DEVELOPMENT OF DOT BLOT ASSAY FOR DETECTION OF WHITE SPOT VIRUS IN SHRIMPS

Dissertation submitted in partial fulfillment of
The requirement for the award of the degree of

MASTER OF FISHERIES SCIENCE
In
INLAND AQUACULTURE

By

P. SRIKALA, B.F.Sc.
1998-2000 Batch

under the guidance of

DR.K.PANI PRASAD

CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
(INDIAN COUNCIL OF AGRICULTURAL RESEARCH)
VERSOVA, MUMBAI - 400 061
2000

केन्द्रीय मत्स्य शिक्षा संस्थान
(समतुल्य विश्वविद्यालय) भारतीय कृषि अनुसंधान परिषद
Central Institute of Fisheries Education
(Deemed University) Indian Council of Agricultural Research

CERTIFICATE

This is to certify that the dissertation entitled " **Development Of Dot blot assay For Detection Of White spot Virus In Shrimps** " is a record of bonafied research work done by **Miss. P. SRIKALA** of the 1998-2000 batch of **M.F.Sc** (Inland Aquaculture) program under our guidance and it has not previously formed the basis for any publication or for the award of any other degree, diploma, or other similar titles.

(Dr. Pani Prasad)
Scientist &MajorAdvisor
Fish Pathology Laboratory

(Dr C.S. Purushothaman)
Principal Scientist & Member
Advisory Committee
Division of Environmental
Pollution & Monitoring

(Dr. Mahesh B.Patel)
Scientist & Member
Advisory Committee
Division of Fish
pathology

सात बंगला , वरसोवा , मुंबई - 400 061
Seven Bungalows, Versova,Mumbai-400061
फोन-Ph.- 6361446/7/8 तार/Grams-फिशईन्स्ट/Fishinst
टेलेक्स/Telex-011-78221 फैक्स/Fax22-6361573
ई मेल / E.mail - cife @ x 400 nicgw.nic in

DECLARATION

I do hereby solemnly affirm that this dissertation entitled " **Development of dot blot assay for detection of white spot virus in shrimps** " is my own work conducted under the guidance of **Dr. K. Pani Prasad,** Fish Pathology Laboratory, Central Institute of Fisheries Education [Deemed University (ICAR)], Mumbai, during the year 1998-2000 and the data has not earlier formed the basis for any other titles, publication or thesis.

P.Srikala

Date: 30th June 2000 **(P.SRIKALA)**

Place: CIFE, Mumbai

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ABBREVIATIONS USED

TESTS

COA	-	Technique cogglutination
FAT	-	Fluorescent antibody
ELISA	-	Enzyme linked immunosorbant assay
DBA	-	Dot blot assay
I FAT	-	Indirect fluorescent antibody test
CIE	-	Counter immune electrophoresis assay
SDS-PAGE	-	SDS -polyacrylamide gel electrophoresis

MEASUREMENT

%	-	Percent
^{0}C	-	Degree Celsius
>	-	Greater than
<	-	Less than
µ l	-	Microlitre
Hr	-	Hour
Min	-	Minutes
Sec	-	Seconds
pH	-	*Puissance de hydrogenion* (Hydrogen ion concentration)
rpm	-	Revolutions per minute
OD	-	Optical density
Cm	-	Centimeter
µg	-	Micro grams

REAGENTS AND CHEMICALS

BSA	-	Bovine Serum Albumin
HCl	-	Hydrochloric acid
Nacl	-	Sodium chloride
$(NH_4)_2SO_4$	-	Ammonium sulphate
NaOH	-	Sodium hydroxide
PB	-	Phosphate buffer
PBS	-	Phosphate buffer saline
PBS-T	-	Phosphate buffer saline
NSS	-	Normal saline solution
SDS	-	Sodium dodecyl sulphate
TEMED	-	Tetraethyl methylene diamine

CLINICAL TERMS

Ag	-	Antigen
Ab	-	Antibody
IgG	-	Immunoglobulin G
Igs	-	Total immunoglobulin
I.P.	-	Intraperitoneal
I.V.	-	Intravenous
NC	-	Nitro cellulose membrane

ACKNOWLEDGEMENTS

It is my privilege to express my deep sense of sincere gratitude to Dr.K.Pani Prasad, Scientist, Division of Fish Pathology, Central Insitute of Fisheries Education (CIFE), Mumbai for his scholarly guidance, encouragement, utmost care, generous help and critical suggestions in carrying out the dissertation work and in the preparation of the manuscript.

I am extremely thankful to Dr S. Ayyappan Director, Dr S.A.H. Abidi, Former Director, Dr S.C. Mukherjee, and Joint Director, for providing me with all the infrastructure facilities needed for the conduct of this work.

Iam very much thankful to Dr.C. S. Purushothaman, Principal Scientist, for his encouragement, help and guidance to carry out the study.

Iam sincerely thankful to my co-guide Dr.K.V. Ragendran, Senior Scientist, Division of Fish Pathology for his valuable advice, guidance, help and encouragement throughout the dissertation.

I wish to thank the library staff of C.I.F.E particularly Mr. K. P. Khalsa for helping me to procure the relevant literature from the library.

I wish to thank my classmates Promod Kiran, Bhasker, Gautam Raj, Madhavi, Jasintha for their valuable help rendered during my dissertaion work and special thanks to Prem Kumar and Absuma V., Ph.D. Scholars. I also express

my thanks to all my class mates for their co-operation and help rendered during my study period.

My thanks are also due to Rajitha, my room-mate and class mate for her affection, care, support and providing me moral support during my stay at C.I.F.E.

I owe everything to my beloved parents and my brother, their support, inspiration and above all their overwhelming love and concern was the drivin I would like to thank Indian Council of Agricultural Research for the financial support throughout the study and my entire tenure of M.F.Sc.

I would like to thank Indian Council of Agricultural Research for the financial support through out the study and my entire tenure of M.F.Sc.

P. Srikala

(P.SRIKALA)

सारांश

जलकृषि के क्षेत्र में लगातार हो रहे विकास ने कृषि प्रणाली की तीव्रता के लिए मार्ग खोला है । उत्पादन की वृद्धि के साथ-साथ रोगों में होने वाली वृद्धि इस उद्योग के लिए एक खतरा बन गया है । इस रोग के कारण संवर्धित जीव में बढ़ रहे अनिश्चितता तथा आर्थिक कर्मी के रोकथाम के लिए आधुनिक परीक्षणों द्वारा प्रेरणार्थक एजेन्टस का पता लगाना आवश्यक हो गया है ।

वर्तमान अध्ययन संस्थान में श्रिंपों में होने वाले संक्रामक रोग wsv की जांच के लिए डोटवोल्टरऐसे का विकास किया गया । इस प्रयोग में एण्टीजन, खरगोश में एण्टीसेरा की वृद्धि, इमिनोग्लोब्युलिन (lgs) को अलग करना, lgs मे इमिनोग्लोब्युलिन को अलग करना आदि की पहचान तथा खरगोश IgGHRPO व एण्टी wsv संयुग्मी को तैयार करना ।

lgG की शुक्ता की पुष्टि अगरजैल परीक्षण मे किया गया । संयुग्मी wsv को पता लगाने के लिए जिस अवमिश्रण का प्रयोग किया गया वह मानक था । कृषि क्षेत्र में संक्रामित श्रिंप तथा कृत्रिम रूप से संक्रामिक श्रिंप में WSV को पता लगाने के लिए HRPIgG संयुग्मी को तैयार किया गया । इस परीक्षण से यह स्पष्ट हो गया कि wsv की मौजूदगी संक्रामित श्रिंप के ऊतक/आर्गन में पाया गया । इमिनोडायगोनिस्टिक परीक्षण से wsv संक्रमित श्रिंप में यह परिणाम सामने आया कि डोटवोल्टऐसे जो था लगातार संवेदनशील विशिष्ट व सस्ता देखा गया ।

ABSTRACT

Rapid development of aquacuture has increased the way for the intensification of the culture system. Along with hike in production, increase in disease outbreaks, also has evolved which is threatening the industry. High degree of uncertainity as well as the huge economic losses associated with the disease of cultured organisms increased the need of advanced tests for identification of causative agents.

The present investigation was carried out to develop an dot blot assay for diagnosis of white spot virus infection in shrimps. The experiment involved the identification of the antigen, raising of antisera (to WSSV) in rabbits, separation of immunoglobulin (Igs), separation of immunoglobulin G (IgG) from Igs and preparation of anti WSSV rabbit IgG HRPO conjugate. The purity of IgG was confirmed by agar gel precipitation test. The dilution of conjugate required for detection of WSSV was standardized.

The HRPO IgG conjugate was prepared and utilized to detect WSSV in artificially infected shrimps with WSSV and in shrimps infected under field condition. The test confirmed the presence of WSSV in infected shrimp tissue/organs. The results indicated that the dot blot assay is a rapid, specific, sensitive, inexpensive and on-site immunodiagnostic test for the detection of WSSV infection in shrimps.

INTRODUCTION

INTRODUCTION

The major species for shrimp aquaculture in the world are the penaeid shrimp, of which there are 318 species belonging to family Penaeidae. Approximately 109 species have presently having potential commercial value (Holthius 1980) and of this *Penaeus monodon* is one of the most important species.

Aquaculture of penaeid shrimps has grown from its experimental beginnings roughly three decades ago into a major industry all over the world. Shrimp aquaculture has today become an established industry in many countries of the World and the penaeid shrimp represents a high value food commodity. Shrimp culture provides a vital source of income, employment, trade and economic importance. The world production of cultured shrimp has a total value of several billion US dollars (Subasinghe, 1996).

Shrimp culture, as practiced today, relies on intensive and semi intensive systems. High stocking densities also increases the development and transmission of many diseases within cultured populations. Penaeid shrimp viruses have assumed great importance because of their effect on the growth and sustenance of the penaied shrimp aquaculture industry. Several of these viruses have been associated with large epizootic and massive mortality in shrimp farms and the development of new management strategies for handling viruses has cut into profits.

Among the diseases, White spot syndrome virus (WSSV) causes the most serious epizootic disease of cultured penaeid shrimp (Wang *et al.*, 1999). WSSV has the ability to cause acute epizootic of 2-7 days duration with mortalities from 10-70% upto 100% and massive systemic pathology (Anon., 1995). The epizootic started in 1992, and spread through East and South East Asia, Indonesia and into India and other shrimp growing countries of the region (Nunan *et al.*, 1998). Since 1995, it was reported from North America (Wang *et al.*, 1999). WSSV is a more serious threat because it infects a wide spectrum of crustaceans, some of which will not die due to the virus, and but act as carriers, and when carriers are more, elimination is difficult (Flegel, 1996 and Rajendran *et al*, 1999). WSSV causes serious losses in culture of *Penaeus chinensis*, *P. monodon* (Wang *et al.*, 1999), *P. japonicus* (Takahashi *et al.*, 1994), *P. pencillatus* (Chou *et al.*, 1995), *Metapenaeus ensis* (Wang *et al.*, 1998), *P. indicus* and *P.merguensis* (Anon., 1995). Infected shrimp exhibits swimming at the water surface, congregation at the pond periphery, reduction in feed consumption, reddening of appendages and conspicuous white spots on the inner side of the cephalothoracic carapace. Histological sections of gills, lymphoid organs, subcuticular ectodermal layer and gut wall showed marked necrosis and eosinophilic to basophilic intranuclear occlussions (Rajendran *et al.*, 1998).

In India, WSSV was reported in early 1993. However, adequate attention was paid to the disease only in 1995 when disease appeared in epizootic proportion. The virus is believed to have been transmitted through seed imported from South East

Asian countries in 1994. Mortality has been reported in shrimp of all ages and sizes from Vishakhapatanam of Andhra Pradesh to Sirkali of Tamil Nadu in the east coast of India with affected shrimp showing typical signs of white spot syndrome, and by 1995 it has spread to Orissa and West Bengal (Mukherjee, 1996). Along the West coast, it was first noticed from Goa in January 1995 and later it spread to Karnataka and Gujarat. The epizootic resulted in heavy mortalities in all types of shrimp culture systems including areas where pond environment was apparently good (Mukherjee, 1996 and Karunasagar *et al.*, 1997). Even crop holidays were not helpful in controlling the outbreak due to the wide host range of the virus. The economic loss due to WSSV during 1995-96 in India is estimated to be over Rs. 600 crores against this backdrop of the disastrous result in the marine shrimp culture due to the panepizootic of WSSV, one of the measures to counter the problem is to switch over to an alternative culture species or use of the shrimp seed free of virus.

Penaeid shrimp viruses can be isolated and observed by electron microscopy but details are necessary because different strains belonging to same family look identical. Until recently, only information on clinical symptoms, histology and electron microscopic thin section structural morphology was available.

In recent years, it has become popular to examine using the techniques of direct and indirect immunosorbant assays. In these cases specific virus can be studied in detail. These methods are

not diagnostic *per se*, but rather confirmatory for a presumptive diagnosis. In depth studies to describe the pathogenesis of the disease process need to be done.

The more rapid development in the aquaculture sector necessitates more rapid disease diagnosis. The simple and field level immunodiagnostic kits are the need of the hour. It will avoid the necessity of highly sophisticated laboratories for disease diagnosis. As the farmer will be in a position to ascertain the incidence of the disease in the system at an early stage, early preventive measures can be taken saving the bulk of the lot.

WSSV is the most virulent virus known to affect cultured shrimps. Till date, no treatment is known to control the white spot virus. Hence, early diagnosis followed by suitable management practices is the only alternative in tackling this disease. A presumptive diagnosis can be done by observing clinical symptoms, such as the presence of white spots on the carapace. These methods can detect white spot disease only in the late stage of infection. While the immunodiagnostic techniques are more powerful and sensitive diagnostic tools for the identification of viral pathogens even at a very early stage (asymptotic/carrier stage) of infection.

Recently, immunoenzyme assays have been developed to detect pathogens. Most common procedures are ELISA and dot

blot assay methods. Dot blot assay can be more applicable than the ELISA in the detection of symptomatic carrier can. All of the dot blot methods used are more sensitive than the fluorescent body techniques or counter immunoelectro phoresis.

In response to these, attempts were made to develop dot blot assay for the detection of WSSV in shrimp with the following objectives.

1. To develop and standardize an dot blot assay for WSSV in shrimps

2. To determine the optimal antigen coating level

3. To test the sensitivity and specificity of dot blot assay

4. To study the shellfire of dot blot assay reagents

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 TAXONOMIC AFFILIATION OF WSSV

The first report of losses due to white spot disease has come from China in 1993, where it lead to a virtual collapse of shrimp farming industry (Flegel, 1996 and Zhan *et al.*, 1998). However, Chen *et al.*, 1995 reported about an epizootic due to a systemic, non-occluded bacculo-like virus from Taiwan during 1992 in cultured *P. monodon*, *P. japonicus* and *P. penicillatus* whose clinical signs included conspicuous white spots on the inside surface of the carapace. The disease has been named as explosive epidemic disease (EEDS) of prawn and the virus responsible was named Hypodermal and Hematopoeitic Necrosis Baculovirus (HHNBV) (Cai *et al.*, 1995).

A similar outbreak of serious mortality among populations of cultured kuruma shrimp, *Penaeus japonicus* has occurred in Japan since early spring of 1993 and the causative virus was tentatively named as rod-shaped nuclear virus of *P. japonicus* (RV- PJ) (Takahashi *et al.*, 1994 and Inouye *et al.*, 1994). Later, the virus has been renamed as Penaeid rod- shaped DNA virus (PRDV) by Inouye *et al.*, 1996.

In 1995, white spot disease was reported in Litopenaeus setiferus from Texas and South Carolina (Wang *et al.*, 1999). A similar epidemic has also been occurred in Thailand in *P. monodon* and the causative agent has been named as Systemic Ectodermal and Mesodermal Bacculo Virus (SEMBV) and as

PmNOBII i.e. The second non occluded bacculovirus reported from *P. monodon* (Wongteerasupaya *et al*, 1995). Wang *et al,* 1995 proposed the name baculovirus associated with white spot syndrome (WSBV) to indicate PmNOBIII (the third non-occluded bacculovirus reported from P. *monodon*) related agents.

However the causative agent has been named differently by different workers. To end all confusions regarding the naming of the viral pathogen, Lightner 1996, regrouped all the non occluded baculoviruses causing white spot syndrome under the name white spot syndrome (WSS).

In all the epidemics, the prominent characteristics of the disease was the presence of white spots /patches in the carapace. The epizootic similar to white spot syndrome has been reported in India in early 1993 and the causative agent was identified for the first time as SEMBV (Anonymous, 1995).

This complex consists of several very similar viruses:

a) Baculoviral hypodermal and haematopoietic necrosis, HHNBV, Shrimp explosive epidermic disease, SEED, China viral disease.

b) Rod-shaped nuclear virus of *Penaeus japonicus*, RV-PJ.

c) Systemic ectodermal and mesodermal baculovirus, SEMBV, Red disease, White spot disease.

d) White spot baculovirus, WSBV, White spot syndrome, WSS, White spot disease.

e) Penaied hemocytic rod shaped virus (PHRI).

It belongs to

Family : Baculoviridae

Subfamily: : Nudibaculovirinae

2.2 GEOGRAPHIC DISTRIBUTION OF WSSV

Following initial detection in North East Asia in 1992-1993, WSBV is now believed to be widely spread throughout most of the shrimp growing regions of Asia and the Indo-Pacific.

a) China.

b) Japan, China and Korea.

c) Thailand.

d) Indonesia, Taiwan, Vietnam, Malaysia, India, and U.S.

2.3 HOST SPECIES OF WSSV

P. monodon, P. japonicus, P. chinensis (=orientalis), P. indicus, P. merguiensis, P. penicillatus and P. setiferus. Experimentally, severe and lethal infections of WSBV from Thailand were produced in *P. vannamei, P. stylirostris, P. aztecus, P. duorarum and P. setiferus*. No significant resistance to WSBV complex has been reported for any of the penaeid shrimp.

2.4 PATHOGENICITY OF WSSV

In all the epidemics, the prominent characteristics of the disease was the presence of white spots /patches in the carapace.. The epizootic similar to white spot syndrome has been reported in India in early 1993 and the causative agent was identified for the first time as SEMBV (Anonymous, 1995), but adequate attention was paid only in 1994 when large scale mortality was involved. Subsequently, various reports of the disease outbreak has been reported from different parts of the country. In the east coast of India, during 1994-95 severe mortality of penaeid shrimps *P. monodon* and *P. indicus* occurred due to white spot disease (Anonymous, 1995). Manohar *et al.*, 1996 reported SEMBV in 40 day old post larvae of *P. monodon* from Tamilnadu, a coastal state of India. Karunasagar *et al.*, 1997 reported the outbreak from the West Coast of India. Rajendran et al, 1998, reported the white spot disease outbreak and large scale shrimp mortality in Orissa with shrimp showing typical symptoms of white spot disease and mortality reaching 100% in a week. Samples collected from Andhra Pradesh and Tamilnadu, India showed basophilic intranuclear inclusion bodies in the cuticular epidermis, connective tissue, epithelium of fore gut, lymphoid organ and gills which is characteristic of WSSV (Shankar and Mohan, 1998).

A wide host range has been reported for WSSV from many parts of the world (Flegel, 1996). In addition to the penaeid shrimps, WSSV was found to infect other marine shrimps like *Trachypenaeus curvirostris*, *Metapenaeus ensis*, *Exopalaemon orientalis*, freshwater prawn *Macrobrachium rosenbergii* and

fresh water cray fish *Procambarus clarkii*. The virus was also detected from the tissue of crabs *Portunus sanguinolentus*, *Charybdis granulatus*, *Calappa lophos*, *Charybdis feriala* and lobster *Panulirus versiclor, P. longipes* and *P. pencillatus* (Chang et al, 1998 and Wang et al, 1998). Kanchanphum et al, 1998 found that common crabs like *Uca pugilator*, *Scylla serrata*, and *Sesrama* sp. were found susceptible to WSSV with high viremia and it was transmitted to the cohabiting shrimps.

Lo *et al.*, 1996 observed that copepods, *epihydridae* insects, pest crab *Helice tridens* and *palaemonid* prawns collected from epizootic areas were found to be one step PCR positive for WSBV, and can act as reservoir host. Rajendran *et al*, 1999, reported that 10% cumulative mortality in experimentally infected *M. idella* in 20 days of experiment period, and proved the presence of WSSV through histological and bioassay methods. The same authors observed that crabs and lobsters also were susceptible to WSSV and act as asymptomatic carriers or reservoirs of the virus.

2.5 IMPACT ON THE HOST OF WSSV

Acutely infected shrimp showed rapid reduction in food consumption, became lethargic, and had high mortality rates with cumulative mortalities reaching 100% within 3 to 10 days of the onset of clinical signs.

2.6 DIAGNOSTIC TECHNIQUES

2.6.1 Gross observations:

Acutely infected shrimp often have a loose cutical with white spots of 0.5 - 2.0 mm in diameter which are most apparent on the inside surface of the carapace. In many cases moribund shrimp displays a pink to reddish-brown colouration due to expansion of the cuticular chromatophores and few if any, white spots.

2.6.2 Squash Preparations

Hypertrophied or vacuolated nuclei usually with a single eosinophilic to bluish inclusion body in squashes or impression smears (stained with Giemsa or other blood smear stains) of epithelia and connective tissues of the gills or stomach of shrimp are seen in shrimps are seen in shrimps with clinical signs. Occlusion bodies are absent. Normal cell nuclei are 4 - 10 µm in diameter and display chromatin threads and a nucleolus.

2.6.3 HISTOLOGY

Prominent eosinophilic to pale basophilic (with H&E stains), Feulgen-positive, intranuclear inclusion bodies in hypertrophied nuclei of, most commonly, the cuticular epithelial cells and connective tissue cells, and, less frequently, the antennal gland epithelium, lymphoid organ sheath cells, haematopoietic cells and fixed phagocytes of the heart. Occlusion bodies are

absent. In the early stages of inclusion body development, they are eosinophilic, centronuclear, with a halo (an artifact with Davidson's fixation) and resemble the inclusion bodies of IHHNV. However, the presence of larger more fully developed (without a halo) pale basophilic inclusion bodies in infected target tissue cells during the advanced stages of infection clearly distinguishes the two diseases.

2.6.4 Electron microscopy

Cytopathology occurs in the appropriate target tissue types and is accompanied by large rod-shaped to somewhat elliptical, non-occluded virions of about 70 - 150 nm in width and about 275 - 380 nm in length in the intranuclear inclusion bodies of infected cells.

2.6.5 DNA Probes:

WSBV infected nuclei can be intensely marked by a DIG-labeled DNA probe for WSBV with in situ hybridization assays. Gene probes for WSBV are being developed in China, Japan, Thailand and at the University of Arizona in the U.S. None of the WSBV complex are reactive to the available gene probes to IHHNV, BP, MBV and HPV.

2.6.6 Raising Of Antisera

Dresser (1986) immunized experimental rabbits by injecting a mature rabbit (>6months) in marginal ear vein with a

suspension of 4X 10^9 formalin killed type III *Pneumcocci* in saline (Subhashish and Pani Prasad (1997)).

2.6.7 Separation of Immunoglobulins

Heide and Schwick (1982) precipitated antibodies out of the milk of a goat immunized against tetanus using concentration of 27-30% of the ammonium sulphate.

Proteins can be gradually salted out from their aqueous solutions using highly soluble salts which after ionization, associate strongly with water molecules. As concentration of salt in the protein solution is increased after a certain level salt begin to compete with proteins for water molecules needed for their solution (Dubey, 1983).

Wilson (1986) reported that ammonium sulphate is the most commonly used in salt fractionation, it has a highly water soluble property and can be obtained in a high degree of purity, is cheap and has no deleterious effect on the structure of proteins.

Ammonium sulphate precipitation is widely used laboratory technique for the preparation of a crude immunoglobulin fraction from the whole serum. The use of ammonium sulphate, rather than sodium sulphate, as the precipitating salt offers the advantage of a high solubility, it varies only about 3% between 0^0C and 25^0C (Hudson and Hay, 1991).

2.6.8 Ion Exchange Chromatography

Peterson and Sober (1956) introduced substituted chromatography to fractionated serum proteins by anion exchange cellulose.

The serum proteins are separated by DEAE cellulose chromatography into a series of fractions. The first peak of protein elution contains only IgG but all the remaining chromatogen peaks contains a mixture of proteins (Fahey *et al.*, 1958).

Fahey and Terry (1982) reported that the fractions can be concentrated by ultra-filtration, by lyophilization or by use of an absorbant such as carbowax or polyglucose granules, outside a dialysis bag containing protein solution.

Dubey (1983) fractionated immunoglobins by ion exchange chromatography technique. Hudson and Hay (1991) reported ion exchange chromatography as an extremely useful method for the separation of proteins and isolation of immunoglobulins. Both cation (CM) cellulose and anion (DEAE) cellulose are available but latter is used widely for the fraction of serum proteins.

2.6.9 Double Immunodiffusion Test

Ouchterlony (1962) first described double immunodiffusion test for detection of soluble antigens. Fish viruses have also identified by gel diffusion test eg.VHS, IPN and IPNV, lymphocyte virus (Jorgensen, 1968; Rodak *et al.*, 1988).

Rio and Recco (1971) reported that for optimum precipitation to occur with serum, correct pH of the buffer and incubation temperature is required. This test is simple to perform rapid and requires small volumes of sample.

Gel diffusion tests have been used to determine the serological relationships between fish bacterial strains eg. *Vibro anguillarum* in Salmonids on the basis of lipopolysaccharides, between *vibro* species form marine teleosts (Harrel *et al.*, 1976 and Johnson, 1977) for comparision antigenicity of *A.hydrophila*, *A.salmoninarum* (Kimura and Yoshimizu, 1981; Salati and Kusuda, 1985; Bullock, 1966; Chen *et al.*, 1974). Crowle (1973) performed immunodiffusion with 0.8% agarose in phosphate buffer, PH 7.2 containing 0.85% sodium chloride.

2.6.10 Western Blot

The anionic detergent SDS was introduced by Shapiro *et al.*, (1967) and subsequently used by Weber and Osborn (1969). In a continuous gel system to restrict protein mobility solely on the basis of continuous gel system to restrict protein mobility solely on the basis of molecular weight.

In 1970, Laemmli combined the effect of SDS with the tris-glycine discontinuous polyacrylamide gel electrophoresis system. Other combinations of detergent and buffers have been described (Neville, 1971) but the Laemmli SDS-PAGE procedure has become the conventional method of protein electrophoresis in to day.

Towbin (1979) introduced a novel method for immunodetection of proteins electrically transferred from a polyacrylamide gel onto a porous nitrocellulose membrane. This technique, widely referred to as immunoblotting or western blotting has since become one of the most prevalent immunochemical methods.

Previous reviews have described the development and application of protein Immunoblotting (Gershoni and Palade, 1982; Towbin and Gordon, 1984; Bers and Garfin, 1985; Tsang *ET al.*, 1985; Besiege, 1986).

Hammed *et al.*, 1998 used western blot and found a distinct band in the Hemolymph of SEMBV infected shrimp.

Nodal *et al.*, 1998 observed three protein bands of WSSV, with approximate molecular weights 19, 23.5 and 27.5 ka. using Western blots and used western bolting for identification of viral proteins using polyclonal hyperimmune serum prepared against purified CBV.

Western blots of purified Taura syndrome Virus (TSV) were used to compare the specificities of the Monoclonal Antibodies (Mabs) by Poulos *et al.*, 1999.

2.6. 11 Dot Blot Assay

The Dot blotting technique was developed for detection of specific DNA and RNA sequences by hybridization (Alwine *et al.*, 1977). Immunobloting, in which proteins are transferred

from a gel after electrophoretic separation on nitrocellulose membranes, was first demonstrated by Towbin *et al.*, (1979). The technique was applied for studies of protein structure (Renart *et al.*, 1979; Bittner *et al.*, 1980; Burnette, 1981), protein-protein interactions (Szewczyk and Kozoloff, 1985), ligand-receptor studies (Gershoni and Palade, 1983; Lauritzen *et al.*,1990) and during recent years for routine clinical testing of antibodies against human immuno deficiency virus (HIV) by immunoblotting of patient sera (Lauritzen and Lindhadt, 1988; Lauritzen and Pluskal, 1988).

A variation of ELISA, the dot blot method, was reported by Hawkers *et al.*, (1982) and was introduced to detect *Vibrio anguillaram* (Cipriano *et al.*,) *Aeromonas salmonicida* (Sakai *et al.*, 1986) or *Renibacterium salmoninarum* (Sakai *et al.*, 1987) considering the field application and the economical advantage, the dot blot method was considered more applicable than the ELISA in the detection of symptomatic carrier fish. In this study, the sensitivity of the dot blot method was compared with that direct and indirect immunoenzyme methods, using either peroxidase-antiperoxidase (PAP) or avidin-biotin peroxidase complex (ABC).

Dot blot assays (DBA) were performed basically as described by Cipriano *et al.*, (1985) for detection of antigen of *Vibrio anguillaram.* Jorge bolinches *et al.*, (1990) performed dot blot assay to determine the existence of separate antigenic markers within Vibrio *angullarum* that are useful for epidemiological and ecological studies, similarity among the 10

established O-serotypes were evaluated by different serological methods. They demonstrated that the strains from serotype O1 constituted a homogenous group by double immunodiffusion test, dot blot assay (DBA) and enzyme-linked immunosorbant assay (ELISA). However, with in serotype O2, two different patterns of serological reactions were detected by the three methods.

Lu *et al.*, (1995) performed dot blot assay for detection of *P. monodon* type baculovirus (MBV) derived from cloned MBV polyhedrin genome in cultured *P. monodon* fabricius.

Sahul Hameed *et al.*, (1998) used the dot blot assay for studies on the pathogenicity of SEMBV and its detection the presence of SEMBV in hemolymph, eyestalk, gills, head soft tissue, abdominal muscle and hepatopancreas of infected P. *monodon*. The antiserum raised against prominent viral protein in mice was used to detect SEMBV in shrimp by dot blot assay. The results indicated that hemolymph, eyestalk and gills are containing more infectious virus.

Poulos *et al.*, (1999) used the dot blot assay to determine three monoclonal antibodies of different immunoglobulin isotypes were compared with reactivity to hemolymph from TSV-infected shrimp during acute and chronic phases of infection and their cross reactivity. The results with chronic phase hemolymph samples were variable and indicated the need to develop an immunoassay in which the virus in a sample is captured by one by one antibody and then detected with a second antibody.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

All the glassware used throughout the experiment were of neutral glass of Borosil or Corning make. All the chemicals that were used for the experiment were of Qualigens (Glaxo), SRL and Sigma, (U.S.A.). Dehydrated media supplied by Hi-Media were utilized. Triple distilled water was used for the preparation of all the media and reagents.

3.1 LOCATION

The experiment was carried out in the Fish Pathology laboratory, Central Institute of Fisheries Education, Mumbai.

3.2 PREPARATION OF GLASSWARE

All the glassware were soaked overnight in a mild detergent solution. Next day, they were washed thoroughly in tap water and finally with triple distilled water. After air drying, they were wrapped in paper and sterilized in hot air oven at 180°C for one and half hour.

3.3 VIRUS

WSSV is obtained from Fish Pathology laboratory, Central Institute of Fisheries Education, Mumbai.

3.4 ANTI WSSV RABBIT SERUM

Anti -WSSV was raised in rabbits using WSSV.

3.4.1 Experimental Rabbits

Clinically healthy NewZealand white rabbits of 8-10 months old, weighing approximately one kg were procured. They were housed in stainless steel cages and kept in a well-ventilated animal room. They were provided with compound pelleted feed and vegetables and *ad libitum*, drinking water.

3.4.2 Immunization of Rabbits

Polyclonal hyperimmune sera against WSSV was prepared in New Zealand white rabbits by the following immunization schedule. 100µg of purified viral protein with Freund's incomplete adjuvant was injected intradermally and subcuntaneously on day 0. Subsequent injection of 100µg of viral protein with Freund's incomplete adjuvant was given on days 7, 13, 27 and 41. The rabbits were exsanguinated on day 63.

3.4.3 Collection of Blood

Blood was drawn as per the method of Herbert and Kistensen (1986). One week after the last injection, the rabbit was bled, through marginal ear vein or through cardiac puncture. About 20-50ml blood was collected in a single bleed.

3.4.4 Separation of Anti WSSV Serum

Blood was dispensed in sterile test tubes, 5ml in each and was allowed to clot by keeping the test tubes in slanting position at room temperature for one to two hours. Free serum was collected with a Pasteur pipette. Then the clot was cut with a blunt glass rod and care was taken not to haemolyse the clot. The tubes with the clots were kept at 4^0C overnight. The test tubes were

centrifuged at 2500rpm for 15min and supernatant (serum) was separated. Proteases and complement were inactivated by heating the serum at 56^0C for 45min in a waterbath. The antiWSSV serum was stored in 1ml aliquots in polypropylene (PP) storage vials (Laxbro) at -20^0C, until further use.

3.4.5 Separation Of Immunoglobulins (Ig s)

Total immunoglobulins (Igs) were separated from anti WSSV rabbit serum by salt fractionation using ammonium sulphate precipitation method (Hudson and Hay, 1991).

For precipitation, 10 ml serum was taken in a beaker and equal volume of saturated ammonium sulphate (pH 8.0) was added dropwise. The suspension was stirred for 30min. The suspension was centrifuged at 10,000 rpm for 30min and the supernatant was discarded. The pellet was dissolved in 10ml of normal saline and precipitated again by gradual addition of equal volume of saturated ammonium sulphate solution. The suspension was dialyzed in a dialysis tubing (sigma, USA) against three changes of phosphate buffer saline.

After dialysis optical density (OD) at 280nm was measured and concentration of immunoglobulins in mg/ml was calculated by OD_{280} X 1.4. It was stored at -20^0C in plastic vials, until further use.

3.4.6 Anti WSSV Rabbit Immunoglobulins G (IgG)

Separation of WSSV rabbit IgG was performed by anion exchange chromatography using DEAE cellulose (SRL Chemicals, Mumbai) according to the procedure of Hudson and Hay (1991). DEAE cellulose of ion exchange capacity 0.6 meq/gm, practicle size 50-20μm was employed for separation of IgG from total Immunoglobins.

3.4.6.1 Regeneration of DEAE Cellulose

25gms of DEAE cellulose powder was swelled in distilled water for 2-3hours (20-25ml/g) and allowed it to stand for 30min The supernatant was discarded and the sediment was stirred in a magnetic stirrer for 30min with 500ml 1M sodium hydroxide. The mixture was allowed to stand for 30min. The supernatant was discarded and the sediment was mixed with 500ml of 1 M Sodium chloride with constant stirring for 30min. The mixture was allowed to stand for 30min and the supernatant was discarded. After the sediment was mixed with 500 ml of 1 N HCl for 30min, it was allowed to stand for 30min. and the supernatant was discarded. The sediment was washed few times with distilled water and finally washed with 0.01 M phosphate buffer pH 8.0 a few times till pH of the DEAE cellulose was that of the buffer.

3.4.6.2 Packing of Column

A glass column 2×45 cms, with sintered glass disc (Agrawal Glassware, Mumbai) was mounted vertically on a stand. 5-cm latex tubing with screw cork fixed to the outlet of the column. The column was filled to about 10cms, from the bottom with 0.01-M phospahate buffer and the outlet was closed. The

thick slurry of DEAE cellulose was poured into the column and was allowed to settle under gravity. Outlet was opened and more slurry was added slowly, until height of packed bed reached to 30cms. The flow rate was adjusted to 35ml per hour.

3.4.6.3 Sample Application and Elution

The dialyzed anti-WSSV rabbit Igs was used as the sample. The buffer was allowed to run down to the level of packed DEAE cellulose bed and the outlet was closed. Circular disc of filterpaper (Borosil, Mumbai) was placed on the bed of the cellulose. 4ml of antiWSSVrabbit IgS was applied carefully on the bed and the outlet was opened till the sample had entered the bed. A few ml of the buffer was used to wash down the sample from column sides into the bed and then a continuous flow of PB through a reservoir attached to the column.

Three ml quantity elutes were collected in clean sterile test tubes. The OD at 280nm of the elutes were measured in a double beam spectrophotometer (Cintra GBC, Australia) and a graph was plotted against the tube number. The fractions representing the first peak were considered as IgG. The first peak samples were pooled and used as antiWSSV gig. It was stored at -20°C, until further use (Flow chart 1).

3.5.1 Double Immunodiffusion Test

Double immunodiffusion test was performed as per the procedure of Oucterlony (1962), for confirming the development of anti WSSV rabbit Igs. 3.5 ml of 1% molten agarose (SRL, Mumbai) in PBS of pH 7.4 was dispensed on a clean glass slide

with a pipette and allowed to solidify. Wells were punched 4 mm apart using a gel puncher. The agarose from the well was removed using a Pasteur pipette and bottom of the well was filled with molten agarose, to prevent seepage of reagents. The wells were charged with WSSV and Anti-WSSV rabbit serum. The slides were incubated at 4^0C for 48 hours in humid chamber and observed for lines of precipitation.

The gel slides were immersed in normal saline with three changes of normal saline over a period of 36-48 hours for deproteinisation. A moist filter paper was kept over the slide and kept at 37^oC, until dried. The dried slides were stained with 1% Coomassie brilliant blue (Sigma B-0149) for three hours and destained in a destaining solution containing 10% acetic acid, 40% ethanol and 50% distilled water, until the background became clear and the precipitation lines were stained dark blue.

3.5.2 Polyacrylamide Gel Electrophoresis

Assemble vertical slab gel apparatus using 1mm spacers. Seal the glass plates on 3 sides with 1%agarose. Pour the separating gel mixture to a level approximately 2.5cm below the top of the glass plates. Gently layer 250µl of TDW over the gel surface. Allow to polymerize after separating gel formation remove water from the top and pour stacking gel mixture, insert the comb between the plates and allow to polymerize. On gel formation, fill both tanks with electrode buffer and remove the comb. Sample preparation is done by mixing 3vols. of protein solution with 1vol. of 4x sample buffer and boil in water bath for 90secs.The maximum sample volume is determined by the slot

capacity. About 1μg protein per band is required for visualization by coomassie blue staining.

Load the prepared samples into the wells in stacking gel by layering them under electrode buffer using a microliter syringe or micropipette. Attach the leads to the unit and connect them to a power supply. Run the gelunder constant current conditions at i mA per slot. Electrophoresis is continued until bromophenol blue dye reaches the bottom of the gel. Dismantle apparatus and remove gel from between the plates and place in a tray containg distilled water. Cut a small corner of the gel to indicate the direction of loading.

3.5.2.1 Blotting Onto Nitrocellulose

Place a wet 3mm Whatman filter paper, slightly bigger than the size of the gel, on a glass plate. Place the gel on the filter paper without trapping any air bubbles. Cover the gel slowly with wet nitrocellulose paper. No air bubbles should be trapped. Mark the nitrocellulose paper with a pencil to indicate the orientation. Carefully cover with wet Whatman No.3.Place the sandwich between wet Scotch Brite pads and position in the grid, such that the gel side of the sandwich is towards the cathode.

Place the grid in the buffer tank and start the transfer at 0.2amps. Run for 3hr. Remove nitrocellulose paper from the sandwich and proceed as follows.

3.5.2.2 Immunostaning Of Nitrocellulose Blots

For western blotting purified virus which was electrophorised on SDS-PAGE was blotted onto nirtocellulose membranes (Schleicher and schnell, NH, USA) at 100v for 1 hour. The nitrocellulose membranes were then washed with PBS, blocked with 5%skim milk in PBS for 1 hour, Washed with PBS and then treated with 1:1000 rabbit anti –WSSV IgG diluted in 1%skim milk /PBS for 1 hour. The NC paper was washed with PBS-T(0.05% Tween-20 in PBS) twice and once with PBS.

The NC paper was treated with 1:2000 goat anti rabbit HRPO (Horse radish peroxidase) Kirkegoard and Perry laboratories, MD, USA) diluted in 1%skim milk /PBS for 1hour.the NC paper was washed three times with PBS and incubated with TMB ($3.3^{1}.5.5^{1}$ tetramethyl benzedine, kirkegoard and perry laboratories, MD, USA) substrate for 3min.washed in distilled water and dried.

Analysis of structural proteins of WSSV by Westren blotting revealed the prominent and consistent protein of 19, 23.5 and 27.5 Kda. These bands probably corresponding to the large, glycoprotein, nucleocapsid and matrix protein.

3.5.3 Western Blotting

For western blotting purified virus which was electrophorised on SDS-PAGE was blotted onto nirtocellulose (NC) membranes (Schleicher and Schnell, NH, USA) at 100V for 1 hour. The nitrocellulose membranes were then washed with PBS, blocked with 5% skim milk in PBS for 1 hour, washed with

PBS and then treated with 1:1000 rabbit anti WSSV IgG diluted in 1% skim milk /PBS for 1 hour. The NC paper was washed with PBS-T(0.05% tween-20 in PBS) twice and once with PBS. The NC paper was treated with 1:2000 goat anti rabbit HRPO (Horse raddish peroxidase) Kirke Goard and Perry laboratories, MD, USA) diluted in 1% skim milk /PBS for 1hour. The NC paper was washed three times with PBS and incubated with TMB (3, 3^1, 5, 5^1 Tetramethyl benzedine, Kirke Goard and Perry Laboratories , USA) substrate for 3 min, washed in distilled water and dired and obseved for protein bands (Flow chart 2).

FLOW CHART 1

SEPARATION OF ANTI WSSV RABBIT IgG BY ION EXCHANGE CHROMATOGRAPHY

Swell 25gm. DEAE cellulose in distilled water for 2-3hrs

↓

Discard the supernatant

↓

Stir sediment with 500ml of 1 M NaOH for 30min

↓

Allow the mixture to stand for 30min

↓

Discard the supernatant

↓

Stir sediment with 500ml of 1 m NaCl for 30min

↓

Allow the mixture to stand for 30min

↓

Discard the supernatant

↓

Stir sediment with 500ml of 1n HCl for 30min

↓

Allow the mixture to stand for 30min

↓

Discard the supernatant

↓

Wash the sediment with distilled water

↓

Wash the sediment with the buffer until the pH of buffer is obtained

↓

Column packing

FLOW CHART 2
WESTERN BLOTTING

Purified virus (WSSV) blotted on to NC paper by SDS-PAGE

↓

Wash in PBS

↓

Blocking with 5% skim milk in PBS for 1hour

↓

Wash NC paper in PBS

↓

Treat with 1:1000 rabbit antiWSSV IgG

↓

Wash NC paper with PBST thrice and PBS once

↓

Treat with 1:2000 goat antiRabbit HRPO

↓

Wash NC paper in PBS thrice

↓

Incubate with TMB for 3min

↓

Wash in distilled water and dry

↓

Analysis of WSSV proteins

3.6 DOT BLOT ASSAY

The dot blot assay performed as per the procedure of Dr. K. Pani Prasad (1999).

3.6.1 Direct Method of Dot Blot Assay

7μg of anti WSSV virus IgG was transferred onto the nitrocellulose membrane as a small dot. (or the nitro-cellulose paper can be loaded approximately with 100μg/cm^2 protein). The IgG was allowed to dry. The free sites were blocked using 5% skim milk powder in PBS pH 7.4 for 2-4hours. Wash with PBS (pH 7.4) three times and store at 4^{0}C, until further use.

The hemolymph was diluted (collected from WSSV suspected shrimp) 1:10 in 1% skim milk powder and the NC membrane was treated with diluted hemolymph (1:10 in PBS) for one hour at room temperature and mixing at periodical shaking. The NC membrane was washed with PBS-T (PBS 500ml and 250μl of Tween- 20) three times and treated with 1:2000 anti WSSV rabbit IgG-HRPO conjugate. It was incubated at room temperature for one hour with periodical shaking The membrane was washed with PBS-T three times and finally treated with TMP substrate and observed for the change in colour within three minutes.

In positive cases a blue colour develops as a dot and in negative cases it is colourless. (Flow chart 3).

3.6.2 Indirect Method of Dot Blot Assay

About 7μg of anti WSSV IgG was transferred onto the nitrocellulose membrane as a small dot. (or the nitro-cellulose paper can be loaded approximately with 100μg/cm^2 protein) and allowed the IgG to dry. The free sites were blocked using 5% skim milk powder in PBS (pH 7.4) for 2-4 hours. The membrane was washed with PBS (pH 7.4) three times and stored at 4^{0}C, until furture use. The hemolymph (collected from WSSV suspected shrimp) was diluted 1:10 in 1% skim milk powder and treated the NC membrane with diluted hemolymph for one hour at room temperature with periodical shaking. The NC membrane was washed with PBS-T three times and treated with 1:1000 αWSSV rabbit anti serum and incubated at room temperature for one hour with periodical shaking.

The membrane was washed with PBS-T three times and treated with 1:2000 anti-rabbit IgG HRPO conjugate and incubated at room temperature for one hour with periodical shaking The membrane was washed with PBS-T three times and finally treated with TMB peroxidase substrate. The membrane was observed for the change in colour within three minutes.

In positive cases a blue colour develops as a dot and in negative it is colourless (Flow chart 4).

FLOW CHART 3

DIRECT DOTBLOT ASSAY

7μg of anti WSSV IgG onto NC membrane as a dot

↓

Blocking with 5% skimmilk in PBS for 2-4hour

↓

Wash NC paper in PBS 3times

↓

Treat with hemolymph 1:10 with skim milk powder for one hour

↓

Wash NC paper with PBS-T thrice

↓

Treat with 1:2000 anti WSSV RabbitIgG- HRPO conjugate for one hour

↓

Wash NC paper in PBS thrice

↓

Incubate with TMB for 3min.

↓

Wash in distilled water and dry

↓

Analysis of WSSV proteins

FLOW CHART 4

INDIRECT DOTBLOT ASSAY

7μg of anti WSSV gig onto NC membrane as a dot

↓

Blocking with 5% skim milk in PBS for 2-4hour

↓

Wash NC paper in PBS 3times

↓

Treat with hemolymph 1:10 with skim milk powder for one hour

↓

Wash NC paper with PBS-T thrice

↓

Treat with 1:1000 rabbit αWSSV IgG for one hour

↓

Wash NC paper with PBS-T thrice

↓

Treat with 1:2000 anti Rabbit WSSV HRPO for one hour

↓

Wash NC paper in PBS –T thrice

↓

Incubate with TMB for 3min.

↓

Wash in distilled water and dry

RESULTS

4. RESULTS

4.1 PROTEIN CONCENTRATION OF TOTAL IMMUNO GOBULINS

The OD at 280nm of total immunoglobulins was found to be 1.33 and the protein calculated was1.862 mg/ml.

4.2 ANTI WSSV RABBIT IgG

Anti WSSV rabbit gig was separated by anion exchange chromatography using DEAE cellulose. The OD at 280nm was plotted against the tube numbers as show in Fig 1. The fractions representing the first peak were tested for their reactivity by agar gel precipitation is shown in Fig.1. Tube numbers 28, 29, 30 and 31 , which gave signal precipitation lines, were pooled. The OD at 280nm of the pooled fractions was 0.941 and The concentration of IgG in mg/ml. was calculated to be 1.317.

4.3 OPTIMAL CONJUGATE CONCENTRATION

The optimal conjugate concentration which gave good results was 1:2000. This concentration was utilized for all the experiments. (Table 1)

4.4 WESTERN BLOTTING OF WSSV

The positive reaction of western blotting is shown in Plate: 1. The positive reaction showed blue colour for while there was no development of colour for a negative result.

4.4 DOT BLOT ASSAY ON WSSV

The positive reaction of dot blot assay is shown in Plate: 2. The positive reaction showed blue colour for while there was no development of colour for a negative result.

4.5 SHELF LIFE OF DOT BLOTASSAY REAGENTS

The stability of the dot blot assay reagents was assessed by storing the reagents at different temperatures viz. 37^0C, R.T and 4^0C. The details of the storage of various components of dot blot assay are shown in Tables 2, 3 and 4.

Table 1: Determination of Optimum Concentration of Conjugate for dot blot assay

Conjugate Dilution	Test (WSSV+Serum+Conjugate +Substrate)	Control PBS+Serum+Conjugate+Substrate
1:10	++++	–
1:100	++++	–
1:250	++++	–
1:500	++++	–
1:1000	++++	–
1:2000	++++	–
1:4000	++	–

It is scored as (4+) in case of distinct reaction.

Table 2: Shelf life of dot blot assay reagents (PBS-T)

Period in weeks	Storage Temperature		
	37^0C	Room temp. ($22\text{-}30^0$C)	4^0C
1	++++	++++	++++
2	++++	++++	++++
3	++++	++++	++++
4	++++	++++	++++
5	++++	++++	++++
6	+++	++++	++++
7	+++	+++	++++
8	+++	+++	++++

It is scored as (+-) in case of distinct reaction.

Table 3: Shelf life of dot blot assay reagents (substrate)

Period in weeks	Storage Temperature		
	37^0C	Room temp. ($22\text{-}30^0$C)	4^0C
1	++++	++++	++++
2	++++	++++	++++
3	++++	++++	++++
4	+++	+++	++++
5	+++	+++	++++
6	++	++	++++
7	++	++	+++
8	++	++	+++

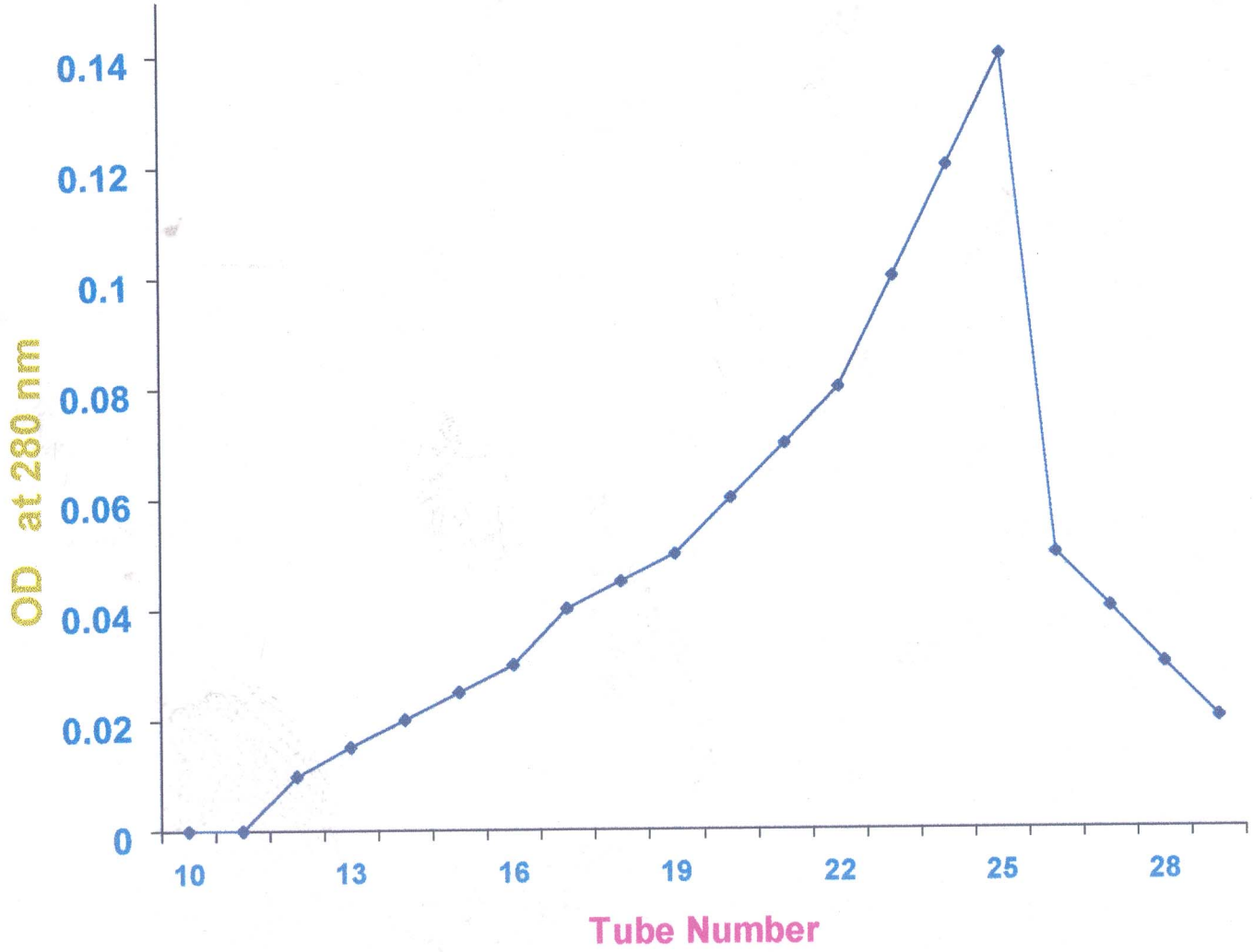
It is scored as(4+) in case of distinct reaction

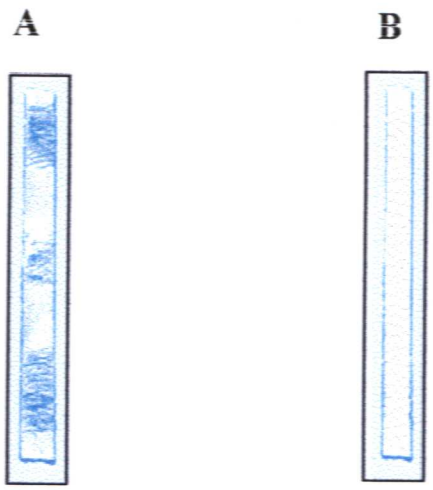
Table 4: Shelf life of Dot blot assay reagents (Conjugate)

Period in weeks	Storage Temperature		
	37^0C	Room temp. ($22\text{-}30^0$C)	4^0C
1	++++	++++	++++
2	++++	++++	++++
3	+++	++++	++++
4	+++	+++	++++
5	+++	+++	++++
6	++	+++	++++
7	++	++	+++
8	++	++	+++

It is scored as (+-) in case of distinct reaction.

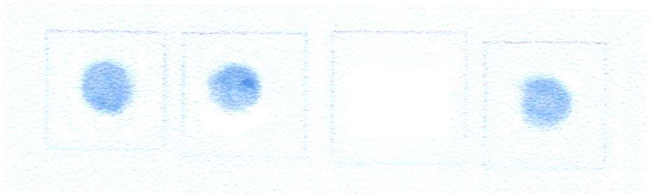
Fig 1.SEPARATION OF ANTI WSSV RABBIT IgG BY DEAE CELLULOSE





A: Positive B: Negative

Plate 2: Westren blotting of WSSV



A, B, D : Positive C: Negative

Plate 3: Dot blot assay of WSSV

DISCUSSION

DISCUSSION

With the intensification of aquaculture practices there has been an increase in the incidence of disease outbreaks. Shrimp diseases in India received importance only during late 90's and still there is large scope for improvement. There is a need for more integrated approach for the control of spread of disease outbreaks. It is not only the high economic risks associated but also the high amount of uncertainty of the conditions prevailing in the system attract the attention towards the diseases of the cultured organisms. Production losses alter the profitability profile decisively and rob the farmer off their daily bread.

Diseases affecting shrimp require rapid, specific and sensitive techniques for early diagnosis, to carry out appropriate control measures. The production aspects of aquaculture have been greatly stressed upon in India but unfortunately a panic stricken approach is adopted towards disease diagnosis and control.

In view of the involvement of WSSV in shrimp rapid detection of this virus would be very important for the aquaculture. Conventional methods of virus diagnosis using culture methods take more time for confirmation and requires trained manpower. These methods require the availability of live infected shrimps and transported to the laboratories for diagnosis. Here lies the need and the importance of immunology as a powerful tool to answer diverse research questions in biological and environmental sciences. Antibody reactions are very specific

and visualized in many ways, offering uniquely sensitive and specific assays (Ward, 1990).

The immunodiagnostic assays frequently used are dot blot assay, ELISA, fluorescent Antibody Technique, gel diffusion test, Coagglutation test, Latex agglutination test and Complement fixation test. Among them dot blot assay scores over others because of its inherent characteristic of rapid, sensitive, inexpensive, specific, ease of performance, field suitability and stability.

In the present study, it is hoped to make available standardized a dot blot assay kit for WSSV to the aquaculture industry. This kit is expected to provide the necessary reagents and procedures for easier diagnosis of WSSV infection in shrimp.

The results of titration of antisera confirm the presence of WSSV antibodies in the sera by the western blot and double immune diffusion test. The precipitation of Igs by ammonium sulphate precipitation by the method of Dubey, 1983, Wilson, 1986b, was followed and immunoglobulin protein concentration of 1.2888 mg/ml was obtained and was further purified to obtain IgG.

The anti-WSSV rabbit IgG was separated by anion exchange chromatography on DEAE cellulose. The OD at 280 nm of the fractions was plotted against tube number and first peak represented IgG. This was in conformity with Hudson and Hay (1991).

The optimal antigen coating level was determined by checkerboard titration method. It was found to be 1:10. The indirect dot blot method using HRPO-anti WSSV Is conjugate was found to be a more sensitive and reliable reaction avoiding the confusion. The conjugate dilution of 1:2000 was found to be optimum for the test indicating cost effectiveness of the test.

The stability of the enzyme conjugate used in dot blot assay is important for reproducible and reliable performance of the kit. Before storing, the conjugate should be stabilized by the addition of BSA to the final concentration of 1%. For long-term storage the conjugate should be mixed with an equal volume of glycerol and stored at -20° C.

Generally the kit is likely to be exposed to various temperature during transportation to distant places. Also, the storage temperature is likely to vary owing to difference in temperature is likely to vary owing to difference in temperature at different sites. Hence, in the present study generally the kit is likely to be exposed to various temperatures during transportation to distant places. Also, the storage temperature is likely to vary owing to difference in temperature is likely to vary owing to difference in temperature at different sites. Hence, in the present study attempts were made to study attempts were made to study the shelf life of the kit at various storage temperatures viz.37^{0}C, room temperature and 4^{0}C. The results of this revealed that specificity, sensitivity and reactivity of the reagent did not change till 8 weeks at different storage temperatures, although a slight decline in reactivity was observed in the 37^{0}C stored kit. Further

stability studies are being carried out in the Division of Fish Pathology.

Conclusively, the present study standardized a procedure for the use of Dot blot assay for WSSV. This specific, cheap and rapid test could be extended to the diagnosis of other bacterial, viral or fungal fish diseases.

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APPENDIX

BUFFERS

STOCK SOLUTIONS:

A: 0.01M Solution of Na2H2PO4.2 H2O = 1.5601gm/1000ml

B: 0.01M Solution of Na2HPO4 = 1.4198gm/1000ml

Take X ml of A and Y ml of B, diluted to a total of 200ml. The required pH was obtained as per the following table.

X	Y	pH
23.0	77.0	7.3
19.0	81.0	7.4
5.3	94.7	8.0

2. PHOSPHATE BUFFER SALINE (PBS) pH 7.2

NaCl	-	8.0gm
NaH2PO4.2H2O	-	1.44gm
KH2PO4	-	0.2gm
KCl	-	0.2gm
Distilled water	-	1000ml

3. PHOSPHATE BUFFER SALINE (PBS-T) pH 7.2

NaCl	-	8.0gm
NaH2PO4.2H2O	-	1.44gm
KH2PO4	-	0.2gm
KCl	-	0.2gm
Distilled water	-	1000ml
Tween-20	-	0.5ml

4. NORMAL SALINE SOLUTION (NSS)

NaCl	-	8.6gm
Distilled water	-	1000ml