NUCLEIC ACID BASED IDENTIFICATION OF NODAVIRUS AND IRIDOVIRUS ASSOCIATED WITH WILD CAUGHT AND CULTURED MARINE FINFISH

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CERTIFICATE

This is to certify that the thesis entitle "***Nucleic acid based identification of nodavirus and iridovirus associated with wild caught and cultured marine finfish***" submitted by **Mr. Santhosh, K.S.** I.D.No. **MFK 918** in partial fulfillment of the requirements for the award of **Master of Fisheries Science** in **Fisheries Microbiology** of the Karnataka Veterinary, Animal & Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, association ship, fellowship or other similar titles.

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INTRODUCTION

I. INTRODUCTION

A virus is a small obligate intracellular parasite that can replicate only inside the living cells of organisms. It is ubiquitous in nature and infects all types of organisms, from animals and plants including both terrestrial and aquatic to bacteria and archaea (Sano *et al.,* 2004). It has been recognized that viruses are an important component of marine aquatic ecosystem and to determine its role in such systems, it is essential to understand virus-host interactions (Jiang *et al.*, 1998). Only a small percentage of the viruses from the ocean have been characterized because it was only 15-20 years ago, scientists realized the key role played by viruses in functioning of the ocean. Due to lack of their host and host cell availability, they cannot be cultivated in the controlled environment of the laboratory.

Aquaculture is one of the fastest growing sector among the food producing sector and is expected to contribute to the reduction in gap between demand and supply of fishery products (FAO, 2010). The contribution of aquaculture to the total production of fisheries has increased from 31.19 % in 2004 to 37.97 % in 2009. With average annual growth rate of 6.6 %, the per capita supply has increased from 0.7 kg in 1970 to 7.8 kg in 2008 (FAO, 2010). Over 350 different species of aquatic animals are being cultured, including 34 finfishes, 8 crustaceans and 12 molluscans with annual production exceeding 1, 00,000 tonnes (FAO, 2009).

Production of marine fishes has been initiated in full scale rapidly due to their economic value and the demand to meet food security. Ample scope has been given to marine fisheries considering the availability of resource and the extent to which it can be used. In

recent years, with the rapid development of intensive aquaculture, infectious viral diseases have severely affected many highly valued fish species causing heavy economic losses (Qin *et al.,* 2006).

The cultured animals are susceptible to infection by several bacterial, viral, parasitic and fungal pathogens. Diseases caused by bacteria and virus alone account for about 80% of all infections which has led to great economic loss to the farmers. Among the infectious diseases, viral diseases often cause heavy damage to production. There are many viral diseases that have been reported for cultured animals from different parts of the world (Oh and Choi, 1998; Sohn *et al.*, 1998; Oh and Jung, 1999; Jung and Oh, 2000).

The family *Iridoviridae* having genus *Lymphocystivirus*, *Ranavirus* and *Megalocytiviruses* (i.e., red seabream iridovirus [RSIV], rock bream iridovirus [RBIV], and infectious spleen and kidney necrosis virus [ISKNV]) infect a wide variety of marine fish in Southeast Asia, including several species that are widely cultured (Nakajima *et al.,* 1998). Besides, fish nodavirus from the family *Nodaviridae* causes viral encephalopathy and retinopathy (VER), which affects a variety of farmed marine teleost (Munday and Nakai 1997). It was found that more than thirty species of marine fishes belonging to 16 families were susceptible to this virus (Munday *et al.,* 2002). It has been reported that morbidity and mortality caused by virus in wild and cultured fish population is very high with nodavirus and iridovirus infection occurring most frequently in Mediterranean countries.

In order to circumvent disease problems in present day aquaculture system, it is important to have highly sensitive and specific diagnostic tools for rapid detection of these pathogens. Formerly, conventional methods such as microscopy and histopathology were used to check the changes that occurred in tissues due to viral infection. Later rapid and sensitive DNA based molecular techniques such as polymerase chain reaction (PCR) and its variants Reverse- transcriptase PCR, Real- time PCR and Loop-mediated isothermal amplification (LAMP) and probe hybridization methods were developed for detection purpose.

With this background, the current study was aimed with the following objective:

1) To study the prevalence of nodavirus and iridovirus in wild caught and cultured marine finfish.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1. VIRUS

Viruses are found where there is life and have probably existed since living cells first evolved. They are the most common biological agents in the sea, typically numbering ten billion per litre (Fuhrman, 1999). Viruses are parasites of living cells which invade host cells and propagate using the host biological machinery. They display a wide diversity of shapes and sizes, are much smaller than bacteria with sizes ranging from 20 to 400 nm (1/100 average bacterium size). They have only one type of nucleic acid, either deoxy-ribonucleic acid (DNA) or ribonucleic acid (RNA) as their genetic material based on which they are referred to as DNA or RNA virus respectively (Fuhrman, 1999).

2.1.1. Mode of reproduction

Viruses have different modes of life cycle, among which lytic and the lysogenic cycles are the main mode of reproduction. Lytic cycle is virulent in nature wherein viruses infect a cell (or tissue), get replicated, lyse host cells and release viral progeny that are ready to infect other cells. In the lysogenic cycle, the virus infecting the host incorporates its genome into the host genome. Whenever the host divides, the viral genome is also replicated and is released from the host cell by budding.

2.1.2. Classification

The classification of viruses was described based on naming, grouping and their similar properties. Andre Lwoff *et al.,* classified the viruses based on the Linnaean hierarchical system in 1962 as:

Order - Virales

Family - Viridae

Subfamily - Virinae

Genus - *Virus*

Species - *Virus*

The International Committee on Taxonomy of Viruses (ICTV) was formed to classify viruses according to their shared properties (not those of their hosts) and the type of nucleic acid forming their genomes. Viruses have not been assigned to any phylum or class due to their relatively small genomic size and high mutation rate which has made it difficult to trace its ancestry beyond the level of "Order" in the hierarchical classification scheme. Nobel laureate David Baltimore devised the Baltimore classification system based on the mechanism of mRNA production and in conjunction with the ICTV classification system. Based on Baltimore classification system there are seven different types of viruses and they are listed in table 1.

Table 1. Baltimore classification of viruses.

Class	Type	Example
I	dsDNA viruses	Iridoviruses, Adenoviruses, Herpesviruses, Poxviruses
II	ssDNA viruses,(+) sense DNA	Parvoviruses
III	dsRNA viruses	Reoviruses
IV	(+) ssRNAviruses (+) senseRNA	Nodaviruses, Picornaviruses, Togaviruses
V	(−) ssRNAviruses (−) senseRNA	Orthomyxoviruses, Rhabdoviruses
VI	ssRNA-RTviruses (+) senseRNA	Retroviruses
VII	dsDNA-RT viruses	Hepadnaviruses

2.2. Virus infecting fish

Viruses infecting fishes are essentially same as those infecting warm blooded vertebrates. Their morphological, biochemical and biological properties seem to fit well to the modern schemes of viral classification. However an important character seen among fish viruses is that they are able to increase replication through a broad temperature range which is not seen among viruses from homeothermic vertebrates (Lauffer and Smith, 1966). A large number of viruses are known to infect a wide range of finfish species. These include, lymphocystis virus, nodavirus (PNN), infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia (VHS), spring viraemia of carp (SVC), catfish iridovirus, catfish herpervirus, eel herpervirus, etc., Among these, lymphocystis and nodavirus are the most frequently reported viral infections in finfish species produced intensively in Mediterranean countries both from marine and freshwater culture

systems. This could probably be an overestimation of the diagnosis of these two viruses in contrast to other viral diseases and is probably due to its easy diagnosis based on clinical signs, macroscopical examination and histopathology.

2.3. Nodavirus

Nodavirus is the smallest among the animal viruses having a genome size of 4.5 kb. It is a single stranded (positive stranded) ribonucleic acid (RNA) virus with a diameter of 25-30 nm, non-enveloped and icosahedral in shape belonging to the genus *betanodavirus* of family *Nodaviridae* (Mori *et al.*, 1992, Comps *et al.*, 1994; Chi *et al.*, 2001). The family *Nodaviridae* includes two genera namely, betanodaviruses, which predominantly infect fish, and alphanodaviruses, which predominantly infect insects (Ball and Johnson, 1999).

2.3.1. Origin and nomenclature of nodavirus

The word "noda" originates from Japanese village Nodamura, where the viral particles were first isolated from mosquitoes (*Culex tritaeniorhynchus*) in 1956. Yoshikoshi and Inoue, (1990) reported nodavirus infection in hatchery-reared larvae and juveniles of Japanese parrotfish (*Oplegnathus fasciatus*). Later Mori *et al.*, (1992) reported in moribund and dead striped jack larvae (*Pseudocaranx dentex*) and designated it as striped jack nervous necrosis virus (SJNNV) in the family *nodaviridae*.

Nodavirus is also known as piscine neuropathy nodavirus (PNN) due to its wide range of susceptible fish hosts and the consistent neuropathology of the disease condition (Frerichs *et al.*, 1996). The disease is characterized by the development of a vacuolating encephalopathy and retinopathy in a variety of larval and juvenile marine fish with high mortality and has also

been described as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) (OIE, 1997; Yoshikoshi and Inoue, 1990).

2.3.2. Genome

The nodavirus genome is organized with a bisegmented positive sense single-stranded RNA. Among the 4.5 kb genome, the larger segment, RNA1 (3.1 kb) encodes the RNA-dependent RNA polymerase (protein A) and the smaller segment, RNA2 (1.4 kb) encodes the capsid protein (Figure 1). It is also observed that RNA3, a sub genomic RNA (0.4 kb) which is formed only in infected cells is transcribed from the 3' end of RNA1 and encodes for B2 protein, a non-structural protein having a suppressor function for post-transcriptional gene silencing (Iwamoto *et al.*, 2005 and Fenner *et al.*, 2006). RNA2 (1.4 kb) forms a major open reading frame encoding capsid protein and consist of a highly conserved region and a variable region.

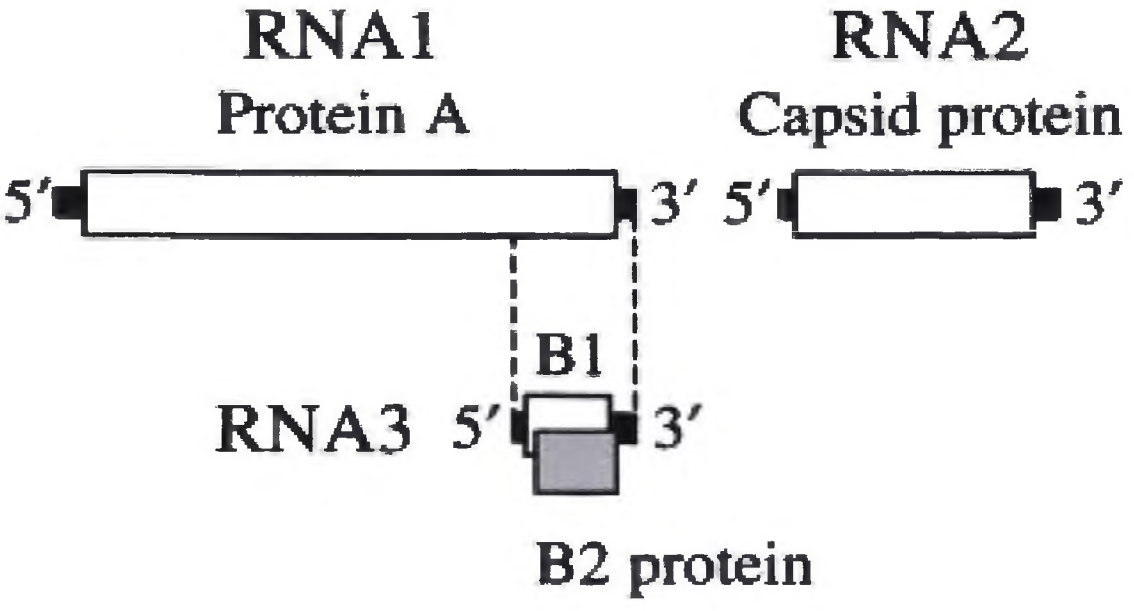

Figure 1. Genomic compilement in nodavirus. (Johnson *et al.,* 2001)

2.3.3. Classification of nodavirus

Based on nucleotide sequences of the variable region of RNA2, fish nodaviruses have been classified into four different genotypes, striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and redspotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.,* 1995, 1997 and Skliris *et al.*, 2001). Among these, host ranges of SJNNV and TPNNV are limited to striped jack (*Pseudocaranx dentex*) and tiger puffer (*Takifugu rubripes*) respectively. The BFNNV has been isolated from cold water species, such as Barfin flounder (*Verasper moseri*) and Pacific cod (*Gadus macrocephalus*) and RGNNV was found to have a broad host range causing disease among variety of warm water fish species, particularly groupers and seabass. This has led to discussions on possible host specificity and temperature dependence in betanodavirus strains (Aspehaug *et al.,* 1999; Chi *et al.* 1999).

2.3.4. Susceptible host and clinical signs

Susceptibility to nodavirus infection was reported in more than thirty species of marine fishes belonging to 16 families (Munday *et al.,* 2002), which includes Japanese flounder (*Parulichthys olivaceus),* striped jack (*Pseudocumnx dentex),* sevenband grouper (*Epinephelus septrmfusciutus),* humpback grouper (*Cromilrptes ultivelis),* seabass (*Lateolabrax japonicus),* shy drum (*Umbrinu cirrosa),* turbot (*Scophthulmus maximus),* Atlantic halibut (*Hippoglossus hippoglossus),* red drum (*Sciaenops ocellatus),* Atlantic cod *(Gadus morhua),* Pacific cod (*Gadus macrocephalus*), barfin flounder (*Verasper moseri*) and common sole (*Solea solea*) (Nishizawa *et al.*, 1994; Fukuda *et al.,* 1996; Tanaka *et al.*, 1998; Pavoletti *et al.*, 1998; Bovo *et al.,* 1999; Watanabe *et al.,* 2000; Zafran *et al.*, 2000 and Oh *et al.*, 2002). It has also been

isolated from freshwater aquarium fish from Singapore (Hegde *et al.,* 2003). The fish nodavirus association with mass mortality in Asian seabass, (*L. calcarifer)* has been reported recently in India (Azad *et al.*, 2005; Parameswaran *et al.* 2008: Shetty *et al.,* unpublished).

Nakai *et al.* (1995) reported that VNN disease is characterized by abnormal swimming behavior such as whirling or corkscrew motion. Heavy vacuolation of the central nervous tissues and spherical viral particles in the cytoplasm of infected nerve cells can also be seen. Other clinical signs observed are darkening of body, bulged eye balls with a prominent white coloration around the eye ball on optic capsule (exophthalmia) and enlargement in belly region due to inflated swim bladder and gas accumulation. Other non-specific signs include anorexia, lethargy and 100% mortality is seen to occur in the early metamorphosis stage. The histopathological observations of infected tissues show neural degeneration and vacuolation usually in retina, brain, spinal cord and ganglia in the peripheral nervous system (Munday *et al.,* 2002). Further studies have also reported the loss of ability to produce brain cells and head kidney leucocytes after the nodaviral infection (Montes *et al.*, 2010).

2.3.5. Transmission

Nodaviruses can be transmitted both horizontally between fishes and vertically from spawners to larvae (Breuil *et al.*, 2000). Horizontal transmission of the disease was demonstrated by immersion of healthy fishes in a homogenate of diseased tissue which caused 100 % mortality (Arimoto *et al.*, 1993 and Nguyen *et al.*, 1996). It was further reported that transmission can also occur by intraperitoneal injection (Mori *et al.*, 1991) or by water and cohabitation (Glazebrook *et al.*, 1990). Arimoto *et al.* (1992) detected nodavirus in gonads of striped jack (*Pseudocaranx dentex)* spawners by a reverse transcriptase polymerase chain

reaction (RT-PCR) technique and established vertical transmission of nodavirus. Infected brood stock serve as carriers and result in infected postlarvae. Though it was believed that only larval and juvenile fishes are susceptible to nodavirus (Breuil *et al.*, 1991), Fukuda *et al.*, (1996) reported infection among adults of seven banded grouper (*Epinephelus septemfasciatus*).

2.3.6. Detection

For the detection and scientific study of nodavirus, specific and sensitive methods have been established. In general molecular, immunological and cell culture methods are commonly used. Molecular methods such as reverse-transcriptase PCR and real-time PCR have played more important role in detecting and characterizing NNV. PCR amplification using primers designed by Nishizawa *et al.,* (1994) based on the RNA2 segment nucleotide sequence of SJNNV is a useful technique in detecting VNN infection. Immunological based assays include enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC) and indirect fluorescent antibody technique (IFAT). ELISA was one of the first immunological methods to be developed by Arimoto *et al.,* (1992) to detect SJNNV but found to be not very sensitive. For virus propagation, characterization and viral infection mechanism studies, cell lines are important. Frerichs *et al.,* (1996) used SSN-1 cell line derived from striped snakehead (*Ophicephalus striatus*) to isolate and propagate betanodavirus from diseased sea bass juveniles. Parameswaran *et al.* (2006) established and characterized India's first marine fish cell line (SISK) from the kidney of Asian seabass (*L. calcarifer*). Azad *et al.* (2005) reported viral nervous necrosis infection in Asian seabass (*L. calcarifer)* from the hatcheries in Tamil Nadu along the east coast of India.

2.4. Iridovirus

Iridoviruses belonging to the family iridoviridae are double stranded DNA viruses, large in size with 120-300 nm in diameter, non-occluded, icosahedral in shape having a genome size of 150-280 kbp. The genome of the viruses was found to be circularly permuted and terminally redundant, which is a unique genome feature among eukaryotic viruses (Delius *et al.,* 1984; Goorha and Murti, 1982 and Darai *et al.,* 1983, 1985). This terminal repetition has added another 10–30% to the size of the genome. Viral particles may be enveloped or non-enveloped depending upon whether they are released from the cell by lysis, or budding from the plasma membrane. Viral DNA replication takes place in both the nucleus and cytoplasm of infected cells (Goorha *et al.,* 1979).

2.4.1. Origin and nomenclature

The name iridoviridae is derived from Greek word "Iris" which means goddess of rainbow. This is due to the appearance of rainbow like iridescence in heavily infected insects and pelleted samples of invertebrates. Iridovirus infection was detected for the first time In Claude river in larvae of crane fly (*Tipula* spp.) which were glowing with patches of blue coloration and recognized as Tipula Iridovirus (TIV) in 1954.

2.4.2. Genome

The iridovirus genome contains approximately 105 potential open reading frames (ORFs), which encode polypeptides ranging from 40 to 1294 amino acids and 20 microRNA candidates (Zhang *et al.*, 2010). It appears unique among the eucaryotic viruses that it is

terminally redundant and cyclically permuted which results in the resolution of genome concatamers during the DNA replication.

A simplistic view of this concept is given in the figure 2. For example; a viral genome consisting of 10 genes (**A**) forms a long concatamer (**B**) and the resolution of this concatamer (**C**) will results in synthesis of packaged DNA lengths (**D**) that contain a complete genome as well as duplicated copies of some terminally redundant genes. Depending on the cyclic permutation, the ends of packaged DNAs differ from one virus particle to other.

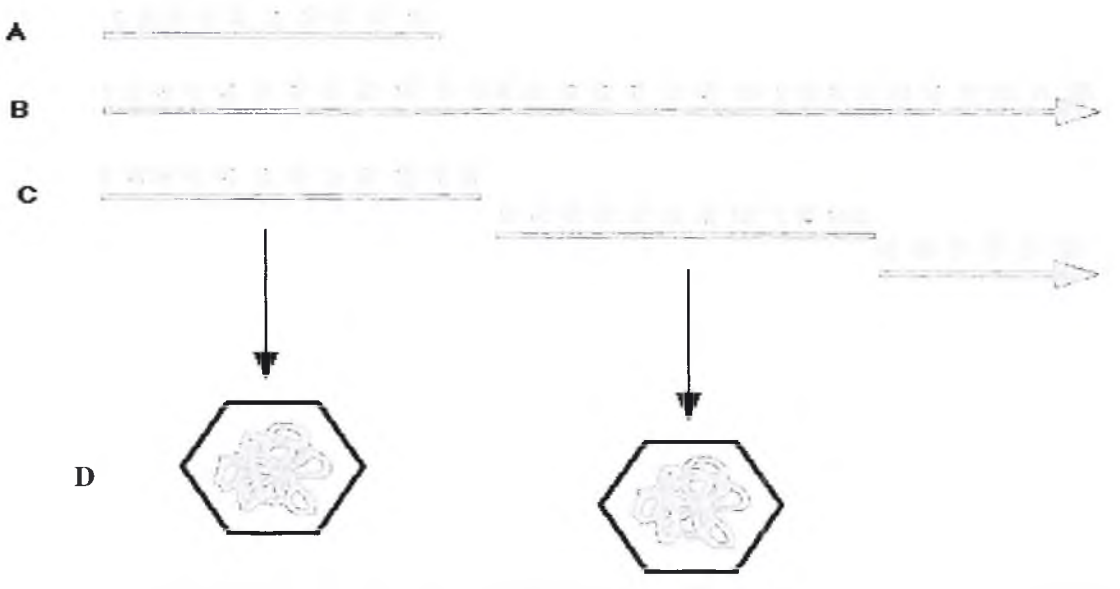

Figure 2. Unique characteristic of iridovirus: cyclically permuted and terminally redundant in nature.

2.4.3. Distinguishing characteristics of iridovirus

The most distinctive feature of the family *Iridoviridae* is the presence of a major capsid protein (MCP). It forms the main structural component of the viral particle comprising 40-45% of the total viral polypeptides and having a molecular weight of about 50 kDa (Flu gel *et al.*, 1985). Since MCP and ATPase genes are relatively conserved within the family *Iridoviridae* these have been recognized as a suitable target to carry out phylogenetic studies (Tidona *et al.,* 1998) and differentiating iridovirus strains and species (Bollinger *et al.,* 1999).

2.4.4. Classification of iridovirus

Members of the family *Iridoviridae* are known to infect not only cold-blooded vertebrates (bony fish, amphibians, and reptiles) or invertebrates (primarily insects), but also crustaceans and mollusks (Trevor *et al.*, 2005). The family was subdivided into five genera in the eighth report of the International Committee on Taxonomy of Virus (ICTV). They are as follows iridovirus and chloriridovirus, which infect mainly invertebrates, ranavirus which infect amphibians, bony fish and reptiles (Chinchar, 2002) and lymphocystivirus and megalocytivirus, which infect cold blooded vertebrates (Chinchar *et al.,* 2005). Apart from host range, vertebrate and invertebrate iridoviruses are also differentiated based on the levels of DNA methylation (Willis *et al.*, 1980). Ranavirus, lymphocystivirus and megalocytivirus are DNA methylated, whereas iridovirus and chloriridovirus are non methylated.

2.4.5. Host susceptibility

The first isolation of lymphocystivirus infecting fish species was reported in gilthead sea bream in cultured fish from Israel by Paperna *et al.,* (1982). Subsequently, the presence of this virus has been reported from more than 30 species of finfishes (Wang et *al.*, 2003) and farmed ornamental fishes like African lamp eye (ALIV) and Dwarf gourami (DGIV) (Sudthongkong *et al.*, 2002). Lymphocystivirus was the first iridovirus genome to be sequenced (Tidona and Darai, 1997). Lymphocystis disease has worldwide geographical distribution and has been described in more than 125 species of teleosts which belong to both marine and freshwater environments, affecting wild and cultured fishes (Anders, 1989). Iridovirus isolated from red sea bream (red sea bream iridovirus- RSIV) in western Japan differ genetically and pathogenically *in vivo* and *in vitro* from other group of iridovirus and grouped them into a new genus megalocytivirus. Nucleotide sequence and phylogenetic analysis revealed that most of the isolates are similar to orange-spotted grouper iridovirus (OSGIV), rock bream iridovirus (RBIV) and grouper sleepy disease virus (GSDIV), demonstrating distinct phenotypic nature of RSIV isolates.

Rasin (1927) reported lymphocystis disease in freshwater ornamental fish, *Macropodus* sp. for the first time. Later it was reported from several other exotic ornamental fishes, such as imported cichlid *Etroplus maculatus* from Singapore to Canada (Armstrong and Ferguson, 1989); dwarf gourami *Colisa lalia* from south east Asia to Australia (Anderson *et.,* 1993); guppy *Poecilia reticulata* and doctor fish *Labroides dimidatus* in the USA, which were imported from south east Asia (Hedrick *et al.,* 1990).

As of now, ten iridovirus genomes have been sequenced completely: **IIV-6**: Invertebrate iridescent virus 6 (Jakob *et al.*, 2001), **LCDV-1**: Lymphocystis disease virus 1 (Tidona and Darai, 1997), **LCDV-C**: Lymphocystis disease virus China (Zhang *et al.*, 2004a), **TFV**: Tiger frog virus (He *et al.*, 2002), **FV-3**: Frog virus 3 (Tan *et al.*, 2004), **ATV**: Ambystoma tigrinum virus (Jancovich *et al.*, 2003), **ISKNV**: Infectious spleen and kidney necrosis virus (He *et al.,* 2002), **RBIV**: Rock bream iridovirus (Do *et al.,* 2004), and **SGIV**: Singapore grouper iridovirus (Song *et al.*, 2004), **RSIV**: Red sea bream iridovirus and **OSGIV**: Orange spotted grouper iridovirus (Cheng *et al.,* 2010).

Iridovirus infects commercially important food and ornamental fish species of both fresh and marine water habitats across the globe. The fish species susceptible for iridovirus is presented in table 2.

Table 2. Fish susceptible to iridovirus infection.

Common name	Scientific name
Red sea bream	*Pagrus major*
Black porgy	*Acanthopagrus schlegeli*
Yellow fin sea bream	*Acanthopagrus latus*
Crimson sea bream	*Evynnis japonica*
Japanese amberjack	*Seriola quinqueradiata*
Greater amberjack	*Seriola dumerili*
Yellowtail amberjack	*Seriola lalandi*
Striped jack	*Pseudocaranx dentex*
Northern bluefin tuna	*Thunnus thynnus*
Japanese Spanish mackerel	*Scomberomorus niphonius*
Chub mackerel	*Scomber japonicus*
Japanese jack mackerel	*Trachurus japonicus*
Japanese parrotfish	*Oplegnathus fasciatus*
Spotted knifejaw	*Oplegnathus punctatus*
Cobia	*Rachycentron canadum*
Snubnose pompano	*Trachinotus blochii*
Chicken grunt	*Parapristipoma trilineatum*
Crescent sweetlips	*Plectorhinchus cinctus*
Chinese emperor	*Lethrinus haematopterus*
Spangled emperor	*Lethrinus nebulosus*
Largescale blackfish	*Girella punctata*
Rockfish	*Sebastes schlegeli*

Croceine croaker	*Pseudosciaena crocea*
Hong Kong grouper	*Epinephelus akaara*
Convict grouper	*Epinephelus septemfasciatus*
Malabar grouper	*Epinephelus malabaricus*
Longtooth grouper	*Epinephelus bruneus*
Orange-spotted grouper	*Epinephelus coioides*
Yellow grouper	*Epinephelus awoara*
Greasy grouper	*Epinephelus tauvina*
Brown-marbled grouper	*Epinephelus fuscoguttatus*
Giant grouper	*Epinephelus lanceolatus*
Murray cod	*Maccullochella peelii*
Gourami	*Trichogaster* spp
Japanese sea perch	*Lateolabrax japonicas*
Barramundi or Sea bass	*Lates calcarifer*
Senegalese sole	*Solea senegalensis*
Largemouth bass	*Micropterus salmoides*
Bastard halibut	*Paralichthys olivaceus*
Spotted halibut	*Verasper variegatus*
Torafugu	*Takifugu rubripes*
Chinese perch	*Siniperca chuatsi*
Red drum	*Sciaenops ocellatus*
Flathead mullet	*Mugil cephalus*

(**Source:** Bloch *et al.,* 1993; Paperna *et al.*, 2001; Tapiovaara *et al.*, 1998; Sudthongkong *et al.*, 2002 and Jeffery *et al.*, 2006).

2.4.6. Clinical signs

The disease caused by iridovirus is characterized by the development of wart-like lesions, generally on the external surface of infected fish (Weissenberg, 1965) and also by the appearance of pearl-like nodules, either singly or in groups, on the skin, fins and tail (Wolf, 1988). Similar nodules may occur internally over the mesenteries, peritoneum and several internal organs (Wolf, 1988). The development of each nodule represents a single infected cell that has undergone a massive increase in cell volume. Rupture of these mature nodules release virus which get transmitted to uninfected fish indicating the horizontal transmission of the disease. Presence of white sturgeon iridovirus in gonadal tissue after exposure suggested the survival of virus in reproductive organs which subsequently get transmitted to progeny during spawning. This vertical mode of transmission from broodstock to progeny is known to infect fishes from juvenile to adult stage (Georgiadis *et al.*, 2001).

The initial sign of disease include weight loss due to reduced feed intake, lethargy, darkening in pigmentation, especially of the fin and ceased swimming (Bloch *et al.*, 1993). Internally, the infected fish have little to no body fat, pale livers and enlarged spleen in addition, to the gastrointestinal tract being empty (Hedrick *et al.*, 1990). Rapid identifying feature of the disease is large epitheloidal cells in gills showing hyperemia and hemorrhage, which turns pink or pale as the infection progresses. Histopathological observation of diseased fish tissues show enlarged cells containing unique inclusion body-bearing cells and necrosis in the spleen and hematopoietic tissue (Sudthongkong *et al.*, 2002). Sano *et al.,* (1994) reported that occurrence of lymphocystis disease is related to the water temperature and is most prevalent in winter season with water temperature ranging from 22-25°C (Kitamura *et al.*, 2007).

2.4.7. Detection

The iridoviral infection can be detected by using single step PCR and quantified using real time PCR. Sudthongkong *et al.,* (2002) designed primer sets for the major capsid protein (MCP) gene and ATPase gene and sequenced the PCR products derived from 5 iridovirus isolates obtained from sea bass in South China Sea, red sea bream from Japan, brown-spotted grouper with a grouper sleepy disease from Thailand, dwarf gourami from Malaysia and African lampeye from Sumatra Island, Indonesia. The five iridovirus sequence showed high homology and a further phylogenic analysis revealed that although these viruses were isolated from various fishes in geographically different regions, they all originated from a single source that was native to south-east Asian region. Jeffery *et al.*, (2006) designed PCR primer pairs to detect and differentiate viruses within the megalocytivirus genus of the family *Iridoviridae*.

Qin *et al.*, (2002) (2002b) produced polyclonal antibodies against Singapore grouper iridovirus (SGIV) by immunizing rabbit with purified SGIV to analyze its antigenic properties using rabbit immunoglobulin G (IgG). The SGIV specific monoclonal antibodies (MAbs) have been produced, characterized and antigenic capture ELISA system developed for the detection of SGIV (Chengyin *et al.*, 2003).

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1. Sampling

3.1.1. Sampling site

Marine and brackishwater finfish were collected from various culture systems (Kumta and Ankola) situated along the south-west coast of Karnataka and wild caught samples from fish landing centre, Mangalore at fortnightly intervals from June 2010 to April 2011. The wild caught samples that were transported from east coast of India to the processing plants situated in the west coast were also obtained and analyzed for the presence of viruses.

3.1.2. Sample collection

Live samples were brought to the laboratory in oxygen packs in plastic bags. If they were in moribund condition, they were brought in chilled state and if they were dead, they were immediately fixed on site using fixative and transported to the laboratory. The collected samples were processed for molecular analysis as detailed in the following section.

3.1.3. Sample type

During the study, moribund fish samples were collected and fixed in corresponding fixative and brought to the laboratory. The samples used in the present study are listed in table 3 and 4.

Table 3. Samples analyzed for nodavirus

Sl.No	Sampling source	Species analyzed	No. of samples
1	Rajiv Gandhi Centre for Aquaculture (RGCA) Chennai, Tamil Nadu	*Lates calcarifer* (Asian seabass)	9
2	Asian seabass fish culture pond Kumta, Karnataka	*Lates calcarifer* (Asian seabass)	7
3	Asian seabass seed rearing center, Kumta, Karnataka	*Lates calcarifer* (Asian seabass)	3
4	Wild caught fishes, fish landing center, West-coast Mangalore	*Epinephelus diacanthus* (Grouper)	3
		Etroplus suratensis (Pearl spot)	4
5	Wild caught fishes, fish landing center, East-coast,T.N	*Epinephelus diacanthus* (Grouper)	5
6*	*Case study on nodavirus outbreak in Asian seabass cage culture demo farm, Neeleswaram, Kerala	*Lates calcarifer* (Asian seabass)	12
Total			**43**

*Case study on disease outbreak of nodavirus in Asian seabass demonstration cage culture farm, Kerala was included separately.

Table 4. Samples analyzed for iridovirus

Sl. No	Sampling source	Species analyzed	No. of samples analyzed
1	Asian seabass seed rearing center, Kumta, Karnataka	*Lates calcarifer* (Asian seabass)	8
2	Aquaculture pond, Ankola, Karnataka	*Mugil cephalus* (Grey mullet)	3
		Etroplus suratensis (Pearl spot)	2
		Sillago sihama (Silver whiting)	1
3	Wild caught fishes from Indo Fisheries processing plant, Mangalore	*Nemipterus japonicas* (Pink perch)	2
4	Wild caught fishes, fish landing center, West-coast Mangalore	*Cynoglossus luctosus* (Tongue sole)	3
		Euryglossa orientalis (Oriental sole)	2
		Platycephalus indicus (Flatheads)	2
		Arius jella (Cat fish)	3
		Psettodes erumei (Indian halibut)	6
		Zebrius quagga (Zebra sloe)	4
		Lagocephalus inermis (Puffer fish)	1
		Rastralliger kanagutta (Indian mackerel)	1
5	Wild caught fishes, fish landing center, East-coast,T.N	*Epinephelus diacanthus* (Grouper)	6
	Total		**44**

3.1.4. Fixation

Collected samples were immediately fixed on site to avoid degradation of RNA and DNA using RNA fixative and Davidson's fixative respectively. Samples were fixed in the ratio of 1:5 with fixative (eg: a 0.5 g sample required about 2.5 ml of fixative) for proper fixation. Fixed samples were maintained at room temperature until further processing.

Composition of fixative

RNA fixative (1L):

- Formalin (100%) – 349 ml
- Ethyl alcohol (95%) – 407 ml
- Ammonium hydroxide – 22 ml
- Distilled water – 222 ml

Store at room temperature (28°±1°C)

Davidson's fixative or DNA fixative (1L):

- Ethyl alcohol (95%) – 330 ml
- Formalin – 220 ml
- Glacial acetic acid – 115 ml
- Distilled water – 335 ml

Store at room temperature (28°±1°C)

3.1.5. Sample processing

Collected samples were taken for RNA or DNA extraction depending on whether the virus was a RNA virus (nodavirus) or a DNA virus (iridovirus).

3.2. Molecular methods

3.2.1. RNA extraction

RNA was extracted using TRIzol LS reagent according to manufacturers' instructions. TRI (Total RNA Isolation) reagent is a complete and ready to use reagent for isolation of total RNA. It is chemically composed of phenol and guanidine thiocyanate in a monophasic solution which rapidly inhibits RNAse activity.

Reagents used for RNA extraction

- TRIzol LS reagent (invitrogen)
- Chloroform
- Isopropyl alcohol
- Ethyl alcohol (75%)
- DEPC (diethyl pyrocarbonate) treated water

Procedure for RNA extraction

The targeted tissue (brain, retina and kidney) was homogenized in 750 µl of TRIzol LS reagent and incubated at room temperature (28°±1°C) for 20 mins. Then 200µl of chloroform was added, mixed vigorously by shaking and incubated at room temperature (28°±1°C) for 15 min. The homogenate was separated into upper aqueous phase, middle inter phase and lower organic phase by centrifuging at 12,000g for 15 min at 4°C. The upper colorless aqueous phase containing RNA was taken in fresh microfuge tube and incubated with 500 µl isopropyl alcohol at room temperature (28°±1°C) for 20 min to precipitate RNA. Precipitated RNA was solubilized in DEPC treated water or RNase free water after washing with 75% ethyl alcohol and stored at -80°C until for further analysis.

Extracted RNA was quantified using NanoDrop® Spectrophotometer (ND 1000, V3.30, Thermo Fisher scientific, USA).

3.2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcription was carried out to obtain complementary DNA from extracted total RNA by using gene specific reverse primer according to manufacturers' instructions (Fermentas International Inc, Burlington, Ontario L7N 3N4, Canada).

cDNA synthesis

10 µl of RNA was mixed with 2 µl of gene specific reverse primer and incubated at 70°C for 5 min to linearize RNA and then chilled on ice for 2 min to allow the primer to bind to RNA. Following this 8 µl of RT-PCR reaction mixture consisting of 4 µl of 5X buffer (0.25 mol/l Tris - HCl pH 8.3, 0.25 mol/l KCl, 0.02 mol/l $MgCl_2$ and 0.05 mol/l DTT), 2 µl of 0.01 mol/l deoxyribonucleotide triphosphate (dNTPs) mix, 20 units of ribonuclease inhibitor (Fermentas International Inc, Burlington, Ontario L7N 3N4, Canada) and 100 units of RevertAid^{TM} H minus M MuLV reverse transcriptase (Fermentas International Inc, Burlington, Ontario L7N 3N4, Canada) was added to the RNA-primer mixture. Then the reaction mixture was incubated at 42 °C for 1 hour followed by heating at 70 °C for 10 min to denature the RNA forming double strand with cDNA and to inactivate reverse transcriptase. The reaction was terminated by incubating on ice for some time. The obtained cDNA sample was used as a template for PCR amplification.

3.2.3. PCR amplification

The synthesized cDNA or extracted DNA was subjected to PCR amplification using gene specific forward and reverse primers. It was carried out in a programmable thermocycler (MJ Research, USA)

PCR was carried out in 30 µl reaction mixture containing 10X buffer (100 mM of Tris-HCl, pH 8.3, 20 mM of $MgCl_2$, 500 mM of KCl and 0.1% gelatin), 200 mM of deoxyribonucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 10 pmol each of primers and 1 U of *Taq* DNA polymerase (Bangalore Genei, India), sterile water. 2µl of cDNA or DNA was used as template for amplification.

The optimized PCR programme consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of specific conditions as described in table 5. The final extension was performed at 72 °C for 10 min.

Table 5. Primers used and cycling conditions followed for PCR amplification.

Primer	Cycling conditions			No. of cycles	Primer specific for
	Denaturation	Annealing	Extension		
T4NV	94^{0}C for 60s	55^{0}C for 60s	72^{0}C for 60s	30	Nodavirus
LCNV	94^{0}C for 60s	50^{0}C for 60s	72^{0}C for 60s	30	Nodavirus
MCP	94^{0}C for 30s	56.5^{0}C for 30s	72^{0}C for 60s	30	Iridovirus
ATPase	95^{0}C for 60s	48^{0}C for 60s	72^{0}C for 60s	30	Iridovirus

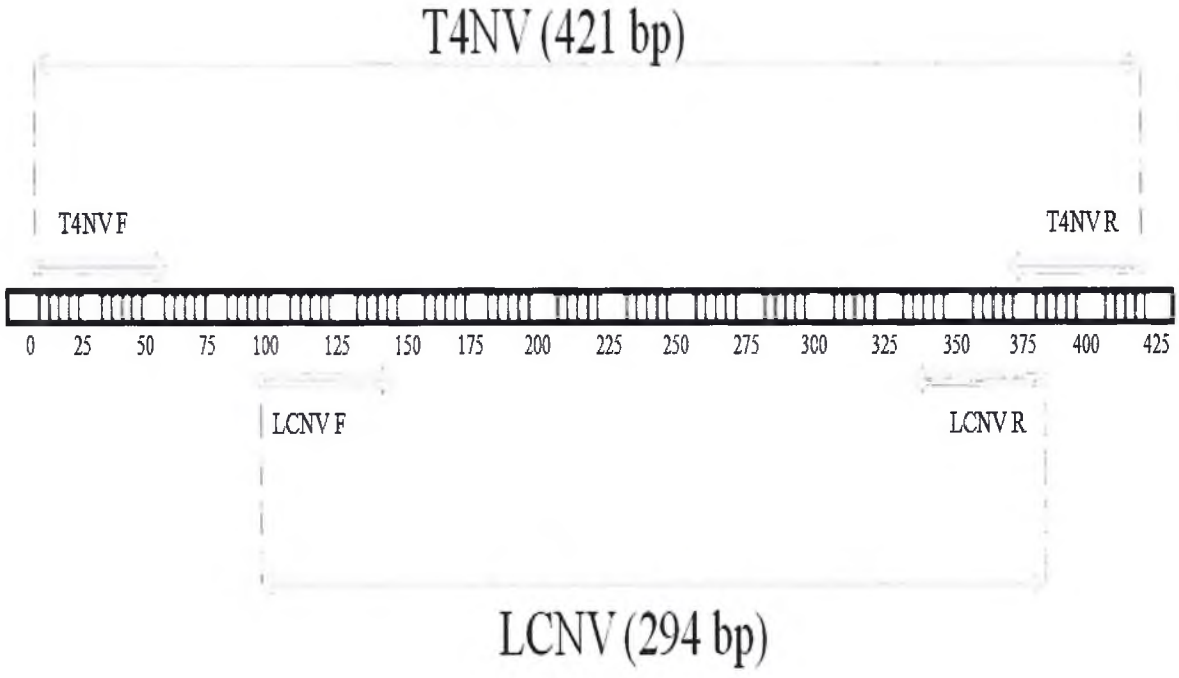

Figure 3. Indicating primer binding site of T4NV and LCNV gene.

Table 6. Primers and cyclic conditions for the detection of nodavirus and iridovirus.

a) For nodavirus

Primer name	Primer sequence (5'-3')	Annealing Temp (°C)	Product length (bp)	Reference
T4NV	F CGTGTCAGTCATGTGTCGCT R CGAGTCAACACGGGTGAAGA	55	426	Nishizawa *et al.*, 1994
LCNV	F GTTCCCTGTACAACGATTCC R GGATTTGACGGG GCT GCTCA	48	294	Parameswaran *et al.*, 2008

b) For iridovirus

Primer name	Primer sequence (5'-3')	Annealing Temp (°C)	Product length (bp)	Reference
MCP	GTTTGATGCGATGGAGACCC ATGCCAATCATCTTGTTGTAGCC	56.5	399	Jeffrey Go *et al.*, 2006
ATPase	CACCACCTGTGTGTATTTGTC CTTCATCCCACCCATTTC	48	237	Jeffrey Go *et al.*, 2006

3.2.4. Gel electrophoresis

The PCR products were resolved by agarose gel electrophoresis. 1.5 % (w/v) agarose gel was prepared in 1X TAE buffer. The molten agarose was cooled to below 65°C, ethidium bromide added to a final concentration of 0.5 µg/l, mixed gently, poured to gel mould with comb and allowed to set. 10 µl of each of the PCR product was mixed with 2 µl of 6X gel loading buffer and loaded into the wells. 100 bp DNA ladder (Bangalore Genei, India) was used as a molecular weight marker. Electrophoresis was carried out at 100-120 V and the bands obtained were visualized under UV transilluminator and photographed using a gel documentation system (Herolab, Germany).

3.3. A case study on the mass mortality observed in cage culture of Asian Seaass (*L. calcarifer*) due to nodavirus disease outbreak in Kasaragod, Kerala

3.3.1. Sample collection

12 moribund diseased Asian seabass juveniles fish samples (6-10cm size) were collected randomly from different fresh water cage culture farms at Neeleshwaram-Kasargode district of Kerala in West coast of India, where severe disease outbreak occurred in October 2009.

3.3.2. Clinical signs; case history and gross pathology

The pond was deepened to a minimum depth of 2 meter and reinforced by stacking sand filled HDPE Sacs around pond periphery. The pond is connected to Thejaswiny river (fresh water) by a 150 meter long channel. A temporary sluice gate with shutter was installed to ensure water level and entry of wild fishes. The pond was 1.2 acre in size, in that 3×3 meter sized two cages were installed with 5 meter apart. To maintain the water quality, 40% of water was exchanged once in a week and cages are regularly monitored and cleaned to remove algae. The Asian seabass larvae (5000 in no.) from RGCA brought in polythene bags were socked in two cages and fed with weaning feed.

Cage no.	No. of seeds	stocking density (no. /m^3)	Size of the fish (in cm)
I	2290	250	3.5
II	2292	250	3.5

While stocking, all the larvae are active in swimming behavior and normal in body coloration. Gradually a few of the juveniles became slightly darker in body coloration (Figure 10) after 10 days after stocking of fishes into the cages. The severe mortality was observed from15 days of stocking and continued upto 50 days. The moderate mortality occurred even after 50 days particularly during the stressful condition like water exchange, size grading of the fishes etc. The smaller size fishes were more prone to disease than bigger size fishes. During the case study, fishes showed clinical signs like succession in feeding, floating on the surface of the cages, pale gills, anorexia, inflated abdomen, exophthalmia, darkening of the whole body whirling movement, erratic swimming, cork-screw type behavior necrosis of the internal organs (brain; kidney) and finally leads to death has been observed. During the present study, the outbreak resulted in severe mortality of upto 70-80% approximately in 2-3 weeks of stocking period.

The moribund juvenile fishes were collected from different cage culture farm and were preserved in RNAlater (Ambion) and brought to the laboratory. Juvenile fish samples were processed immediately upon collection for extraction of RNA. Total RNA was extracted from the target organs like brain, retina and kidney tissue using TRIzol method and Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted as mentioned in the section 3.2.2. The T4NV and LCNV primers were used for the detection of nodavirus.

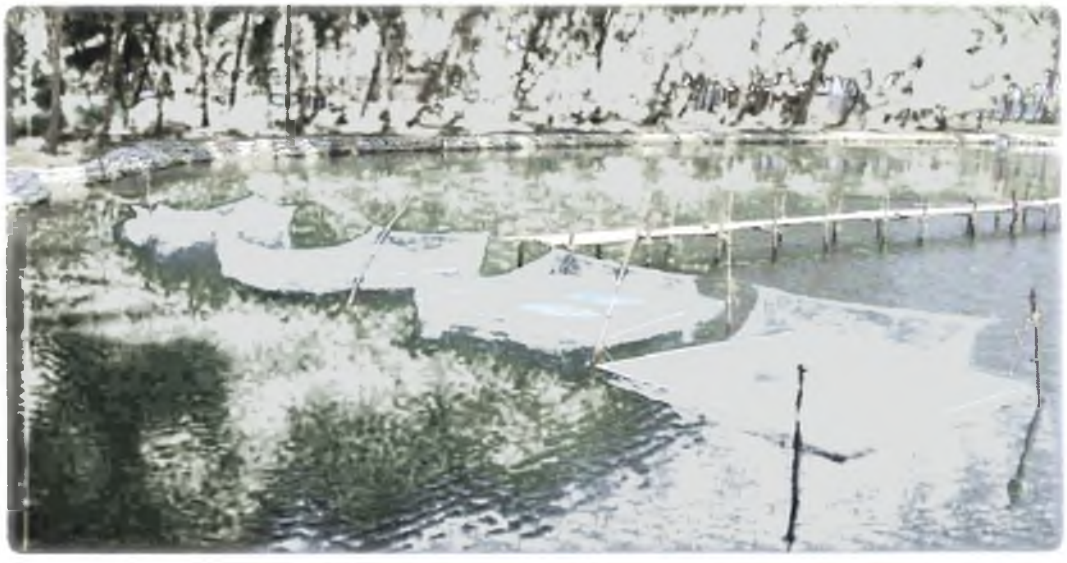

Figure 3. Nodavirus disease outbreak farm area (Asian seabass cage culture pond) in Kasaragod, Kerala

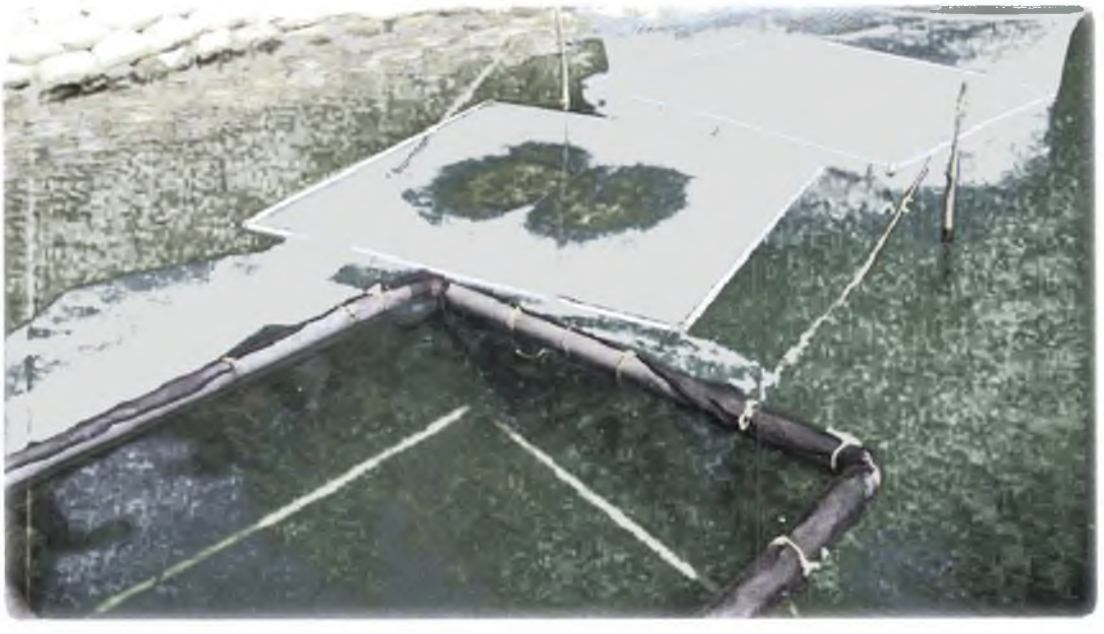

Figure 4. Cages used in nodavirus disease outbreak farm in Kasaragod, Kerala

3.4. Sequencing of case study samples

3.4.1. Purification of PCR products

The T4NV and LCNV genes were PCR amplified to obtain 120 µl using the primers and conditions as mentioned earlier. The PCR products were purified before ligation to the cloning vectors to remove *Taq* DNA polymerase, primer dimers, excess dNTPs which may interfere with subsequent process, using QIAquick PCR Purification Kit (Qiagen, Germany).

To one volume of the PCR product, 5 volume of buffer PB was added and mixed and then transferred to QIAquick spin column placed in a 2 ml collection tube. The flow through was discarded after centrifugation at 13,000 × *g* for 1 min, washed by adding 750 µl buffer PE containing ethanol followed by centrifugation at 13,000 × *g* for 1 min. The DNA was eluted by adding 30 µl of elution buffer (10 mM Tris-Cl, pH 8.5) or distilled water to the centre of the QIAquick column placed in a fresh microcentrifuge tube and spinning the column at 13,000 × *g* for 1 min.

Figure 5. Schematic diagram of purification of PCR products using QIAquick PCR Purification Kit.

3.4.2. Cloning

Ligation of PCR products into the cloning vector

The pDrive cloning vector (Qiagen, Germany) was used for cloning and sequencing. The vector map is shown in figure 7. The ligation reaction was carried out according to the manufacturer's instructions. The ligation reaction mixture was prepared by mixing 4 µl purified PCR product (T4NV and LCNV each) and 1 µl vector (50 ng/µl) in 200µl PCR tubes. 5 µl of 2X ligation master mix was added to each tube, mixed gently and incubated at 16°C in a thermal cycling block for 2 hours.

Transformation

Transformation was carried out according to the protocol described by Qiagen. An aliquot of the ligation mix was transferred to tube containing 100 µl of competent cells. Frozen -80°C QIAGEN EZ competent cells were used for transformation of T4NV and LCNV genes of nodavirus.

After adding the ligation mix to the competent cells, the contents were mixed gently and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 90 seconds in a water bath. The tubes were then immediately transferred to ice and cells were allowed to chill for 1-2 mins. 500 µl of SOC broth maintained at room temperature (29±1°C) was added to the tubes and incubated at 37°C for 2 hr with vigorous shaking (200 rpm) in horizontal position. 25 µl, 50 µl and 100 µl of the transformed broth were spread on LB agar plates containing kanamycin (30 µg/ml). The plates were incubated overnight at 37°C.

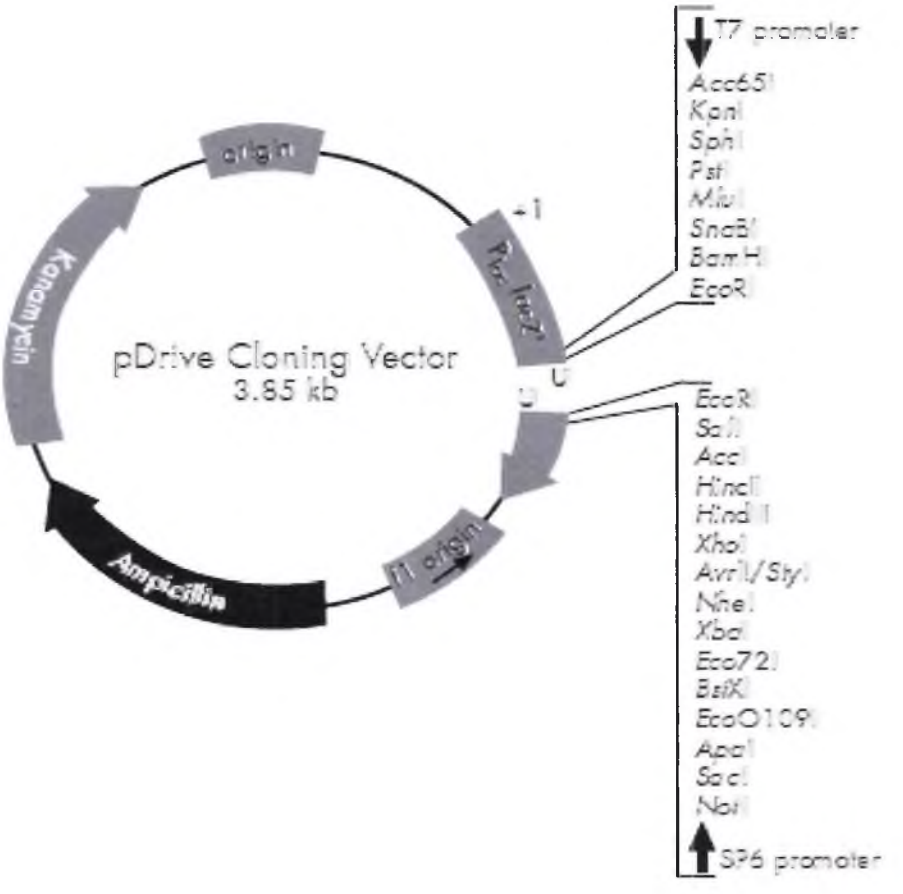

Figure 6. Vector map of the pDrive prelinearized vector. Reproduced from Qiagen, Germany.

Preparation of competent cells

Competent cells were freshly prepared according to the supplier's protocol (Qiagen, 2003). A loopful of *E. coli* EZ competent cells was removed from the supplied vial and streaked on to LB agar plate and incubated overnight at 37°C. A single colony was picked and inoculated into 10 ml of LB broth and incubated overnight at 37°C. 1 ml of the overnight grown culture was added to 100 ml of pre-sterilized LB broth in a 250 ml flask and incubated for 90-120 min with shaking at 200 rpm at 37°C. When the absorbance at OD_{600} reached 0.5, the culture was cooled on ice, transferred to 50 ml polypropylene tubes and centrifuged at 4000 × g for 10 min at 4° C. The supernatant was discarded and the pellet resuspended in 30 ml of ice cold TFB1 buffer (30 ml for a 100 ml culture), incubated on ice for an additional 90 min and centrifuged at 4000 × g at 4°C for 10 min. The supernatant was discarded and cell pellet was resuspended in 4 ml of ice cold TFB2 buffer. 150 µl quantity of the suspension of competent cells was aliquoted to several 1.5 ml microcentrifuge tubes and stored at -80°C.

Screening of transformants

Transformants were randomly selected and screened by using the vector and gene specific primers for the presence of insert by preparing crude lysate of the transformant cultures. A few selected colonies were inoculated to 4 ml of LB broth containing kanamycin antibiotic (30 µg/ml) and incubated overnight at 37°C. A 1:10 dilution of the overnight culture was prepared by adding 50 µl of the culture to 450 µl of 1X TE buffer in a 1.5 ml microcentrifuge tube. The cells were lysed by heating at 98°C for 10 min, snap chilled on ice and centrifuged briefly for 5 min. 2 µl of the crude lysate was used as template for PCR using gene specific primers.

The analysis of transformants was done using gene specific primers (T4NV and LCNV primer) and vector specific primer (M13 primer). To determine the correct insertion of the gene cloned, a combination of vector specific primers and the gene specific primers were used *i.e.*, vector specific forward primer and gene specific reverse primer or the *vice versa*. All the positive clones were stored at -80°C in 30% glycerol broth.

Table 7. Vector primers and sequence used for confirmation of transformant

Vector primer	Sequence
M13 forward	5' GTAAAACGACGGCCAGT 3'
M13 reverse	5' AACAGCTATGACCATG 3'

3.4.3. Sequencing of cloned genes

The cloned T4NV and LCNV genes in PCR positive clones were sequenced to determine the orientation and correctness of nucleotide sequence using vector specific primers (M/s Chromous Biotech Ltd, Bangalore). The sequences of T4NV and LCNV gene generated in this study were submitted to GenBank.

3.4.4. Phylogenetic analysis

The sequence of the cloned product was obtained and aligned against other betanodaviruses coat protein gene sequences available in the GenBank and phylogenetic tree was constructed using MegaAlign program (Windows version 5.05; DNASTAR, WI, USA).

3.5. DNA extraction

DNA was extracted according to the protocol of Wang *et al.*, (2006) with slight modifications.

Reagents used for DNA extraction

- Lysis buffer
- Proteinase K (100 µg/ml)
- Phenol-chloroform-iso amylalcohol (25:24:1)
- Absolute alcohol
- 1X TE buffer
- Sterile distilled water

Procedure for DNA extraction

The targeted tissue (kidney, liver, spleen, viscera) was removed aseptically from the fish and placed in sterile 1.5 ml microfuge tube and homogenized in 1 ml sterile distilled water and placed on ice. The homogenate was centrifuged at 1500g for 10 min at 4 °C and the supernatant obtained was transfered to fresh tube and mixed with equal volume of lysis buffer and 5 µl of proteinase K.

The mixture was incubated at 55 °C for 3 hours followed by the addition of 200 µl of PCIA (Phenol-chloroform-iso amyl alcohol) and centrifuged at 1500 g for 10 min at 4 °C. The supernatant was then taken in a separate tube and incubated with 1 ml of absolute alcohol at -20°C for 30 min to precipitate DNA. Precipitated DNA was sedimented by centrifuging at 1500 g for 10 min at 4 °C and washing with 70% ethyl alcohol. The obtained DNA pellet was vacuum dried, dissolved in 50-100 µl 1X TE buffer and stored at -20°C until further use.

PCR amplification was carried out (cyclic conditions is mentioned in table 5) and amplified products are observed through gel electrophoresis as mentioned in section 3.2.3 and 3.2.4 respectively by using the primers mentioned in the table 6 for iridovirus.

3.6. Reagents

3.6.1. For cloning

Glycerol broth

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g
Glycerol	30 ml
Distilled water	70 ml

The medium was autoclaved at 110°C for 10 min.

Luria Bertani (LB) medium

Tryptone	1.5 g
Yeast extract	5 g
Sodium chloride	10 g
Distilled water	100 ml

The medium was autoclaved at 121°C for 15 min.

LB agar

Tryptone	1.5 g
Yeast extract	5 g
Sodium chloride	10 g
Agar	1.5 g
Distilled water	100 ml

The medium was autoclaved at 121°C for 15 min.

LB agar with antibiotics

4 g of LB agar was dissolved in 100 ml of distilled water and sterilized at 121°C for 15 min. When the medium cooled to 50°C, known concentration of antibiotic was mixed and poured into plates.

Kanamycin stock solution (30 mg/ml)

1.5 g kanamycin monosulfate salt was dissolved in 50 ml deionized water. The solution was filter sterilized using 0.22 μm syringe filter (Pall Corporation, USA) and stored in aliquots at 4°C.

SOC medium

Tryptone	2%
Yeast extract	0.5%
Sodium chloride (NaCl)	10 mM
Potassium chloride (KCl)	2.5 mM
Magnesium chloride ($MgCl_2$)	10 mM
Magnesium sulphate ($MgSO_4$)	10 mM
Glucose	20 mM

All the components except glucose were dissolved in 100 ml distilled water and sterilized by autoclaving at 121°C for 15 min. When the medium was cooled, filter sterilized glucose was added and stored at 4°C.

TFB1 buffer

Rubidium chloride	100 mM
Manganese Chloride	50 mM
Potassium acetate	30 mM
Calcium chloride	10 mM
Glycerol	15%

All the chemicals used were of molecular biology grade. pH was adjusted to 5.8 to avoid precipitation of insoluble manganese. After adjusting the pH, the buffer was filter sterilized and stored at 4°C.

TFB2 buffer

MOPS	10 mM
Rubidium chloride	10 mM
Calcium chloride	75 mM
Glycerol	15%

Note : MOPS – (3[N-Morpholino] propanesulhonic acid)

All the chemicals used were of molecular biology grade. pH was adjusted to 6.8. After adjusting the pH, the buffer was filter sterilized and stored at 4°C.

Phosphate buffered saline (PBS)

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen orthophosphate	0.24 g
Distilled water	1000 ml

The pH of the buffer was adjusted to 7.4 with conc. HCl, sterilized by autoclaving at 121°C for 15 min and stored at room temperature.

3.6.2. For DNA extraction

Lysis buffer (1L)

10mM Tris–HCl (pH 8.0)	1.21 gm
100mM EDTA	37.0 gm
RNase (20 µg/ml)	0.2 mg
SDS - 0.5% (w/v)	5.0 gm
Distilled water	1000 ml

All the chemicals were dissolved in 1 L of distilled water and filter sterilized before using.

Phenol Chloroform Isoamyl-alcohol (25:24:1)

phenol	25 ml
chloroform	24 ml
isoamyl-alcohol	1 ml

PCIA was prepared by adding 25 ml of equilibrated phenol, 24 ml chloroform and 1 ml of isoamyl-alcohol, mixed well and stored at 4 °C.

3.6.3. For RNA extraction

1x DEPC treated water

Diethylpyrocarbonate(DEPC)	1 ml
Distilled water	1000 ml

1 ml of DEPC was added to 1L of distilled water (at a ratio of 0.1% v/v) and incubated overnight at room temperature followed by autoclaving at 121˚C for 15 mins.

3.6.4. For gel electrophoresis

TAE buffer - Stock solution (50 X)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water	1000 ml

242 gm of Tris base was added to 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA mixture. The volume was made up to 1 liter with distilled water and stored at room temperature (28°±1°C). The working solution (1000 ml of 1× TAE) was prepared by diluting 20 ml of 50× stock solution to 1000 ml with distilled water.

6X Gel loading buffer

Bromophenol blue (0.25 %)	25 g
Sucrose (40 %)	4 g
Distilled water	10 ml

25 gm of Bromophenol blue and 4 gm of sucrose was mixed with 10 ml of distilled water and stored at 4 °C.

Ethidium bromide (10 mg/ml)

Ethidium bromide	1 g
Distilled water	100 ml

1 gm of Ethidium bromide was dissolved in 100 ml distilled water. The solution was either added to agarose during gel preparation or to the appropriate buffer (1X TAE buffer) to yield a final concentration of 0.5 µg/ml.

EXPERIMENTAL RESULTS

V. EXPERIMENTAL RESULTS

4.1. Analysis for nodavirus

4.1.1. Clinical signs

Fishes sampled in the present study were found to be swimming sluggishly on the surface of the pond; few of them were slightly darker in body coloration exhibited whirling movement and erratic swimming behavior. Fishes infected with the virus showed clinical signs such as discoloration of gills, anorexia and bulged eye balls with a prominent white coloration around the eye ball on optic capsule (Exophthalmia). The belly region was observed to be enlarged, probably due to inflated/swollen swim bladder with gas accumulation. The dead fish were spongy in texture and smelled foul due to rapid decay. The mortality rate was around 80-90 %.

4.1.2. RT-PCR amplification

cDNA was synthesized using extracted total RNA from the sampled tissue and taken for reverse transcriptase PCR using T4NV and LCNV primers.

A total of 43 samples were analyzed for nodavirus which included Seabass (*L. calcarifer),* Six barbed grouper *(Epinephelus diacanthus) and* Pearl spot *(Etroplus suratensis).* 17 of 43 samples analyzed were found positive for nodavirus by both sets of primers used in the study. All the samples generated amplicons of 426 bp and 294 bp for T4NV (figure 11) and LCNV (figure 12) respectively.

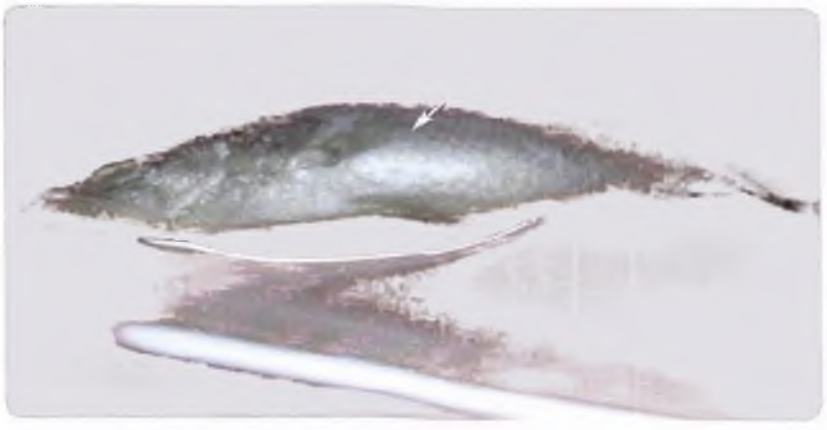

Figure 7. Nodavirus infected moribund seabass fish showing the clinical signs of inflated abdomen.

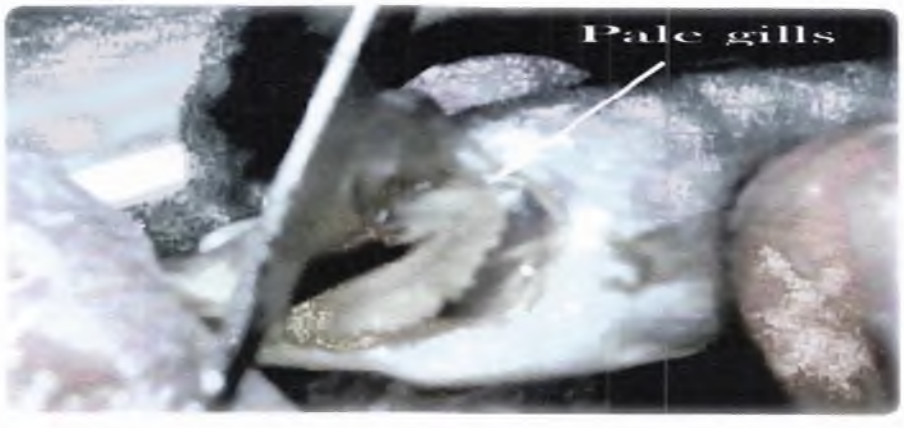

Figure 8. Nodavirus infected sea bass showing the clinical signs of pale gill symptom.

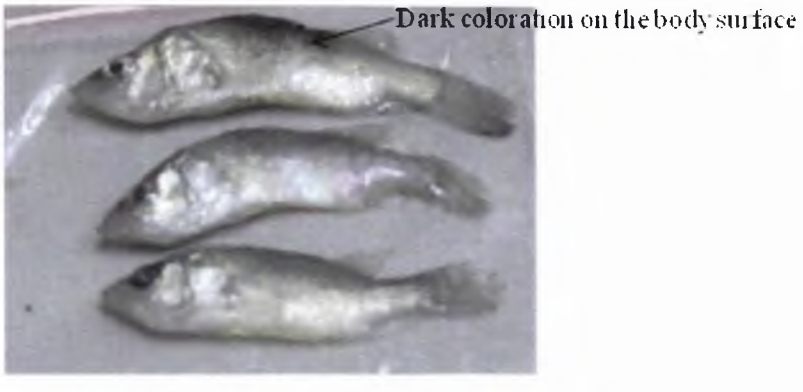

Figure 9. Nodavirus infected moribund sea bass showing the dark colouration on the body surfaces.

Table 8. RT-PCR detection of nodavirus from the samples analyzed.

Sl.No	Sampling source	Species analyzed	No. of samples analyzed	T4NV primer	LCNV primer
1	Rajiv Gandhi Centre for Aquaculture (RGCA) Chennai, Tamil Nadu	*Lates calcarifer* (Asian sea bass)	9	5	5
2	Asian sea bass fish culture pond Kumta, Karnataka	*Lates calcarifer* (Asian sea bass)	7	0	0
3	Asian sea bass seed rearing center, Kumta, Karnataka	*Lates calcarifer* (Asian sea bass)	3	0	0
4	Wild caught fishes, fish landing center, West-coast Mangalore	*Epinephelus diacanthus* (Grouper)	3	0	0
		Etroplus suratensis (Pearl spot)	4	0	0
5	Wild caught fishes, fish landing center, East-coast,T.N	*E. diacanthus* (Grouper)	5	0	0
6*	* Case study on nodavirus outbreak in Asian sea bass cage culture demo farm, Neeleswaram, Kerala	*Lates calcarifer* (Asian sea bass)	12	12	12
	Total		**43**	**17**	**17**

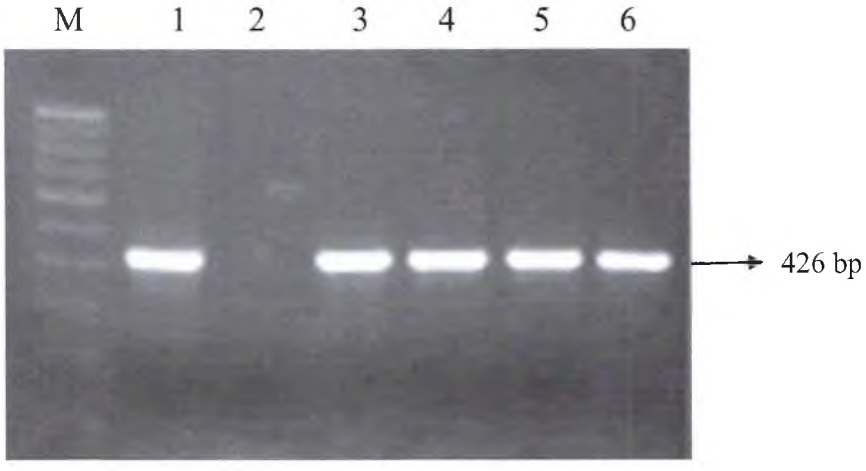

Figure 10. Detection of fish nodavirus by Reverse transcriptase PCR using T4NV-F/R primer set.

M: 100 bp DNA ladder, Genei Bangalore,

Lane 1: Postive with T4NVF/R primer

Lane 2: Negative with T4NVF/R primer

Lane 3-6: nodavirus positive samples

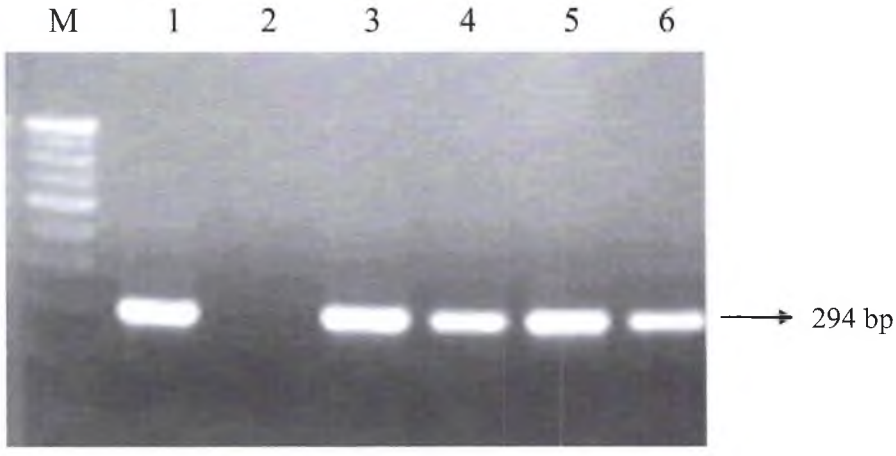

Figure 11. Detection of fish nodavirus by Reverse transcriptase PCR using LCNV-F/R primer.

M: 100 bp DNA ladder, Genei Bangalore,

Lane 1: Postive with LCNV primer

Lane 2: Negative with LCNV primer

Lane 3-6: nodavirus positive samples

RT-PCR analysis for the case study samples

All the 12 samples were analyzed for nodavirus and found to be positive for nodavirus through RT-PCR by using 2 set of detection primers. All the 3 organs like brain, retina and kidney samples taken from the individual fish were positive for RT-PCR. The RT-PCR amplification of the T4 region of the coat protein gene (by T4NV-F/R primers) gave a PCR product of 426 bp. The RT-PCR analysis of another set of primer (LCNV-F/R) gave an amplicon size of 294 bp in all 12 individual fish samples. Amplified samples were considered for further study.

4.1.3. Nodavirus nucleotide sequences obtained during the study

A) Nodavirus T4NV coat protein gene, partial cds

1 CGTGTCAGTC ATGTGTCGCT GGAGTGTTCG ACTGAGCGTT CCGTCTCTTG AGACACCTGA
61 AGAGACCGTC GCTCCCATCA TGACACAAGG TCCCCTGTAC AACGATTCCC TTGCCACACA
121 CGACTTCAAG TCCATCCTCC TTGGATCCAC ACCATTGGAC ATTGCCCCTG ATGGCGCAAT
181 CTTCCAACTG GACCGTCCGC TGTCCATTGA TTACAGCCTT GGAACTGGAG ATGTTGACCG
241 TGCTGTCTAT TGGCACCTCA AGAAGTTTGC TGGAACTTCC GGCACACCTG CAGGCTGGTT
301 TCGCTGGGGC ATCTGGGACA ATTTCAATAA AACGTTCATA GATGGCGTTG CTTACTACCC
361 TAATCAGCAG CCTCGTCAAA TCCTGCTGCC TGTCGGCACT GCCTTCACCC GTGTTGACTC
421 G

B) Nodavirus LCNV coat protein gene, partial cds

1 GTTCCCTGTA CAACGATTCC CTTGCCACAC ACGACTTCAA GTCCATCCTC CTTGGATCCA
61 CACCATTGGA CATTGCCCCT GATGGCGCAA CCTTCCAACT GGACCGTCCG CCGTCCATTG
121 ATTACAGCCT TGGAACTGGA GATGTTGACC GTGCTGTCTA TTGGCACCTC AAGAAGTTTG
181 CTGGAACTTC CGGCACACCT GCAGGCTGGT TTCGCTGGGG CATCTGGGAC AATTTCAATA
241 AAACGTTCAC AGATGGCGTT GCTTACTACC CTGATGAGCA GCCCCGTCAA ATCC

4.1.4. Nucleotide sequence submission to the GenBank

The T4NV and LCNV products were purified and the gene sequences generated in this study were submitted to GenBank were assigned accession numbers HM017077 (nodavirus T4NV coat protein gene, partial cds) and HM017076 (nodavirus LCNV coat protein gene, partial cds). The sequences were analyzed by BLAST program of NCBI (http://blast.ncbi.nlm.nih.gov/). The sequences of our seabass nervous necrosis virus gene showed more than 90% homology to the nucleotide sequence of piscine nodavirus isolated from other countries.

4.1.5. Phylogenetic analysis

DNA sequence of the coat protein gene of T4NV (HM017077) and LCNV (HM017076) were compared with 21 coat protein gene sequences of piscine nodavirus available in the GenBank (Table 9). The phylogenetic tree (Figure 13) constructed based on the coat protein gene sequence of betanodavirus revealed that our T4NV and LCNV sequences clustered together with the redspotted grouper nervous necrosis virus (RGNNV) sequence.

Table 9. GenBank nucleotide sequences of coat protein gene of nodavirus from different fish species used for phylogenetic tree construction.

Accession number	Host species/ (Scientific name)	Country
HM017076	Asian sea bass (*L. calcarifer*)	India
HM017077	Asian sea bass (*L. calcarifer*)	India
EU380202	Asian sea bass (*L. calcarifer*)	Malaysia
FJ617262	Asian sea bass (*Lates calcarifer*)	Malaysia
FJ617265	Asian sea bass (*L. calcarifer*)	Malaysia
AF245004	Dragon (*Epinephelus lanceolatus*)	Taiwan
EF591371	Barramundi(*L. calcarifer*)	Australia
EF591372	Barramundi(*L. calcarifer*)	Australia
D38636	Redspotted grouper (*Epinephelus akaara*)	Japan
EF558369	Redspotted grouper (*Epinephelus akaara*)	China
AF499774	Guppy (*Poecilia reticulate*)	Singapore
GQ120525	Asian sea bass (*L. calcarifer*)	Malaysia
AF175518	Brownspotted Grouper (*Epinephelus malabaricus*)	Thailand
AF175516	Barramundi(*L. calcarifer*)	Singapore
AF318942	Greasy grouper (*Epinephelus tauvina*)	Singapore
D38637	Tiger puffer (*Takifugu rupripes*)	Japan
EU236149	Tiger puffer (*Takifugu rupripes*)	Japan
D38635	Barfin flounder (*Verasper moseri*)	Japan
EU236147	Barfin flounder (*Verasper moseri*)	Japan
EU826138	Barfin flounder (*Verasper moseri*)	Japan
AF175519	Striped jack (*Pseudocaranx dentex*)	Japan
D30814	Striped jack (*Pseudocaranx dentex*)	Japan
AB056572	Striped jack (*Pseudocaranx dentex*)	Japan

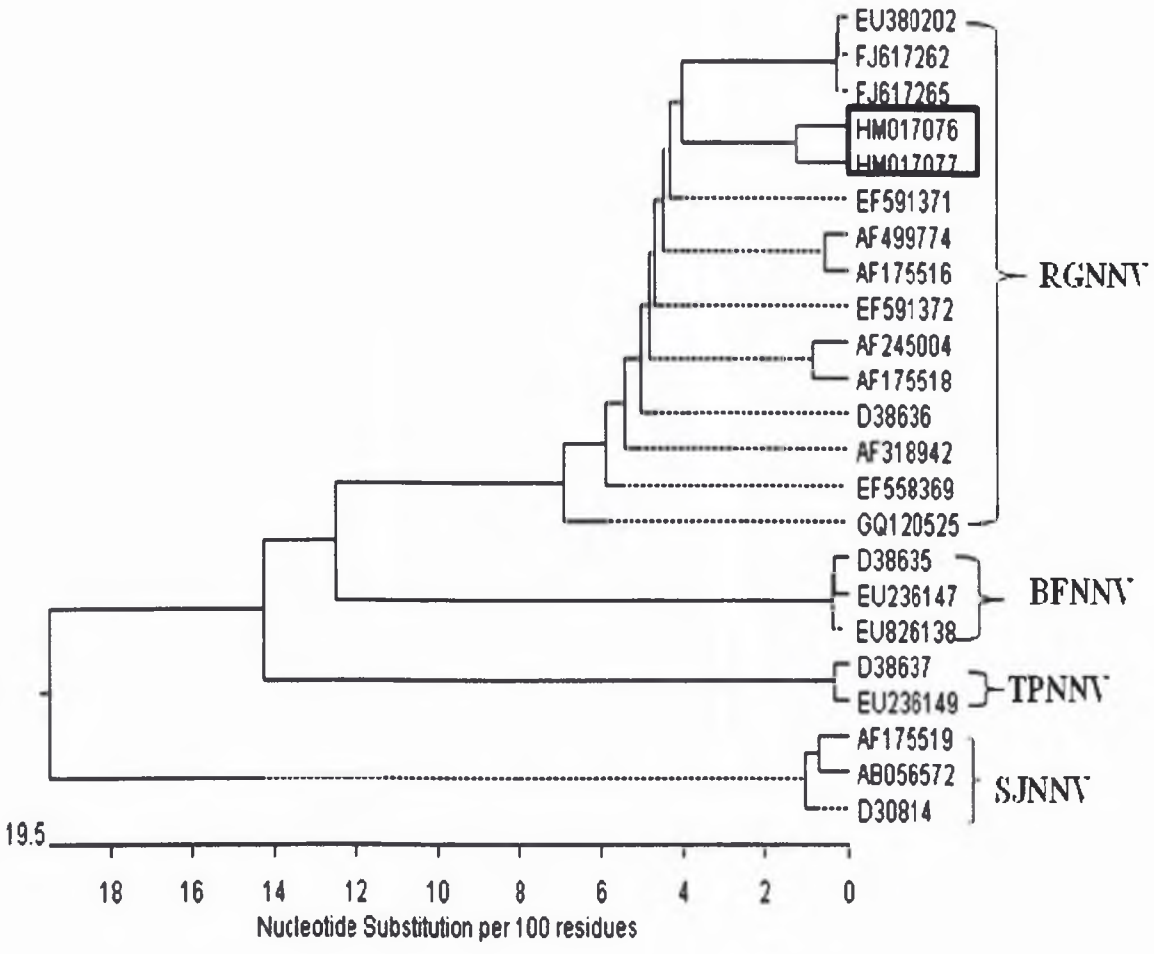

Figure 12. Phylogenetic tree constructed from the variable region of coat protein sequences of 23 betanodaviruses. The distance between sequences is represented by the length of each pair of branches.

4.2 Analysis of iridovirus

DNA extracted from the sampled tissues was subjected to single step PCR using MCP and ATPase primer for MCP and ATPase gene. None of the 44 samples analyzed in this study was found to be positive for this virus.

Table 10. PCR detection of iridovirus from the samples analyzed.

Sl. No	Sampling source	Species analyzed	Number of samples analyzed	Iridovirus positive
1	Asian sea bass seed rearing center, Kumta, Karnataka	*Lates calcarifer* (Asian sea bass)	8	0
2	Aquaculture pond, Ankola, Karnataka	*Mugil cephalus* (Grey mullet)	3	0
		Etroplus suratensis (Pearl spot)	2	0
		Sillago sihama (Silver whiting)	1	0
3	Wild caught fishes from Indo Fisheries processing plant, Mangalore	*Nemipterus japonicas* (Pink perch)	2	0
4	Wild caught fishes, fish landing center, West-coast Mangalore	*Cynoglossus luctosus* (Tongue sole)	3	0
		Euryglossa orientalis (Oriental sole)	2	0
		Platycephalus indicus (Flatheads)	2	0
		Arius jella (Cat fish)	3	0
		Psettodes erumei (Indian halibut)	6	0
		Zebrius quagga (Zebra sloe)	4	0
		Lagocephalus inermis (Puffer fish)	1	0
		Rastralliger kanagutta (Indian mackerel)	1	0
5	Wild caught fishes, fish landing center, East-coast,T.N	*E. diacanthus* (Grouper)	6	0
	Total		**44**	**0**

DISCUSSION

V. DISCUSSION

Marine viruses are pathogens which are known to infect many of the aquatic organisms and known to be present upto ten billion per liter (Fuhrman, 1999 and Liu *et al.,* 2009). Viruses are successful parasites and utilize a wide variety of habitats with their host ranging from bacteria and algae to specialized cells of highest vascular plants and warm blooded animals (Lauffer and Smith, 1966). Fish are affected by several viruses both in the ocean and culture system. Generally infected fish doesn't feed and it results in a rapid increase in mortality which is a sign of viral infection. This results in huge economic loss and affects the sustainability of culture systems. Therefore it is important to detect viral infection in early stages and adopt better management practices to create stocks that are free from infections.

5.1. Prevalence of nodavirus

Seabass, *L. calcarifer*, is considered a potential candidate species for farming in India, due to its high growth rate, tolerance to wide changes in environmental conditions and its huge demand in domestic and export markets (Parameswaran *et al.,* 2008). VNN associated with mass mortalities in cage-reared seabass (*D. labrax*) was reported by Le Breton *et al.*, (1997) from Europe. Azad *et al.,* (2005) reported incidence of viral nervous necrosis-related mass mortalities in hatchery produced Asian seabass *L. calcarifer* from India. The present study is the first to record the incidence of nodaviral infection in seabass *L. calcarifer* from west coast of India.

Mori *et al.,* (1992) purified the disease causing agent for the first time in striped jack, *Pseudocaranx dentex* and designated it as striped jack nervous necrosis (SJNNV) belonging to the family *Nodaviridae.* Nishizawa *et al.*, (1994) designed PCR primers based on the nucleotide sequence of variable region of RNA2 gene of SJNNV and used it to detect VNN infection by RT-PCR. Azad *et al.,* (2005) designed primers specific for T4 region of coat protein gene and reported that it could be used for the detection of piscine nodavirus from different geographical locations. In the present study, primers designed by Nishizawa *et al.,* (1994) for variable region of T4 gene and nested primers designed by Parameswaran *et al.,* (2008) were used which gene amplicons of size 426 bp and 294 bp respectively.

During the study, the fish samples from various culture systems and captured stocks along south-west coast of India were analyzed for nodavirus. Though there are sporadic outbreak reports of nodavirus, there is no systematic study on the prevalence of nodavirus infection in Indian waters.

During the case study, the disease outbreak occurred in mass mortalities in cage cultured Asian seabass, was found to be nodavirus infection (viral nervous necrosis; VNN) by clinical symptoms, RT-PCR and DNA sequencing. The clinical symptoms like exophthalmia, darkening of the whole body, whirling movement, erratic swimming and cork-screw type behavior are the typical symptoms of seabass nervous necrosis virus infection (Zafran *et al.* 1998; Azad *et al.* 2005; Parameswaran *et al.* 2008; Ransangan *et al.* 2010). Nodavirus mainly targets nervous tissues and cause heavy vacuolation which result in loss of equilibrium and abnormal swimming behavior before death (Parameswaran *et al.*, 2008). Similar diseases with

same clinical signs have been reported from different parts of the world (Glazebrook *et al.,* 1990; Yoshikoshi and Inoue, 1990; Mori *et al.,* 1991; Munday and Nakai, 1997; Skliris and Richards, 1999; Oh *et al.,* 2002 and Maeno *et al.,* 2004). Le Breton *et al.* (1997) reported the VNN associated with mass mortalities in cage-reared seabass (*D. labrax*). Hegde *et al.* (2003) reported the nodavirus infection in fresh water ornamental fish guppy (*P. reticulate*) by experimental infection study and in our case we are reporting the VNN infection in fresh water cage culture system. It was observed that seabass juveniles were more susceptible to VNN at 10 days onwards with high mortalities was recorded about 80% during 15-30 days of stocking. From our observation, smaller size fishes are more prone to VNN infection than the bigger size fishes in the cages. This could be due to the various stress factor like overcrowding in the cages, cannibalism nature of the seabass, size grading procedure applied in that cage culture system etc. Azad *et al.* (2005) observed larger larvae of Asian seabass are less affected than smaller fishes due to the stress factor like competition for the food, crowding, water quality and poor nutrition (OIE 1997).

In the study, Asian seabass fingerlings from RGCA, Chennai were also analyzed for nodavirus. The fishes of size 8.5 to 9.5 cm were found swimming sluggishly with overall darkening in the body coloration. Eye balls were bulged with a prominent white coloration on optic capsule. The fishes were hardly able to accept feed due to anorexia and fatigue. Gills were pale in color, abdomen inflated and the dead fishes smelled foul due to rapid decay.

Earlier it was believed that only larval and juvenile fish were susceptible to nodaviral infection (Breuil *et al.*, 1991), Later Fukuda *et al.* (1996) reported that adult groupers are also susceptible. In this study, samples brought from RGCA, Chennai (size 12-14 cm) were initially screened for nodaviral infection before stocking in the laboratory and they were

negative for the virus by PCR. After 15 days of stocking, slight mortality was observed and most of the fishes were found in moribund condition. Analyses of dead and moribund fishes showed them to be positive for nodaviral infection. Thus we assume that adult fishes can be causes of nodavirus without showing clinical signs. However, various stress associated factors like variation in salinity, temperature and overcrowding may lead to severe viral infection resulting in severe mortality of the fish.

Arimoto *et al.,* (1993) and Nguyen *et al.,* (1996) reported that the disease could be reproduced in the larvae of striped jack, *Pseudocaranx dentex*, by immersion in a homogenate of diseased tissue. Mushiake *et al.,* (1994) found gonads, eggs and larvae of striped jack, *Pseudocaranx dentex* carrying VNN and reported vertical transmission of VNN from mother to offspring. VNN can be transmitted both vertically and horizontally in Asian seabass in culture systems (Azad *et al.*, 2006 and Ransangan *et al.*, 2010). The absence of viral transmitting agents in the culture environment of fish but the presence of nodaviral infection in fish suggests that the disease is possibly transmitted from carriers onto their progeny by vertical transmission.

During the study, T4 region of the coat protein genes have been sequenced and our sequence showed high similarity (more than 90%) with the other Asian strains of nodavirus. The phylogenetic analysis of our sequence with other available sequences revealed our viral strain as belonging to the redspotted grouper nervous necrosis virus (RGNNV) genotype (Figure 13). According to Nishizawa *et al.* (1997) RGNNV is one of the widely distributed nodavirus genotype in piscine nodavirus group.

5.2. Prevalence of iridovirus

Lymphocystis disease is geographically distributed worldwide and has been reported to infect more than 125 species of wild caught and cultured teleost fish from marine and freshwater environments (Anders, 1989). As there are no cases of iridovirus reports from India, in this study 44 samples from captured and culture systems were collected along Southwest coast of India and screened for presence of this virus. Our results were negative by PCR for all the samples tested. From these results it appears that the lymphocystis disease is probably not there in Southwest coast of India.

Due to the increased demand for aquaculture production, there is increasing pressure for development of more efficient production systems. Major improvements have already been achieved through enhanced management, nutrition, water quality maintenance and genetic improvement of production traits. Since the industry still depends on wild broodstock, it is important to know the prevalence status of infectious agents. Viral disease outbreaks are one of the major threats for efficient aquaculture production.

To control viral infections in aquaculture it is important to understand the epidemiology and pathogenic mechanisms of the virus. Generally, the virus can be transmitted in aquaculture system through the carrier animals, inlet water; feed, broodstock via the eggs/sperm. It has been proved that piscine nodavirus can infect both marine water fishes and freshwater fishes through experimentally demonstrated infection studies (Hegde *et al.*, 2003; Skliris and Richard. 1999). The vertical transmission of VNN from brood fish to offspring has been well demonstrated by Breuil *et al.* (2002). Nerland *et al.* (2007) reported the horizontal

transmission of nodavirus in Atlantic halibut *(Hippoglossus hippoglossus)* larvae. Some of the VNN infected larval fish can survive and act as a carrier for the next generation (Nerland *et al.* 2007). In our study, though fishes survived even after 50 days of culture they may act as the viral carrier animals and cause infection both by vertical and horizontal transmission to the next generation. Screening procedure (through PCR and ELISA) and better management practices to avoid stress, must be the control strategy for VNN infection in seabass aquaculture.

SUMMARY

VI. SUMMARY

This study was carried out to look for the prevalence of nodavirus and iridovirus in cultured and wild caught marine finfishes from south-west and east coast of India. In the present study, 43 fish samples were analyzed for piscine nodavirus, of which 17 sample were positive for nodavirus by reverse transcriptase PCR. None of the 44 fish samples analyzed for iridovirus were positive. This could be due to the low prevalence of iridovirus infection in Indian waters or a variant which is missed by the primers used in this study.

The main conclusions drawn from this study are:

- Prevalence of nodavirus is high in juveniles of seabass, *(L. calcarifer).* Adult fishes are also prone to VNN infection.
- The nodavirus infection could be detected by PCR from the target organs like brain, retina, and kidney.
- Piscine nodavirus was detected from brain, retina and kidney of the fish sampled.
- The piscine nodavirus infection in seabass was observed in both fresh and marine water culture system and involvement of this virus in massive mortality of seabass, *L. calcarifer* is being reported for the first time from west-coast of India.
- Vertical transmission of VNN disease in juveniles of seabass has been observed.
- Phylogenetic analysis of RNA 2 coat protein coding sequences revealed that our nodavirus belongs to the Red spotted grouper nervous necrosis virus (RGNNV) and showed more than 90 % homology with other previously isolated and characterized betanodavirus.
- All samples analyzed for iridovirus were negative by PCR.

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ABSTRACT

VII. ABSTRACT

Production of marine fish in India has been initiated in recent years because of their economic value and to address food and nutritional security. Rapid expansion and intensification of aquaculture, however, has been accompanied by increased incidence of disease outbreaks. The morbidity and mortality caused by viruses in cultured fish population is very high with nodavirus and iridovirus infection occurring most frequently. So the present study was carried out to understand the prevalence of nodavirus and iridovirus in cultured and wild caught marine finfish from southwest coast of India.

In the present study 43 fish samples were analyzed for nodavirus by PCR of which 17 were positive. The risk associated with nodavirus outbreaks is seen from the results of the study. VNN disease in seabass, *L. calcarifer* caused massive mortality and is being reported for the first time from the west-coast of India. During the study period, piscine nodavirus was detected from the target organs like brain, retina and kidney of the moribund seabass. RNA2 coat protein coding genes were cloned and sequenced. Sequence analysis showed 90% homology to the RNA2 coat protein gene sequences of piscine nodaviruses isolated from different geographical regions. The phylogenetic analysis based on the RNA2 coat protein gene partial nucleotide sequences revealed that our strain of virus belonged to the red-spotted grouper nervous necrosis virus (RGNNV). It has been reported that RGNNV is one of the widely distributed genotype of four known genotypes of piscine nodavirus.

During the study, 44 samples of both the cultured and wild caught fish samples were also tested for iridovirus and none were positive for lymphocystis iridovirus. The present study indicates the absence of lymphocystis disease in samples from southwest coast of India.