generally, the tiny hatchlings remain clinging to the nest, so precaution has to be taken while transferring the nest from the breeding pool.

(ii) Egg-depositors

These species deposit their eggs on a substrate (tank glass, wood, rocks and plants). Egg depositors usually lay less egg than egg-scatterers, although the eggs are larger.

Depending on the type of egg depositor, the tank should be furnished differently. For those egg-depositors that care for their young, the parents can remain in the tank after spawning.

Substrate spawners, depending on the species, should be given a tank furnished glass panes, broad-leafed plants, or flat stones for spawning sites. Some species such as Discus and Angelfish prefer vertical surfaces. From a group of such fishes a pair is

selected by providing a slanting serrated glass surface plate, where a suitable pair comes near the plate for cleaning the surface for laying eggs. At that time the pair is carefully removed for further breeding in a separate tank. For cavity spawners like *Badisbadis*, Chameleon fish, flower pots turned on their side, coconut shells, and rocky caves are suitable spawning sites. The tank should be furnished with either live or plastic plants to give the fish a sense of security.

Egg-depositors that do not care for their young should be given a tank furnished with fine and broad-leafed plants. After spawning the parents or plants with the eggs are to be removed. If the plants containing eggs are removed, new plants should be placed in the tank for future spawning. The very common ones are *Rosboradaniconius*(Slender rasbora). They require soft, slightly acidic (pH 5.5-6.5) and temperature at 28°C. After conditioning male and female, they are placed in a tank planted with flat leaved plants. Rasbora's prefers peace and quiet environment for breeding with low lighting conditions. The male and female brooders are placed together in breeding tank. Once spawning has occurred, as indicated by the slimness of the female fish, both parents from the breeding tank need to be removed. The eggs laid on the underside of the flat levels will hatch after 25-30 hours. Generally from a larger female up to 250 eggs are produced and resultant hatchlings become free swimming after 3-4 days. At this stage the tiny hatchlings should fed infusorians and newly hatched brine shrimp. As they grow bigger they should be fed zooplanktons. They can also bred by another method by putting the gravid brood in a mesh net through which eggs are dropped in to the bottom and hatching takes place.

(iii) Egg-buriers

These species usually inhabit waters that dry up at some time of the year. The majority of egg buriers are Killifish which lay their eggs in mud. The mature parents lay their eggs before dying when the water dries up. The eggs remain in a dormant stage until the fresh rains and stimulate hatching. These fishes are *Aplocheiliuslineatus, A. panchax A. dayi and A. parvus.* A peat-moss substrate is one of the best substrates for egg-burying species as mentioned by some of the authors. In order to initiate hatching, the stored peat can be immersed in soft water.

(iv) Nest-builders

Nest builders build the nest for their eggs. The nest is usually in the form of bubble-nest formed with plant debris and saliva-coated bubbles (labyrinth fish), or an excavated pit in the substrate (cichlids). Nest builders practice brood care. Therefore, they should be provided with a wide leafy material with which to build their nests and the tank should have no water current to disturb the nest. Species that build nests are coming under Gouramies (*Colisalalia, C fasciata*) and fighter fish (*Bettasplendens*).

For breeding such fishes the sexes are kept separately for few weeks. As the abdomen of female becomes grossly distended with eggs it is transferred to a smaller breeding tank containing few floating plants with water level of 5-6" at a temperature of 28-30°C. After one or two days a good male is introduced in the

breeding tank. A transparent perforated plastic sheet or a glass is covered over the tank to keep the humidity and temperature at high level and help to maintain the bubble nest in good condition. The male soon begins building a bubble nest. This is possible by taking a large gulp of air at the water surface and converting it into many smaller bubbles that are passed into gill chamber and coated with an anti-burst agent before release. During and after making the nest, the male displays to the female, which usually ends with both fish embracing near the nest resulting deposition of a large numbers of eggs in the nest. After making the bubble nest if the female do not lay eggs then the male become very aggressive and may kill the female. After breeding, the female is removed. The male guards the eggs which remain attached to the floating bubble nest. Within 24 hours, hatching takes place. The moment, the fry begin leaving the nest, the male is also removed from the tank. After 36 hours, the young ones remain in free swimming stage, they are provided with infusorians as starter feed. After a week the fry starts taking newly small zooplanktons. During this stage fry requires vigorous feeding. Subsequently when they grow little bigger they can be stocked in bigger cement tanks for further growth.

(b) Mouth-brooders:
These are species that carry their eggs or larvae in their mouth. Mouth brooders can be divided into ovophiles and larvophiles group. Ovophile or egg-loving mouth-brooders lay their eggs in a pit, which are sucked up into the mouth of the female. The small number of large eggs hatches in the mother's mouth, and the fry remain there for a period of time. Fertilization often occurs with the help of egg-spots, which are colorful spots on the anal fin of the male. When the female sees these spots, she tries to pick up the egg-spots, but instead gets a mouthful of sperm, fertilizing the eggs in her mouth. Many cichlids and some labyrinth fish are ovophile mouth brooders.

Larvophiles or larvae-loving mouth-brooders lay their eggs on a substrate and guard them until the eggs hatch. After hatching, the female picks up the fry and keeps them in her mouth. When the fry can fend for themselves, they are released. Ovophile mouth-brooders can be bred in the main aquarium because the eggs are protected in the mouth cavity. However, it is better to separate mouth-brooders with eggs because of their potentially aggressive behavior. There are no special breeding tank requirements other than the usual tank set-up for the species. Larvophile mouth-brooders should be placed in a breeding tank because the eggs are not protected in the mouth, but laid on a surface where they are open to predators. For mouth brooders open cement cisterns of 2 feet water depth with planted long leafed plants are generally used for commercial production.

(c) Ovo-viviparous (Livebearers)

Livebearers are fish that bear live young. They are ovoviviparous in nature, where the eggs form and hatch within the female before birth. Livebearers are often prolific, easily bred species. Breeding of most of the livebearers is relatively easy. They are mostly molly, platy, swordtail and platy. Development of young ones takes

place inside the female body and they released after about four weeks. The species of livebearers include guppy (*Poeciliareticulata*), black molly (*Poeciliasphenops*), swordtail (*Xiphophorushelleri*) and platy (*X. maculatus*). The number of babies produced by a livebearer is normally 50-60 only, though some larger swordtails and guppies may produce as many as 100 nos. If the livebearers are fed properly with natural feed and supplemented with better artificial feed, the mother produces more than 100 young ones. Soon after the baby comes out from the mother, they have to be separated out to reduce mortality caused by predation. Small livebearers can be bred in breeding traps where the newborns come out of the reach of the brood mother. However, a more traditionally preferable set-up is a separate, densely planted tank where only gravid females are kept. As the young ones are visible in the water surface, they are to be removed and fed the powdered feed and zooplanktons. As soon as all the young are born, the mother is to be removed and fed suitably.

Fish health and care

Many aquarists/ farmer/ hobbyist do not have access to laboratory techniques that would allow a specific diagnosis and a targeted treatment of fish diseases. In most cases, symptoms are discovered during routine tank clean-up or fish feeding, and sometimes this leads to inappropriate use of medications that compound the problem.

Origin of diseases

Any kind of disease to the fish may be due to following reasons.

1. During transportation, if a fish is stressed due to bad living conditions at the retail outlets this may contaminate other fishes.
2. Introduction of a healthy carrier or diseased fish
3. Introduction of polluted water from the source it is drawn
4. Accidental introduction of various harmful substances like pesticides
5. Sudden decrease in temperature or thermal shocks
6. Sudden decrease in oxygen levels
7. Excess of nitrogenous matter due to a filter malfunction or by stocking of too many fishes.
8. Injury caused due to fight by tank mates, collision against the decorative materials or over excited mating rituals
9. Underfeeding or a poor nutritional disorders due to feeding of inbalanced diets

Preventive measures

An old proverb is always applicable to fishes also; Prevention is always better than cure. Preventing disease is much more economical than providing expensive treatments following a disease outbreak. There is not a single, ideal, universal

preventive programme that can be applied. Specific considerations must be taken into account for individual enterprise, however, some general preventive recommendations are as follows:

1. Adequate and non-polluted water
2. Sufficient space
3. Adequate and well balanced feed
4. Prevent high temperature fluctuations
5. Removal of dead fish, faecal matter as often as possible and of organic matter such as uneaten feed and prevention of accumulation of algae on tank walls.
6. Regular cleaning and disinfection of the system
7. Provision of compatible species in the tanks
8. Reduction of handling stress
9. Parasite control and preventive measures are required to be followed.
10. Provision of diligent surveillance to recognize early signs of disease
11. All new incoming fishes should be quarantined from resident stock.
12. Movement of fishes should be restricted from a suspected or unknown disease status area.
13. Separate the infected fishes
14. Provide adequate nursing for diseased fishes
15. After diagnosis, begin treatment of diseased fishes as soon as possible

Common diseases and treatment

Generally, there are two types of diseases. They are infectious and non-infectious. The former are caused by bacteria, fungi or viruses. The main triggering factor is stress, poor dietary quality and decrease in temperature. Some pathogens have a dual lifecycle: one in the fish and the other in the water. Non infectious diseases are not caused by pathogens, but by the poor environmental conditions. It is important to avoid medication with out proper consultation. However, some of the common diseases are mentioned below along with the treatments. It is always safe to consult a fish pathologist before any treatments to fish.

Table-1 Common fish diseases and treatments

Sl. No.	Symptoms	Disease	Causative agent	Treatment	Observations
1.	Pin head size white spots on the body and fins	Ichthyoph-thyriasis	Protozoan parasites (*Ichthyoph-thirius*)	Increase the temperature and treat with 5% Methylene blue (1 drop/L)	Contagious, observed during sudden drop of water temperature

				Treat the fish for 1 week	
2.	White spots smaller than above	Oodinium	Unicellular parasite (mono flagellate)	Copper sulphate 0.1 mg/l for 10-15 minutes bath	Contagious
3.	White clumps with cotton like appearance	Saprolegni asis0	Fungi including *Saprolegnia*	1tsp salt/2L water, 1-2 drops of 5% Methylene blue or malachite green 2 mg/L for 30 min. dip or 0.1 mg/L for permanent bath	Favoured by wounds
4.	Swollen eyes	Exophthal mus	Bacteria, viruses, fungi, sometimes together	1% Silver nitrate on poped eyes followed by 1% potassium permanganate	Treatment is difficult
5.	Gradual disintegratio n of fins	Pseudomo-niasis	Bacterial disease	Surgical removal of ragged portion by a fine sterilised scissor, paint the cut wounded portion by Iodine solution. Repeat the same at 12 and 24 hours	Unusual swimming behaviour
6.	Swollen abdomen, erected scale	Dropsy	Bacterial disease	No known cure. Antibiotics may be tried	Contagious, difficult to treat
7.	Opercula sticking out, unusual swimming , tiny flukes on the gills	Gyrodacty-liasis	Parasite fluke, *Gyrodactylu s*	Formaldehyde 5-6 drops/l water dip treatment for10 minutes. Repeat for 3 days.	Not easy to detect

8.	Red patch in the body,	Argulosis/ learnaeasis	*Argulus/ Lernaea* Ectoparasite	Physical removal of parasite, 15 minutes bath in 1-2% potassium permanganate, painting the region with Iodine solution. Repeat the same after 12 and 24 hours	Violent rubbing due to irritation
9.	Unusual swimming behaviour	Longer exposure to poor water quality	Oxygen problems, poor water quality due to nitrogenous substances	Increase of oxygen, one third water exchange, check pH and nitrates level	Symptoms can also correspond to an infectious disease
10.	Gasping for air at the surface	Lack of oxygen	Defective aeration,	Increase the aeration, check all water parameters	This can be a symptom of an infectious disease
11.	Weight loss and poor growth	Nutritional problems	Underfeeding or lack of balanced diet	Fresh feed and live food	Possibility of commercial vitamins in the feed
12.	Skeletal deformity	Hereditary problem or lack of vitamin C in the feed	Genetic origin or Nutritional disorder	If mass deformity occurs separate the parents , check the Vitamin C level	It is not unusual for a few of the fry in a batch to be affected

Table-2Adverse environmental conditions and related problems

Sl. No.	Problem	Symptom in fish	Remedial measures
1.	Chlorinated water	Restless movement, loss of balance	Vigorous aeration for 24 hours.
2.	Ammonia load	Inflamed gills, and fin edges blood spot, loss of balance	Water exchange and aeration required.

3.	Nitrites and Nitrates	-do-	Partial water exchange.
4.	Oxygen and nitrogen (excess)	Gas bubble disease. Small bubbles visible under skin, in fins and around eyes, Exophthalmia (Pop eye)	Keep the tank away from sunlight, if algae or plants exist.
5.	Insufficient oxygen	Gasping at surface, loss of body colour.	Vigorous aeration is required with partial water exchange.
6.	pH		
	(a) Acidosis (pH 04-05)	Fast swimming movements; Gasping at surface. Fish jump out from water.	Avoid over stocking, Partial water exchange. Stop feeding temporarily.
	(b) Extreme Acidosis (pH > 04)	Sluggishness tendency to settle in bottom and to hide, loss of appetite and colour.	Stop feeding, exchange water, and provide aeration.
	(c) Alkalosis (pH 09-10)	Serious damage to gills, disintegration of fin edges, body slime is excessive.	Avoid direct sunlight and partial water exchange.
	(d) Temperature (low)	Sluggish movement, resting at bottom. Fin and body movement gradually slows down. Loss of colouration.	Add ground water drawn through deep bore well, as part of water exchange exercise.
	(e)Temperature (high)	Increased level of activity (above normal) Increase in metabolism, increased rate of respiration, gasping at surface.	Addition of water or in an extreme situation thermostat also can be used if the tank is small. Add well water. Water exchange is very much essential.

STUDY OF INNATE IMMUNITY: PARASITIC INFECTIONS IN FISH

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Parasite:

A parasite is an organism that lives in or on another larger organism of a different species (the host), upon which it depends for nourishment. Although the parasite benefits from the association, the host is harmed. Depending on the species, the host/parasite relationship may be temporary or permanent.

An organism causing harm to a fish either externally or internally and deriving nutrients from it are referred as fish parasites.

Types of parasite:

1. **Based on site of attachment:**
   a. Ectoparasite and
   b. Endoparasite

2. **Based on type of attachment:**
   a. Obligate and
   b. Facultative

3. **Based on cell type:**
   a. Protozoan parasite
   b. Metazoan parasite

Ectoparasite:

A parasite that lives or feeds on the outer surface of the host's body (including the gills, mouth, skin and fin surfaces) is called an **ectoparasite**. Ectoparasites do not usually cause disease themselves although they are frequently a vector of disease and leads to many secondary infections. It includes fish louse (*Argulus* sp.), Lernea, and flukes.

Endoparasites:

A parasite that lives inside the body (including tissues, blood, organs and gastro intestinal tract) of its host is called an **endoparasite.** Endoparasites include organisms such as tapeworms, hookworms, and trypanosomes.

Obligate parasite:

The parasite which cannot complete its life cycle without exploiting a suitable host is called an obligate parasite**.** They are entirely dependent on their host for the completion of their life cycle and survival.

Facultative parasite:

The organism that may be parasitic upon another organism but can exist independently is known as facultative parasite. They does not rely on any host for completion of its life cycle and survival.

Protozoan parasite:

The name 'proto-zoa' literally means 'first animals'. Protozoa exist as structurally and functionally independent individual cells (unicellular organisms). Four main groups of protozoan parasites are recognized on the basis of their locomotary organells. They are as follows:

1. Amoebae
2. Fagellates
3. Ciliates
4. Sporozoa

Protozoan parasite			
Amoebae	**Fagellates**	**Ciliates**	**Sporozoa**
Apiosoma sp.	*Piscinoodinium* sp.	*Ichthyophthirius multifiliis*	Microsporidia (Glugea sp.)
Ambiphyra sp.	*Icthyodinium* sp.	*Trichodina* sp.	Myxobolus sp.
	Trypanosoma sp.	*Chilodonella* sp.	Thelohallanus
	Costia sp.		Heneguya
	Ichthyobodo sp.		

Metazoan parasite:

It basically covers any multicellular parasites. These parasites are broadly divided into the Nematodes or round worms, Platyhelminthes or flat worms which again are comprised of trematodes or flukes and cestodes or tape worms and Copepods.

Metazoan parasite			
Platyhelminthes			**Nematodes**
Trematodes		**Cestodes**	
Monogenetic trematodes	**Digenetic trematodes**		
Dactylogyrus sp.	*Sanguinicola* sp.	*Diphylobothrium sp.*	*Capillaria* sp.
Gyrodactylus sp.	*Posthodiplostomum* sp.	*Protocephalus* sp.	*Contracaecum* sp.
	Diplostomum sp.	*Ligula* sp.	*Eustongylids* sp.
	Neascus sp.		*Camallanus* sp.
	Uvulifer sp.		*Philometra* sp

Protozoan parasite infection in carp:

Many fish's disasters in fish farms were caused by different considerable protozoa, which have direct life cycle and facilitate translocation from host to host making huge damages to fish wealth, i.e. *Ichthyophthirius multifiliis*, *Trichodina*, *Chilodonella, Myxobolus* etc. Parasites with direct life cycles can be important pathogens of cultured fish; parasites with indirect life cycles frequently use fish as intermediate hosts. Knowledge of specific fish hosts greatly facilitates identification of parasites with marked host and tissue specificity, while others are recognized because of their common occurrence and lack of host specificity. Examination of fresh smears that contain living parasites is often diagnostic.

Ambiphyra and ***Apiosoma*** are sessile amoebae that can be found on the skin, gills, and fins of fish. These seem to be more common in pond fish than tank-reared fish and have a predilection for organically rich environments. They are not generally found on marine fish. When examined from a lateral view, *Ambiphyra* is the shape of a tin can and *Apiosoma* spp are vase-shaped. Neither Ambiphyra spp nor Apiosoma spp are particularly pathogenic if present in low numbers (no more than 1–2/low-power field); however, when present in high numbers, these parasites can cause significant epithelial damage, predisposing fish to opportunistic pathogens in the environment and compromising respiration and osmoregulation. Infested fish demonstrate flashing, decreased appetite, loss of condition, and hyperplasia of infested epithelial surfaces. Severe infestation of the gills is particularly damaging. The organisms can be controlled with a single treatment of formalin, copper sulfate, potassium permanganate, or a salt dip. Excessive crowding and poor sanitation are frequently associated with heavy infestations and should be corrected.

Ciliated protozoa are among the most common external parasites of fish. Most ciliates have a simple life cycle and divide by binary fission. Ciliates can be motile, attached, or found within the epithelium. The most well-known organism in the latter group is *Ichthyophthirius multifiliis*. The infection caused by *I multifiliis* is referred to as "ich" or "white spot disease." The parasite is an obligate pathogen that cannot survive without the presence of living fish. All fish are susceptible, and a similar appearing parasite, Crytocaryon irritans, occurs in marine species. The parasite is readily transmitted horizontally via direct exposure to infected fish or via fomites (nets, etc). Fish that survive an outbreak may be refractory in future outbreaks, but may also serve as a source of infection to previously unexposed individuals. The parasite invades epithelial tissue of gills, skin, or fins, leaving a small wound and visible white spot or nodule where each parasite encysts. The organism causes substantial damage because of its unique life cycle (*see* below), which allows a rapid intensification of infection. Infected fish are extremely lethargic and covered with visible white dots. Mortality can be rapid and catastrophic. Infections that are confined to gill tissue may not be recognized by nonprofessionals (because white spots are not grossly visible), but are easily diagnosed using gill biopsy techniques. The organism is identified using a light microscope at magnification of 40× or 100×. It is large (0.5–1 mm), round, covered with cilia, and has a characteristic horseshoe-shaped macronucleus. Its characteristic movement varies from constantly rotating to ameboid-like.

Ich infections require immediate and thorough medical treatment. Formalin or copper are often drugs of choice. Over-the-counter medications for pet fish often contain formalin and malachite green and are effective, but due to regulatory concerns regarding the use of malachite green, should not be dispensed by the veterinarian. Multiple chemical treatments (with intervals determined by water temperature) are required for successful treatment of *I multifiliis*. At warm temperatures typical of home aquaria (eg, >26°C), infected fish should be treated every 2–3 days. Constant chemical exposure for at least 3 wk is generally recommended to control Cryptocaryon in marine systems; lowering salinity to 16–18 ppt is often helpful.

Ichthyobodo spp, Costia spp are some of the most common and smallest (~15 × 5 µm) **flagellated protozoan** parasites of the skin and gills. They are flattened, pear-shaped organisms with 2 flagellae of unequal lengths. These parasites can be found on freshwater or marine fish from a broad geographic range. Ichthyobodo move in a jerky, spiral pattern, and free-swimming organisms are fairly easy to identify in direct smear preparations. Once attached, the organism can be difficult to see, but movement typical of a flickering flame

may be seen under 400× and is characteristic. Affected skin often has a steel-gray discoloration due to copious mucus production ("blue slime disease"), and gills may appear swollen. Behavioral signs of infestation include lethargy, anorexia, piping, and flashing. Ichthyobodo is readily controlled with salt, formalin, copper sulfate, or potassium permanganate baths. Because the parasite has a direct life cycle, a single treatment should be adequate. If reinfestation occurs, sanitation and quarantine practices should be evaluated. Oodinium spp, is less common but can also result in high mortality. These parasites produce a disease that has been called "velvet," "rust," "gold-dust," and "coral disease" because of the brownish gold color they impart to infected fish. The pathogenic stages of the organism are pigmented, photosynthetic, nonflagellated, nonmotile algae that attach to and invade the skin and gills during their parasitic existence. When mature, these parasites give rise to cysts that contain numerous flagellated, small, free-swimming stages that can initiate new infections. Control of Amyloodinium is challenging, and the prognosis is guarded. Copper sulfate is the only therapeutic option for food animals in the USA, and repeated treatments are necessary to break the life cycle. The disease is particularly problematic in clown fish. The treatment of choice in ornamental fish is chloroquine, delivered at 10 mg/L as an indefinite bath.

Myxosporidian diseases significant in aquaculture include whirling disease and proliferative kidney disease of salmonids and proliferative gill disease ("hamburger gill disease") of channel catfish. Whirling disease is caused by *Myxobolus cerebralis*. Fish are infected as fingerlings when the parasite infects cartilage in the vertebral column and skull, resulting in visible skeletal deformities. Affected fingerlings typically show rapid tail-chasing behavior (whirling) when startled. The disease is also sometimes called "blacktail" because the peduncle and tail may darken significantly. Recovered fish remain carriers. Adults do not show behavioral signs, but skeletal deformities associated with infection do not resolve. The disease can be prevented by purchasing uninfected breeding stock and maintaining them in an environment free of the intermediate hosts (tubifex worms). A presumptive diagnosis of whirling disease is made by detection of spores from skulls of infected fish. Diagnosis may be confirmed histologically or serologically. Whirling disease is of regulatory concern in some states. **Microsporidians** are tiny, intracellular, spore-forming organisms with single polar filaments that are common parasites of finfish. They are host- and tissue-specific and can also infect helminth parasites of fish. The spores are extremely resistant, and microsporidian diseases are considered non-treatable. Microsporidians have a direct life cycle; therefore, horizontal transmission in an aquarium is likely. Depopulation and disinfection are recommended for elimination of microsporidian infections.

Metazoan parasites infecting carp:

A majority of freshwater fishes carry heavy infection of parasites which cause deterioration in the food value of fish and may even result in their mortality. Besides these, there are a number of helminth parasites which are transmitted to human beings only through fish. The study of helminth parasites, its frequency and distribution in fishes, is very scant in Bangladesh, compared to the considerable progress achieved by Japan, India and other oriental regions. These parasites use the fish for their shelter and food and destruct more or less each and every organ resulting in pathogenic effects (Lilley et al.1992). Parasites interfere with the nutrition, metabolism and secretary function of alimentary canal, damage nervous system and even upset the normal reproduction of the hosts (Rahman et al.1998a,b). The distribution of helminths of the same host and their incidence and intensityof infestation varies from one place to another. One should know these variations in infestation and the taxonomic details of these worms for proper management of the fish crop in ponds.

Helminths are common in both wild and cultured fish. Fish frequently serve as intermediate or transport hosts for larval parasites of many animals, including humans. Helminths with direct life cycles are most important in dense populations, and heavy parasite burdens are sometimes found. In general, heavy parasite burdens seem to be more common in fish originating from wild sources.

Monogeneans have direct life cycles and are common, highly pathogenic, and obligatory parasites of the skin and gills. Freshwater parasites tend to be ~0.1–0.8 mm long and are best seen microscopically; however, several important species parasitizing marine fish are significantly larger and may be visible grossly. The worms can be identified by their characteristic hold-fast organ, the haptor, which is armed with large and small hooks. Aquarium and cultured fish are subject to a rapid buildup of parasites by continuous infection and worm transfer to other fish in the tank or pond. Although many species are host-specific, the more common types seen in aquaria are less selective. The 2 most common genera in freshwater aquaria are *Gyrodactylus* and *Dactylogyrus*. *Gyrodactylus*, a common parasite of goldfish, gives birth to live young and is usually found on skin; *Dactylogyrus* lays eggs and is principally a parasite of the gills. Monogenean infected fish may show behavioral signs of irritation, including flashing and rubbing the sides of their bodies against objects in the aquarium. Fish become pale as colors fade. They breathe rapidly and distend their gill covers, exposing swollen, pale gills. Localized skin lesions appear with scattered hemorrhages and ulcerations. Ulceration of the cornea may become evident if the eyes are involved. Mortality may be high or chronic. Praziquantel (2 mg/L, prolonged bath) is the treatment of choice for monogenean infection in freshwater and marine ornamental fish. Formalin is the only treatment option for food fish. Multiple treatments at weekly intervals are recommended for Dactylogyrus because eggs may be resistant to chemical treatment. Organophosphates (0.25 mg/L, prolonged bath) have been used successfully in ornamental fish in the past, but treatment with praziquantel is considered more effective. Organophosphates should be avoided in systems containing elasmobranchs. Monogenes on marine fish can be removed using freshwater dips for 1–5 min, depending on the tolerance of the species; however, eggs will not be damaged or removed. To prevent the disease, introduction of infected fish should be avoided.

Digeneans have complicated life cycles, with several larval stages that infect one or more hosts. With rare exceptions, the first intermediate host is a mollusc, without which the life cycle generally cannot be completed. A diagnosis usually can be established by gross or microscopic examinations that reveal the cercarial, metacercarial, or adult worms in any of the tissues or body cavities of the fish. Fish tend to form pigmented tissue encapsulations that encyst the parasites. Depending on the color of the cysts in the skin, the condition is called black, white, or yellow grub disease. Heavily parasitized fish often are weak, thin, inactive, and feed poorly. Treatment is not recommended. Pond-reared, juvenile, tropical fish may develop severe gill disease from metacercarial cysts in gill tissue. Although acute death is occasionally seen, infected fish more commonly die during harvest or shipping when they may be exposed to suboptimal dissolved oxygen concentrations. Treatment of infected fish has not been successful; however, prevention of the disease by elimination of the intermediate host, a freshwater snail, has been effective.

Both larval and adult **cestodes** are common in fish. Larval forms encyst in visceral organs and muscle, while adults usually are found in the intestinal tract. Aquatic Crustacea are the most common intermediate host for fish; accordingly, wild and cultured pond fish may be heavily infected. *Diphyllobothrium latum*, the broad fish tapeworm infection of humans, is acquired by eating larval tapeworms in the flesh of food fish. Aquarium fish may

be purchased with heavy cestode infections but have limited exposure once in the aquarium (unless fed infected intermediate hosts). There is no safe, effective treatment for larval tapeworm infections. The Asian tapeworm, Bothriocephalus acheilognathus, is occasionally seen in carp and aquarium fish. It is usually found in the anterior intestine and may be associated with enteritis and degeneration of the intestinal wall. Praziquantel is the drug of choice for treatment of cestodes in ornamental fish, but it is not approved for any aquatic use and cannot be used in food animals.

Nematodes are common in wild fish that are exposed to the intermediate hosts. Fish may be definitive hosts for adult nematodes, or they may act as transport or intermediate hosts for larval nematode forms (anisakids, eustrongylids, and others) that infect higher vertebrate predators, including humans. Encysted or free nematodes can be found in almost any tissue or body cavity of fish. Aquarium and cultured pond fish may be heavily infected if crustacean intermediate hosts are present. Cyclops and Daphnia spp are common intermediate hosts for *Philometra* sp, a nematode that is pathogenic for guppies and other aquarium fish. These blood-red worms can be seen in the swollen abdominal cavity and protruding from the anus of affected fish (red worm disease). Capillaria sp are commonly found in aquarium fish, particularly freshwater angelfish. Heavy infections in juvenile angelfish have been associated with poor growth rates and an inability to withstand shipping and handling. Treatment with fenbendazole (25 mg/kg for 3 days) is recommended, but efficacy has not been firmly established. Levamisole (10 mg/L) administered as a bath treatment for 3 days has also been recommended. Ivermectin is highly toxic to aquarium fish, particularly cichlids, and its use is not recommended.

Some **Copepods**, such as the anchor worm, are obligatory parasites of finfish during specific stages of their complicated life cycle. They lose their copepod form, including their appendages, and become rod- or sac-like structures specifically adapted for piercing, holding, feeding, and reproducing. Grossly, they appear as barb-like attachments to the skin or gills, where they feed on blood and tissue fluids. They can cause hemorrhage, anemia, and tissue destruction, as well as provide a portal of entry for other pathogens. Many different species of these parasites can be found on freshwater and marine fish. The anchor worms, Laernea sp, are commonly found in a wide variety of aquarium- and pond-reared fish, including goldfish and other cyprinids. Ergasilus sp infest the gills. Organophosphates are effective in controlling copepodid parasites, but legal restrictions constrain clinical use. Some success has been achieved in freshwater fish by giving infected fish a 3% (30 ppt = 30 g/L) salt dip (<10 min, remove fish when it rolls) followed by 5 ppt (5 g/L) salt added to the affected tank for 3 wk. The increased salinity kills immature forms as they hatch.

Lice (Branchiuria) are related to the parasitic copepods and have flattened bodies adapted for rapid movement over the skin surface. By means of hooks and suckers, they periodically attach for feeding by inserting the piercing mouth part (stylet) into the skin. Sea lice (Lepeophtheirus salmonis) are a significant disease problem of pen-reared salmonids. Consultation with a salmonid health specialist is suggested if these parasites are encountered, as treatment options are limited and environmental concerns are significant. Argulus spp are lice commonly found on aquarium, pond-reared, and wild freshwater fish. Organophosphates (0.25 ppm, prolonged bath) are used for treating infested aquarium fish but are not approved for use in food fish.

Comparative host-parasite immunology

Fish are the first organisms with an immune system showing the same basic aspects of specificity and memory formation as observed in higher vertebrates. There are more then 55 000 fish species that, by inhabiting highly different environments, are challenged by all kinds of pathogens. While their immune system evolved under constant pressure from infectious micro-organisms such as viruses, bacteria and larger parasites, it is fascinating to see how the immune system of such an evolutionary successful group of animals evolved under so many different environments. Comparisons (e.g. with the human immune system) may not only give an idea of the history of the immune system, but may also distinguish what has been conserved from what has varied, which will shed light onto what is essential and what is accessory to the human immune system.

Studies on parasitic infections of fish enable the evaluation of different modes of adaptation that allow parasites to infect and persist in vertebrates. Antigenic variation observed in the Salivaria (e.g. *T. brucei*) and intracellular hiding (e.g. *T. cruzi*) are the best known examples. In analogy to the salivarian trypanosomes both *T. danilewskyi and T. borreli* are believed to live exclusively extracellularly in the blood and tissue fluids of their fish hosts but, differently, show no evidence of antigenic variation. Analogous to the stercorarian trypanosomes, the surface coat of *T. danilewskyi* shows a clear resemblance with the carbohydrate dominated surface coat of *T. cruzi* highly glycosylated mucin-like surface proteins are abundant, anchored in the plasma membrane by glycosylphophatidylinositol (GPI) residues. A co-ordinated study of the co-evolution of fish parasites with the fish immune response will certainly provide new insights in immune evasion mechanisms used by parasitic species that have not yet been studied in great detail.

Of course, host and parasite are not entities that can be considered separately. Instead, host parasite interactions need to be considered. It is not of benefit to a parasite to kill the host, at least not until transmission to another host has been ensured. For the fish it is important that the immune response adapts to different developmental stages of the parasite. A highly activated immune system must be down regulated immediately after control of initial parasite replication. In fact, it is becoming increasingly clear there is a delicate balance between an effective, protective immune response and one that causes more damage that it prevents. Indeed, in the case of some parasite infections it is not clear whether morbidity should be ascribed to the parasites or is the result of an uncontrolled immune response. For example, trypanostatic effect of nitric oxide (NO) on *T. borreli invitro*, NO appeared to have an immunosuppressive effect *in vivo*. The immunosuppressive (crytostatic) effect was strongest on carp lymphocytes, that were especially susceptible to No, while high levels of the antioxidant glutathione in neutrophilic granulocytes protected this cell type from NO mediated cytotoxicity. This is a clear example of a disturbed balance between an immune response (stimulation of macrophages) that should be effective and protective and the same response causing more damage (overproduction of NO) than it prevents.

To date, it is well recognized that the innate immune responses against parasites in fish are at least as important for their protection as are the adaptive responses. A parasite differs from its host in many thousands of molecules and if a fish were to recognize and respond to this large variety of antigens, the resulting massive lymphocyte activation would hinder an effective immune response. Thus, the immune system generally reacts to a limited range of antigenic determinants only, the initiation of the adaptive response being controlled by innate immune recognition. The innate immune system has to differentiate between the

large number of pathogen associated molecular patterns (PAMPs) found in nature, distinguishing these structures from self and discriminating between different pathogens. Protozoan PAMPs that are predominantly re recognized as foreign are the GPI anchors. It appears that district PAMPs from various pathogens are recognized by distinct members of the roll like receptors (TLR) family for initiation of the immune response. TLRs can establish a combinatorial repertoire to discriminate among the large number of PAMPs found in nature (e.g. TLR2 and TLR6 together recognize peptidoglycan, TLR2 alone recognizes lipopetides). Interestingly, recent findings indicate that the recognition system for proteozoan GPI has much in common with the recognition system of LPS, while the use of TLR2 or TLR4 knockout mice indicated an essential role of TLR2, but not TLR4, in the induction of IL012 TNF_a and No by GPI activated macrophages.

For *T.borreli* infections in carp, a heat labile fraction of *T. boreli* CpG motifs in its DNA are PAMPs responsible for the induction of NO and most likely also for the induction expression of TNF_a, IL-$_1$b and $_1$b and $_i$NOS by carp head kidney phagocytes *in vitro*. Probably, in the signal transduction pathway leading to activation of phagocytes, protein tyrosine kinase (PTK) and protein kinase C (PKC) are activated and collaborate in activation of the transcription factor NF-$_K$B. Activation of NF-$_K$B then leads to downstream expression of the NF_KB dependent loci iNOS, IL-1b and TNFa. The heat labile fraction of *T. borreli* responsible for phagocyte activation could be GPI-anchored proteins.

T. danilewskyi PAMPs did not seem to induce as much NO production as *T. borreli* PAMPs. Probably, divergent GPIs have evolved to the advantage of the various parasitic protozoa to activate or down regulate the endogenous signaling of the host. Most likely, in fish, pattern recognition receptors such as TLRs on cells of the monocyte-macrophage lineage recognize PAMPs on parasites and transduce a signal. In fact, many partial sequences from a variety of fish species have been reported as putative TLR like receptors. More recently, we described the first full length TLR cDNA in goldfish, predicted to encode a type II transmembrane protein with an extracellular domain containing leucine rich repeats and cytoplasmic tail encoding a toll/interleukin-1 receptor domain.

In conclusion, no matter where a parasite enters the body it will encounter resident but highly active macrophages that can recognize PAMPs by their TLRs. These macrophages are maintained by local division and immigration from blood and not only are able to phagocytose large numbers of parasites but also participate in chemotaxis, cytokine mediated intercellular communication and regulation of coagulation of the complement cascade and of acute phase protein production. As such, macrophages perform a key function in the immune response against parasites.

Macrophages

Natural resistance associated macrophage protein (NRAMP)

Macrophages play a critical role in iron metabolism. Transition metals such as iron participate in many cellular functions such as (i) regulation of transcription (ii) the function of hundreds of different enzymes including metalloproteases, superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS); and (iii) cellular functions such as endosomal fusion. Any dysfunction in the metal sensing/ transporting pathway will have pleiotropic effects and may cause disease. For that reason, iron concentrations in body tissues are tightly regulated, excessive iron can lead to tissue damage as a result of the formation of free radicals. The co-ordinate control of iron uptake and storage is tightly regulated by the feedback system of iron responsive element containing gene products and iron regulatory proteins that modulate the expression levels of the genes involved in iron metabolism.

Macrophages are responsible for the re-utilization of iron from haemoglobin by phagocytosing senescent erythrocytes. In non-erythroid cells, iron is either stored as ferritin or targeted to iron containing molecules, such as iron regulatory proteins. Also, in macrophages, iron is necessary for the production of hydroxyl radicals (OH) via the Fenton reaction, and iron levels control the production of nitric oxide (NO) after activation by antigenic stimuli.

In plasma, iron is transported by transferring, with high affinity for ferric iron, a 80 kDa glycoprotein consisting of two globular domains, both containing a high affinity binding site for a single iron molecule. These binding sites are highly conserved through evolution. Transferrin exists as a mixture of iron free (apo), single iron (monomeric) and two iron (deferric) forms. Although the relative percentage of each form depends on the concentration of iron and transferring in plasma, under normal conditions most of the iron molecules are bound to transferring. Most cells (so not only macrophages) acquire iron from transferring first by binding of transferring to transferring receptors. Following binding, the complex consisting of iron, transferring and transferring receptor is internalized via a classic receptor mediated endocytic pathway. Iron is released from transferrin within acidic endosomal compartments and then transported across the endosomal membrane into the cytoplasm by the iron transporter, NRAMP2, or solute carrier family 11 memebr 2 (Sclc11a2). Aportransferrin and the transferring receptor both return to the cell surface, where they dissociate at neutral pH.

There are two different views on how exactly NRAMP1 polymorphisms influence disease resistance/susceptibility. One hypothesis is that NRAMPI1 functions by increasing intraphagosomal Fe^{2+} in order to provide the catalyst for the Haber-Weiss/Fenton reaction, generating highly toxic hydroxyl radicals for5 bacterilcidal activity. The other hypothesis is that NRAMPI1 functions by depriving the intraphagosomal pathogen of essential Fe^{2+} (critical for growth) and other divalent cations (Zn^{2+}, Mn^{2+}, for superoxide dismutase), both critical to pathogens for mounting an effective anti-oxidant defence. The broad specificity divalent cation transporter activities of NRAMP provide a rational explanation for the pleiotropic effects of NRAMP1 on macrophage activation and function. (e.g. regulation of IL-lβ,iNOS, MHC class II, TNFα, NO release, L-arginine flux, oxidative burst and tumouricidal as well as anti-microbial activity)

As described above, cells acquire iron from transferring by release of iron within acidic endosomal compartments, using the NRAMP2 protein to transport iron across the endosomal memebrane into the cytoplasm. Many cell types show this ability, in contrast to the transport of iron across the membrane of late endosomes/ lysosomes by NRAMP1, which is restricted to macrophages. Thus, expression of NRAMP1 is limited to macrophages while NRAMP2 expression is ubiquitous. There are, however, indications that no NRAMP1 exists in fish but that a duplicated NRAMP2 gene exists with different functions. We described the first NRAMP gene in fish as most probably being NRAMP2 gene. Likewise, in trout (O*ncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), catfish (*Ictalurus punctatis)* and puffer fish (*Fugu rubripes*) NRAMP2 genes have been found (for trout for fathead minnow, accession nr. AF190773; for catfish, for puffer fish. So far, no fish NRAMP1 gene has been identified.

However, in fish, in contrast to the situation in mammals, the NRAMP2 gene is often duplicated. For example, in rainbow trout two NRAMP proteins have been described (α and β) , both of them clustering with the mammalian NRAMP2 proteins (as did the carp NRAMP). Also in puffer fish, two NRAMP2 protiens have been found, one of them localizing to late endosomes/lysosomes, consistent with a divergence towards an NRAMP1

like function. In rainbow trout, NRAMP2α expression was limited to the head kidney and ovary, while NRAMP2β expression was ubiquitous. Three NRAMP transcripts were identified in catfish but these were due to alternative splicing in the 3 UTR and alternative polyadenylation and did only yield a single functional protein. However, injection of catfish with LPS increased transcription of NRAMP2 in the kidney and spleen. Furthermore, expression could be induced in a catfish monocyte/ macrophage cell line. In carp, NRAMP2 expression in the head kidney was affected during *T. borreli* infection as shown by an early down regulation that could not easily be explained by a change in cell types populating the head kidney. These data suggest a differential regulation of NRAMP2 expression supportive of the existence of an NRAMP1 like function in fish. Further research will be needed, however, to verify expression of an NRAMP1 like transcript in fish macrophages.

Transferrin

As described before, cells acquire iron from tranferrin first by binding of transferring to transferring receptors. Transferrin itself, however, may also exert effects that are not directly linked with maintaing iron levels. These include the inductin of neutrophilic end stage maturation and the up-regulation of chemokine synthesis by human proximal tubular epithelial cells, while transferring may also be an important component of inflammatory serum that modulates oxygen and nitrogen radical production by chicken macrophages.

Recently, in goldfish, we showed that tranferrrin cleavage products act as macrophage activating factor (MAF) by stimulating macrophages to produce large amounts of nitric oxide. Possibly, neutrophilic granulocytes, that are generally the first immune cells recruited to the site of inflammation, initiate the cleavage of transferring via the production of neutrophil derived proteases. Likewise, activation by recognition of a proteolytic fragment from a self-protein has also been found in *Drosophila* (a proteolytically processed products of the sp"tzle gene activates Toll). The recognition of endogenous 'danger' signals (e.g. moleculaes produced by stressed cells, or products that are usually found inside a healthy cell) by the immune system is the basis of the 'Danger model'. At this moment research on a putative goldfish macrophage receptor able to detect these transferring cleavage products is ongoing.

Carp transferring is highly polymorph and more than seven different alleles have been identified according to differences in electrophoretic mobility. So far, no differences in iron-binding capacity have been reported for the transferrin alleles. However, transferring polymorphisms might give rise to cleavage products with different three dimensional conformations. Subsequently, these cleavage products might have different abilities to bind to the putative macrophage receptor recognizing transferring cleavage products. This could result in different capacities to induce nitric oxide production in cyprinid macrophages and would certainly provide a functional rationale for the transferring polymorphism observed in fish.

Considering host parasite interactions, pathogenic micro-organisms such as trypanosomes also have evolved a range of mechanism from one host species to another, trypanosomes have to deal with the diversity of host transferrins. For *T. brucei* it has been found that the genome has up to 20 expression sites that encode similar but not identical transferring receptors, one being active at a given time. By switching between different expressions sites, the parasite can express different host –specific transferring receptor. Transferrin uptake by *T. brucei* involves transferring binding to a hetero-dimeric tranferrin (TF) binding protein protein complex (TFBP). The TFBP-TF complex is internalized and

transported to lysomsomes, where transferring is proteolytically degraded. Most probably, the receptor is recycled to the membrane of the flagellar pocket. The resulting large peptide fragments are released from the trypanosomes while iron remains cell associated. The possibility that such parasite induced fragments of transferring also might stimulate NO production in activated macrophages is purely speculative but could provide an explanation for the strong induction of NO by *T.borreli* observed.

Nitric oxide

In mammals, macrophages are considered the prime source nitric oxide (NO), which has been recognized as one of the most versatile players in the immune system. No is involved in the pathogenesis and control of infectious diseases, tumours, auto immune processes and chronic degenerative diseases. NO can react with a variety of reaction partenrer (DNA, proteins, thiol, prosthetic groups, reactive oxygen intermediates (ROIs)) and is widespread produced (by three different NO synthases). Considering the fact that many of the targets of NO are regulatory molecules (e.g. transcription factors) it is not surprising that NO has pleiotropic effects.

As described above, iron availability is a major factor modulating the immune response. Many of the cytotoxic effects of No can be explained by its reactivity with iron at the active sites of enzymes. Reactive nitrogen intermediates (RNIs)and ROI such as No and H_2O_2 can modulate iron availability within the cell by activating iron responsive element (IRE) binding by iron regulatory proteins (IRP) -1 and IRP-2. These IRPs bind to mRNA translation or stability. IRPs are sensitive to changes in iron concentrations. At highiron concentratin, a 4Fe-4S cluster is inserted in the IRP, liganded to three cysteine residues, inactivating its IRE binding. At iron deprivation, IREs in the 5UTR of *ferritin* (iron storage) inhibit ferritin mRNa translation, while IREs in the 3UTR of the transferring receptor *TFR* (iron uptake) and NRAMP2 confer stability to these mRNAs and increase TfR and NRAMP2 protein. Notably, also mRNAs encoding erythroid 5-aminolaevulinic acid synthase (5-ALA synthase), the first enzyme in haem synthesis for haemoglobin, harbour an IRE in the 5′-UTR. High NO levels, by activating IRP, might inhibit 5′-ALA *synthase* translation. This may be relevant to the role of nitric oxide in parasite induced anaemia and may explain lowered blood haemoglobin concentrations associated with e.g. parasitic infections. The regulatory cross talk between iron and No in macrophages is further highlighted by the transcriptional regulation of iNoS by iron.

What has become clear during the last few years is the importance of the amino acid L-arginine as a factor that can influence immune responses: L-arginine serves not only as substrate for No production but also as substrate for arginase activity. Arginase converts L-argnine in ornithine that is the precursor for polyamines, important for cell replication. In this respect, L-arginine is also of vital importance to the parasite. Parasites need L-arginine is also of vital importance to the parasite. Parasites need L-arginine is also of vital importance to the parasite. Parasites need L-argnine for synthesis of polyamines, required for DNA and synthesis of trypanothione(the parasite equivalent of glutathione, the main protectant against nitrosative and oxidative stress) . In fact, it has been demonstrated that polyamine biosynthesis inhibitors can lead to the destruction of fish trypanosomes. Trypanosomes actively compete with their hosts for this essential nutrient. As fish cannot synthesize L-arginine they present an excellent model for studying the role of L-arginine in the immune response.

Macrophage polarization

As mentioned above, arginse converts L-arginine in ornithine that is essential to cells for replication. It is this arginase activity that has provided more insight into the existence of two macrophage subsets, activated macrophages metabolize L-arginine by two pathways involving either the enzyme iNOS or arginase. The balance between the two macrophage subsets is under influence of a competitive regulation by T helper (Th)1 and Th2 cells via their secreted cytokines. In mammals, it is now clear that a wider variety of parasite induced responses show a dominant Th1 or Th2 cytokine production profile that is associated with either an excerbative or protective effect on infection. The discovery that cytokines produced by respective Th1/Th2 lymphocyte subsets cross inhibit each other's development and provides a widely applicable, molecular based rationale for the understanding of the polarization of immune responses observed in many infectious diseases. Th2 immune responses are associated with the production of interleukin (IL)-4, IL-10 and IL-13, which are essential for the production by B cells of antibodies, while Th1 immune responses are particularly associated with tumour necrosis factor (TNF)α and interferon (IFN)y. Immune responses can often be assigned a type I or type II designation based solely on the cytokine production profile without directly implicating the cellular source. Classically activated (or M1) macrophages, occur in a type I cytokine environment and are inhibited by type I cytokines. Classically activated macrophages possess cytotoxic, antimicrobial and antiproliferative functions based on their ability to secrete NO, and play a defensive role in several diseases. However, the same classically activated macrophages also secrete inflammatory mediators (TNFα, IL-1β, IL-6 and NO) that are involved in the setting of immunopathologies. In contrast, alternatively activated macrophages secrete anti-inflammatory molecules (IL-10, TGF-β) that down-regulate inflammatory processes and counteract No synthesis by expressing arginase. Accordingly, the typeI/typeII cytokine balance may influence the development of these different subsets of macrophages that are antagonistically regulated. The level of iNOS or arginase activity in these macrophage subsets may reflect their type I or type II designation. For fish, this allocation would allow for typing immune response as type I or type II, based on the production of NO or arginase activity, respectively.

To date, there is no clear evidence for existence of Th1/Th2 cells or classically/alternatively activated type of macrophages in fish. However, since, IFN y, TNFα, IL-1β, IL-6 and No characterize the type I response, it is worthwhile to consider these molecules in fish. No data on the existence of IL-6 like molecules in fish have been reported. In addition, although several authors have claimed functional evidence for the existence of interferon (IFN)-y activity as macrophage activating factor (MAF), so far no IFN-y but only IFN induced genes but have been cloned and sequenced in fish. In fact, as discussed above, we previously suggested that the MAF-like activity of lymphocyte culture supernatants might not ascribed to IFN-y activity but might be due to activation by transferring cleavage products. In contrast, both IL-1β and TNFα have been identified in fish, not only in carp, but in several other fish species. Also, NO production by, e.g. carp macrophages, is well recognized in fish.

For the type II response, IL-4, IL-10, IL-13 and TGFβ are considered the main players. No data on the existence of IL-4 and IL-13 like molecules is only just emerging. In contrast, several TGF-β homologues have been cloned in fish and although additional functional studies have to be done with TGFβs, studies with recombinant mammalian TGF-β1demonstrated inhibition of trout macrophage functions. In conclusion, an assignation of fish immune responses as type 1 or type II, based on cytokine production profiles, even

without directly implicating the cellular source, is still in its infancy but could tentatively be based on the profiles of those mediators that have been described for fish.

Carp macrophages

Studies on macrophages in common carp (Cyprinus carpio) so far have relied on density gradient centrifugation resulting in leucocyte fractions enriched for macrophages. Subsequent adherence to plastic allows for a final enrichment for macrophages up to 60% purity. However, the presence of neutrophilic granulocytes, forming the main impurity, hinders definite conclusions on macrophage functions, for which reason most studies in carp cautiously refer to 'phagocytes'. No macrophage cell line exists for carp.
Previously, in goldfish, we established and characterized an *In vitro* derived kidney macrophage (IVDKM) culture system. Flow cytometric analysis of this culture system indicated the presence of three distinct macrophage sub-populations, while functional, morphological, cytochemical and developmental characterization of these sub-populations suggested that they represent putative early progenitors (R1), monocytes(R3) and macrophages (R2) cells are morphologically similar to mature tissue macrophages of mammals and contain acid phosphatase, localized nuclear myeloperoxidase and non specific esterase. Both R2 (macrophages) and R3 (monocytes) sub-populations exhibit distinct No and respiratory burst activities. In addition, the R2 mature macrophage like cells were capable of self proliferation, which capacity makes this sub-population of cells especially interesting for studies on macrophage function in (cyprinid) fish. Here, in addition to the goldfish system, the establishment and characterization of a similar *in vitro* derived culture system for carp macrophage like cells is described.

Initial isolation of carp kidney leucocytes and the generation of a first carp IVDKM culture were done as previously described. To establish a primary carp kidney leucocyte culture we used 25 %(v/v) cell-conditioned medium (CCM) from established goldfish IVDKM cultures. Control kidney leucocyte cultures without CCM did not develop well. Four subsequent culture cycles the concentration of the original goldfish CCM was reduced to 0.8% only and CCM could be considered carp derived. In order to find the optimal seeding density and optimal percentage of CCM for carp IVDKM cultures, different seeding densities and different CCM percentages were tested. Development of carp IVDKM cultures was evaluated by eye and flow cytometry.

Typical carp IVDKM cultures were comparable to similar cultures established for goldfish but different from those previously established for rainbow trout. Flow cytometric analysis indicated the presence of three distinct macrophage sub-populations (R1, R2, R3) which were similar for both cyprinid fishes, while for the salmonid rainbow trout three distinct but different population were observed. In rainbow trout, the R3 population (monocytes) was most abundant.

Having established that carp IVDKM cultures behave similarly to goldfish IVDKM cultures and that carp R1,R2,R3 sub-populations are morphologically indistinguishable from goldfish R1,R2,R3 sub populations, carp IVDKM R2 and R3 sub populations from a 7-10 days old culture were sorted by flow cytometry and used for cytochemical and functional characterization. Sorted carp R2 cells were morphologically similar to macrophages, they are large irregularly shaped with a low nucleus to cytoplasm ratio and often have extensive vacuolization and membrane ruggling. Preliminary results suggest that the carp R2 population is positive for esterase (a-naphthyl acetate esterase) and acid

phosphatase but negative for myeloperoxidase. Sorted carp R3 cells were morphologically similar to monocytes, they are large round cells (12-15µm in diameter) with a low nucleus to cytoplasm ratio and often have eccentrically placed kidney shaped nuclei. The R3 cells seem positive for acid phosphatase but negative for both esterase and myeloperoxidase activity. Functional evaluation was done by measuring the nitric oxide production by carp R2 and R3 cells after appropriate stimulation. Both R2 and R3 sub-populations were capable of producing No; R2 (macrophage-like) cells produced higher amounts than R3 (monocyte like) cells, which produced only small amounts of No. in conclusion, cyprinid IVDKM cultures are comparable in form and function and are useful tools for studying fish macrophage function.

Arginase activity in fish

As described above, activated macrophages metabolise L-arginine by two alternative pathways involving either iNOS or arginase. Two isoforms of arginase exist in terrestrial (ureotelic) vertebrates from duplicated genes. One form, essentially present in the liver, is a cytosolic enzyme of the urea cycle and eliminates execess ammonia through the excretion of urea. The extrahepatic (mitochondrial) arginase, more ubiquitous, plays a role in macrophage polarization.

Fish are uricotelic in that they can directly excrete ammonia living in an aqueous environment. Therefore, the synthesis of urea, which is a less efficient nitrogen end product than ammonia, would not only be an unnecessary step in waste conversion but would be a waste of energy. Thus, not surprisingly, although an ancient trait in animals, expression of the ornithine urea cycle (OUC) does not occur in teleosts, except for some air breathing fish species. The genes for the OUC are not believed to be suppressed nor deleted but silenced. Indeed, the fifth step in the OUC cycle; the hydrolysis of dietary L arginine to urea and ornithine by arginase is one step of the OUC that occurs in fish even if the rest of the cycle is incomplete. The role of urea formed is stillpoorly understood in most fish species. Since, like all vertebrates, fish do not have urease for the breakdown of urea, it is difficult to understand the expenditure of irretrievable energy for no logical reason that accompanies the production of urea. What is clear, however, is that fish do express arginase activity in many tissues but mostly in liver and kidney, but also, that the arginse activity in fish without a functional urea cycle may not be centred on ammonia detoxification.

We have found partial carp arginase cDNAs in a head kidney macrpphage library and are presently sequencing ath remainder of the cDNA sequences. Further, for fish a number of expressed sequence tags for zebrafish , rainbow trout and pufferfish (F. rubripes) arginase cDNAs are reported in the database. The fish cDNA sequences were used along with several evertebrate arginase and mammalian arginase-1 (liver form) and arginase-2 (extra hepatic form) sequences to create a neighbour-joining tree. The fish arginases formed two separate clusters together with the mammalian arginase-1 and arginse -2 sequences. Thus, the arginse gene duplicatin that gave rise to two arginase genes in the vertebrates likely occurred before the separation of vertebrates and invertebrates. Our findings that arginse in carp is expressed in head kidneymacrophages suggests the existence of atleast an extra hepatic and functional arginase-2 like form in fish. In conclusion, teleost fish can be expected to hve tow arginse genes that likely encode for functional arginases that converts L-artinine to urea and ornithine but that are not centred on ammonia detoxification.

The above findings on arginse cDNAs of fish suggest fish macrophages should express arginase activity. We continued our experiments by using in *vitro* cultures of carp macrophages to study arginase activity in fish.Having established that at 20°C both goldfish and carp IVDKM cultures result in macrophage like and monocyte like sub-populations, we proceeded with characterizing carp leucocytes isolated from the head- rather than the mid kidney and comparing the effect of *in vitro* culture at a higher temperature. Flow cytometric analysis showed that the use of carp head rather than mid kidney leucocytes made no difference to the development of R1-R2-R3 macrophage sub-populations. However, preliminary observations suggested that at higher temperature (27↑8) carp macrophages primarily develop into macrophage sub populations . This latter cell population (IVDHKM, 27°C) could be readily stimulated to produce nitric oxide and to show arginase activity. Thus, carp IVDHKM, the majority being macrophage like cells, can be induced to express arginase activity after appropriate stimulation. This supports the notion of an extrahepatic functional arginase gene in fish macrophages. Whether this is the result of a polarized alternatively activated like macrophage phenotype remains to be confirmed.

Future perspective

Cells belonging to the monocyte macrophage lineage have long been recognized as heterogeneous, the heterogeneity reflecting the plasticity of the system's response to cytokines and microbial products *a.o.* To date, the existence of polarized (warm blooded vertebrate) macrophages that differ in terms of receptor expression, effector function and cytokine and chemokine production is rapidly acknowledged. For cold blooded vertebrates such as teleost fish, the information on T. lymphocytes is sparse and prototypical type I or type II cytokine profiles may not even exist. Macrophages are considered an evolutionary ancient cell type, however, and if macrophage polarization does indeed occur in fish it may for a large part determine the general pattern of innate immune response against foreign stimuli. No, doubt, the differentiation of fish macrophages such as seen during our *in vitro* culture system will allow us to perform detailed examinations of this phenomenon in cyprinid fish. If indeed macrophage polarization exists in teleosts, the study of infections with different fish parasites that have evolved with this aspect of the innate immune system in place will certainly bring new insights into the evolution of host parasite relationships.

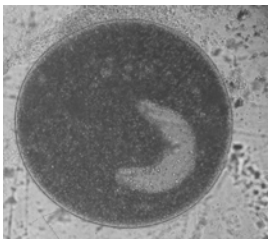

Fig.1. *Ichthyophthirius multifiliis*

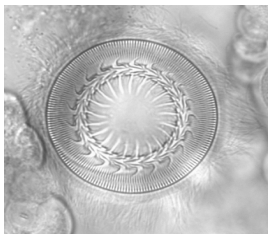

Fig.2. *Trichodina*

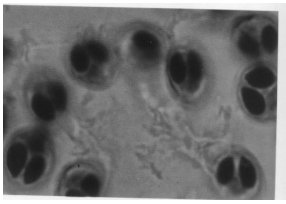

Fig.3. *Myxobolus*

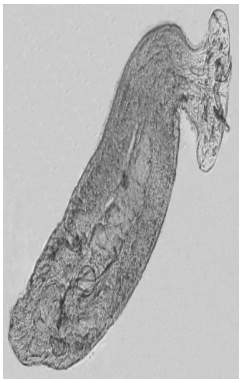

Fig.4. *Dactylogyrus*

Fig.5. *Gyrodactylus*

Fig.6. *Diphyllobothrium latum*

Fig.7. *Capillaria* sp.

Fig.8. *Lernea*

Fig.9. *Argulus* (Fish lice)

APTAMERS: A PROMISING TOOL FOR IMAGING, DIAGNOSIS AND THERAPY

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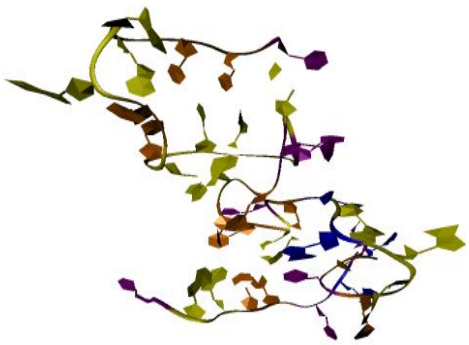
Introduction

Aptamers are a group of molecules, which can specifically bind, track, and inhibit target molecules, comprising DNA aptamers, RNA aptamers, and peptide aptamers. The term aptamer is derived from the Latin word "aptus", meaning "to fit", As the name implies, aptamers are suitable for applications based on molecular recognition needs that include diagnostics and therapeutics. On the therapeutic front, aptamers have made tremendous progress and are already in clinical trials only 8 years after the inception of the technology. Another area of research that is being actively pursued is the application of aptamers for in vivo diagnostics or in vivo imaging. In this report, I have limited the discussion of aptamers to in vitro diagnostics and summarized some promising developments in the field.since aptamers are specific three-dimensional *in vivo* structures that are capable of binding to specific target proteins. So far, there are much progress about developing novel aptamers and their expansile applications. This prospect systematically introduces the composition and technological evolution of aptamers, and then focuses on the application of aptamers in diagnosis, imaging, and therapy. **Aptamers are generated in a process named SELEX or cell-SELEX. They resemble antigens in respect of their highly specific binding in the lower nanomolar and/or picomolar range.** These molecules can assume a variety of shapes due to their propensity to form helices and single-stranded loops, explaining their versatility in binding to diverse targets. They are used as sensors, and therapeutic tools, and to regulate cellular processes, as well as to guide drugs to their specific cellular targets. Contrary to the actual genetic material, their specificity and characteristics are not directly determined by their primary sequence, but instead by their tertiary structure.

Structure of aptamer



The detection and quantification of molecules play an essential role in basic discovery research as well as in clinical practice. Technologies that allow specific detection and precise quantification of molecules are evolving to cater to new analytes as well as to improve existing techniques. As a result, novel approaches that challenge traditional methods are being discovered. Today, technological advancements have enabled researchers to change the properties of molecules on demand, thereby generating variants that suit various applications. This is quite contrary to what Nature has designed these molecules to be. For example, the antibody molecule that evolved to recognize antigens with high affinity and specificity is now being intentionally modified to catalyze different reactions. This is the task that enzymes have been designed to undertake. In a different area of research, the development of the in vitro selection and amplification technique has allowed the discovery of specific nucleic acid sequences that bind to a wide array of target molecules with high affinity and specificity. The technique by which these oligonucleotide ligands are obtained is called the systematic evolution of ligands by exponential enrichment (SELEX) process.

Aptamer identification: the SELEX process

The identification of rare nucleic acid sequences with unique properties from very large random sequence oligonucleotide libraries was described in 1990. This method, called the SELEX process, is a technique for screening very large combinatorial libraries of oligonucleotides by an iterative process of in vitro selection and amplification. Combinatorial libraries based on replicable biopolymers such as nucleic acids (and peptides) offer the convenience of iterative amplification of their members, making the screening process fast and easy.

The SELEX process begins with a random sequence library obtained from combinatorial chemical synthesis of DNA. Each member in a library is a linear oligomer of a unique sequence. The complexity, or the molecular diversity, of a library is dependent on the number of randomized nucleotide positions. Theoretically, a library containing a 40-nucleotide random region is represented by 1.2×10^{24} individual sequences ($4^{20} = 1.2 \times 10^{24}$). However, in practice, the complexity of a typical combinatorial oligonucleotide library obtained from 1-µmol scale solid-phase DNA synthesis is limited to 10^{14} to 10^{15}individual sequences. The success of finding unique and rare molecules that interact with a target parallels the diversity of the libraries used. The degree of molecular diversity present in random sequence oligonucleotide libraries supersedes that of other combinatorial libraries used for screening. These include peptide libraries used for phage display as well as the libraries made up of small organic molecules.

In the screening process, a random sequence oligonucleotide library is incubated with a target of interest in a buffer of choice at a given temperature (Fig. 1). During this step, a very small fraction of individual sequences tends to interact with the target, and these sequences are separated from the rest of the library by means of any one of the physical separation techniques. Typically, nitrocellulose filter partitioning is used with protein targets that are retained on nitrocellulose. Small molecular targets are generally immobilized on a solid support to generate an affinity matrix, in which case sequences that do not interact with the

target on the solid support can be removed easily by a simple washing step. The population of sequences bound to the target is isolated and amplified to obtain an enriched library to be used for the next selection/amplification cycle. The enrichment efficiency of high-affinity binders is governed by the stringency of selection at each round. The progress of the enrichment of high-affinity binders can be determined by carrying out binding analysis of enriching populations against the target. Once affinity saturation is achieved after several rounds of selection/amplification, the enriched library is cloned and sequenced to obtain the sequence information of each member. Individual sequences are further characterized on the basis of their ability to bind to the target. Usually, the majority of individual sequences, >90%, in an enriched library are "winners", aptamers that bind to the target used for selection

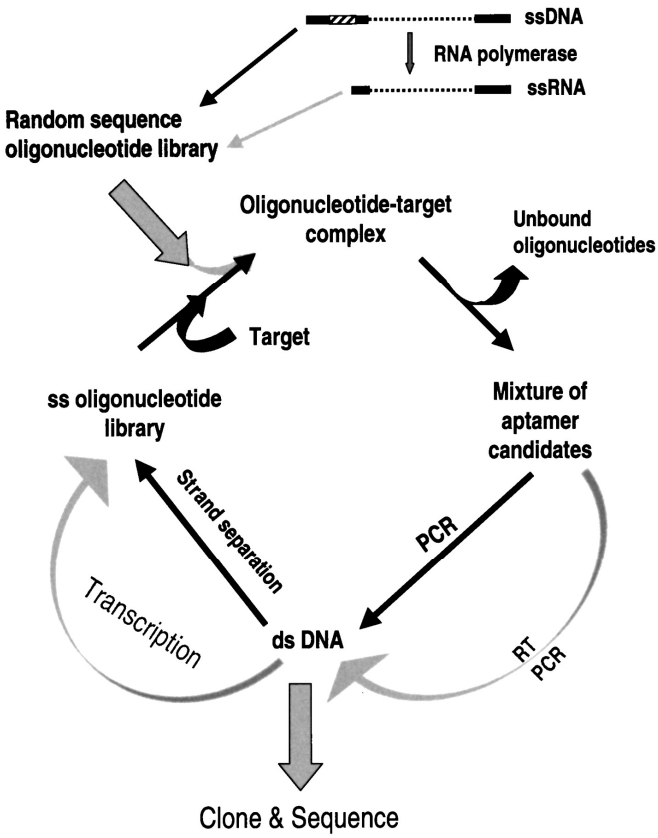


Fig. 1 Generalize scheme indicating the key steps in the SELEX process

Aptamers that come out of a SELEX experiment are full-length sequences containing the fixed sequences that were included to aid the amplification process. These full-length aptamers are generally 70–80 nucleotides long and could be truncated to eliminate nucleotide stretches that are not important for direct interaction with the target or for folding into the structure that facilitates target binding. The identification of truncated aptamers restricted to the minimal target-binding domain requires some effort, but it has been successfully carried out to obtain functional aptamers less than 40 nucleotides long. In the majority of cases, the fixed sequence regions used for primer binding are unimportant for aptamer function and can be eliminated. Technological advances have already been

made to eliminate the requirement for the fixed regions in random sequence libraries used for the SELEX process, thereby producing short aptamer sequences.
The number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as on the nature of the target. For most targets, affinity enrichment is reached within 8–15 cycles. In general, a researcher could accomplish one cycle of SELEX every 2 days. Including cloning and sequencing, a typical SELEX experiment may take approximately 2–3 months. Once the sequence is identified, an aptamer is produced by chemical synthesis. Small-scale synthesis and purification of an aptamer do not take more than 3 days and provide ample quantity of aptamer (several nanomoles) for the design and optimization of a diagnostic assay. This whole process is faster than the amount of time typically spent to generate a cell-line to produce a specific monoclonal antibody and purification of the antibody. The SELEX process recently was automated to make aptamer discovery even faster and more economical [24]. The automated platform carries out the iterative SELEX process around the clock with little or no human intervention and intuition. It has parallel processing capabilities to handle multiple SELEX experiments run on microtiter plates, allowing fast and high-throughput discovery of aptamers.
Aptamers are known for their remarkable specificity. Aptamers can discriminate targets on the basis of subtle structural differences such as the presence or absence of a methyl or a hydroxyl group and the D- vs L-enantiomer of the target. The high degree of specificity often seen in aptamers, sometimes even better than antibodies, is a result of the selective demand in the SELEX process that eliminates sequences that bind to closely related analogs of the target. Practically, this is achieved by the process called "counter-SELEX" that effectively discards ligands that have ability to bind the target as well as closely related structural analogs of the target. During selection, the population of aptamers bound to the target is subjected to affinity elution with structural analogs and the sequences eluted are discarded. In some diagnostic applications of small molecule targets, it may be important to measure the analyte as well certain structural variants of the analyte. In that case, aptamers retained on the target could be specifically eluted with the structural analogs to select the species that do not discriminate them.

The counter-SELEX strategy could be a valuable tool in identifying aptamers aimed at a unique target in a complex mixture, probably even without knowing what the target is, for example, in the search for aptamers that bind to an "epitope" present exclusively on the surface of cancer cells but not in healthy cells, or to find aptamers that interact with molecules present in the serum of patients infected with a pathogen but not present in uninfected individuals. In these applications, it would be possible to use the cells from healthy tissue (or serum from a healthy individual) to remove sequences that bind to the background that does not contain the epitope of interest before the library is challenged with cancer cells (or serum from the patient).

Aptamer- Microarrays : A cutting edge technology for the future

Microarray technology provides a means of analyzing a large number of molecules simultaneously. For example, the entire yeast genome has been analyzed on a single array

confined to a microscope slide. Such analyses carried out at the DNA level are facilitated by the high affinity and specificity of interactions driven by complementary base pairing. For proteomics, ligands that could capture proteins with high affinity and specificity provide the basis for an array technology aimed at protein capture and detection. Although antibody-based microarrays are being developed for the analysis of a large number of proteins, microarrays based on high-affinity aptamers would be very attractive for the following reasons:

- Aptamer identification is a rapid process that can be performed on an automated platform.
- Aptamers can be immobilized at a defined density at precise locations on a solid surface with existing technologies used to generate DNA microarrays.
- Homogeneous preparations of aptamers are readily available from chemical synthesis.
- Microarrays based on aptamers are robust and are expected to have a long shelf life.
- Aptamers containing 5-halo-uracil analogs could be used to lock the bound proteins irreversibly.
- The formation of irreversible cross-linking between aptamers and proteins is highly specific and provides another dimension of specificity in addition to the specificity provided by affinity. This dual specificity provided by cross-linkable aptamers eliminates the need for secondary ligands specific for each protein for detection. Thus, it is conceivable that proteins captured on a cross-linkable aptamer microarray could be detected by a reagent that is specific to proteins and does not react with nucleic acid.

In the near future, aptamer microarrays are expected to play a dominant role in the arena of proteomics that not only will facilitate better disease management by analyzing the expression of proteins by patients but will also help discover new therapeutics by target validation.

Conclusion

Over the past three decades, antibodies have been the reagent of choice for the development of diagnostic assays. Consequently, diagnostic platforms that are commonly used today were evolved to better suit antibodies. The discovery of aptamers whose affinity and specificity parallel those of antibodies is expected to have a future impact on diagnostics. Aptamer research is in its pre-puberty age and will require time before examples of aptamer products are available. The aptamer research already moving in a fast pace is expected to get even better with a completely automated SELEX process that allows high-throughput aptamer identification. It is important to point out that aptamers may not offer solutions for all molecular recognition needs. The value of aptamers in most cases will be in applications or formats where the performance of antibodies is inadequate rather than in replacement of assays that work well with antibodies. The ability of aptamers to function well in combination with antibodies in diagnostic formats will have considerable impact in the designing of assays that have not been feasible before. Covalent capture of targets is

unheard of in antibody research but feasible with aptamers. It is reasonable to expect that microarrays generated by aptamers with the ability to covalently capture proteins will make inroads to proteomics in the near future.

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