

SEROLOGICAL CHARACTERIZATION OF *VIBRIO HARVEYI* PROTEIN ANTIGENS

A Thesis

Submitted to the

*West Bengal University of Animal and Fishery Sciences,
in partial fulfilment of the requirements for the degree of*

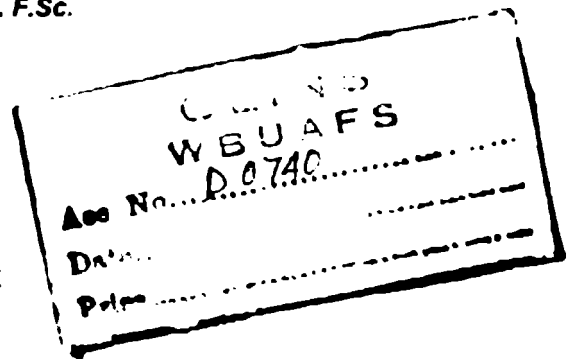
Master of Fishery Science

in

Fishery Pathology and Microbiology

By

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**DEPARTMENT OF FISHERY PATHOLOGY AND MICROBIOLOGY
FACULTY OF FISHERY SCIENCES
WEST BENGAL UNIVERSITY OF ANIMAL AND FISHERY SCIENCES**

2002



TO MY
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CERTIFICATE

This is to certify that the work embodied in the thesis entitled “**SEROLOGICAL CHARACTERIZATION OF *VIBRIO HARVEYI* PROTEIN ANTIGENS**” submitted by **Mr. P. Ganesan** in partial fulfilment of requirement for the Degree of **Master of Fishery Science (Fishery Pathology and Microbiology)** in the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my supervision and guidance. The results of the investigation reported in this thesis have not so far been submitted for any other Degree or Diploma. The assistance and help received during the course of investigation have been duly acknowledged.

Date :

The 14th Aug., 2002

Mohanpur, Nadia



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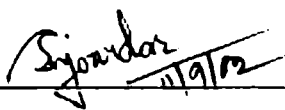
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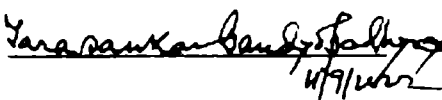
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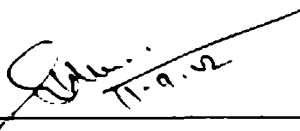
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ABBREVIATIONS

AGPT	:	Agar gel precipitation test
APS	:	Ammonium per sulphate
CIE		Counter current immuno electrophoresis
DID		Double immuno diffusion
ECP		Extracellular product
ELISA	:	Enzyme linked immunosorbent assay
FCA	:	Freund's complete adjuvant
H₂O₂		Hydrogen peroxide
IFA		Incomplete Freund's adjuvant
KH₂PO₄		Potassium dihydrogen phosphate
KOH	:	Potassium hydroxide
MR	:	Methyl red
NA	:	Nutrient agar
Na-K-tartarate	:	Sodium potassium tartarate
Na₂HPO₄		Disodium hydrogen phosphate
NSS	:	Normal saline solution
OPD		Orthophenylene diamine
PBS	:	Phosphate buffer saline
SDS		Sodium dodecyl sulphate
SDS-PAGE	:	Sodium Dodecyl Sulphate Poly Acrylmide Gel Electrophoresis
TCBS	:	Thiosulphate citrate bile salt sucrose agar
TEMED	:	Tetramethyl ethylene diamine
TSA		Tryptic soy agar

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Chapter - 1

INTRODUCTION

1. INTRODUCTION

Aquaculture is a dependable year round source of animal protein with quite high yield compared to that of agriculture or livestock. Of all aquaculture practices shrimp aquaculture is quite promising throughout tropical and subtropical countries. The past twenty years has seen substantial investment in the development of aquaculture production system particularly in Asia. Driven by substantial profits available on world markets, there has been heavy investment in shrimp production systems in marginal coastal lands at the environmentally sensitive-land water interface in several Asian countries including India.

Shrimp farming in India showed rapid growth since 1991 after the introduction of liberalization policies. Cultured shrimp production in India showed increasing trend from 35,000 mt during 1991-1992 to 62,000 mt during 1993-94 and 82,850 mt during 1994-95. Faster expansion of small-scale shrimp ponds particularly in coastal Andhra Pradesh, raised the Indian cultured shrimp production to attain a better level registering 82,700 mt production during 1998-99 and 8400 mt during 1999-2000 (Anon, 2001). In the beginning the shrimp farming was traditional system, extensive or semi intensive scale due to low capital investment. Later with the development of industry, it has been closely followed by the occurrence of infectious and non-infectious diseases. Initially, a primitive technology was in existence for several decades, based on natural water bodies, often enclosed at key times of the year. The combinations of production economics and profit expectation have driven a process of intensification in aquaculture and pushed it to limits beyond the boundaries of current knowledge. As a result, the underlying science of current production technologies is often not well understood. But production cycles are often interrupted by sudden outbreaks of disease and adversely affected by degradation of the production environment.

Such changes cannot easily be predicted or managed because there is little basic understanding of production system interactions.

The single largest cause of economic loss in aquaculture Industry is disease problems with bacterial diseases the most significant. Bacterial diseases in cultured shrimp occur as primary diseases or as secondary diseases. Many of these bacteria causing the diseases are the normal inhabitants of water. They cause the disease only when they cross the threshold level or when shrimps are under stress. Another predisposing factor for aquatic animal health to diseases is overcrowding (Noga, 1996). Shrimp aquaculture is often a typical example of these conditions.

One of the most common disease syndrome in aquacultured marine and estuarine finfish, molluscs and crustaceans is vibriosis. It is a major scourge of marine fish and shell fish world wide (Egidius 1987; Lightner 1988; Austin and Austin, 1993). This infection is more common in hatcheries, but is becoming more of a problem in grow out ponds and has been referred to as 'luminescent bacterial syndrome'. Vibriosis is found so often in association with other problems, that it is almost true to say, that any dead or dying shrimp will have some form of vibriosis. Because vibrios are also isolated from healthy penaeid shrimp, the hypothesis of the opportunistic nature of vibriosis are associated with penaeid shrimp has become widely accepted (Ruangpan and Kitao, 1991). Opportunistic vibrios may cause serious problems in larvae when they are suffering from stress caused by

1. Suboptimal or unstable environment.
2. High stocking densities.
3. Inadequate management.

Vibrio harveyi, the major casual agent of luminous vibriosis, affects a diverse range of marine vertebrates and invertebrates over a wide geographical area. The organism has been reported to be pathogen of cultured penaeid shrimp (Sunaryanto and Mariam, 1986; Karunasagar *et al.*, 1994; Pizzutto and Hirst,

1995; Liu *et al.*, 1996; Robertson *et al.*, 1998; Vandenberghe *et al.*, 1998) and fish (Kraxberger *et al.*, 1990; Ishimaru and Muroga, 1997; Alvanez *et al.*, 1998) of cultured penaeid shrimp (Sunaryanto and Mariam, 1986) fish (Kraxberger – Bealty, 1990). Moribund shrimp *P. mondon* larvae (Latchford, 1995), eggs, larvae and post-larvae of tiger shrimp *P. mondon* (Anon, 1995a).

Vibrio vulnificus, *V. parahaemolyticus* are some luminous and non-luminous strains of *V. harveyi*. In most ecological studies of marine luminous bacteria *V. harveyi* has not been distinguished from the *V. splendidus*. The organism is synonymous with *V. charchariae* (Pedersen *et al.*, 1998).

Although the virulence factors of *V. harveyi* are not completely understood, Liu *et al.* (1996b) considered that proteases, phospholipase, haemolysins or exotoxins might be important for pathogenicity. Chen (1997) considered that cystine protease was the major exotoxin. Identification of suitable antigens of any pathogen is considered to be having diagnostic importance. But unfortunately, reports regarding antigens of *Vibrio harveyi* is meagre. Thus, in the present background this study was undertaken with the following objectives:

- To prepare particulate and soluble somatic antigens of *Vibrio harveyi*.
- To assess sero-reactivity of soluble somatic antigens of *Vibrio harveyi*.
- To characterize *Vibrio harveyi* protein antigens at the polypeptide level.

Chapter - 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Vibriosis

According to Emmy Egidius (1987), vibriosis is a term for group of well known fish diseases reported from a large number of marine fish species. General external disease symptoms are haemorrhages and superficial skin lesions and in most cases there is a general septicaemia. Even though disease caused by *Vibrio* spp have been known for nearly a century and have been extensively studied, their pathogenesis and pathology are not fully known. Until around 1980, *V. anguillarum* was the only *Vibrio* species connected with diseases in fish. During the last few years several new fish pathogens belonging to this genus have been described.

Vibrio spp, both pathogenic and non-pathogenic are very common natural microbial flora in the marine and estuarine environments (Baumann and Schubert, 1981; Hung *et al.*, 1984).

A variety of *Vibrio* spp is associated with vibriosis (Austin and Austin, 1985; Kinne, 1990; Sinderman, 1990a and 1990b; Anderson and Convoy, 1970). Therefore, aquaculturist who use sea water or brackish water for their culture systems are potentially at risk of *Vibrio* infections. Although vibriosis is a disease usually associated with marine and estuarine species, it can occur in freshwater species, but much less commonly.

More than 40 aquatic species have been reported to suffer from *Vibrio* associated disease syndromes, including the salt water eel, dolphin fish, turbot, grouper, sole, roach, sea scorpion, salmon, trout, carp, young frogs, crabs, crayfish, oyster, shrimp (Kinne, 1990; Lavilla-Pitago *et al.*, 1992b; Sinderman, 1990a).

The majority of bacterial infections in penaeid shrimps are attributable to *Vibrio* spp (Bell and Lightner, 1991; Lightner, 1988).

Vibriosis is primary bacterial disease in shrimp and is usually caused by *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* (Lightner, 1988; Lightner *et al.*, 1992). Other species occasionally involved with infections include *V. damsela*, *V. fluvialis* and *V. hollisae* (Lightner *et al.*, 1992). Two luminescent species *V. harveyi* and *V. splendidus* have also caused significant shrimp disease problems especially in shrimp hatcheries (Baticados *et al.*, 1990; Lavilla-Pitago *et al.*, 1992a).

Vibrio spp have been reported to cause serious disease of larval and post-larval stages of *Penaeus monodon* (Johnson, 1983; Lightner, 1988). Mortalities of *P. monodon* and *P. merguensis* larvae associated with the luminescence have been observed in hatcheries in Indonesia (Sunaryanto and Mariam, 1986), in Thailand (Tansutapanit and Ruangpan, 1987) and in Philippines (Lavilla-Pitago *et al.*, 1990; Baticados *et al.*, 1991). Most of these *Vibrio* spp are part of autochthonous flora and marine water (Baumann and Baumann, 1981) and therefore, the source of infection is suspected to be near shore sea water (Lightner *et al.*, 1992).

Otta *et al.* (1999) reported on the presence of bacterial flora associated with shrimp culture ponds, where the total bacterial count ranged from 10^3 to 10^5 /ml of *Vibrio* count ranged from 10^1 to 10^4 /ml. *Vibrio* spp were found to be the largest group in all the ponds.

Felix and Sundararaj (1993) reported the presence of pathogenic luminescent bacteria, which was epizootic, causing mortality of 5.2 million larvae of *Penaeus indicus* in a commercial shrimp hatchery in Tuticorin, India. Jayabalan *et al.*, (1996) reported that *V. harveyi* and *V. splendidus* were the predominant pathogens for luminescent vibriosis in shrimps.

A number of *Vibrio* spp including *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. damsela* and *V. alginolyticus* are involved in shrimp disease

(Raungpan and Kitao, 1991). Anderson *et al.* (1988) reported that vibriosis caused 70-95% reduction in the expected harvests in some farms in Malaysia.

Since 1950, *V. parahaemolyticus* has been recognized as a potential enteropathogen all over the world. Data on the incidence of this pathogen in fish, fish products and aquatic environments are available (Nair *et al.*, 1975; Victor and Freda, 1976; Natarajan *et al.*, 1979; Nair *et al.*, 1980; Karunasagar and Mohankumar, 1980; Pradeep and Lakshmanaperumalsamy, 1984; Sanjeev and Iyer, 1986; Sanjeev and Stephen, 1994).

Georgekutty (1989) made a survey on the distribution of *Vibrio* spp in water and infected parts of diseased fish in Trivandrum coast. Maya *et al.* (1995) observed the proliferation of *Vibrio* spp in relation to weight of alimentary canal of *Etroplus suratensis* and *Etroplus maculatus*.

A great variety of extracellular substances have been associated with the virulence of pathogenic bacteria. They include a variety of proteases (Kothary and Kreger, 1985), lipase and nuclease (Janda *et al.*, 1984). Similarly haemolysin (Miyamoto *et al.*, 1969) and arylsulfatase (Baum *et al.*, 1959) are also involved in the pathogenesis.

Inamura *et al.* (1985) regarded protease produced by *Vibrio* spp as one of the virulence determinants in infection of fish.

2.2. Characterization of *Vibrio*

The genus *Vibrio* represents motile, Gram negative, oxidase positive, fermentative rod shaped bacteria, which grow on thiosulphate citrate bile salt sucrose (TCBS) agar. The species of genus *Vibrio* are facultative anaerobes (C+ for O/F test) and belongs to the family vibrionaceae (Babu *et al.*, 2000).

The vibrios are identified by following the main key established by Alisna and Blanch (1994). According to them, the cluster of vibrio species including *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. harveyi* and *V. vulnificus* exhibit arginine dehydrolase negative, lysine dicarboxylase positive and ornithine

dicarboxylase positive. The identification feature for another cluster includes arginine dihydrose negative, lysine decarboxylase positive, ornithine decarboxylase negative (comprising *V. alginolyticus*, *V. orientalis*, *V. campbelli* and *V. vulnificus*).

The sucrose fermentation reaction is very important identification of vibrios. The reaction gives yellow colour colonies in thiosulphate (TCBS) agar if ferments and resulted in acid production. If it is not fermented, it gives green colour colonies in TCBS agar (Lavillo-pitago and De pena, 1998). Many vibrios such as *Vibrio harveyi*, *V. damsela*, *V. vulnificus*, *V. parahaemolyticus*, *V. penaeicida* are sucrose non-ferments. (Song *et al.*, 1993; Alsina and Blanch, 1994; Lavilla-pitago *et al.*, 1998).

2.3. *Vibrio harveyi*

Bacterial diseases have been implicated to be one of the most devastating diseases, which can completely destroy hatchery productivity for extended periods. Significant larval mortalities in Asian shrimp hatcheries, including India, are often associated with luminescent vibriosis caused by *Vibrio harveyi* or *V. splendidus*. The disease is widely known as 'Luminous disease'. Because this problem is similar throughout Asia, scientists from several countries including the Philippines, Indonesia, Malaysia, India, Thailand and Taiwan are focusing their research inputs to determine the cause, diagnosis and to develop technologies to control and prevent these luminescent bacterial infections. (Babu *et al.*, 2001).

The *Vibrio harveyi* is a fish pathogen (Grimes *et al.*, 1984; Bertone *et al.*, 1996; Yii *et al.*, 1997)

Prayitno and Latchford (1995) have shown that the pathogenicity of *V. harveyi* and *V. splendidus* strains is related to the age of the larvae of *Penaeus indicus* and *P. monodon* suggesting different pathogenic strains might be involved at different larval developmental stages.

Vibrio harveyi was isolated from diseased penaeid shrimp (Lavilla-Pitago *et al.*, 1992; Owens *et al.*, 1992; Lightner, 1993; Prayitno and Latchford, 1995) and other marine animals (Kraxberger-Beatty and Mc Garey, 1990).

Large scale losses of larval and juvenile penaeids have been associated with epizootics of *Vibrio* sp (Lightner *et al.*, 1992; de la Pena *et al.*, 1993; Song *et al.*, 1993; Xu *et al.*, 1993; Hammed and Rao, 1994; Lee *et al.*, 1996).

Disease outbreaks attributed to luminous *V. harveyi* have been observed in Philippines (Lavilla-Pitago *et al.*, 1990), Indonesia (Sunaryanto *et al.*, 1986), Thailand (Jiravanichpaisal *et al.*, 1994), India (Karunasagar *et al.*, 1994), Australia (Pizzutto and Hirst, 1995) and Taiwan (Liu *et al.*, 1996).

The disease caused by *V. harveyi* has been successfully controlled with antibiotics, although antibiotic resistant strains of *V. harveyi* have been recorded (Karunasagar *et al.*, 1994).

Vibrio harveyi has not been previously described as fish pathogen (Austin and Austin, 1987; Colwell and Grimes, 1984; Munro, 1982). Results of this study suggested that *V. harveyi* may be an opportunistic pathogen which infects previously injured ocular and dermal surfaces. An opportunistic pathogen such as *V. harveyi* could have adverse effects on snook brood stock survival and propagation programmes.

Anon (1995) also reported on the sources of luminescent bacteria *V. harveyi* in *Penaeus monodon* hatcheries especially in the midgut contents of black tiger shrimp. Prayitno and Latchford (1995) isolated luminous bacteria from moribund shrimp *Penaeus monodon* larvae, closely related to *V. harveyi* and *V. phosphoreum* at concentrations of 10^3 /g. The virulence of the bacteria was related to age of larvae such that 25.28% of zoea, 47.08% mysis and 51.50% of PL which survived 48 h exposure to the pathogen. They reported that the larval prawns are particularly susceptible to *V. harveyi* infections.

Vibrio harveyi and *V. splendidus* affect eggs, larvae and post-larvae of the tiger prawn *P. monodon* (Anon, 1995).

Robertson *et al.* (1998) isolated a culture of *V. harveyi* from diseased *Penaeus vannamei*. It was pathogenic in penaeid shrimp larvae when used in a bath at 10^5 cells ml⁻¹ for 2 h. The resultant disease has the characteristics of *Bolitas negricans*. Large numbers of *V. harveyi* was found in intestine and hepatopancreas.

According to Alabi *et al.* (1999), luminescent vibriosis epizootics led to reduced shrimp production due to mortalities and slow growth of affected stocks. During disease outbreaks at 18 to 32 days of culture, luminous bacterial load of affected shrimps were higher than healthy shrimps. The majority of bacteria were dominated by *Vibrio* sp viz., *V. harveyi* (27.91%), *V. splendidus* (13.37%) and *V. parahaemolyticus* (10.46%) in descending order.

Baticados *et al.* (1991) suggested that the presence of luminous vibrios in rearing waters of shrimp farms and spawners, served as carriers of pathogens. The vibrios mainly isolated were *V. harveyi* and occasionally *V. splendidus*. *Penaeus monodon* (PL5) and *P. penicillatus* (PL4) mass mortalities were reported owing to the presence of *V. harveyi* at a rate of 1×10^3 numbers/ml in culture water.

According to Austin (1999), *V. harveyi* has emerged as a serious pathogen of penaeids especially in South America and Southeast Asia.

V. harveyi is a marine bacterium that causes luminous vibriosis (Zhang and Austin, 2000) and is important pathogen of cultured penaeid shrimp (Lavilla-Pitago and Baticados, 1990) and has been isolated from diseased marine fish such as *Acanthopagrus cuvieri* (Saeed, 1995), seabream, *Sparus aurata* (Balebona *et al.*, 1995) and dentex, *Dentex dentex* cultured on Mediterranean coast of Spain (Company *et al.*, 1999).

Abraham *et al.* (1997) studied the pathogenicity of luminous *V. harveyi* associated with diseased penaeid shrimp. The mean lethal doses of two of the strains were found to be 1.41×10^6 cells/animal and 1.02×10^7 cells/animal.

2.4. Agglutination Test

The possibility of using the agglutination test in the diagnosis of vibriosis was suggested by McFadyeen and Stockman (1909, 1913) who reported agglutination titres of 1:40 to 1:1000 in the blood of 4 ewes at the time of abortion and Smith *et al.* (1920), who found that the blood serum of 3 cows that aborted from vibriosis agglutinated *V. fetus*.

The significance of agglutinin titres depends upon the sensitivity of antigen. Plastringe *et al.* (1951) interpreted titres that were twice the average titre obtained with normal sera as suspicious and titres that were four or more times the titres given by normal sera as positive. They used *Vibrio fetus* antigens that gave titres of 1:50 to 1:100 with normal sera in testing sera from 405 of the cows using agglutination test.

The diagnostic significance of the agglutination reaction for vibriosis in sheep was discussed by Marsh and Tunnicliff (1955).

Shiomi *et al.* (1989) reported galactose-specific agglutinin from skin mucus of conger eel, *Conger myriaster* agglutinate but not inhibit growth of *Vibrio anguillarum*.

Ewart *et al.* (1999) showed that lectin from salmon serum, *Salmo salar* could bind to *Vibrio anguillarum* and *Aeromonas salmonicida*.

According to Austin (1988) agglutination test is used as the most common test for detection of bacteria from fish and the test is simple and specific.

Correlations have been shown between agglutination titre and ELISA readings (Rieque and Laurencin, 1987; Waterstrat *et al.*, 1989).

de le Pena *et al.* (1993) identified 75 strains of vibrios using plate agglutination test. They noted comparatively higher antibody titre with formalin-killed antigen than with heat killed antigen, indicating that the types of antigen can affect the agglutination titre.

Mishra and Shekhar (1997) noticed higher antibody titre with formalin-killed *V. parahaemolyticus* than with live antigen.

2.5. Immunodiffusion

In the double diffusion method of Ouchterlony (1949) antigen and antibody are placed in different wells in agar-gel plates and allowed to migrate towards each other, precipitation taking place where they meet.

The molecular weight of antigen will affect the shape of the precipitation line (Korngold and Leeuwen, 1957); if it is greater than that of antibody, the line will curve towards the antigen well because of its slower rate of migration through the agar.

Immunodiffusion is a valuable technique for comparative studies of antigen in different animal proteins. It has also been applied to diagnose a number of bacteria and virus, causing disease in domestic animals (Darbyshire, 1964 and 1965).

According to Blachman *et al.* (1978), when *Vibrio cholerae* strain 569 B inoculated onto a antiserum agar, a double ring halo of toxin-antitoxin precipitate is formed.

Immunodiffusion techniques were used to study toxin and antitoxin reactions of three human enteropathogenic strains of *Eschericia coli* viz., 334, 408-3 and 10407 and 1 strain of *Vibrio cholerae*. The reaction resulted in the formation of converging arch around the central well which shows immunodiffusion reactions when antiserum to *Vibrio cholerae* 569 B was placed in the central well while corresponding enterotoxin was in well no 1, 3 and 5 (Singh *et al.*, 1977).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Bogwald *et al.* (1990) tested the specificities of salmon antisera made against *V. salmonicida* and *V. anguillarum* by ELISA. Immunizations were performed with formalin killed whole cells of *V. salmonicida* and *V. anguillarum* serovar 1 and 2. Antibody specifications were tested by ELISA using whole cells

more antigenic in nature than other preparations indicating their suitability of use as coating antigen in ELISA and DIA.

According to Lee (1990), the immunogenicity of *Vibrio* spp is the reflection of the presence of a number of heat stable and heat liable antigens. The flagellar H-antigens are heat liable, while the cellular O-antigens are heat stable.

Oliver *et al.* (1992) stated that bacterial soluble antigens are mostly the flagellar H-antigens and are released into the supernatant due to the heat shock. These are antigenic in nature and help in protection and react in vitro with specific antisera. Mishra and Shekhar (1997) reported that in DIA no cross reactions were noted with bacteria belonging to *Aeromonas* sp and *Pseudomonas* sp. But cross reaction was visible with *V. alginolyticus* and it was mild with *V. harveyi* and *V. anguillarum*. Out of 60 samples screened by DIA, 35 showed positive colour reaction indicating that these belong to *Vibrio* spp.

Mishra and Shekhar (1997) noted that in ELISA cross reactions was visible with *Vibrio* spp but not with *Aeromonas* spp and *Pseudomonas* sp.

Reports of use of DIA for aquatic bacteria affecting shrimps are not available. But Mialhe *et al.* (1992) used dipstick ELISA, that has the similar methodology and principle as DIA.

Adams (1991) reported that using higher serum dilutions in ELISA no cross reactions have been found between *V. alginolyticus*, *V. anguillarum* and *V. parahaemolyticus*. Castro *et al.* (1995) showed that using higher dilution of antiserum and conjugate cross reaction can be minimized and even eliminated with other vibrios.

de le Pena *et al.* (1993) differentiated vibrio strains from kuruma shrimp and fish by using higher dilution of antiserum and conjugate.

Lee (1990) showed that *V. alginolyticus* and *V. parahaemolyticus* share common H-antigens on their polar flagellum and hence they cross react.

Hoel *et al.* (1998) measured the antibody responses in salmon and rabbit sera against whole cells of *A. salmonicida* and *V. salmonicida* by ELISA described

by Erdal and Reits (1992) with the following modifications: (i) sonicated cells *A. salmonicida* and *V. salmonicida* at concentrations of $\mu\text{g protein ml}^{-1}$ were used as coating agents instead of whole cells and (ii) the fish plasma sample were diluted 1 : 80. For analysis of the rabbit sera, the secondary antibody was a peroxidase-conjugated donkey-anti-rabbit antibody (Amershar, Buckinghamshire, U.K.) at a dilution of 1 : 3000.

Hoel *et al.* (1998) showed *V. salmonicida* reacted significantly with *A. salmonicida* measured in ELISA. These results are in contrast to the results achieved by Havarstein *et al.* (1990).

Hoel *et al.* (1997) showed that vaccination of Atlantic salmon with *V. salmonicida* induced antibody responses measured in an ELISA against *A. salmonicida* whole cells and LPS, but not against A-layer suggesting that LPS epitopes are involved.

Hastings (1998) reported that, antibody responses to *A. salmonocida* LPS are not correlated with protection.

Havarshein *et al.* (1990) did not find any cross reaction between *V. salmonicida* and *A. salmonicida* in salmon antisera in ELISA.

2.7. Crossed immunoelectrophoresis (CIE)

Hoel *et al.* (1998) characterized the levels of specific antibodies against *A. salmonicida* antigens in cross immunoelectrophoresis (CIE). Rabbit vaccinated against *V. salmonicida* produced antibodies and cross reacted with antigen preparations of *A. salmonicida*. In cross immunoelectrophoresis, electrophoresis and staining gels were performed according to Dalsgaard (1984). Grubb (1983) reported that the CIE does not allow the molecular characterization of the antigens, and the sensitivity is not good. Nevertheless, the CIE is capable of demonstrating the presence in antisera of very minute amounts of precipitating antibodies, and the amount of cross reacting antibodies is not necessarily very high.

Ellis *et al.* (1988) used crossed immunoelectrophoresis to compare immune responses in rabbits and rainbow trout. The results showed that *V. salmonicida* induced precipitating antibodies in rabbits that cross reacted with *A. salmonicida* antigens. Another observation was antibody reactivity of antisera produced in rabbits against *V. salmonicida* (RaVs) recognized two antigens from *A. salmonicida* not recognized by antibody reactivity of antisera produced in rabbits against *A. salmonicida* (RaAs).

Bussard (1959) originally described the immune precipitation methods. He performed electrophoresis test simultaneously with the immune gel precipitation. Calliford (1964) developed the counter immunoelectrophoresis as a tool for determining the species origin of body fluids, which ultimately enables it for detecting antigen and antibodies.

According to Singh (1977) three human enteropathogenic strains of *Escherichia coli* viz., 334, 408-3 and 10407 and one strain of *Vibrio cholerae* 569 B were employed to examine the antigenic relationship between their enterotoxin preparations. Immunoelectrophoretic techniques were used to study toxin-antitoxin cross reactions. Cross reactions between *V. cholerae* 569 B enterotoxin and antitoxin to *E. coli* 333 was evidenced by the presence of precipitation reaction of the reactants. But there was lack of interaction between *V. cholerae* 569 B enterotoxin and *E. coli* 408-3 antitoxin.

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Montero and Austin (1999) examined the extra cellular products of *V. harveyi* using SDS-PAGE and revealed the presence of protease. Bands with molecular weights of 70, 47, 34 and 24 kDa were detected in gels containing 0.1% gelatin as substrate. They recorded the bands of caseinolytic activity with molecular weights of 109, 40 and 25 kDa in all extracellular products of

V. harveyi. Thus it appeared that a total of seven distinct proteases were present in extracellular products. They demonstrated SDS-PAGE analysis of extracellular products of *V. harveyi* from cellophane overlays, which revealed the presence of 10 bands with activity towards gelatin (160, 81, 70, 38 and 33 kDa) and casein (120, 109, 50, 38 and 36 kDa). However, it is possible that a number of the lower molecular weight bands represented, breakdown products of the higher molecular weight proteases.

Protease production by *V. harveyi* has been investigated previously, by Fukasawa *et al.* (1988a). Fukasawa *et al.* (1998b) reported the presence of 3 metal chelator sensitive proteases in extracellular products produced from environmental (water) isolates of *V. harveyi* with molecular weights of 84, 49 and 46 kDa.

Liu *et al.* (1997) reported the purification of a 38 kDa cysteine protease from a prawn pathogenic isolate of *V. harveyi* and suggested that this molecule plays a major role in infections of tiger prawns.

Henriette *et al.* (1999) examined the importance of lipopolysaccharides composition and structure for the resistance of the fish pathogen *V. anguillarum* to direct complement mediated killing by rainbow trout serum. *V. anguillarum* strains of serogroups 01 and 02 and with different lipopolysaccharide structure, as demonstrated by their lipopolysaccharide profiles in SDS-PAGE were selected for analysis. This study demonstrated that most strains of both serogroup 01 and serogroup 02 were resistant to rainbow trout NS. The same serum-resistant strains were, in a recent study found to be pathogenic for Atlantic salmon, whereas, all of the serum sensitive strains, except for RVAU 910614 -11 were found to be weakly pathogenic or non-pathogenic. These results indicated that the serum resistance of *V. anguillarum* contributed to its ability to survive and induce disease in infected fish.

According to Zhang and Austin (2000) extracellular products of *Vibrio harveyi* are pathogenic to rainbow trout, *Onchorhynchus mykiss* and Atlantic salmon, *Salmo salar*. Extracellular products protein profile showed a very high

diversity between the isolates, although most shared a 34 kDa protein. The extracellular product of E18 and M3G31, which were recovered from diseased shrimp in China, demonstrated a banding profile with bands at approximately 41, 37, 34, 33 and 27 kDa. They observed *Vibrio harveyi* isolates VIB 571 and VIB 572 which originated from fish in Spain, demonstrated banding profile with bands at 43, 38, 34 and 28 kDa. The extracellular product of VIB 661 generated a very thick band at 52 kDa. The extracellular product derived from the most pathogenic isolate VIB 645 produced >15 bands, including those at 76, 51, 46, 41, 34, 31, 27 and 23 kDa. The lipopolysaccharide profile for the majority of the strains were similar with three bands of approximately 34, 24 and 13 kDa. Bogwald *et al.* (1990) reported the differences in the structure of lipopolysaccharides from 2 serovariants as observed on SDS-Polyacrylamide gel.

Lipopolysaccharides from serovar 2 has a broad low molecular weight band and an O-polysaccharide chain of heterogenous chain length. Lipopolysaccharide from serovar 1 appeared to be more homogenous both in the low and high weight region.

Hoel *et al.* (1998) performed SDS-PAGE in a Mini Protean Multi Casting Chamber (Bio Rad, Richmond, California, U.S.A.) as per the method of Laemmli (1970) in *Vibrio salmonicida*.

Chapter - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Bacteriological culture media

For the cultivation of the organisms in this study, the common culture media used include Nutrient Agar (NA), Tryptic Soy Agar (TSA), Thiosulphate citrate bile salt sucrose agar (TCBS) and transport medium.

All the dehydrated media (NA, TSA and TCBS) were procured from Hi media, Mumbai (India). The composition of all these media is given in Annexure (1). 1 M sodium hydroxide solution was used before sterilization of the medium to adjust the pH. For the biochemical identification of the bacteria many other media were prepared following the standard procedures of Abraham *et al.* (1998); using the required ingredients. The composition of all these media is given in Annexure (1). Sodium Chloride level of 1% (W/V) was maintained in all the medium, except in the specified places. 0.85% sodium chloride (Normal saline solution) was used as diluent for the enumeration of the bacteria.

3.2. Sterilization of culture media, containers and instruments

Bacterial culture media, diluents and autoclavable containers and instruments were sterilized in autoclave at 121°C (15 lb/in² gauge pressure) for 15-20 minutes unless specified otherwise. Some media used for the biochemical test were sterilized by steaming for an hour or at 110°C for 15 min as applicable. The glasswares were sterilized in hot air oven at 180°C for 1 hour, and polythene bags were sterilized by ultraviolet (UV) rays for 30 min.

3.3. Biochemical screening

The *V. harveyi* slant obtained from the Laboratory of Department of Fishery Pathology and Microbiology, WBUAFS was maintained on the nutrient

agar slants at room temperature with sub-culturing every 14 days. It was confirmed upto the species level by biochemical screening following the method described by Abraham *et al.* (1998).

The following biochemical tests were performed for the confirmation of *V. harveyi*.

3.3.1. Gelatinase test (West and Colwell, 1984)

The test was used to detect the proteolytic activity of bacteria by seeing the gelatin liquid fraction. The test was done by plating method and the medium used in the test was gelatin agar which consists of nutrient agar (NA), 5% gelatin and 1% sodium chloride. Young culture was spot inoculated onto the agar and was incubated at 37°C for 24 hrs. After incubation, the plates were flooded with saturated ammonium sulphate, which was allowed to stand for 2 minutes. The solution was decanted and observation was noted.

3.3.2. Oxidase test

Oxidase test detects the presence of cytochrome 'C' oxidase that is mainly responsible for the oxidation of the dye, called N-tetramethyl para-phenylene diamine dihydrochloride (oxidase reagent). This test was done by paper method. 1% solution of oxidase reagent was prepared and Whatman No. 1 filter paper strips were soaked into it. The young culture was picked up with a platinum loop and placed on the paper strip and the colour developed was noted.

3.3.3. Arginine dihydrolase and Lysine and Ornithine decarboxylase reactions on decarboxylase medium (A/L/O test)

1% solution of arginine, lysine, ornithine amino acids were prepared and taken in test tubes. A loop full of young culture was inoculated in all the tubes. After inoculation, the test tubes were overlaid with sterile paraffin, and incubated at 37°C for 24 hrs. The colour changes in the tubes were observed.

3.3.4. Growth in different salt concentrations

This test is done to find out the ability of bacteria to tolerate salt (NaCl) at various concentrations. The organism was streaked onto the nutrient agar medium containing 0%, 3%, 6%, 8% and 10% sodium chloride, respectively. Then the plates were incubated at 37°C for 24 hrs. The growth on the agar plates was observed.

3.4. Preparation of bacterial bulk culture

A loop full of young culture (*Vibrio harveyi*) from nutrient agar slant was transferred into several test tubes containing normal saline solution (0.85%) and were incubated at 37°C for 24 hrs. The bacterial broth culture in the normal saline solution was swabbed on several tryptose soy agar (TSA plates) and were incubated at 37°C for 24 hrs. The bacterial growth on these plates were harvested by adding sterile normal saline solution, and were transferred into the sterile test tubes. The bulk culture of the bacteria thus prepared were maintained at 4°C for further works.

3.5. Preparation of bacterial antigen

Heat killed antigen was prepared according to Mishra and Sekhar (1997) with some modifications. Live cultures were taken in a sterile tube and kept in a water bath for 30 minutes at 60-65°C. Then the culture was cooled and kept at 4°C.

The somatic sonicated antigen was prepared by disintegration in a ultrasonicator using a titanium probe operated at 810 μm peak to peak at 0°C. Soluble sonicated extracts were centrifuged at 10,000 rpm for 30 minutes at 40°C. Soluble supernatant was filtered using 0.22 μm filter and the protein content was determined by the method of Lowry *et al.* (1951).

3.6. Protein estimation

The protein content of *V. harveyi* was estimated by using the standard method of Lowry *et al.* (1951). Six test tubes were taken and 100 µl of Bovine serum albumin (BSA) solution, 100 µl of 0.85% normal saline solution (blank) and 100 µl of sample were added into each 2 tubes. 3 ml of Lowry's reagent was added to all tubes and mixed properly. After 10 minutes, 0.3 ml of Foline reagent (1N) was added to all tubes and again mixed well. After 30 minutes, the reading was taken at 750 nm in spectrophotometer. The protein level was estimated by using the OD value and calculation was made by the following formula;

$$\text{Protein value (mg/ml)} = \frac{\text{O.D. of test sample}}{\text{O.D. of standard BSA}} \times \text{concentration of BSA solution}$$

3.7. Hyper immunization of rabbits

Apparently healthy rabbits (two nos.) were inoculated intramuscularly with 5 doses of heat killed *Vibrio harveyi* antigen mixed with equal volume of adjuvant (Sisco Research Laboratory, Mumbai) given at 10 days interval. As a first dose 500 µgm of antigen was given with Freund's complete adjuvant and for subsequent 4 doses, 750 µgm of antigen was given with Freund's incomplete adjuvant. The animals were bled 10 days after last injection by cardiac puncture. The immune serum was harvested and stored at -20°C in eppendorf tubes. One control rabbit was maintained without immunization and serum was collected and kept at -20°C for further use.

3.8. Agglutination test

The specificity of rabbit antiserum against the *V. harveyi* antigen was tested by using the agglutination test. The clear, grease free sterile glass slide was taken, the corners were marked 'T' (test antiserum) and 'C' (control antiserum). 20 µl of

V. harveyi antigen poured on both sides separately. 20 µl of antiserum raised against the *V. harveyi* antigen was added with antigen in the side of the slide marked as 'T'. The antiserum taken from the control rabbit was added with the antigen in the side of the slide marked as 'C'. It was kept for 5 minutes and the result was noted.

3.9. Agar gel precipitation test

The specificity of rabbit antiserum against the *V. harveyi* antigen was tested by using the Ouchterlony immunodiffusion technique. The immunodiffusion gel was prepared at a concentration of 1% with normal saline solution (0.85% NaCl) in a sterile conical flask. The agar was dissolved in normal saline solution in a boiling water bath with constant stirring with careful observation. Now 4 ml of this molten agar was poured on a clear and grease free glass slide to give a thickness of 2 mm. Agar should be poured on the slide in such a way that no agar came out of the slide during pouring. This slide was then kept at room temperature for half an hour for solidification. When agar became harden, the wells were punctured by using template puncher. 20 µl of *V. harveyi* antigen was added in the control well and neat, 1:2, 1:4, 1:8 diluted rabbit antiserum raised against the *V. harveyi* antigen were added to each peripheral wells (20 µl).

3.10. Enzyme Linked Immunosorbent Assay (ELISA)

Antibody responses against sonicated whole cells of *Vibrio harveyi* were detected by ELISA as described by Erdal and Reita (1992) with some modifications. Briefly, the polystyrene microplate (96 wells) was coated with 20 µgm/well of sonicated antigens of *V. harveyi* and incubated for 2 hrs at 37°C [The plate was washed 3 times with PBS containing 0.05% Tween 20 (PBS-T)]. The plate was blocked for overnight at 4°C with PBS containing 5% of skim milk powder and washed 3 times with PBS-T. Serum samples diluted (1:50, 1:100,

1:200) in PBS were added to the wells and incubated for 2 hrs at 37°C. After incubation the plates were washed 3 times in PBS-T.

The ELISA micro-titreplates were kept in 1:1000 dilution of anti-rabbit HRP conjugate for 2 hrs at 37°C temperature. After 2 hrs, the plates were further washed with washing solution (PBS-T) to remove the unbound conjugate. Substrate solution was added (100 µl/well) and kept for 30 min in dark at room temperature. After development of colour, the reading was taken using the ELISA reader.

3.11. Countercurrent immuno electrophoresis

a) Preparation of agar gel plates

Firstly, the agarose gel was prepared at a concentration of 1% with tris-borate buffer (0.05 M, pH 8.6) in a sterile conical flask. The agar was dissolved in the buffer in a boiling water bath with constant stirring with careful observation so that the agar did not become yellowish due to too much of heating. Now 7 ml of this molten agar was poured on a clear and grease free glass slide, to give a thickness of 2 mm. Agar should be poured on the slide in such a way that no agar come out of the slide during pouring. These slides were then kept at room temperature for half an hour for solidification. When agar became harden two rows of wells were punched using template puncher. 20 µl of *V. harveyi* antigen was added in first row of wells and 20 µl of rabbit antiserum, raised against *V. harveyi* antigen was added to the wells parallel to the first row.

b) Electrophoresis

For proper electrophoretic migration firstly, the electrophoretic tank was filled with tris-borate buffer (0.05 M, pH 8.6). The slide was then placed over the bridge and the 2 ends of the slides were connected to the buffer solution in the chamber with blotting paper strips soaked with buffer solution.

After completing the connection, the electrophoretic module was so operated that a steady current of 3 mA was achieved. The connection of the slide was done so that antigen was charged at the cathodic well where as hyper immune serum was added to the anodic wells. The set was operated for 1 hour following which the slides were kept at room temperature for 2 to 5 minutes then the slide was observed for development of precipitation lines as a result of antigen-antibody reaction between antigen-antibody wells.

3.12. Cross-reactivity of *Vibrio harveyi* antigen with related antisera by electrophoretic technique

The cross-reactivity was assessed by *V. harveyi* antigen with different antisera raised against the *V. alginolyticus*, *V. fluvialis*, *Pseudomonas* sp using counter current electrophoresis by the following procedure.

The agarose gel was prepared by melting 1% of agarose in tris-borate buffer solution (0.05 M, pH 8.6). The melted agar solution was carefully pipetted onto a glass slide allowed to cool before using the template puncher. 20 µl of *V. harveyi* antigen was added in 1 row of wells and 20 µl of different rabbit antisera raised against the particulate antigens of *V. harveyi*, *V. alginolyticus*, *V. fluvialis* and *Pseudomonas* sp were added separately to each well parallel to the first row. The glass slide was placed on the electrophoretic chamber such that the antibody was on the anode side and antisera were on the cathode side. The tanks of electrophoretic chamber were filled with tris-borate buffer solution. The operation was carried out for 1 hour at 3 mA constant current.

3.13. Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Soluble *Vibrio harveyi* protein antigens was analysed by SDS-PAGE according to Laemmli (1970) with some modifications.

A vertical minislab gel electrophoresis system (Bangalore Genei) was used with 12.5% polyacrylamide gel containing 0.1% SDS. The samples were mixed with sample buffer in a proportion of 1:1. The quantity of protein was 40 μg per track. The samples were heated at 100°C for 2 minutes before loading. Proteins were run at 40 μA for 90 minutes and then stained by Coomassie Blue staining solution. Standard molecular weight markers (medium range) were run parallel along with sample proteins to determine the relative molecular weights of the poly-peptides.

Chapter - 4

RESULTS

4. RESULTS

4.1. Biochemical Screening

In gelatinase test, clear zones were formed around the colonies immediately, after flooding the plates with saturated ammonium sulphate by liquefaction or hydrolysis of gelatin. So the organism showed positive result to the test. In oxidase test, after placing the culture on the paper strip, the colour changed to violet immediately, indicating the test was positive. In arginine dihydrolase and lysine and ornithine decarboxylase reactions on decarboxylase medium, there was no colour change in arginine dihydrolase test tubes. The colour change from red to yellow was observed in lysine and ornithine decarboxylase test tubes. Therefore '- + +' result was observed in the A/L/O test.

In growth in different salt concentrations, the bacterial growth was observed in 0%, 3%, 6%, 8%, 10% salt concentrations.

4.2. Preparation of Hyper Immune Sera

After fifth inoculation of *V. harveyi* antigen, the heart blood was collected from the rabbit and the serum was prepared and AGPT was conducted to know whether the serum is having specific antibody against *V. harveyi* antigen. Visible bands were observed in between antigen and antibody wells indicating the antiserum contain specific antibodies against *V. harveyi* antigens.

4.3. Protein Estimation of *Vibrio harveyi* Antigen

Protein content of *Vibrio harveyi* sonicated soluble antigen was estimated as per the method of Lowry *et al.* (1951) and the OD value was found to be 1.19. The calculated protein value of the antigen was 3.38 mg/ml. In the same method

V. harveyi particulate antigen was estimated and OD value observed was 1.18 and calculated protein value was 3.32 mg/ml.

4.4. Agglutination Test

To determine the sero-reactivity of particulate antigen of *V. harveyi*, slide agglutination test was performed using *V. harveyi* particulate antigen with the *V. harveyi* antisera raised in rabbit. The antigen and antiserum was mixed properly and kept for 5 minutes. Clear agglutinated mass was observed showing specific antigen and antisera reaction (Fig 1).

4.5. Agar Gel Precipitation Test

Immunodiffusion test was conducted to study the *V. harveyi* soluble protein antigen with antiserum raised in rabbits. Moreover to know titre of the antiserum, it was diluted from 1:2 to 1:8. Dilutions were made with normal saline solution (NSS). When neat, 1:2, 1:4 and 1:8 diluted antiserum was kept in peripheral well and *V. harveyi* antigen in the central well, precipitating bands were observed in all the cases, revealing complete circle surrounding the center well. (Fig 2). It indicated that titre of antiserum was 8.

4.6. Enzyme Linked Immunosorbent Assay

Seroreactivity of *V. harveyi* sonicate soluble antigen was assessed by ELISA using antisera raised in rabbits. The antiserum was diluted in different dilutions with PBS (1:50, 1:100, 1:200). The diluted sera were used in duplicate in ELISA plate. It was observed that more the dilutions of antiserum, less the reactivity with antigens. The reactivity ranged from 1.170 to 1.194 in terms of OD. The average OD values in different dilutions are shown in Table 1 and expressed in Fig 3.

4.7. Countercurrent Immuno Electrophoresis

To assess sero-reactivity of *V. harveyi* sonicated antigen with the antiserum raised in rabbits, counter current immuno electrophoresis was performed to reduce the time of antigen and antibody reaction by applying electrophoretic technique. Clear precipitating bands were observed in between the antigen and antibody wells (Fig 4).

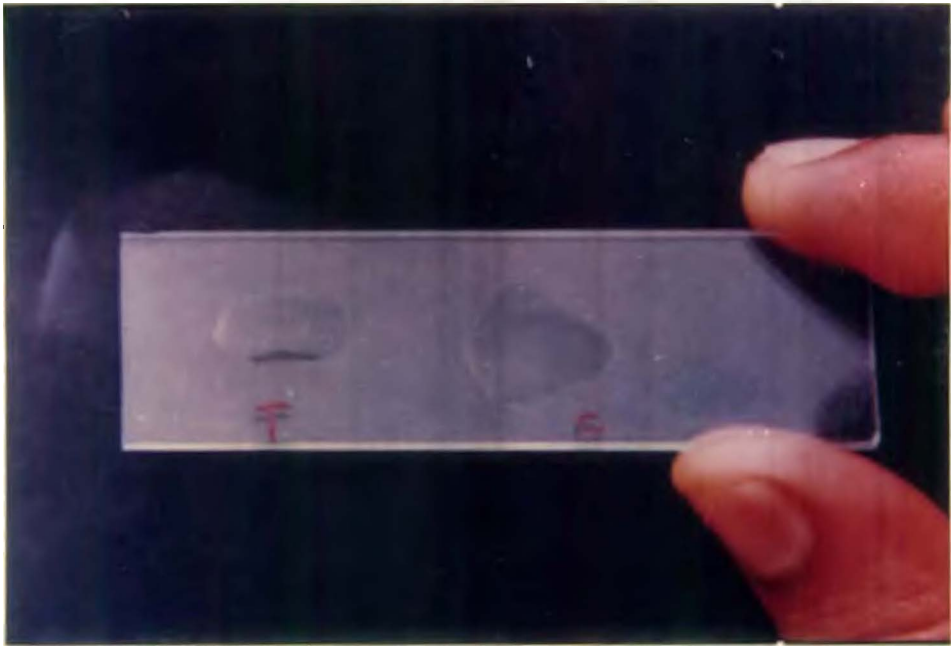
4.8. Determination of Cross-Reactivity of *Vibrio harveyi* Antigens with Related Antiserum by Countercurrent Immuno Electrophoresis

When cross-reactivity was assessed by *V. harveyi* antigen with various antisera raised against the *V. alginolyticus*, *Vibrio fluvialis*, *Pseudomonas* sp using counter current electrophoresis, it was observed that precipitation band was only formed when *V. harveyi* antigen reacted with its antiserum. But no precipitation band was observed when *V. harveyi* antigen with the other antisera reacted, indicating no cross-reactive proteins were present in *V. harveyi* antigen preparation. The precipitation band is shown in the Fig 5.

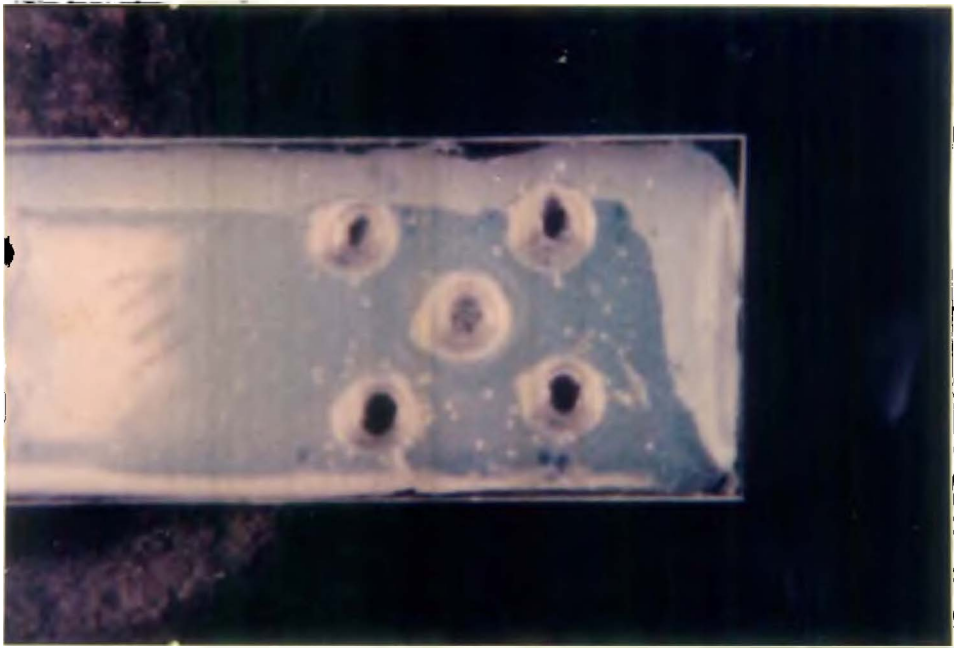
4.9. SDS-PAGE

When *Vibrio harveyi* soluble sonicated antigen were resolved in 12.5% poly acrylamide gel and stained by Coomassie blue, 25 polypeptide bands were observed. Critical analysis of 8 major and 17 minor bands out of 25 bands. The molecular weight of the polypeptide was calculated by comparing the known molecular weight marker, run parallel in the same gel. The individual molecular weight of the polypeptide was 103, 102, 100, 98, 96, 93, 88, 84, 72, 90, 65, 62, 60, 52, 50, 40, 38, 35, 30, 28, 25, 24, 22, 19 and 15 kDa (Fig 6).

Fig. 1. Assessment of sero-reactivity of *Vibrio harveyi* whole cell protein antigen with hyperimmune serum by slide agglutination test.



**Fig. 2. Determination of titre of hyperimmune serum by
agar gel precipitation test.**



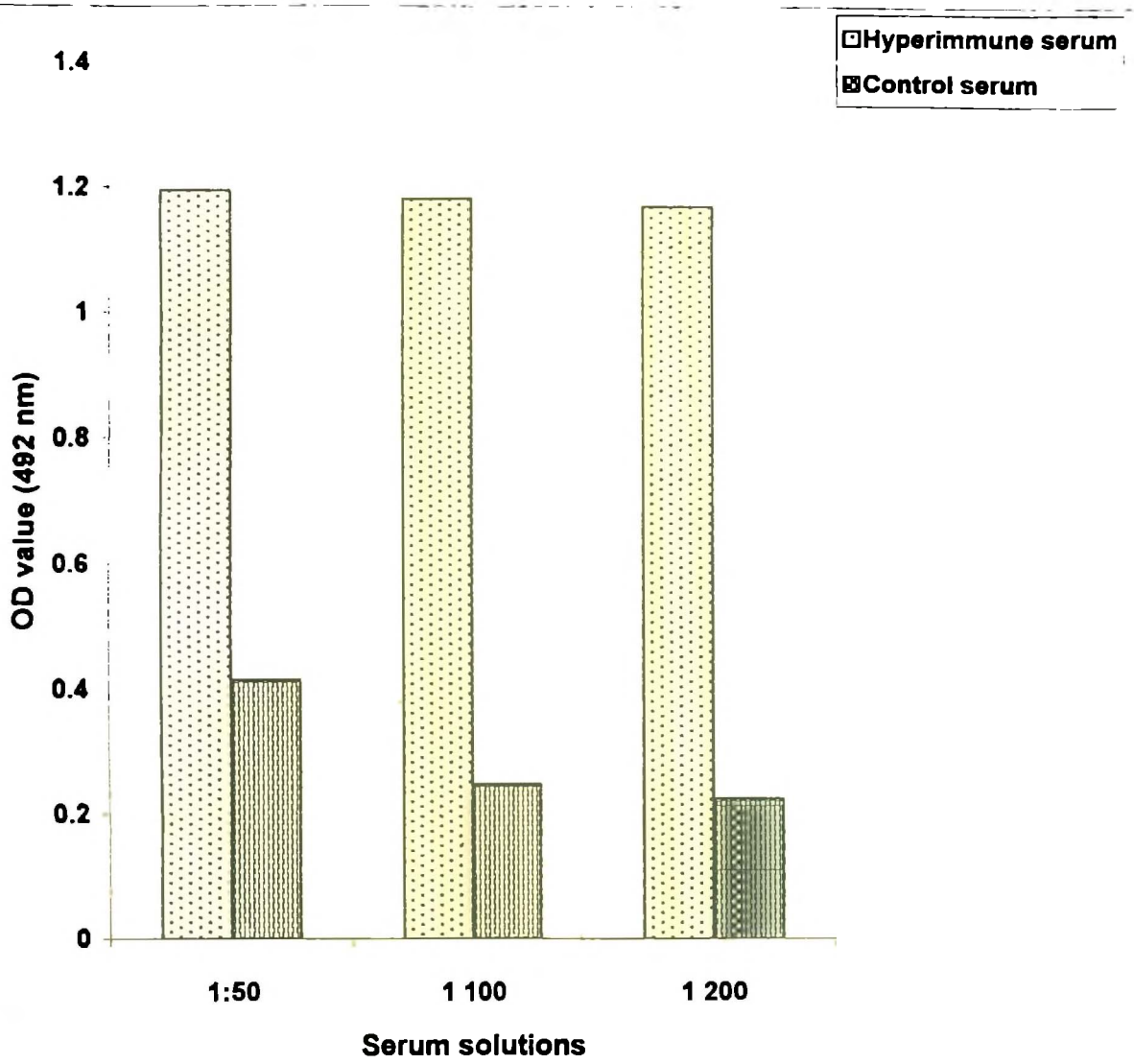


Fig (3). Sero-reactivity of *Vibrio harveyi* sonicated antigens with hyperimmune and control sera as assessed by ELISA

Table 1. Sero-reactivity of *Vibrio harveyi* sonicated protein antigen with hyperimmune and control sera as assessed by ELISA (at 492 nm).

Serum dilutions	1:50	1:100	1:200
OD value of hyperimmune serum	1.194	1.182	1.170
OD value of control serum	0.415	0.248	0.224

Fig. 4. Assessment of sero-reactivity of *Vibrio harveyi* sonicated protein antigen with hyperimmune serum by countercurrent immuno electrophoresis.

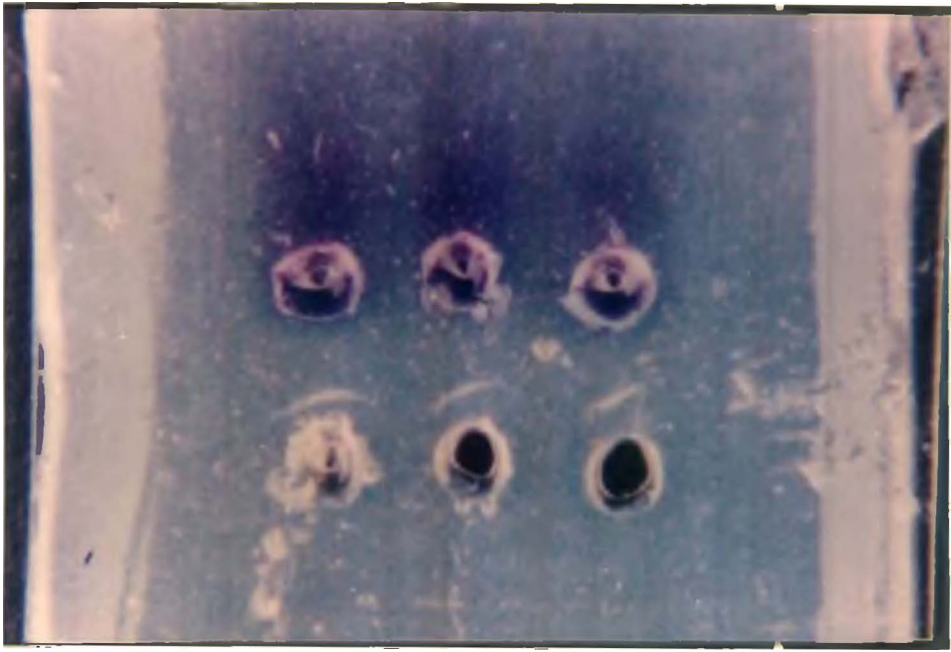


Fig. 5. Determination of cross-reactivity of *Vibrio harveyi* sonicated protein antigen with the antisera against *Vibrio* spp and *Pseudomonas* sp by countercurrent immuno electrophoresis.



Fig. 6. Determination of molecular weights of polypeptides of *Vibrio harveyi* sonicated protein antigen by SDS-PAGE.

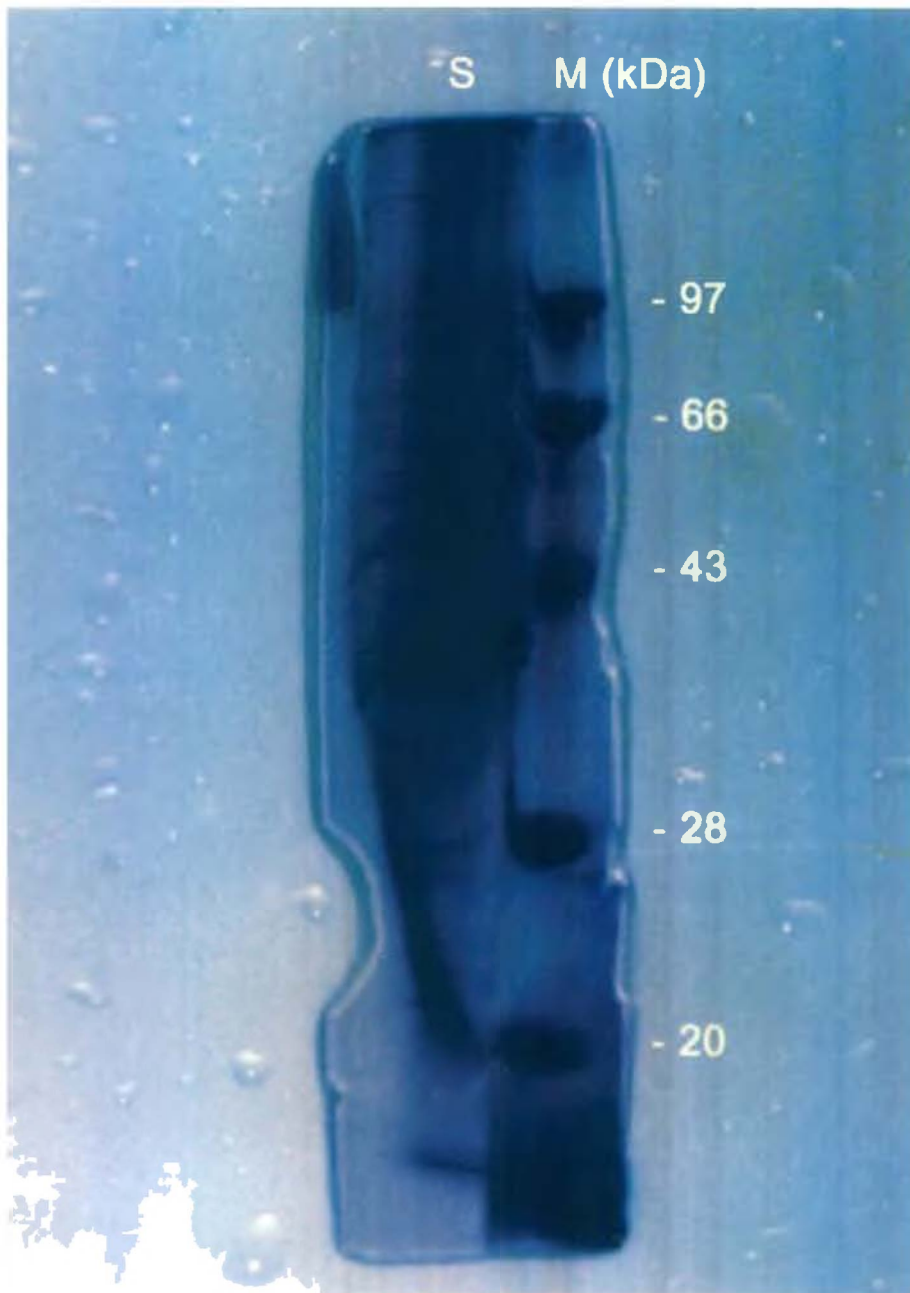


Fig.4 Coomassie blue R-250 stained polypeptide *V.harveyi* protein antigen preparation.

S - Sample. M - Marker

Chapter - 5

DISCUSSION

5. DISCUSSION

In the present study, protein antigens were characterized serologically by using some serological techniques such as AGPT, ELISA, countercurrent immuno electrophoresis, SDS-PAGE etc. The antiserum against the *Vibrio harveyi* antigen was prepared by hyperimmunization of rabbit by using FCA and IFA adjuvants.

First the sero-reactivity between the *Vibrio harveyi* antigen and the antisera raised in rabbit was determined by using the agglutination. The antigen and antiserum were mixed properly and kept for 5 minutes, clear agglutinated mass observed showing specific antigen and antiserum reaction.

Austin (1988) opined agglutination test can be used as the most common test for detection of bacteria from fish and the test is simple and specific. The specificity of *Vibrio* antisera was adjusted by de le Pena *et al.* (1993) by agglutination test. He also performed the agglutination test and noted comparatively higher antibody titre with formalin killed antigen than with heat killed antigen, indicating that the types of antigen can affect the agglutination titre.

Immunodiffusion test has been applied to diagnose a number of bacteria and virus causing diseases in domestic animals (Darbyshire, 1964, 1963). In the present study the agar gel precipitation test was done by Ouchterlony (1949) was kept in peripheral well and *V. harveyi* antigen in the central well, precipitating bands were observed in all the cases, revealing complete circle surrounding the central well, which indicated that titre of antiserum was 8. Blachman *et al.* (1978) revealed, when *Vibrio cholerae* strain 569 B was inoculated onto a antiserum agar, a double ring halo of toxin-antigen precipitate is formed.

To access sero-reactivity of *Vibrio harveyi* sonicated antigen ELISA was performed using different dilutions of antiserum raised in rabbits. As the antiserum was specific to *Vibrio* antigen, colour was developed which was quite brighter than the colour formed by controlled rabbit serum used. The OD values of

antiserum were 1.194, 1.182 and 1.170 in 1:50, 1:100 and 1:200 dilutions, whereas in control it was 0.415, 0.248 and 0.224 in those dilutions, respectively.

Bogwald *et al.* (1990), tested the specificities of salmon antisera made against *V. salmonicida* and *V. anguillarum* by ELISA. Immunizations were performed with formalin killed whole cells of *V. salmonicida* and *V. anguillarum* serovar 1 and 2. Antibody specifications were tested by ELISA using whole cells and purified LPS as antigen. The results showed that individual antisera from salmon immunized with *V. anguillarum* serovar 2 contained lower titre of antibodies to homologous whole cell antigen, and had almost background activities towards lipopolysaccharides.

Specificity of *Vibrio* antigens was assessed previously by several workers using ELISA (Bogwald *et al.*, 1990; Mishra and Shekar, 1997). Using ELISA specific seroreactivity was assessed in *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus* (Adam, 1991) as also in *V. salmonicida* (Hoel *et al.*, 1998).

To assess sero-reactivity of *V. harveyi* sonicated antigen with the antiserum raised in rabbits, countercurrent immuno electrophoresis was performed. Clear precipitating bands were observed in between the antigen and antibody wells. The precipitating band formed towards antisera wells. This might be due to less migration of antibody passively towards cathode wells.

To assess cross-reactivity of *V. harveyi* antigen with related antiserum raised against the *V. alginolyticus*, *V. fluvialis* and *Pseudomonas* sp using countercurrent electrophoresis. It was observed that precipitating band was only formed when *V. harveyi* antigen reacted with antiserum. But no precipitation band was observed when *V. harveyi* antigen with other antisera reacted, indicating no cross reactive proteins was present in *V. harveyi* antigen preparation.

Hoel *et al.* (1918), characterized the levels of specific antibodies against *A. salmonicida* antigen in cross immuno electrophoresis (CIE). Rabbit vaccinated against *V. salmonicida* produced antibodies and cross reacted with antigen preparation of *A. salmonicida* in crossed immuno electrophoresis.

Ellis *et al.* (1988) used crossed immuno electrophoresis to compare immune responses in rabbit and rainbow trout. The results showed that *V. salmonicida* induced precipitating antibodies in rabbits that cross reacted with *A. salmonicida* antigens. Another observation was antibody reacting of antisera produced in rabbits against *V. salmonicida* (RaVs) recognized two antigens from *A. salmonicida* not recognized by antibody reactivity of antisera produced in rabbits against *A. salmonicida* (RaAs). Previously, three human enteropathogenic strains of *Eschrechia coli* viz., 334, 408-3 and 10407 and one strain of *Vibrio cholerae* 569 B were employed to examine the antigenic relationship between their enterotoxin preparation by Singen in 1977. Immuno electrophoretic techniques were used to study toxin-antitoxin cross reaction. Cross reaction between *V. cholerae* 569 B enterotoxin and antitoxin to *E. coli* 333 was evidenced by the presence of precipitating reaction of the reactants. But there was lack of interaction between *V. cholerae* 569 B enterotoxin and *E. coli* 408-3 antitoxin.

In the present study, species specificity of *V. harveyi* was observed in countercurrent immuno electrophoresis. Hence it can be inferred that the serological technique (CIE) can be used for species specific diagnosis in *Vibrio harveyi* infections.

To perform molecular characterization of *V. harveyi* soluble protein antigen SDS-PAGE was performed using 12.5% acrylamide gel. Twenty five polypeptides were detected after staining the gel with Coomassie blue stain. Out of these 8 major and 17 minor bands in molecular weight range of 15 to 103 kDa.

Montero and Austin (1999) examined the extracellular products of *V. harveyi* using SDS PAGE and revealed the presence of protease. Bands with molecular weights of 70, 47, 34 and 24 kDa were detected in gel containing 0.1% gelatin as substrate. They recorded the bands of caseinolytic activity with molecular weights of 109, 40 and 35 kDa in all extracellular products of *V. harveyi*. Thus it appeared that a total of seven distinct proteases were present in extracellular products. They demonstrated SDS PAGE analysis of extracellular

products of *V. harveyi* from cellophane overlays revealed the presence of 10 bands with activity towards gelatin (160, 81, 70, 38 and 33 kDa) and casein (120, 109, 50, 38 and 36 kDa). The presence of three metal chelator sensitive proteases in extracellular products from environment (water) isolates of *V. harveyi* with molecular weights of 84, 49 and 46 kDa was reported by Fukasawa *et al.* (1998). Extracellular product protein profile showed a very high density between the isolates, although most shared a 34 kDa protein. The extracellular product of two isolates, recovered from diseased shrimp in China demonstrated a banding profile with bands at approximately 41, 37, 34, 33 and 27 kDa (Zhang and Austin, 2000). The same group observed *V. harveyi* isolates VIB 571 and VIB 572, which originated from fish in Spain, demonstrated banding profile with bands at 43, 38, 34 and 28 kDa. The extracellular product of VIB 661 generated very thick band at 52 kDa. The extracellular product derived from the most pathogenic isolate VIB 645 produced >15 bands, including those at 76, 51, 46, 41, 34, 27 and 23 kDa. The lipopolysaccharide profile for the majority of the strain were similar with three bands of approximately 34, 24 and 13 kDa. SDS PAGE of cell envelope proteins of *V. anguillarum* showed that regardless of the serogroup of source of isolation, all the strains possessed protein components with molecular masses ranging between 94 to 14 kDa (Santosh *et al.*, 1995). Analysis of cell envelope proteins showed that *V. anguillarum* related organisms possessed major protein components with molecular weight ranging from 32 to 45 kDa (Santosh *et al.*, 1997).

From the present study it can be concluded that

- 1) Seroreactivity exists in *Vibrio harveyi* soluble protein antigen.
- 2) *Vibrio harveyi* soluble protein antigen is the mixture of 25 polypeptides in the range of 15 to 103 kDa.
- 3) Species specific diagnosis of *Vibrio harveyi* is possible using *V. harveyi* antiserum at the field level.

Chapter - 6

SUMMARY

6. SUMMARY

To characterize the sero-reactivity of *Vibrio harveyi* soluble protein antigens certain serological techniques were used in the present studies. First, *V. harveyi* isolate obtained from the laboratory was tested for its characteristics. Then bulk culture was prepared to produce significant bacterial antigens. Heat killed particulate and sonicated soluble antigens were prepared and protein content was estimated in the antigen preparation. Hyperimmunization performed in rabbit with *V. harveyi* whole cell proteins by using Freund's complete adjuvant and Freund's incomplete adjuvant. Specificity of hyper immune serum was assessed by slide agglutination test. Titre of the hyper immune serum was determined by agar gel precipitation test. Upto 1:18 dilutions of antiserum precipitation band was observed indicating high titre of the antiserum. When seroreactivity of antiserum was assessed by Enzyme Linked Immunosorbent assay, it was observed that OD values in case of hyperimmune sera were 1.194, 1.182 and 1.170 in 1:50, 1:100 and 1:200 dilutions of antiserum, respectively, whereas, in controlled rabbit serum the OD values were found to be 0.415, 0.248 and 0.224. Specific precipitation was observed in *V. harveyi* soluble antigen and antiserum when countercurrent immuno electrophoresis was performed. To assess cross reactivity of *V. harveyi* protein antigen with other related bacterial antigens, countercurrent immuno electrophoresis was performed. Only *V. harveyi* antigen showed immune reaction with specific antiserum. But no precipitating band was observed when *V. harveyi* antigen was tested with *V. alginolyticus*, *V. fluvialis* and *Pseudomonas* sp were used.

To resolve *V. harveyi* soluble protein antigen at the polypeptide level, SDS PAGE was performed at 12.5% poly acrylamide gel. After Coomassie blue staining it was observed that twenty five polypeptides were present in the antigen preparation in the range of 15 to 103 kDa. The present study revealed the

seroreactivity property of *V. harveyi* antigens. Moreover the *V. harveyi* antigen was mixture of twenty five polypeptide antigens. It can be concluded that, species specific diagnosis of *V. harveyi* was observed to be possible using serological techniques.

Chapter - 7

BIBLIOGRAPHY

7. BIBLIOGRAPHY

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Chapter - 8

ANNEXURE

8. ANNEXURE

ANNEXURE – 1

Gelatinase test

Saturated ammonium sulphate	
Gelatin	5 g
Sodium chloride	1 g
Distilled water	100 ml

Nutrient agar (NA)

Hi Media, 1991

	g/l
Peptone digest of animal tissue	5.0
Sodium chloride	10.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25°C)	7.4±0.2

Oxidase reagent

N, N, N', N' – Tetramethyl para-phenylene Diamine dihydrochloride	10 mg
Distilled water	1 ml

Whatman No. 1 filter papers were cut into strips and put into the reagent solution. After soaking, the strips were dried at 37°C for about 15-30 minutes.

Thiosulphate citrate bile salt sucrose (TCBS) agar

Hi Media, 1991

	g/l
Peptone special	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Sodium cholate	3.00
Oxagall	5.00
Sucrose	20.00
Sodium chloride	10.0
Ferric citrate	1.00
Bromothymol blue	0.04
Agar	15.00 g
Final pH (at 25°C)	8.6±0.2

Tryptic soy agar (TSA)

Hi Media, 1991

	g/l
Tryptone	15.0
Soya peptone	5.00
Sodium chloride	10.0
Agar	15.0
Distilled water	1000 ml
Final pH (at 25°C)	7.3±0.2

ANNEXURE – II

AGPT

Agarose gel

	G/100 ml
Agarose	1.00
Sodium chloride	0.85

CIE

Trisborate buffer

	g/100 ml
Tris	0.657
Boric acid	Required amount
pH	8.6

ELISA

i) COATING BUFFER

Sodium carbonate	0.159 gm
Sodium bicarbonate	0.293 gm
Sodium azide	0.020 gm
Distilled water	Upto 100 ml
PH	9.6

ii) BLOCKING SOLUTION

PBS	100 ml
Skimmed milk powder	5 g

iii) WASHING SOLUTION

PBS	100 ml
Tween – 20	0.05 ml

iv) SUBSTRATE SOLUTION

1. Citrate buffer - 0.1 (M)
(pH - 4.6)

SOLUTION A + B

1. SOLUTION A

Citric acid	2.1 g
Distilled water	100 ml
Store at 4°C	

2. SOLUTION B

Sodium citrate	2.94 g
Distilled water	100 ml
Store at 4°C	

Mix 12.75 ml of Solution A with 12.25 ml of Solution B to prepare citrate buffer.

SUBSTRATE BUFFER

Citrate buffer	100 ml	
H ₂ O ₂	12 ml	Prepare before use

25 ml of citrate buffer + 3 ml H₂O₂ + 0.025 gm of OPD

PROTEIN ESTIMATION

Lowry's reagent

	g/100 ml
Solution 'A'	
Sodium carbonate	2
Sodium hydroxide	0.4
Solution 'B'	
Sodium potassium tartarate	1
Solution 'C'	
Copper sulphate	1
Stock solution	
Solution A, B and C mixed in the ratio of 50:1:1	
Standard BSA	1 mg/ml

Foline reagent

Foline reagent	1 ml
Distilled water	2 ml

SDS-PAGE REAGENTS

1. 4X LOWER BUFFER (1.5 M TRIS/HCL, pH 8.8)

- Dissolve 90.85 g Tris base in 450 ml dH₂O
- Adjust pH with concentrated Hcl to 8.8 (takes ~9-11 ml)
- Add 2 g SDS, dissolve
- Fill with dH₂O to 500 ml

2. 30% ACRYLAMIDE, 8% BISACRYLAMIDE

- 90 g acrylamide
- 2.4 g bisacrylamide
- Add 180 ml dH₂O
- Stir until dissolved, fill to 300 ml
- Add 2 spoonfuls activated charcoal, stir 5 minutes
- Filter until clear

3. 10% SDS

- Dissolve 50 g SDS in 450 ml dH₂O

4. Upper buffer

- Dissolve 25-30 g tris base in ~ 400 ml dH₂O
- Adjust pH to 6.8 with concentrated Hcl
- Fill to 500 ml.

5. 10% AMMONIUM PERSULPHATE (APS)

- Dissolve 0.1 g APS in 1.0 ml dH₂O
- Make fresh each time

6. 2X SAMPLE BUFFER

- 2 ml glycerol
- 1 ml 2-mercaptoethanol
- 4.5 ml 10% SDS
- 1.7 ml upper buffer
- 0.2 ml 0.1% bromophenol blue
- 0.6 ml dH₂O
- Mix, store at - 20°C.

7. ELECTRODE BUFFER (pH 8.3)

- Dissolve 12.0 g Tris base and 57.6 g glycine in 3000 ml dH₂O
- Check pH, if not 8.3, adjust with 1 N HCl
- Add 4 g SDS (or 40 ml 10% SDS)
- Fill to 4 l.

8. SDS-PAGE GEL STAIN

- 1.25 g Coomassie blue (R 250)
- 500 ml Methanol
- 100 ml Acetic acid
- 400 ml dH₂O
- Mix, stir for 30 minutes.
- Filter.

9. SDS-PAGE GEL DESTAIN

- 600 ml Methanol
- 140 ml acetic acid
- fill to 2 l with dH₂O (1260 ml)

ANNEXURE – 3

Instruments used

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1. Autoclave
 2. Deep freezer
 3. Electrophoretic apparatus
 4. ELISA reader
 5. Incubator
 6. Hot air oven
 7. SDS-PAGE apparatus
 8. Spectrophotometer
 9. Ultra sonicator
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