STUDIES ON THE PATHOBIOLOGY OF PENAEID LARVAE AND POSTLARVAE

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JULY 1989

DEDICATED

TO MY

PARENTS AND TEACHERS

DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON THE PATHOBIOLOGY OF PENAEID LARVAE AND POSTLARVAE"** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recongnition.

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON THE PATHOBIOLOGY OF PENAEID LARVAE AND POSTLARVAE"** is the bonafied record of the work carried out by Mr. **A.SAIT SAHUL HAMEED** under my guidance and supervision and that no part thereof has been presented for the award of any other Degree.

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CONTENTS

PREFACE

Over the last two decades, intense interest has been generated in India to develop aquaculture in the coastal waters. This growing awareness has stemmed in the context of ever increasing demand for protein food, increasing fishing pressure on certain marine fish resources particularly the prawns to meet the demand from foreign markets and consequent stagnation and/or declining trend in the fish catch. Besides, it is realised that coastal aquaculture would help considerably towards integrated rural development of coastal areas, providing employement oppurtunities and the use of the underutilised or unutilised coastal derelict waters. In view of these, coastal aquaculture is now assigned high priority in the national fishery development programmes.

Among the extensively cultivated species of fish and shellfish in the coastal waters of India, penaeid prawns occupy the foremost place. A traditional practice of aquaculture of prawns and fishes in the brackishwaters is prevalent in the country since ancient times. The basic technology of prawn farming in this practice entails stocking of the field by the seeds brought in by the incoming tide, growing them for a short period by feeding on the natrual food available in the field and harvesting of the stock. The quality and quantity of production from this farming system however, are found to be low due to indiscriminate and uncontrolled stocking of the seed, short time allowed to grow the seed before harvesting and little managerial procedures involved by the way of eradication of predatory and competitive species and control of water quality. This

system during past decade is improvised through eradication of undesirable organisms from the field and its preparation, stocking with species of prawns that grow fast and command good price and demand, and gorwing them to marketable size with supplementary feeding and water supply management. The yield as well as the quality of prawns harvested by this system is found to be of higher unit value. This semi-intensive practice is now rapidly spreading and gaining importance in the country.

In the development of aquaculture of prawns in the country, one of the major constraints encountered by the farmer is the non-availability of quality seeds of desirable species as and when required for culture. To meet the ever increasing demand for seed, the technology of seed production of penaeid prawns has recently been developed and several commercial scale hatcheries are now being established in different maritime states.

One of the major factors which influences the production and quality of seed in the hatchery and their subsequent culture in the grow-out system is the diseases and parasites affecting the stock. Under certain unfavourable conditions, diseases due to biotic and abiotic factors affect the larvae and postlarvae of penaeid prawns. Further, these developing stages are found to be more susceptible to diseases than the adult. From the published literature on the subject, it is apparent that most of the imformations on diseases of prawns relate to the adult and studies on the pathology of larvae and postlarvae are limited to a few description of

parasites and reports on their incidence. It is in this context and in the endeaover of providing reliable information on the diseases encountered in the hatcheries, the present investigation on the pathobiology of larvae and postlarvae of penaeid prawns of India is taken up.

The Thesis is presented in nine chapters. Chapter 1 surveys the literature on the diseases of penaeid larvae, postlarvae and adult prawns from India and abroad. This is followed by a chapter on the material and methods employed during the present investigation. In the third chapter, seven cases of diseases and abnormalities encountered in the larvae and postlarvae of Penaeus indicus and P. semisulcatus during the survey carried out in the hatcheries located at different centres of Central Marine Fisheries Research Institute are presented and discussed. The clinical signs, seasonal occurrence and incidence of each of the seven cases are provided along with the information on environmental factors such as salinity, dissolved oxygen, temperature and pH of the rearing medium. The fourth chapter contains the results of the studies on normal heterotrophic bacterial flora associated with eggs, larvae and postlarvae of P. indicus. The fifth chapter deals with the review of the literature on the pathogenic vibrios from the available informations. Morphological, biological, physiological and biochemical characters of the new isolate of Vibrio isolated from the diseased larvae of P. indicus are studied and discussed in the sixth chapter. In the seventh chapter, the pathogenicity of the new isolate of Vibrio on the larvae and postlarvae of P. indicus, P. semisulcatus, P. monodon and adult P. indicus is studied. The eighth

chapter presents the result of histopathological observations made on the various vital organs of uninfected and infected larvae and adult P. indicus, and postlarvae of P. monodon. Finally in the ninth chapter, eleven antimicrobial agents were tested against the new isolate of Vibrio and the results discussed.

The disease syndromes such as Nitzschia closterium infestation, parasitic protozoan infection, parasitic dinoflagellate infection and appendage necrosis encountered in the larval and postlarval stages of P. indicus are reported for the first time from India. N. closterium was proved harmful to the larvae of P. indicus experimentally. Detailed studies on a bacterium responsible for appendage necrosis in larvae of P. indicus, which is found to be different from the known vibrios in the literature on the basis of its morphological, biological, physiological and biochemical characters; pathogenic mechanism of the new isolate of Vibrio; histopathological observations on the vital organs of the infected larvae, postlarvae and adult prawns, and control of Vibrio - infection by antibiotics constitute original contributions in the thesis. The information gathered and the results presented would not only add to the present knowledge on the pathology of penaeid prawns of India, but also would greatly help in the management of hatcheries to produce quality seeds averting severe losses attributable to diseases.

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CHAPTER 1

GENERAL INTRODUCTION

In the context of increasing interest in penaeid prawn culture in coastal waters, establishment of commercial hatcheries for large scale production of seed is being planned by all the maritime states of India. In the hatchery operations, adopting different systems, mortalities (ranging from 1% to 100%) of larvae and postlarvae have been reported frequently. Such mortalities are brought forth by several biotic and abiotic factors, among which diseases contribute significantly to the cause of large scale mortality.

Most of the scientific studies on the diseases of marine animals have come forth only during the past four decades. Sindermann (1970) has given an excellent review and a bibliography on the diseases of commercially important marine fish and shellfish. A perusal of this literature reveals that the significant contributions published prior to 1970 on the diseases of crustaceans relate to the works by Reinhard (1956) on parasitic castration and to the accounts by Gordon (1966), Sindermann and Rosenfield (1967) and Johnson (1968). Anderson and Conroy (1968) discussed the role of diseases in the aquaculture of crustaceans.

Several Institutes and workers are actively involved in the investigations on penaeid prawn diseases and this paved the way for

accumulation of valuable information and considerable expansion of our knowledge about their diseases and the technology of disease control. The most important studies in this field since 1970 were by Bang (1970,1983), Johnson (1970), Rosen (1970), Sprague (1970,1978), Sindermann (1971a,b, 1977, 1979, 1981), Alderman (1973), Unestam (1973), Pauley (1974,1975), Stewart (1974,1983), AQUACOP (1977), Overstreet (1978, 1979, 1983), Lewis and Leong (1979), Lightner (1981, 1983), Couch (1981, 1983), Johnson (1983a,b, 1984) and Lightner et al. (1987a).

Among the different groups of crustaceans, much emphasis of disease investigations has been on prawns, obviously due to their economic value and demand. Knowledge of the disease of penaeid prawns has been reviewed a number of times within the past 15 years (Overstreet,1973,1983; Sindermann, 1974; Johnson, 1978; Lightner,1977, 1983,1985; Couch,1978,1983). Besides these, the valuable studies by Villela et al. (1970), Barkate (1972), Feigenbaum (1973), Barkate et al. (1974), Johnson (1974a), Lightner et al. (1975), Delves-Broughton and Poupard (1976), Gacutan et al. (1977), Liao et al. (1977), Nurdjana et al. (1977) and Perez Alvidrez (1977) have greatly contributed to the fund of data on the diseases of prawns. While the knowledge on the diseases of crustaceans in general and of penaeid prawns in particular is fairly developed and progressive in the advanced countries and as revealed from the above cited investigations and reviews, the information on the subject from India is limited. Among the earlier works, the most significant contribution to the knowledge of crustacean parasites

was by Chopra (1923). Further studies in this field came forth only since the last decade.

In the following section, an attempt is made to briefly review the most valuable studies carried out on penaeid prawn diseases abroad and in India.

An overview of the studies carried out abroad

Viruses, bacteria, fungi, protozoans, trematodes, cestodes, nematodes and parasitic crustaceans cause diseases in penaeid prawns. Apart from these, dietary deficiencies, environmental stress as well as pollution and toxic algal blooms in the water also bring forth diseases.

Viral diseases

Six viral diseases have been reported in cultured penaeid prawns and several additional diseases have been noted to have associated with virus - like or rickettsia - like structures. Three baculoviruses namely Baculovirus penaei, baculoviral midgut gland necrosis virus(BMNV) and Penaus monodon type baculovirus(MBV), the picorna-like virus, infectious haematopoietic and hypodermal necrosis virus(IHHNV) and hepatopancreatic parvo-like virus(HPV) and a reo-like virus in the hepatopancreas have been recognised to cause disease in cultured penaeids (Couch, 1974; Sano et al., 1981; Lightner and Redman 1981,1985b; Lightner et al., 1983a, 1985; Tsing and Bonami 1987).

The occurrence of baculoviruses has been reported in several penaeids such as Penaeus duorarum, P. aztecus, P. japonicus, P. setiferus, P. vannamei, P. stylirostris and P. plebejus cultured on the Northern Gulf of Mexico, the Pacific coast of Central America and New South Wales, Australia (Lightner,1983; Lester et al., 1987; Momoyama,1988). MBV has been encountered in P. monodon in Philippines, Taiwan, Tahiti, Hawaii, Mexico, Malaysia and Indonesia (Lightner and Redman, 1981; Lightner et al., 1983a; Anderson et al., 1987; Nash et al., 1988) and the BMNV has been reported in P. japonicus cultured in southern Japan (Sano et al., 1981). These baculoviruses infect epithelial cells of the hepatopancreas of protozoea through adult life stages and the midgut epithelium of larvae and postlarvae, often resulting in high mortalities. B. penaei and BMNV have often caused serious epizootics in the larval and early postlarval stages in the hatcheries (Couch, 1981; Sano et al., 1981). The viral attack on the epithelial cells causes nuclear hypertrophy, proliferation of nuclear membrane, chromatin diminution and nuclear degeneration. In nature, the transmission of B. penaei probably takes place by feeding of the infected prawn by the non-infected ones (Couch, 1978) or by waterborne exposure (Sano et al., 1981). Recently Momoyama and Sano (1988) successfully transmitted BMN virus to the mysis larvae of P. japonicus exposed to the medium inoculated with the virus. Couch (1976) however was not able to enhance Baculovirus prevalence in P. duorarum by exposing them to low levels of Aroclor 1254, Mirex or Cadmium.

Infectious hypodermal and haematopoietic necrosis virus(IHHNV) has been reported in P. stylirostris (Lightner et al., 1983b; Bell and Lightner, 1987). Positive IHHN infections have been achieved in juvenile P. aztecus, P. duorarum, P. setiferus and P. japonicus following experimental exposure to IHHNV (Lightner et al., 1985). This viral disease is diagnosed by the presence of eosinophilic inclusion bodies within the nuclei of cuticular hypodermis, haematopoietic or connective tissue cells which are completely destroyed in acute cases.

Parvo-like virus(HPV) was first recognised in P. merguiensis cultured in Singapore and in Malaysia (Lightner and Redman,1985b). In addition to P. merguiensis, HPV has caused high mortalities in cultured populations of juvenile P. orientalis from Quing dao, Peoples Republic of China, in P. semisulcatus from Kuwait and in P. monodon from Philippines (Lightner et al., 1985). This disease has been diagnosed by necrosis and atrophy of the hepatopancreas, accompanied by the presence of large prominent basophilic, PAS-negative, Feulgen-positive intranuclear bodies in affected hepatopancreatic tubular epithelial cells.

Tsing and Bonami (1987) isolated and characterised a reo-like virus associated with high mortalities in tank-reared P. japonicus in South France. It was found in the cytoplasm of F. cells and R. cells of the hepatopancreatic tubular epithelium, where it formed large cytoplasmic viral inclusion. The disease experimentally transferred by inoculation of new

hosts with purified virus, or by feeding pieces of hepatopancreas from infected shrimp to new hosts.

Bacterial diseases

A number of diseases caused by bacteria have been reported from penaeid prawns. The majority of bacterial diseases are of a secondary etiology (Lightner, 1977). In most of the cases of bacterial infections in penaeid prawns, motile, Gram-negative, oxidase-positive and fermentative rods have been isolated (Barkate, 1972; Lewis, 1973a,b; Lightner and Lewis, 1975; Lightner 1977; AQUACOP, 1977; Zeng, 1986a,b). Most isolates have been Virbio species, usually V. alginolyticus, V. parahaemolyticus or V. anguillarum. Certain other Gram-negative rods including Pseudomonas spp. and Aeromonas spp. may occasionally be involved in bacterial syndromes in penaeid prawns.

The bacteria affect all the life stages of penaeid prawns (Lightner, 1977). Bacterial infections in prawns are of two types, localized pits in the cuticle (Anderson and Conroy, 1968; Cook and Lofton, 1973; Cipriani et al., 1980) or localized infections in the body and generalized septicaemia (Lightner, 1983). Vibriosis has been implicated as a frequent mortality factor in juvenile and larval penaeid prawns in culture (Sindermann, 1971b; Lightner and Lewis, 1975). The signs of bacterial infected prawns were gradual change from the usual colourlessness to increasing opaqueness of abdominal muscles, prolongation of clotting time of haemolymph and reduction of haemocyte number (Lightner, 1977; Lightner and Lewis, 1975).

V. parahaemolyticus is the causative agent of gastroenteritis associated with the consumption of raw sea food during warm summer months (Vanderzant et al., 1970; Thatcher and Clarke, 1968). V. parahaemolyticus has caused death of the blue crab (Callinectes sapidus) (Krantz et al., 1969) and of the Gulf of Mexico shrimp (P. aztecus) (Vanderzant et al., 1970). Vanderzant et al. (1970) have reported that addition of 3% inoculum of V. parahaemolyticus (24 hr culture in BHI broth) to an aquarium caused the death of the brown shrimp (P. aztecus) in a few hours.

Other vibrios such as V. anguillarum, V. alginolyticus, and V. algosus have been found to be pathogenic to shrimps (Lightner and Lewis, 1975; Leong and Fontaine, 1979). Most of the strains of V. alginolyticus have caused death of all the shrimps tested so far within 24 hr of inoculation (Lightner and Lewis, 1975). Leong and Fontaine (1979) have assessed the virulence of four species of Vibrio in penaeid prawn (P. setiferus) and reported V. parahaemolyticus to be the most virulent species to white shrimp, followed by V. anguillarum, V. algosus and V. alginolyticus in that order. Larval mortalities due to V. harveyi and V. splendidus have been reported in P. monodon hatcheries in many parts of Panay Island, Philippines (Pitago, 1988).

Vibrio spp. as well as members of the genera Beneckea and Pseudomonas with chitinolytic capacities are also responsible for another significant shell disease in the cultured penaeids (Cook and Lofton, 1973).

P. aztecus, P. japonicus and P. merguiensis succumb often due to white pleura disease, whereas P. monodon does not get affected by this disease even if it is reared in the pond containing the bacteria carrying the disease or fed with the infected prawns (AQUACOP, 1977). Recently Takahashi et al. (1984, 1985) have isolated Vibrio from the dieseased postlarvae of Kuruma prawn P. japonicus, and have reported it to be pathogenic as revealed from the inoculation experiments. The efficiency of antibiotic therapy seems to indicate a bacterial orgin for an abnormal swimming behaviour seen in P. merguiensis and P. aztecus, where the prawns whirl with confused movements and then die lying on their backs (AQUACOP, 1977).

Besides the above mentioned bacteria, Leucothrix mucor and Leucothrix - like filamentous ectocommensal bacteria occur on many species of marine and estuarine crabs, shrimps, prawns, their eggs, and on cultured Artemia salina (Johnson et al., 1971; Shelton et al., 1975). L. mucor is a saprophyte and does not penetrate the cuticle (Shelton et al., 1975; Couch, 1978). The thick mat formed by filamentous bacteria on eggs and on gills interferes with respiration and other metabolic exchanges. The larvae and postlarvae get entangled with the filaments, which inturn interferes with their normal behaviour and moulting (Nilson et al., 1975; Lightner and Supplee, 1976). This filamentous bacteria appear in culture systems particularly when the stocking density is high, the water is rich with organic substrate and high temperature prevails (Ishikawa, 1966,1967;

Barkate et al., 1974; Johnson, 1974a; Lightner, 1975, 1977, 1978a, 1983; Lightner et al., 1975; Steenberger and Schapiro, 1976).

Fungal dieases

Fungal diseases are very common in penaeid prawns, particularly in larval and postlarval stages. Several species belonging to phycomycetes fungi and a single genus of the imperfect fungi are involved in causing fungal disease in all the life stages of penaeid prawns. Two general types of fungal diseases, systemic mycosis and localised mycosis, occur in cultured penaeid prawns. The systemic mycosis of larval and postlarval penaeids causes severe mortalities in penaeid hatcheries throughout the world (Lightner, 1977; AQUACOP, 1977; Lightner and Fontaine, 1973; Barkate et al., 1974; Bland, 1975). Chytriodinium parasiticum is found to be parasitic on the eggs believed to belong to penaeid shrimps in the Mediterranean region (Cachon, 1968). Lagenidium callinectes and related species including Sirolpidium like fungus belonging to the phycomycetes fungi have been responsible for epizootics in eggs and larvae of cultured penaeid prawns (Cook, 1971; Lightner and Fontaine, 1973; Barkate et al., 1974; Bland, 1974; 1975; Lightner, 1975,1977,1981,1983,1985; Baticados et al., 1977; Gacutan and Baticados, 1979). Other phycomycetes fungi such as Atkinsiela dubia in P. aztecus (Lightner, 1983), Halipthoros milfordensis in P. duorarum and P. setiferus (Lightner, 1977; Tharp and Bland, 1977), H. phillippinensis in P. monodon (Hatai et al., 1980) and an unidentified phycomycete in P. aztecus (Overstreet, 1973) have also been reported.

L. callinectes is apparently a very active pathogen in larvae of the brown shrimp P. aztecus (Lightner, 1975). It replaces the larval tissues and produces extramatrical germtubes and mortalities may reach 100% within two days (Gacutan and Baticados, 1979; Lightner, 1977). The pathogenesis of the disease has been described in detail by Lightner and Fontaine (1973) and Lightner (1981). P. aztecus is the most sensitive to fungal disease followed by P. monodon, P. merguiensis and P. japonicus in the decreasing order (AQUACOP, 1977). The infection by Lagenidium and Sirolpidium to the larval shrimp occurs through the parent brood stock or through the carrier hosts in the sea water supply, when the fungal zoospore attaches to and encyst in the egg or the larva (Lightner, 1983).

Only one member of imperfect fungus Fusarium solani has been responsible for mortalities in captive populations of several penaeid prawns (Johnson, 1983a). This fungus has been reported from P. japonicus (Egusa and Ueda, 1972; Fukuyo, 1974; Fukuyo and Egusa, 1974; Guary et al., 1974; Hatai et al., 1978; Momoyama, 1987), P. aztecus (Johnson, 1974b), P. setiferus, P. occidentalis (Lightner, 1977), P. californiensis, P. stylirostris and P. vannamei (Lightner, 1975; Laramore et al., 1977; Lightner et al., 1979b). This is an opportunistic pathogen (Lightner, 1981) and has been responsible for mortalities in several species of captive penaeids in North and Central America and Tahiti (Lightner et al., 1975; Lightner, 1977).

Egusa and Ueda (1972) have described a serious disease known as "Black gill disease" in P. japonicus caused by F. solani. Lesions in the gills,

at the bases of the appendages and on the cuticle are the internal symptoms of this disease (Lightner, 1981; Egusa and Ueda, 1972; Shigueno, 1975).

The pathogenesis of F. solani has been studied in artificially infected penaeid prawn (Lightner et al., 1981). The histopathology of "Black gill disease" caused by F. solani in P. japonicus has been worked out by Bian and Egusa (1981) while Solangi and Lightner (1976) have studied the cellular inflammatory response of P. aztecus and P. setiferus to injected suspension of conidia of F. solani.

Protozoan diseases

Prawns serve as hosts of symbiotic, commensal, parasitic, and pathogenic protozoans. Sprague and Couch (1971) published an annotated list of protozoan parasites, hyperparasites and commensals of decapod crustacea. A disease, observed by Couch (1978) in protozoeal and mysis stage of brown shrimp (Penaeus aztecus), is caused by an amoeboflagellate placed in the genus Leptomonas. This organism eventually fills the blood spaces and replaces certain soft tissues of the shrimp. It invades the appendages, including eye stalks and eyes (Couch, 1983).

Gregarines are common inhabitants of the guts of wild and pond-reared P. aztecus, P. duorarum, P. setiferus, P. vannamei and P. brasiliensis (Hutton et al., 1959; Kruse, 1959; Sprague and Couch 1971; Overstreet, 1973, 1978; Feigenbaum, 1975; Johnson, 1978; Couch, 1978). Gregarines

were not causing any disease in penaeids even when present in large numbers in the gut (Johnson, 1978). Two genera, Nematopsis and Cephalobolus have been known from penaeids (Kruse, 1959; Overstreet, 1973; Johnson, 1978; Feighenbaum, 1975).

Microsporidians have caused a characteristic disease called as "Cotton" or "Milk shrimp disease" both in the wild as well as pond cultured prawns incurring considerable loss to the production and value (Kruse, 1959; Overstreet, 1973; Lightner, 1977; Johnson, 1978). Microsporidian infected prawns have distinctly opaque body muscle with dark blue or blackish discolouration due to expansion of the cuticular chromatophores (Lightner, 1983). Incidences of cotton shrimp has been reported in penaeid prawns in different parts of the world (Hutton et al., 1959; Iversen and Manning, 1959; Iversen and Vanmeter, 1964; Baxter et al., 1970; Overstreet, 1973). Four species of pathogenic microsporidian are known to occur in the penaeid prawns: Perezia (=Nosema) nelsoni has been found in the muscle of P. aztecus, P. duorarum and P. setiferus (Sprague, 1950; Hutton et al., 1959; Overstreet, 1973; Couch, 1978; Lightner , 1985); Agmasoma (=Thelohania) penaei, has been found infecting the blood vessels, foregut, hindgut, gonads and occasionally the muscle of P. setiferus (Sprague, 1950; Hutton et al., 1959; Overstreet, 1973; Rigdon et al., 1975); a similar but unnamed species infecting ovaries of P. merguiensis has been described by Baticados (1980); a third microsporidian, Thelohania duorara has been reported to infect muscle, gonads and other organ tissues of P. aztecus, P. duorarum and P. brasiliensis (Iversen and Manning, 1959; Kruse, 1959;

Iversen and Vanmeter, 1964; Overstreet, 1973, Iversen et al., 1987) and the fourth microsporidian, Pleistophora sp. and P. penaei have been found infecting the different tissues of P. aztecus, P. setiferus and P. duorarum (Baxter et al., 1970; Constrasitch, 1970; Sparague, 1970; Overstreet, 1973). An unrecorded haplosporean has been found in the hepatopancreas of P. vannamei (Dykova et al., 1988).

A number of species of protozoan have been reported to cause fouling and/or gill disease in all life stages of cultured penaeids (Overstreet, 1983, Couch 1983; Lightner, 1983). The most commonly reported protozoans include stalked peritrichs such as Zoothamnium spp., Epistylis spp. and Vorticella spp., the loricate ciliate, Lagenophrys sp., an undescribed apostome ciliate and the suctorean Acineta spp. (Couch, 1978; 1983; Overstreet, 1978, 1983; Meng and Yu 1980, 1983; Lightner, 1983). These protozoans have been generally found attached on the gills, appendages and body surface of the larval, postlarval, juvenile and adult penaeids in the culture systems and when abundant on the surface of the gills, could cause hypoxia and death (Overstreet, 1973, 1978; Johnson, 1974a; Lightner, 1975, 1977; Lightner et al., 1975; Couch, 1978). Johnson et al. (1973) reported the loss of an estimated 2000 numbers of pond held brown and white shrimp in a single day due to the presence of large numbers of Zoothamnium sp. on the gills. An unidentified apostome, which caused black gill disease in penaeid shrimp, has been explained by Couch (1978). A pathogenic suctorean, identified as Ephelota gemmipara, has been reported in the larvae of P. monodon (Gacutan et al ., 1979b).

Metazoan parasites

The metazoan parasites of penaeid prawns comprise of helminth parasites such as worms, and bopyrid isopods. Worms that have been found in the prawns are trematodes, cestodes and nematodes which may be found in various parts of the body. Most of the species reported to date, appear to have little effect on individual shrimp infested and probably little significant effect on populations of penaeids (Couch, 1978). Hutton et al. (1959) reported an undescribed species of microphallid trematode metacercariae from pink shrimp. Overstreet (1973) also reported an unidentified microphallid metacercaria from abdominal muscles of white shrimp. Opecoeloides fimbriatus is a very common parasite of penaeids.

The encyst of this parasite is found in hepatopancreas, other internal organs and beneath the exoskeleton of prawns. Prochristianella hispida (=P. penaei) is found mainly in the hepatopancreas of the host (P. duorarum). Kruse (1959) described two other trypanorhynchan pleorcercoid larvae from P. duorarum. Hutton et al. (1959), Kruse (1959), Overstreet (1973), Feigenbaum (1975) and Couch (1978) found a small pyriform cestode larval stage commonly in the intestine of penaeid prawns.

Norris and Overstreet (1976) have found that at least two species of Thynnascaris occurred in penaeid prawns of North America. Overstreet (1973) reported two specimens of Spirocamallanus pereirai in the intestine of P. setiferus. Specimens of Leptolaimus sp. and Croconema sp. have been found by Overstreet (1973) in the brown and white prawns. The

bopyrid isopods have been reported to parasitise the brancial chamber of penaeid prawns in nature (Dawson, 1958; Tuma, 1967; Ahmed, 1978; Cheng and Tseng, 1982; Abu-Hakima, 1984). Although the bopyrid infestations have not generally inhibited the growth of the hosts, they have affected the gonadial development, often causing parasitic castration in the hosts (Tuma, 1967; Abu-Hakima, 1984).

Nutritional disease

In addition to the diseases caused by pathogens and parasites, only one nutritional disease syndrome of cultured penaeids has been identified. This disease occurs due to the ascorbic acid deficiency and is popularly known as 'black death disease'. The disease occurs in penaeid prawns which are reared in closed systems, aquaria or flow-through systems in which most or all of the diet is artificial (Lightner, 1977; Deshimaru and Kuroki, 1976; Lightner et al., 1979a). The disease of black death has not been reported in prawns cultured in ponds, tanks or race ways in which there is atleast some algal growth (Lightner, 1977; Lightner et al., 1979a).

Prawns affected by black death disease typically display blackened lesions in the stomach wall, the hind - gut wall, in the gills and in the subcuticular tissues at various locations especially at the junction of the body and appendages (Lightner, 1983). The disease has been observed in P. californiensis, P. stylirostris, P. aztecus and P. japonicus (Deshimaru and Kuroki, 1976; Lightner, 1977, 1983; Lightner et al., 1977, 1979a; Magarelli et al., 1979). Deshimaru and Kuroki (1976), Lightner et al. (1979a) and

Magrelli et al. (1979) have reported that a dietary requirement of 2000 to 3000 mg of the ascoribic acid per kg. of feed is necessary to control the disease.

Diseases caused by environmental stress

Environmental stress such as supersaturation of atmospheric gases low dissolved oxygen levels, sudden temperature or salinity changes, over crowding and rough handling lead to unhealthy state in prawns and in severe cases, lead to large scale mortalities. `Gas bubble´ disease has been reported to occur in penaeid prawns as a result of supersaturation of atmospheric gases, particularly when the dissolved oxygen level reaches or exceeds 250 per cent of the normal saturation of medium (Lightner et al., 1974; Supplee and Lightner, 1976; Lightner, 1983, 1985). The first sign of gas-bubble disease in shrimp is a rapid erratic swimming behaviour, followed by a stuporous behaviour (Lightner, 1983). Examination of fresh preparations of gills or whole tissue under the microscope revealed the presence of gas bubbles (Lightner, 1983). Several other diseases such as spontaneous muscle necrosis (Rigdon and Baxter, 1970; Venkataramiah, 1971a,b, Lakshmi et al., 1978; Nash et al., 1987), cramped tail condition (Johnson, 1975, 1978; Lightner, 1977) and broken back syndrome (Couch 1978) occurred due to changes in environmental conditions. Muscle necrosis was characterised by whitish opaque areas in the striated musculature, especially of the distal abdominal segments (Rigdon and Baxter, 1970). The condition follows periods of severe stress such as over crowding, low dissolved oxygen levels, sudden temperature or salinity changes and rough

handling (Lakshmi et al., 1978). The cramped tail condition appears to be related to sudden increase in the temperature of water and air (Lightner, 1983), while the broken back syndrome which displays a characteristic dorsal separation of the pleural plates covering the third and fourth abdominal segments (Couch, 1978), appears due to a combination of severe salinity, cold temperature and handling stresses.

Mortalities due to toxic agents

A number of algae belonging to the family Oscillatoriaceae have been reported to cause mortalities in cultured penaeid prawns. Blooms of the diatom Chaetoceros gracilis have been reported to be toxic to the larval stages of P. stylrostris and P. vannamei (Simon, 1978). Filamentous blue green algae such as Schizothrix calcicola, Spirulina subsala and Microcoleus lyngbyaceus are also toxic to the cultured populations of P. stylirostris, P. vannamei and P. californiensis (Lightner 1978b, 1983; Lightner et al., 1978; Simon, 1978; Lightner et al., 1980). The blooms of blue-green algae have been shown to cause haemocytic enteritis (HE), particularly in juveniles, when necrosis and haemocytic inflammation of the mucosal epithelium of those portions of gastrointestinal tract that lack a chitionus lining occur (Lightner, 1978b; 1983; Lightner et al., 1978). This leads not only to osmotic imbalance and poor absorption of nutrients, but also to secondary bacterial infection (Lightner, 1978b, 1983; Lightner et al., 1978, 1980). The occurrence of a toxicity syndrome called "Blue shrimp syndrome unknown" (BSX) in P. californiensis and P. stylirostris farmed in Mexico (Lightner, 1983) has been correlated with the occurrence of red tides. A

dinoflagellate, Amphora sp. may infect the prawn and cause melanisation in the gills (Overstreet and Safford, 1980).

Toxic responses of penaeid prawns to pollutants have been reviewed in depth by Couch (1978, 1979). Organochlorines such as DDT, dieldrin, mirex and PCBS; organophosphates such as baytex, dibrom, malathion and parathion and carbamate such as sevin, have adverse effects on penaeids, usually affecting the physiological processes of hepatopancreas and resulting in death of the animal (Butler, 1966; Nimmo et al., 1970; Lowe et al., 1971; Nimmo et al., 1971a,b ; Nimmo and Blackman, 1972; Parrish et al., 1973; Coppage and Mathews, 1974; Couch and Nimmo, 1974a,b; Hansen et al., 1974; Conte and Parker, 1975; Couch, 1978; Schoor and Brausch, 1980). Although the information available on the effects of petroleum products to penaeid prawns is limited, they are known to cause necrotic lesions on the body, gills, lining of the gastric mill and eyes (Mills and Culley, 1971; Anderson et al., 1974; Cox et al., 1975; Yarbrough and Minchew, 1975; Minchew et al., 1979; Neff et al., 1976).

Penaeid prawns are also sensitive to certain heavy metal pollutants. Exposure of prawns to cadmium causes black gill syndrome by impairing the gill cells and consequently leading to the death of the animal (Bahner, 1975; Couch , 1977; Nimmo et al., 1977). Mercuric salts and methylated mercury are extremely toxic with both short term and long term chronic effects to the prawns (Couch, 1978). Mercury is accumulated by prawns and may interfere with their osmoregulatory abilities (Couch, 1978). Nitrogen, which

enters culture systems primarily as organic compounds that are metabolised to ammonia, nitrite and nitrate by resident culture species and/or bacteria, has also been found to be toxic to cultured crustaceans including penaeid larvae and adult prawns when present in excess (Armstrong, 1979; Chin and Chen , 1987). Nitrite is the most toxic of these three compounds.

Toxic effects of chemotherapeutic chemicals

Certain chemotherapeutic agents, which are used routinely in the treatment of aquatic animal, are found to be toxic to penaeid prawns at certain concentrations (Johnson, 1976a; Hanks, 1976). The optimum exposure time of P. monodon larvae to furanace has been determined (Gacutan et al., 1979b). Moulting dealy and morphological defects have been observed in the larvae of P. monodon resulting from a 24 hour exposure to 1.0 and 2.0 mg/1 furanace bath (Gacutan et al., 1979a). Schnick et al . (1979) have given a list of chemotherapeutants and anaesthetics with their relative toxicity to crustaceans including penaeid prawns, while Hatai et al. (1974) dealt with the toxicity of a number of fungicides. Lightner (1977) and Lightner and Supplee (1976) have reported that the concentrations of 5 -10 ppm of $KMnO_4$ or 0.5 and 1.0 mg/l of cutrine plus were toxic to P. californiensis respectively.

Miscellaneous diseases

Besides the above mentioned diseases, several other diseases have been reported, these include tumor like growth (Sparks and Lightner, 1973; Lightner et al., 1987b) lymphoma - like neoplasm (Lightner and Brock 1987),

hamartoma (Overstreet and Van Devender, 1978), blisters (Lightner, 1977; Johnson 1978), `Golden shrimp´ (Johnson, 1978; Lightner, 1983),blue or white eye disease (Lightner, 1983), amoebosis of larvae (Laramore and Barkate, 1979), larval encrustation, multifocal opacities (Lightner, 1983), gut and nerve syndrome or GNS (Lightner et al., 1984), deformed nauplii, appendage necrosis in larvae, white pleura disease (AQUACOP, 1977; Lightner, 1983), red disease (Liao et al., 1977; Lightner and Redman, 1985a) nerve disease syndrome (Katzen et al., 1984), aflatoxicosis (Lightner et al., 1982; Wiseman et al., 1982) and fatty acid infiltration of hepatopancreas (Salser et al., 1978; Lightner, 1983).

Studies carried out in India

Although information on the capture and culture fisheries of prawns of India and on the biology of economically important penaeid prawns is avaliable from a number of contributions, studies on the diseases of prawns are not many. Chopra (1923) in his excellent monograph entitled "Bopyrid isopod parasitic on India Decapod Macrura", described several bopyrid parasites of Palaemon and Penaeus along with their geographical distribution and keys for identification. Following this, there have been only occasional and isolated studies on diseases of prawns except one Ph.D. thesis with well documented information on adult prawn diseases by Soni (1986).

Various bacterial diseases such as myxobacteriosis, hemorrhagic septicaemia, vibriosis and enteric bacterial infection have been reported in

penaeid prawns in India (Mahadevan et al., 1978). Among the bacterial diseases, vibriosis caused by V.anguillarium is the most frequent disease found in P. indicus cultivated in the brackish water fields (Mahadevan et al., 1978). Recently, brown spot disease caused by Vibrio and Aeromonas sp. is also reported in P. indicus (Chandramohan et al., 1980; Lakshmanaperumalsamy et al., 1982). The bacterium Escherichia coli is found to infect the larvae of P. indicus (Mahadevan et al., 1978). The myxobacterial infection caused by Chondrococcus sp. is reported in P. indicus, P. monodon, M. affinis and M. dobsoni cultured in earthern ponds in the brackishwater areas while Pseudomonas fluorescence causing haemorrhgic septicaemia, is encountered mainly in P. indicus and M. monoceros (Mahadevan et al., 1978) Decay of body surface caused by Staphylococcus aureus and E. coli in P. indicus has been observed by Mahadevan et al. (1978).

Among the diseases caused by fungi, large scale mortality in larvae and juvenlies of P. monodon raised in the hatchery has been reported due to heavy infection by fungus Lagenidum sp. (CMFRI unpublished data). Similarly, the fungi Saprolegnia parasitica and Leptolegnia marina have been recorded from the juvenile of P. monodon caught from the backwaters of Cochin (Gopalan et al., 1980). Five different fungi namely Saprolegnia sp., Achlya sp., Aphanomyces sp., Pythium sp. and Leptomitus sp. have been reported in the freshwater giant prawn Macrobrachium rosenbergii (Shah et al ., 1977).

Santhakumari and Gopalan (1980) have reported the protozoan parasites Zoothamnium rigiduro and Stenter coerulens in M.monoceros. Besides these, Epistylis sp. together with Zoothamnium sp. have been encountered in P. monodon causing hypoxia (Issac Rajendran et al ., 1982; Venkatesan et al ., 1985). Occasionally, these parasites have been found to affect the juvenile prawns in the culture ponds where dissolved oxygen level in pond water decreased to 1.0 ppm due to non- flushing of pond water with tidal water (Issac Rajendran et al., 1982).

The "cotton" or "milk shrimp" disease caused by microsporidian parasites in the natural populations of P. indicus, P. semisulcatus, M. monoceros and M. brevicornis caught off Madras, Mandapam, Tuticorin and Cochin has been reported on several occasions (Subrahmanyam, 1974; Thomas 1976; Santhakumari and Gopalan, 1980. Gopalan et al., 1982; Palaniappan et al., 1982; CMFRI unpublished data). Taxonomy, pathogenicity and histopathology of microsporidian parasites have been studied in detail by Soni (1986).

Large number of metacercarian cysts infecting M. monoceros inhabiting the Cochin backwater have been reported by Gopalan et al. (1982) and Syed Ismail Koya and Mohandas (1982). Instances of isopod bopyrid parasites infecting the branchial chamber or attaching to the appendages have been reported in P. indicus, P. semisulcatus, P. merguiensis, P. japonicus, Parapenaeopsis stylifera, M. monoceros, M. dobsoni, M.

breviconis, M. lysianassa and Palaemon tenuipes from natural population (Chopra, 1923; Menon, 1953; Sawant and Kewalremani, 1964; Thomas, 1977; Soni, 1986).

The `soft prawn´ syndrome in P. indicus has been reported (Mahadevan et al., 1978; Rajamani, 1982; Rao, 1983; Soni, 1986, Ramesh,1988) and being studied at the Central Marine Fishereies Research Institute. This syndrome in cultured prawns is generaly encountered during adverse ecological conditions such as low salinities and combinatins of higher temperature and salinities. A tumour on the carapace of P. indicus from grow-out ponds of Prawn Hatchery Laboratory at Narakkal has been reported (Soni, 1986).

At the symposium on the diseases of finfishes and shellfishes in India held at the College of Fisheries, University of Agricultural Sciences, Mangalore in 1982, 6 papers relating to the diseases of prawns were presented. Later, the Central Marine Fisheries Research Institute, Cochin, oraganised a work shop on "Approaches to finifish and shellfish pathology investigation" in January, 1983, where the guidelines for the indentification of disease problems and the rational approaches to be undertaken to tackle the same were discussed.

The foregoing review of the literature shows that the studies on the diseases of larvae and postlarvae of penaeid prawns in India are scanty. However this aspect assumes great importance in the context of large scale production of penaeid prawn seed in the hatcheries and their subsequent

culture in nurseries to meet the quality seed requirements for the rapidly expanding prawn culture industry in the country. This thesis, therefore, focuses to identify the diseases encountered in the larvae and postlarvae of penaeid prawns of India , to describe their characteristics and pathological significance and finally attempt on their control measures. The results obtained are pressented and discussed.

CHAPTER 2

MATERIAL AND METHODS

The present work was carried out from September, 1985 to April, 1988 at the Central Marine Fisheries Research Institute (CMFRI), Cochin. It involved a general survey of the diseases/abnormalities occurring in the larvae and postlarvae of penaeid prawns in nature and in the hatcheries of CMFRI located at three centres and a detailed study on the vibriosis of larvae and postlarvae of Penaeus indicus H. Milne Edwards. The methods of collection of samples for environmental parameters and of data pertaining to infected/abnormal larvae and postlarvae of prawn as well as the techniques involved for microscopic examination of the specimens, common to all studies, are presented in this chapter. Besides these, the methods employed for isolation, purification, preservation and identification, and experimental pathogenicity of bacterial pathogens, and histopathology are also given in detail in this chapter. Material and specific methods employed for antiserum production and evaluating the antimicrobial compound to control the infection caused by vibrios are described in detail in the relevent chapters.

Collection of samples

To study the larval and postlarval diseases of penaeid prawns from nature, plankton samples were collected from fixed stations located at 10m, 20m and 30m depths in the inshore sea of Cochin (Pl. I, Fig.1). A 50cm diameter zooplankton net, made of bolting silk with a mesh size of 0.1mm was employed to collect the zooplankton samples. The net was operated

PLATE I

Fig.1. Map showing the location of collection sites at 10m, 20m and 30m depths off Cochin.

Fig.2. Map showing the location of hatcheries of CMFRI from where the samples were collected.

PLATE I

Fig.1

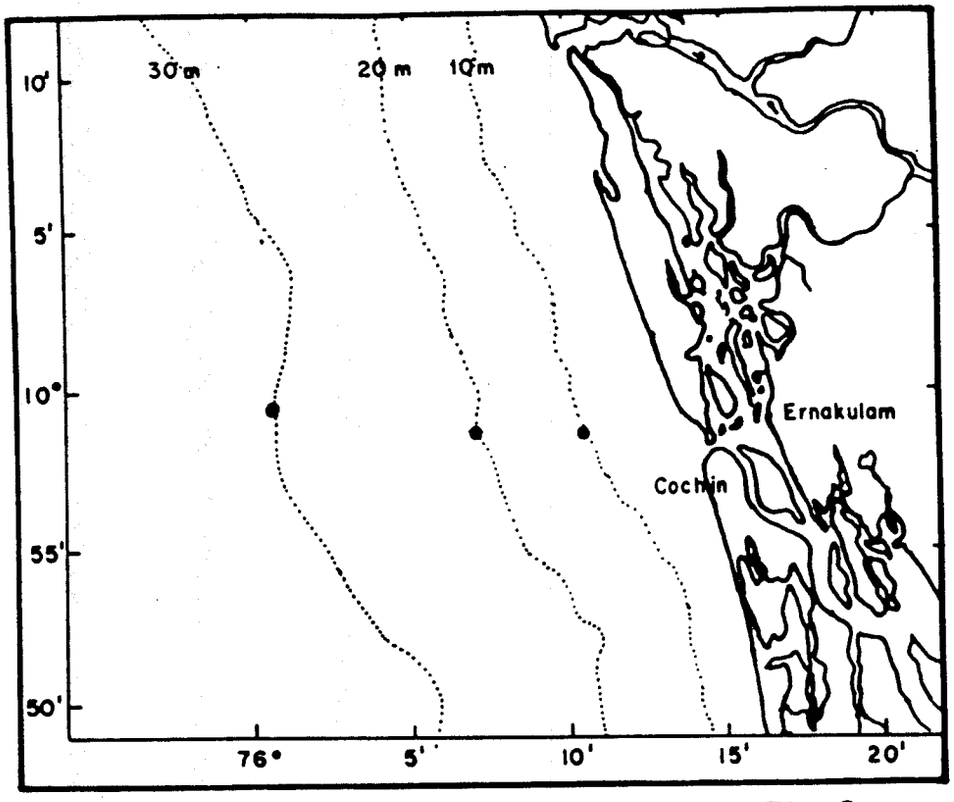
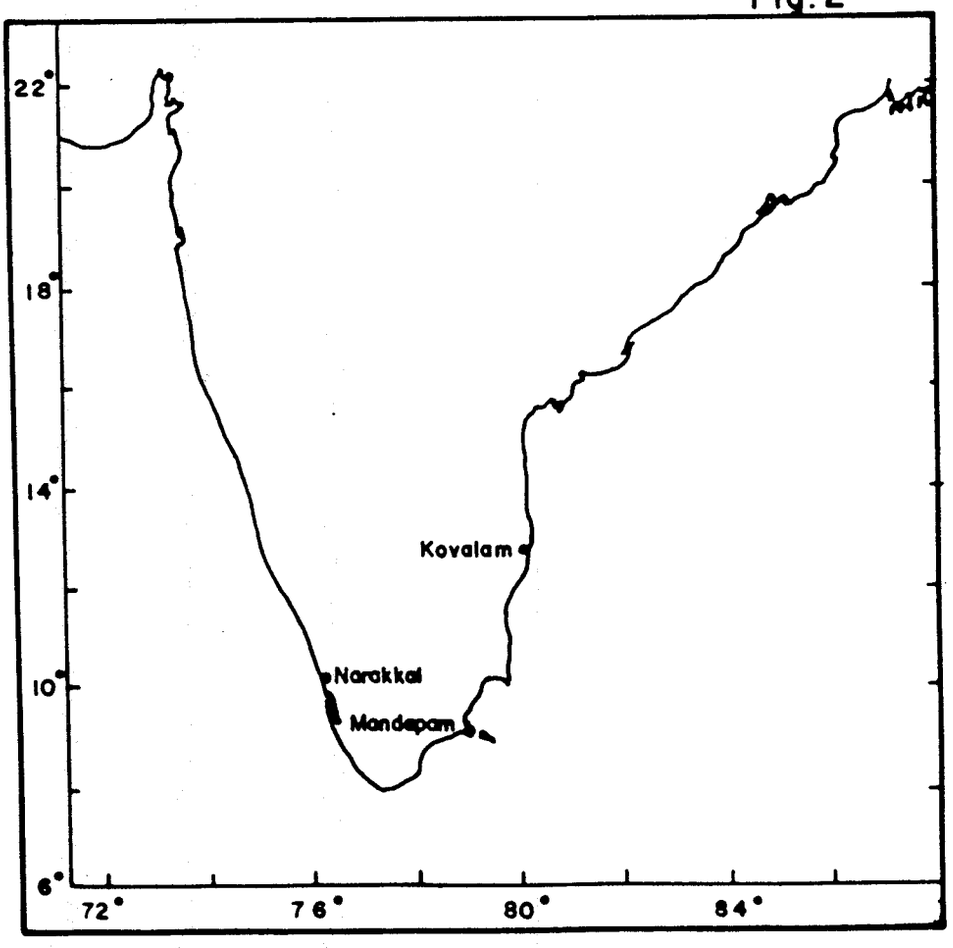


Fig. 2



against the current at subsurface water for about 10 to 15 minutes onboard the research vessel Cadalmin (13.05m OAL) powered with 93 HP engine belonging to the CMFRI. The live zooplankton thus collected was transported to the laboratory where it was analysed for larvae and postlarvae of penaeid prawns. The plankton samples were collected twice in a week during May-June, `86 and September-November, `86. Temperature of the sea water at the collection site was measured onboard the vessel by an ordinary immersible mercury thermometer graded upto 50° C. Water samples for determination of dissolved oxygen and salinity were collected in 125 ml clean glass BOD bottles. To determine the oxygen, the water was collected without agitation following the usual procedure and precautions, and the water samples were fixed immediately with Winkler's solutions. Later, in the laboratory, the salinity of water samples was estimated by argentometric method (Strickland and Parson, 1968) and the dissolved oxygen by the Winkler method (Strickland and Parson, 1968).

The penaeid larvae and postlarvae collected from the plankton were examined carefully with the aid of a stereoscopic dissection microscope (25.2 X). The penaeid larvae and postlarvae observed in the collections were found to be healthy and abnormal or diseased specimens were not encountered among those screened in the plankton samples collected during the period.

The CMFRI has established experimental penaeid prawn hatcheries at Narakkal near Cochin (Pl.II Fig. 2), at Kovalam near Madras (Pl. II, Fig. 2)

and at Mandapam Camp (Pl. I, Fig. 2). The diseased material presented and discussed in the thesis came principally from these hatcheries. At these hatcheries, penaeid prawn seeds are produced following the modified Galveston system. The details of methods of breeding, rearing and related aspects are described by Silas et al. (1985). The author during the course of the study participated in the various aspects of breeding and larval rearing to obtain not only the practical experience in seed production technology, but also to get a greater insight of etiology and larval disease problems in the hatchery. Thus the author participated in the seed production runs at Narakkal for three seasons, during October 1985 - April 1986, October 1986 - April 1987 and October 1987 - April 1988; at Madras he worked for two months from July to August 1986 and at Mandapam Camp, from May to June 1987.

Screening for microbial pathogens and parasites

During the course of rearing, the eggs/larvae/postlarvae of P. indicus/P. semisulcatus were closely and carefully examined with naked eye with the aid of a view pointer to note the general well- being, activity and behavioural pattern. The body surface, rostrum, eyes, appendages, uropod, telson, chromatophore pattern, condition and contents of the gut were scrutinized on the basis of subsample of the larvae taken from the rearing tank for external signs of any disease, parasitic or pathogenic infection or infestation or abnormality. The diseased/abnormal larvae and postlarvae, whenever encountered, were immediately collected with the aid of a scoop net made of bolting silk (50 micron pore size) from the tanks. The

collected specimens were washed thoroughly with sterile sea water and kept in sterilised screw cap bottles containing sterilised sea water for isolating pathogens. The bottles were kept in ice and transported to the laboratory. The isolation of pathogens was carried out within 3 hours of collection. Some of the infected larvae were preserved in fixatives such as 10% neutral buffered formalin, Davidson's fixative or Bouin's fluid for histopathological investigation. In certain cases live larvae were also transported to the laboratory in small polythene transportation bags. The larvae/postlarvae that were suspected to be infected or infested by pathogen other than bacteria or fungi, were fixed in 10% neutral buffered formalin or Davidson's fixative for further studies.

Although monitoring of water quality of the rearing medium was undertaken at regular intervals, particular attention was given to collect the environmental parameters such as temperature, pH, salinity and dissolved oxygen of the rearing in the hatchery at the time of collection of diseased/abnormal larvae from the rearing tank. For temperature measurement, an ordinary immersible mercury thermometer graded upto 50° C (accuracy 0.1° C) was used. An "ITL" make pH meter was employed for determination of hydrogen ion concentration. Besides the collection of data on the water quality of the rearing medium, information on the source and condition of mother prawns used for breeding, number of larvae stocked in the tank, kind and quality of feed given to the larvae and the percentage mortality at different stages were also collected. In addition, the general behaviour of the larvae/postlarvae was also noted.

Isolation and identification of microbial pathogens

Microbial pathogens were isolated from the infected egg, larvae and postlarvae. The procedures followed for isolation and identification of microbes are described below.

Sterilisation

During the present investigation, ultra-violet hood was used to carry out the work of isolating the pathogen to avoid contamination from the environment. The hood was first rendered dust free and cleaned with a neat towel. It was then disinfected with absolute alcohol by the swab method, sealed and sterilised by switching on ultraviolet tube light for a period of twenty minutes.

The glasswares such as petri dish (10 cm in diameter), pipettes (1 ml, 2 ml and 5 ml capacity), test tubes (10 ml and 20 ml capacity) and conical flasks (100 ml, 150 ml and 250 ml capacity) used in microbial analysis of samples were sterilised in hot air oven at 160° C for one and half hours. All the surgical instruments such as scissors, blade, needle, forceps and glass rods were either autoclaved or dipped in absolute alcohol and the excess burned off.

Culture media

The culture medium for isolating the pathogens was chosen after observing the morphology and motility of the pathogens present in the infected larvae/postlarvae under Carl Zeiss-binocular microscope (600X)

(Bullock, 1971; Van Duijn, 1973; Roberts and Shepherd, 1974). The following media were used for isolation.

1.Seawater nutrient agar

Bacto-peptone (Difco)	1.0 g
Beef extract (Difco)	0.3 g
Bacto-agar (Difco)	2.0 g
Aged and filtered seawater	100.0 ml
pH	7.2

2.Seawater nutrient broth

Bacto-peptone (Difco)	1.0 g
Beef extract (Difco)	0.3 g
Aged and filtered seawater	100.0 ml
pH	7.2

Seawater nutrient agar and broth were used for primary isolation of pathogens, purification and maintenance of the isolates.

3. ZoBell's agar (Hi-Media)

4. MacConkey agar (Hi-Media)

5. TCBS agar (Thiosulfate citrate bile salt) (Hi-Media)

6. Pseudomonas agar (Hi-Media)

7. Alkaline seawater peptone

Bacto-peptone (Difco)	1.0 g
Aged and filtered sea water	100.0 ml
pH	8.6 + 0.2

8. Mycological agar (Hi-Media)

9. Sabouraud dextrose agar (Hi-Media)

10. Peptone-yeast extract glucose agar

Bacto-peptone (Difco)	0.125 g
Yeast extract (Difco)	0.125 g
Glucose	0.300 g
Bacto-agar (Difco)	1.5 g
Aged and filtered seawater	100.0 ml
pH	6.8 + 0.2

All the media were sterilised at 115° C for 10-15 minutes and allowed to cool. About 15 ml of the cold sterile medium was then poured to sterilised petri dish and allowed to solidify.

The infected parts of the larvae/postlarvae were cut and kept in sterilised embryo cup along with sterilised sea water and were homogenized with aid of blunt portion of sterilised glass rod. One or two drops of homogenized samples were kept on suitable culture media plates and

streaked on the surface of the agar plates by sterilised bend glass rod. In certain cases, the samples were diluted with sterile sea water to avoid over growth of the bacterial isolates as given by Bullock (1971,1972). After inoculation, the agar plates were incubated at 30° C for 24 to 48 hours along with control plates without inoculam. After the incubation period, the inoculated petri plates were examined carefully for bacterial growth. Morphologically similar and dominant bacterial colonies were selected and streaked on nutrient agar plates to obtain pure culture. For broth culture, one or two drops of homogenized samples were added into the test tubes containing sterilised liquid broth. After inoculation, the test tubes were incubated at 30°C for 24-48 hours. After 48 hours, one or two drops were kept on suitable culture media and streaked on the surface of the media to obtain pure culture. After obtaining the pure culture, isolates were preserved for further study. In the present study, two methods of preservation, namely, oil sealing and preservation in semisolid medium were followed to preserve the bacterial isolates. Bacterial isolates were preserved on seawater nutrient agar by sealing them with sterile paraffin oil (Weiss, 1957). In the other method, the isolates were maintained by stab inoculation of the organism on semi solid seawater nutrient agar in screw cap bottles. After incubation for 24 hours the bacterial isolates were stored at 4°C.

Identification of pathogen

Morphological, biological, physiological and biochemical characters of the bacterial isolates isolated from the diseased specimens were studied.

The bacteria were grown on sea water nutrient agar for biochemical and physiological tests. Liquid cultures used as inocula for various tests performed were grown in peptone seawater.

Morphological characters of the isolates

Colonial morphology of the bacterial isolates was examined on ZoBell's agar and TCBS agar after 24 hours incubation, according to the criteria described by Colwell and Weibe (1970).

Gram-staining: After 24 hours of incubation at 30°C on ZoBell's agar, the organisms were stained by Hucker's modification of the Gram-stain to study the micromorphology and Gram-staining reactions of the bacterial isolates. A small amount of surface growth of the isolate was removed from ZoBell's agar medium and mixed well with a drop of sterilised distilled water on a clean microscope slide with the aid of an inoculation needle to make a smear. The smear was the air dried, heat-fixed and stained with crystal violet and safranin as described by Hucker and Conn (1923, 1927). The stained slides were examined under the microscope (1000 X) to study the micromorphology and Gram- staining reactions of the isolates.

Motility test: The motility was determined by the `hanging drop' method (Collins and Lyne, 1976). A small drop of liquid bacterial culture was placed in the centre of a square glass cover slip with the aid of an inoculating loop. A drop of water placed at each corner of the cover glass. A microscope cavity slide was inverted over it so as to obtain bacterial

suspension in the cavity. The slide with hanging drop of bacterial culture was observed under the microscope (600 X) to determine the motility of bacteria.

For testing swarming, the cultures were inoculated on ZoBell's agar along with a known positive organism (V. alginolyticus) and observed after overnight incubation at room temperature.

Physiological characters of the isolates

a) Temperature tolerance test: The ability of the organism to grow at 5, 10, 15, 30, 37 and 42°C was tested by inoculating a drop of 24 hours broth culture into the test tube containing 1% peptone seawater, which had been preincubated at the temperature of incubation. The tubes were examined after 24 hours for the presence of growth. The growth of the organism was measured by colorimeter (Erma, Japan) at 530 nm and peptone seawater without inoculam was treated as control.

b) pH tolerance test: The growth of bacterial isolates at pH 5, 7, 8, 9 and 10 was determined using 1% peptone seawater adjusted to the appropriate pH with HCl or NaOH.

c) NaCl tolerance test: NaCl tolerance ability of the bacterial isolates was tested by growth in 1% peptone water containing 0, 1, 2, 3, 6, 8 and 10% (W/V) NaCl. The growth of the organism was measured by colorimeter at 530 nm as mentioned above.

d) The ability to tolerate brilliant green (0.00125%), Pyronin G (0.001%), neutral red (0.001%) or crystal violet (0.001%) was tested on seawater nutrient agar, with appropriate additions. Cultures were also tested for ability to grow on CLED agar (Hi-Media), MacConkey agar and Teepol broth.

e) Sensitivity to antibiotics: The reaction to antibiotics was determined by placing sensidiscs of the antibiotics (Hi-Media) on sea water nutrient agar plates streaked with the test organism. The isolates were tested for susceptibility to bacitracin (10 units) chloramphenicol (30 µg), cloxacillin (1 µg), nalidixic acid (30 µg) nitrofurazone (100 µg), oxytetracycline (30 µg), olendomycin (15 µg), penicillin (10 units), polymyxin B (300 units), streptomycin (10 µg) and tetracycline (30 µg).

f) Sensitivity to 0/129: Sensitivity to the vibriostatic compound, 2, 4 - diamino-6, 7 diisoprophyl pteridine phosphate (0/129) was determined by dropping a crystal of 0/129 (Sigma, U.S.A.) into a seawater nutrient agar plate immediately after seeding, as for antibiotic testing. Sensitivity to 0/129 was recorded when a zone of clearing was noted (Shewan et al., 1954).

Minimal inhibitory concentrations (MIC) of 0/129 was determined by using plates of seawater nutrient agar amended with 0, 5, 10, 50, 100, 150 and 300 µg of 0/129 per ml of the medium.

Biochemical characteristics

Arginine dihydrolase, lysine and ornithine decarboxylases were detected using the medium of Moller (Lovelace and Colwell, 1968). Nitrate reduction was determined in 5-day old peptone water cultures containing 0.1% KNO_3, using the Griess-Ilosvay reagents and zinc powder test for false negatives (ZoBell, 1932). Pheylalanine breakdown was tested by the method of Shaw and Clarke (1955). Voges - Proskauer and methyl red tests were performed by the method of Mackie and McCartney (1953). Catalase was detected by adding bacterial culture from seawater nutrient agar to a drop of 2% hydrogen peroxide (Colwell and Wiebie, 1970). Oxidase was determined using the method of Kovacs (1956). Hydrogen sulphide production was detected on Triple Sugar Iron agar (Hi-Media) after 2 days of inoculation (Collins and Lyne, 1976). Christensen's medium was used to detect the production of urease (Collins and Lyne, 1976). Indole production was tested in 1% peptone broth using the Kovacs reagent (Kovacs, 1928). Ammonia production was detected by the method of Colwell and Wiebe (1970).

Cholera-red reaction was tested by adding conc. H_2So_4 (0.5 ml) to a 2 day old culture in peptone seawater containing 0.001% KNO_3, (Beam, 1959).

Production of reducing compounds from gluconate was tested by the method of Haynes (1951).

The production of acid from a variety of carbohydrates was detected by the method of Hugh and Leifson (1953). The carbohydrate solution was

filter-sterilised and added to the OF basal medium at a final concentration of 1.0% . The following carbohydrates were tested: adonitol, arabinose, cellobiose, dextrin, dulcitol, ethanol, fructose, galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. Production of gas from glucose was detected using the OF basal medium of Hugh and Leifson (1953) without agar. Inverted Durham tube was inserted into the broth to capture any gas produced by the culture. Formation of gas was recorded after incubation for 7 days.

Degradation of organic compounds

Gelatin hydrolysis was tested by inoculating seawater nutrient agar containing 0.4% gelatin. After incubation, the plates were flooded with acid mercuric chloride (Smith and Goodner, 1958). A positive reaction was detected by appearance of clear zone around the bacterial colonies.

Starch hydrolysis was tested by growing the cultures on starch agar medium. The cultures were incubated for 24 hours and then flooded with a dilute iodine solution. Positive reaction was detected by a clear unstained zone around the colony.

Casein hydrolysis was determined on seawater nutrient agar to which 5% skim milk had been added (Collins and Lyne, 1976). Positive reaction was detected by clearing around colonies of casein hydrolyzing organisms. Chitin digestion was detected by using the method of Lingappa and Lockwood

(1962). The chitinoclastic activity was detected by a clear zone around the bacterial colonies.

Aesculin hydrolysis was done using the method of Sneath (Collins and Lyne, 1976). Positive reaction showed the blackening of the cultures in 2 - 7 days. Tributyrin hydrolysis was detected using agar after 24 hours incubation (Collins and Lyne, 1976). Positive reaction was determined by the appearance of clear zone around the colonies of lipolytic organisms. The alginase activity was detected by adding sodium alginate to the seawater nutrient agar (Furniss et al., 1979). Alginolytic activity was detected by pitting around the cultures.

The activity of deoxyribonuclease was detected on deoxyribonuclease test agar incorporated with DNA (0.2%) (Collins and Lyne, 1976). Positive reaction was detected by the appearance of clear zone around the bacterial culture 2-5 minutes after flooding the plate with 1 M HCl.

Utilization of citrate was tested on Simmons citrate agar (Hi- Media) (Collins and Lyne, 1976).

Utilization of sole carbon source

Sole carbon source utilization tests were carried out using the method of Stevenson (1967). The carbon sources were filter-sterilised and adjusted to the final concentration of 1% . The final 1% sterile carbon source was added to the sterilised medium of following composition: Ammonium

dihydrogen phosphate 0.1 g; Potassium chloride 0.05 g; Magnesium sulphate 0.05 g; agar 1.5 g, aged sea water 100 ml and 0.5 ml of 0.2% bromothymol blue. The positive reaction was recorded by the production of acid or growth of bacterial culture in the inoculated medium. The carbon sources included adonitol, alanine, arabinose, cellobiose, dextrin, dulcitol, ethanol, fructose, galactose, gluconate, glycerol, inositol, lactose, mannitol, mannose, melibiose, phenol, phenylalanine, putrescine, raffinose, rhamnose, salicin, sodium acetate, sodium alginate, sodium citrate, sorbitol, sucrose, trehalose, tyrosine and xylose.

All the tests carried out in this study were conducted in triplicates. The composition of the media used in this study is given below.

Aesculin Hydrolysis Medium

Bacto-peptone (Difco)	10.0 g
Aesculin	1.0 g
Ferric citrate	0.5 g
Bacto-agar (Difco)	15.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.5

Agar	15.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.4

Dextrose Phosphate Medium

Dipotassium hydrogen phosphat	5.0 g
Bacto-peptone (Difco)	5.0 g
Dextrose	5.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.5

The Voges-Proskauer and methyl red tests were performed on bacterial isolates in this medium.

DNAse Test Agar(Hi-Media)

Tryptose	20.0 g
Agar	15.0 g
Deoxyribonucleic acid (Sigma)	2.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.3

Gelatin Hydrolysis Medium

Bacto-peptone (Difco)	2.0 g
Beef-extract (Difco)	2.0 g
Gelatin (Hi-Media)	12.0 g

Bacto-agar (Difco)	15.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.2

Gluconate Test Medium (Hi-Media)

Peptone	1.5 g
Yeast extract	1.0 g
Dipotassium hydrogen orthophosphate	1.0 g
Potassium gluconate	40.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.0

Hugh and Leifson's Medium or OF Medium

Bacto-peptone (Difco)	10.0 g
Dipotassium hydrogen orthophosphate	3.0 g
Bacto-agar (Difco)	8.0 g
Phenol red	10 ml of 0.1% solution
Aged and filtered seawater	1000.0 ml
pH	7.2

The medium was autoclaved (115° C) for 15 minutes and allowed to cool to 50° C. The sterile (filtered) carbohydrate solution was added to give a final concentration of 1 per cent. The medium was poured into the test tubes aseptically for test.

MacConkey's agar

Bacto-peptone (Difco)	17.0 g
Proteose-peptone (Difco)	3.0 g
Lactose	10.0 g
Bile salts No.3	1.5 g
Bacto-agar (Difco)	15.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Aged and filtered seawater	1000.0 ml
pH 7.5	

Moller's Medium

Bacto-peptone (Difco)	5.0 g
Yeast extract (Difco)	3.0 g
Dextrose	1.0 g
Aged and filtered seawater	1000.0 ml

The ingredients were dissolved in the seawater by heat and pH was adjusted to 6.7. The medium was autoclaved (115° C) for 15 minutes and allowed to cool to 50 C. 10 ml of 0.2% bromocresol purple was added. To 100 ml of sterile medium, 0.5 g of the appropriate amino acid (arginine, lysine and ornithine) was added. Again the medium was sterilised by steaming.

Seawater Peptone

Bacto-peptone (Difco)	10.0 g
Potassium nitrate	2.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.) 7.5	

Simmon's Citrate Agar

Sodium ammonium phosphate	0.8 g
Ammonium dihydrogen phosphate	0.2 g
Magnesium sulphate	0.2 g
Trisodium citrate	2.0 g
Bromothymol blue	0.08 g
Bacto-agar (Difco)	15.0 g
Aged and Filtered seawater	1000.0 ml
pH (approx.) 7.5	

Starch Agar Medium

Starch (soluble)	2.0 g
Beef-extract (Difco)	3.0 g
Bacto-agar (Difco)	15.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.) 7.2	

Teepol Broth

Bacto-peptone (Difco)	40.0 g
Yeast extract (Difco)	6.0 g
Lactose	30.0 g
Phenol red	0.2 g
Teepol 610 (BDH)	4.0 ml
Aged and filtered seawater	1000.0 ml
pH (approx.) 7.5	

Tributyrin Hydrolysis Medium

Bacto-Peptone (Difco)	5.0 g
Yeast extract (Difco)	3.0 g
Tributyrin (Hi-Media)	10.0 g
Bacto-agar (Difco)	15.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.) 7.5	

Triple Sugar Iron Agar (Hi-Media)

Peptone	5.0 g
Proteose peptone	15.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Lactose	10.0 g
Saccharose	10.0 g
Dextrose	1.0 g

Ferrous sulphate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulphate	0.3 g
Phenol red	0.024 g
Agar	12.0 g
Aged and filtered seawater	1000.0 ml
pH (approx.)	7.4

Experimental Pathogenicity

Test animals and seawater

The larvae and postlarvae of Penaeus indicus, P. monodon and P. semisulcatus used for the various experiments were obtained from the Marine Prawn Hatchery Laboratory, Narakkal; Regional Shrimp Hatchery, State Fisheries Department, Azhikode and Regional Centre of CMFRI, Mandapam respectively. The adult P. indicus (90-110 mm) were collected from the grow-out ponds attached to the Prawn Hatchery Laboratory, Narakkal. Detailed informations such as larval stages used, larval density, number of replicates, and duration of pathogenicity experiments are given in the Table 2.1.

Table 2.1. Details of larval stage, duration of experiment and larval density employed in the pathogenecity experiments.

Larval Stage	No. of replicates	Duration of experiment (hrs)	No. of larvae used in each concentration
Nauplius	3	24	75
Protozoea	3	80	60
Mysis	3	72	30
Postlarva I	3	96	18
Postlarva	3	96	12

Table 2.2. Concentrations of different species of *Vibrio* used in the pathogenecity experiment on the larvae and postlarvae (immersion method) of *Penaeus indicus*, *P. monodon*. *P. semisulcatus* and on the adult *P. indicus* (injection method).

Bacteria/ Host	Concentrations used (No. of bacterial cells per ml of filtered seawater)				
	1	2	3	4	5
Vibrio sp. 2448-88					
Larvae & Postlarvae	35×10^4	35×10^5	35×10^6	35×10^7	35×10^8
Adult	$70 \times 10^{5*}$	$70 \times 10^{6*}$	$70 \times 10^{7*}$	$70 \times 10^{8*}$	$70 \times 10^{9*}$
V. alginolyticus	37.8×10^4	37.8×10^5	37.8×10^6	37.8×10^7	37.8×10^8
V. parahaemolyticus	34.2×10^4	34.2×10^5	34.2×10^6	34.2×10^7	34.2×10^8

* No. of bacterial cells injected per adult prawn.

The active and healthy larvae and postlarvae were collected, washed with sterile seawater to remove the food and other adsorbed detritus adhering to the body and maintained in 5 litre clean glass beaker containing approximately 3.5 litres of sterilised seawater for nearly 3-5 hours for acclimatisation before experiments. The water was provided with good aeration. No food was given to nauplius larvae since they depend on the yolk material present in the body. The protozoea and mysis stages were fed with Chaetoceros sp. and Skeletonema sp. twice in a day. The postlarvae were fed with a mixture of crushed and cooked prawn meat and egg yolk. The adult prawns were maintained in one tonne fibreglass tank, equipped with air-lift biological filter till their use for the experiment. The prawns were fed with cooked clam meat.

Natural seawater was used in all the experiments. The seawater pumped from the adjacent sea was initially stored for sometime to settle the sand and particulate matter. This seawater was filtered again in the laboratory through Sartorius filter paper (0.25µ mesh size). The salinity of the seawater used in the experiment with larvae and early postlarvae was adjusted to 30-34 ‰. for postlarvae X and adult prawn, it was maintained at 20 ‰. with the addition of required amount of tap water. The temperature of the rearing medium during the experiments was varying between 28° C and 30° C.

Challenging organisms

Three species of Vibrio and one species of Alcaligenes were used in

the experiments.

The new isolate of Vibrio was isolated from the diseased mysis larvae of P. indicus obtained from the hatchery at Narakkal. V. alginolyticus and V. parahaemolyticus were isolated from P. indicus showing the soft-shell disease syndrome and collected from the grow- out ponds attached to the Marine Prawn Hatchery Laboratory, Narakkal. The methods followed for the isolation of the above isolates were similar to those discussed earlier in the Chapter II. The identification of these bacteria were confirmed by Dr. G.B. Nair, National Institute of Cholera and Enteric Diseases, Calcutta. Alcaligenes sp. was isolated from the normal and healthy larvae of P. indicus. This organism was used as a positive control in the experiments assessing the pathogenicity and virulence of vibrios.

All vibrios were grown on TCBS agar plates and Alcaligenes was grown on nutrient agar plates prepared with sea water. After incubation at room temperature for 24 hours, the culture was harvested by sweeping it into sterile seawater with a folded bend inoculated needle. The bacterial suspension was pipetted up and down in a sterile test tube to break up clumps and aggregates.

Determination of bacterial cell concentration

To determine the level of concentration of bacteria at which 100% mortality of the larvae occurring an experiment was conducted initially. The bacterial suspension prepared at different concentration adjusting with

sterile seawater as discussed above and the optical density at 0.1, 0.3, 0.5, 0.7 and 1.0 was measured by photocolorimeter (Erma, Japan) at 530 nm. Each of these bacterial concentrations was added into the rearing medium of larvae and postlarvae and found out at which O.D. of bacterial concentration caused 100% mortality. It was observed that the bacterial suspension of 1.0 O.D. caused 100% mortality in mysis stage of P. indicus in 72 hours after the inoculation of the new isolate (Vibrio sp. 2448-88). This standard suspension of bacteria contained approximately 1.4 x 10^{12} bacterial cells per ml. This was diluted further by ten fold serial dilutions for 4 times for calculating lethal concentration $_{50}(LC_{50})$ of Vibrio sp. 2448-88 cells for each larval stage (Table 2.2). The pour plate method was used to determine the number of bacterial cells in each dilution used in the pathogenicity experiments.

Pathogenicity experimental set up

a) Immersion method of infection

To determine the optimum density of nauplius, protozoeae, mysis and postlarvae that could be reared respectively for 36, 72, 72 and 120 hrs without changing the water in 500 ml beakers containing 400 ml of seawater, experiments were conducted initially at different larval densities. The result of the experiment showed that 25 numbers of nauplii, 20 protozoeae, 10 mysis, 6 postlarvae I and 4 postlarvae X could be reared without any mortality in 500 ml beaker containing 400 ml of seawater without changing the water during the test period .

In the pathogenicity experiments, the larvae/postlarvae were maintained in sterilised 500 ml glass beaker containing 400 ml filtered sea water. The water was provided with mild aeration throughout the experiment without harming the larvae. The air stones and air tubes were sterilised by immersing in 2.6% sodium hypochloride and then washed thoroughly with sterilised tap water. The beakers were covered with lid in order to prevent contamination.

Protozoea and mysis were fed with Chaetoceros sp. and Skeletonema sp. which were cultured aseptically in 2 litre capacity conical flask containing one litre of filtered seawater fertilized with potassium nitrate, potassium orthophosphate, sodium silicate and EDTA di-sodium salt at the rate of 12 mg, 3 mg, 6 mg and 6 mg respectively. To ensure the experimental level of concentration of bacterial cells in the rearing medium of larvae, the phytoplankton culture was sieved through Whatman No. 41 filter paper and plankton thus collected was added to the rearing medium for feeding the larvae. The postlarvae were fed with a mixture of crushed and cooked prawn meat and egg yolk.

The bacterial suspension of 1.0 O.D. and its four dilutions (Total 5 concentrations) were used in the experiment. For the immersion method of infection, one ml of bacterial suspension from each of these five concentrations was added to the rearing medium of larvae/postlarvae. The number of bacterial cells in the five sets of rearing media of larvae/postlarvae are given in the Table 2.2. One ml of sterilised seawater

was added to the control. Three replicates in each concentration and control were carried out. Animals were checked twice daily for clinical signs of disease and mortality. Dead animals were removed.

b) Infection via intramuscular injection

The adult prawns at the rate of five prawns per tank were maintained in 200 litre capacity fibreglass tanks. The water was provided with good aeration and it was changed daily. The prawns were fed with cooked clam meat.

The same five concentrations of bacterial suspension as mentioned above were used for this experiment. For adult prawn, 0.05 ml of bacterial suspension from each of these five concentrations was injected intramuscularly between the fourth and fifth abdominal segments using 1 cc tuberculin syringe. The number of bacterial cells in 0.05 ml of bacterial suspension used in the experiment is given in Table 2.2. In control, the animals were received 0.05 ml of sterilised normal saline. Animals were examined twice daily for clinical signs of disease and mortality. Dead animals were removed.

c) Oral infection method

Five prawns were individually isolated in aquarium tanks and starved for 24 hours. Each of them was then fed with a piece of prawn meat which was injected with 1 ml of bacterial suspension (1.0 O.D) of the new isolate (Vibrio sp. 2448-88). The prawns were fed three times at 24 hour

interval. After the last feeding with infected meat, the animals were maintained on non-infected meat for a week. In the control, the prawns were fed only with non-infected meat.

Confirmation of pathogenicity

The specific action of Vibrio sp. 2448-88 as a pathogen was confirmed by isolating the isolate of present organism from the experimentally infected moribund and dead larvae, postlarvae and adult prawn to satisfy Koch's postulates. The isolates were isolated from the homogenised samples of experimentally infected ones by spread- plate technique on TCBS agar. The isolates were identified using the procedures described above.

Statistical analysis

LC_{50} (which is defined as that concentration at which 50 % of the population is expected to sustain mortality) values of the bacterial cells of the new isolate of Vibrio (Vibrio sp. 2448-88), V. alginolyticus and V. parahaemolyticus for the nauplius, protozoea, mysis, early postlarva and late postlarva of P. indicus, P.monodon and P. semisulcatus, and adult P. indicus were estimated by probit analysis (Finney, 1952). The method followed involves the following steps: For each x the logarithm of concentration, proportion of mortality (p) (number died/number exposed) is calculated. Empirical probit `y´ corresponding to each `p´ is read from the table 5; Finney, 1952. Basing on these values of x and y regression of y on x, is fitted. $y = a+bx$, where a and b have the usual meanings. Working probit and weighing coefficients are read out from the table 6; Finney,

1952. Regression of working probit on x using

$$Sxx = (Snwx^2 - \frac{(Snwx)^2}{Snw})$$

$$Sxy = (Snwxy - \frac{(Snwx) - (Snwy)}{Snw})$$

$$Syy = (Snwy^2 - \frac{(Snwy)^2}{Snw}) \text{ and}$$

$$\frac{Sxy}{Sxx} \text{ (slope = b) and } \frac{Snwy}{Snw} - b.\frac{Snwx}{Snw}$$

(the intercept = a) is fitted and obtained minimum expected probit. Repeating the iteration till the values of `b´ and `a´ at successive stages do not differ by a pre-determined quantity (in this case the difference is 0.01), X_0 is found out as follows:

$X_0 = \bar{X} + \frac{5 - \bar{Y}}{b}$. Then the estimate of LC_{50} is obtained as

$\hat{LC}_{50}$ = antilog (X_0)

Let $\chi^2_{(k-2)df} = Syy - \frac{(Sxy)^2}{Sxx}$

If $\chi^2_{(k-2)df}$ is not significant then S = 1

If $\chi^2_{(k-2)df}$ is significant then

$$S^2 = \frac{\chi^2}{k-2}$$

The upper and lower limits of $(x_0 - \bar{x})$ are calculated as follows.

$$(x_0 - \bar{x})_u = \frac{1}{1-g}\left[(x_0 - \bar{x}) + \frac{t_s}{b}\sqrt{\frac{1}{\Sigma nw} + \frac{(x_0 - \bar{x})}{Sxx} - \frac{9}{\Sigma nw}}\right]$$

$$= Ru$$

and

$$(x_0 - \bar{x})_l = \frac{1}{1-g}\left[(x_0 - \bar{x}) + \frac{t_s}{b}\sqrt{\frac{1}{\Sigma nw} + \frac{(x_0 - \bar{x})}{Sxx} - \frac{9}{\Sigma nw}}\right]$$

$$= Lu$$

Where $g = \frac{t^2 s^2}{b^2\, Sxx}$

Upper limit of $\bar{x}_0 = \bar{x} + Ru = U\, x_0$

Lower limit of $x_0 = \bar{x} + Lu = Lx_0$

Upper limit for LC_{50} = antilog (Ux_0)

Lower limit for LC_{50} = antilog (Lx_0)

Estimation of LC_{50} and its upper and lowr limits were obtained using the above method by a computer programme developed by Mr. K.Narayana Kurup (Head of FRAD, CMFRI, Cochin).

Histopathology

Healthy, uninfected and experimentally infected protozoea, mysis and adult Penaeus indicus and postlarvae of P. monodon used for the histopathological investigations were fixed in 10% neutral buffered formalin or Davidson's fixative. To ensure appropriate fixation of adult prawn, the fixative (either 10% neutral buffered formalin or Davidson's fixative) was injected to the body of the specimen at the carapace and abdomen with a hypodermic syringe prior to immersing the whole specimens in the fixative. Later, the internal organs such as hepatopancreas, heart, alimentary canal, muscle and gills of the specimens thus preserved were cut and stored in screw cap bottle containing the fixative. In the case of larvae, the whole animal was immersed in the fixative at room temperature. When 10% neutral buffered formalin was used, the fixative was changed after 24 hours and then stored in fresh fixative. For Davidson's fixative the initial fixation time was 48 hours, thereafter the materials were transferred to 70% alcohol and stored.

Decalcification

To facilitate proper sectioning, the specimens, which were fixed in 10% neutral buffered formalin, were decalcified following the decalcification method described by Sanderson (unpublished laboratory techniques, Galveston Laboratory, U.S.A.).

Processing of tissues and staining

For cutting sections of the different tissues in paraffin, dehydration

and clearing of the tissues were carried out at room temperature. The tissues were first washed in two changes of 70% alcohol for one hour each, dehydrated for two hours in two changes of 70% alcohol for one hour each, further dehydrated for one hour each in two changes of 80% alcohol, graded twice in 95% alcohol and in absolute alcohol, cleared through a mixture of absolute alcohol and chloroform (1.1 v/v) and then passed twice in pure chloroform for one hour each. Chloroform was preferred over xylene as the former did not cause the tissue hard and brittle. The tissues, after clearing, were left in a mixture of chloroform and paraffin wax (approximately 1:1) at room temperature overnight. Before embedding, the tissues were impregnated in three changes of paraffin wax with ceresin of 58 to 60 ° C melting point for one hour each. The transverse sections were cut at 5 to 7 µm thickness using a manual rotatory microtome. After deparafinishing in xylene, the sections were hydrated through graded series of alcohol upto 70% and stained with Harris alum haematoxylin and counterstained with 1% alcoholic eosin (Preece, 1972). Some of section were also stained with special stains such as giemsa, and crystal violet and basic fuchsin (Collidge and Howar, 1979) for bacteria wherever necessary. Applying the routine procedure, stained sections were dehyrated through the graded series of alcohol and mounted with glass cover slip in DPX mount through xylene.

Processing and staining of frozen sections

For cutting frozen sections of organs, the fixed materials were impregnated in 6% gelatin at 37°C for overnight and 12% gelatin at 37° C

for 24 hours. After impregnation, the organs were embedded in 12% gelatin. The sections were cut at 15 µm thickness using a histostat (American opticals, U.S.A.) at -20 C. The sections were stained with Oil red-O (Pearse, 1968) and also Sudan-black B (Pearse, 1968). The stained sections were mounted with glass coverslip in glycerin gelatin mounting medium.

Light microscopy and photomicrography

The histological sections were studied using an Olympus binocular compound microscope. Cellular measurements were taken with Olympus microscope fitted with a caliberated ocular micrometer scale having an accuracy upto 10 µm. Photomicrographs were taken with camera (Minolta) attached to American Opticals microscope with projection eye piece 10 X and objectives 10, 20, 40 and 100X using 24 x 36 mm negative film of 125 ASA. The magnification of the enlarged prints was calculated with ocular and stage micrometer.

CHAPTER 3

A SURVEY OF THE DISEASES AND ABNORMALITIES OF LARVAL AND POSTLARVAL PENAEID PRAWNS OF INDIA

INTRODUCTION

Among the penaeid prawns occurring in the coastal waters of India, those belonging to the genera Penaeus, Metapenaeus and Parapenaeopsis are important as they principally support the commercial prawn fisheries of the country. During April 1987 - March 1988, the total annual prawn production was estimated at 184956 tonnes in which the contribution of penaeid prawns was 152767 tonnes (CMFRI Annual Report, 1987-88). In the export trade of fish and fishery products from the country, the penaeid prawns form an important commodity. In the total export of 89125 tonnes of fishery products valued at Rs. 489.55 crores in 1987, this group contributed to 51643 tonnes, valued at Rs.401.00 crores (Nair, 1988). The species belonging to the genera Penaeus and Metapenaeus, besides contributing to the capture fisheries, are farmed in the brackishwater areas following the traditional extensive system of culture, and in recent years, the former group is increasingly sought after for culture, due to high market demand and price, faster rate of growth and larger size.

The biology and fishery aspects of most of the commercial species of penaeid prawns of India have been investigated in detail (George, 1970a,b,c,1972,1978; Kunju 1970; Mohamed, 1970a,b,1973; Rao,1970, 1973;

Kurian and Sebastian, 1975; Silas et al., 1984). The general pattern of their life cycle is that they breed in the sea, and the eggs on further development pass through different larval stages such as nauplius (6 substages), protozoea (3 substages), mysis(3 substages) and reach the postlarval stage within a period of 10-12 days (Pl.II,Fig. 1). As they transform to postlarval stages, they move to shallow productive inshore waters and the adjacent estuaries and brackishwaters wherever available. In these ecosystems, they undergo further development and become juveniles. After spending a part of their life in these regions, they migrate back to sea for growth, maturation and breeding.

Most of the investigations carried out so far on the larvae and postlarvae of penaeid prawns of India relate to the larval development, nutrition, abundance and seasonal variation of prawn seed resources. Larval development of most of the commercially important penaeid prawns such as Penaeus indicus, P. monodon, P. semisulcatus, Metapenaeus affinis, M. brevicornis, M. dobsoni, M. monoceros and Parapenaeopsis stylifera has been studied in detail respectively by Muthu et al. (1978a); Silas et al. (1978); Devarajan et al. (1978); Muthu et al. (1978c); Rao (1978); Muthu et al. (1978b); Mohamed et al. (1978) and Muthu et al. (1978d). Distribution pattern, abundance and seasonal variations of penaeid prawn seeds along the east and west coasts of India have been studied (Rao, 1972; Kuttyamma, 1975; Gopinathan, 1978; Chakraborti et al., 1982; Ramamurthy, 1982; Suseelan and Kathirvel, 1982; Anil, 1983; Ganapathy, 1987). Recently Gopal(1986) and Chandge (1987) have investigated the nutritional

PLATE II

Fig.1. Larval stages of Penaeus indicus

a and b : Developing eggs.

c : Nauplius I.

d : Protozoea I.

e : Protozoea II.

f : Protozoea III.

g : Mysis I.

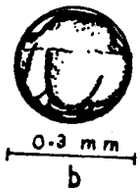
h : Mysis II.

i : Mysis III.

j : Postlarva I.

PLATE II Fig. 1

a



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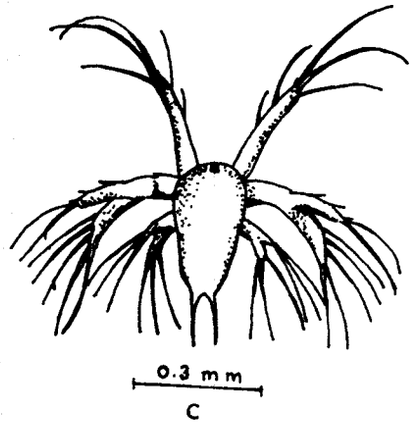

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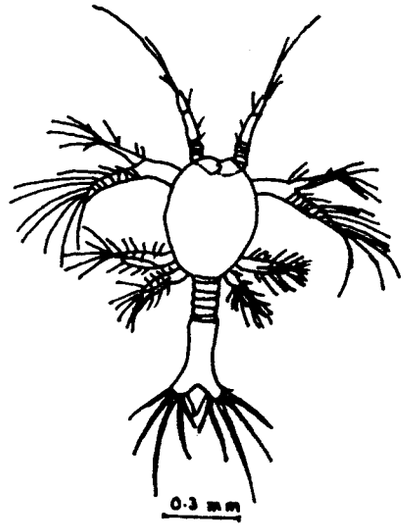

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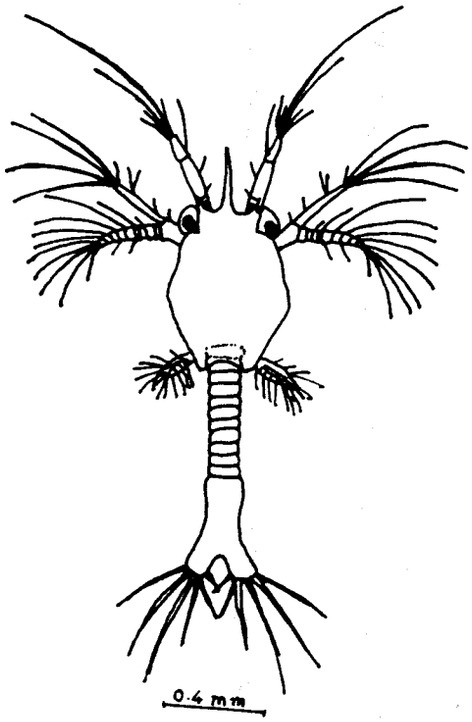

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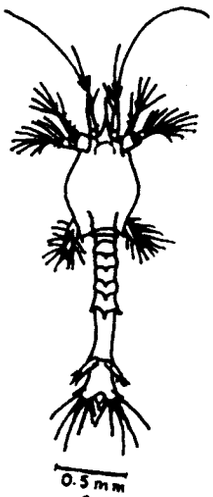

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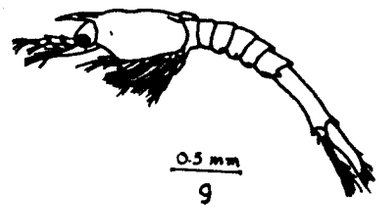

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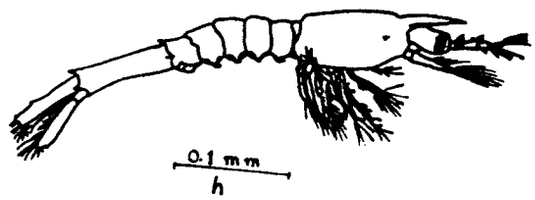

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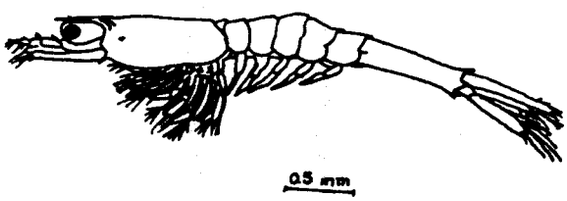

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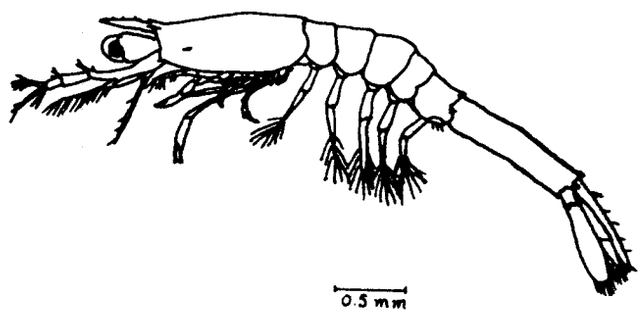

j

requirements of larvae, postlarvae and juveniles of P. indicus.

With the increasing interest in the culture of penaeid prawns for augmenting the prawn production of the country, the technology of seed production in hatcheries has been developed (Silas and Muthu, 1977; Alikunhi et al., 1980; Hameed Ali, 1980; Hameed Ali and Dwivedi, 1980, 1982; Hameed Ali et al., 1982; Silas et al.,1985). Although these studies and reports describing the hatchery production of seed have identified several factors affecting the production and survival, information on diseases as the causative factor for larval and postlaval mortalities is very much limited (Mahadevan et al., 1978; CMFRI, unpublished data). This situation may be due to the non- occurrence of noteworthy diseases so far in the hatcheries operated in the country and in the larval and postlarval population in nature, or inadequate knowledge on the identification or diagnosis of diseases or poor documentation of disease incidences. However mortalities of larvae and postlarvae due to diseases have been attributed as an important factor for the production and economical loss in the hatcheries. In view of these it was felt desirable that a survey was carried out initially to make an inventory of the various diseases/abnormalties encountered in the natural population and in the hatcheries, and to understand their incidence, characteristics and pathological significance so as to obtain the basic information on the subject, and subsequently to select the most important disease affecting the larvae and postlarvae for detailed study. The results of the survey conducted with this objective are presented in this section.

Seven cases encountered in the present survey are grouped into two categories, namely diseases caused by biotic factors and abnormalities. Since abnormality is considered as an anatomical deviation from the normal (Runnells et al., 1960), such cases are also briefly considered here. The seven cases of diseases and abnormalities reported and studied are:

Category I: Diseases caused by biotic factors

1. Ciliate infestation
2. Diatom infestation (Nitzschia closterium)
3. Parasitic infection by Leptomonas
4. Parasitic dinoflagellate infection
5. Appendage necrosis.

Category II: Abnormalities

1. Heteromorphic eye
2. Abnormal eggs and deformed nauplii

3.I. DISEASES CAUSED BY BIOTIC FACTORS

3.I.1. CILIATE INFESTATION

(Plate III, Figs. 1 to 3)

Host	: Protozoea III of Penaeus indicus.
Locality	: Narakkal Prawn Hatchery Laboratory.
Date of collection	: 24-12-1985.
Incidence	: Occurs whenever the organic load of the rearing medium increases.
Season	: Throughout the year.
Environmental parameters of the rearing medium	: Temperature 25-28°C; salinity 31-34 ‰; pH 8.1-8.3; dissolved oxygen 2.6-3.0 ml/l; ammonia 15-20 µg/l; rearing medium turbid due to phytoplankton bloom.
Material studied	: 25 numbers of protozoea III measuring 2.4-2.6mm in total length.
Clinical signs	: Larvae weak and appear fobby.

Observation: Healthy nauplii of P.indicus were stocked in the rearing tanks on 19th December 1985. While examining the condition of larvae on 24th December 1985, protozoea III larvae in one of the rearing tanks were found to be weak and appeared fobby due to ciliate infestation.

The infested larva when examined under the microscope revealed the presence of a large number of dichotomously branching, contractile colonies of peritrich ciliate attached to cephalothorax, abdomen and uropod (Pl.III, Fig. 1). The ciliate was most abundant on the cephalothoracic region of the host on the lateral aspect(Pl. III, Fig. 2). Each of the colonies was comprised of several trophonts of inverted bell shape with contractile stalk (Pl. III, Fig. 2). Trophonts measured 35 to 40 x 25 to 35 µm in size and the diameter of stalk was 8 to 12 µm. A central contractile fibril or myoneme was seen traversing throughout the stalk. Each trophont possessed an adoral ciliature, one or more vacuoles and a horse-shoe shaped macronucleus located near the centre. A closer examination of the infested larvae showed that the attachment of the colonies to the host was superficial and there was no mechanical damage to the cuticle or underlying tissues. Host haemocytic response to the infestation was totally absent.

The heart beat was counted in normal and infested protozoea III. The heart beat was very low in the infested protozoea (100-120 per minute) when compared to that of the normal protozoea (200-220 per minute). Further, in the infested larvae, the heart stopped its continuous beating frequently and started functioning again after an interval of 5 seconds.

This duration was seen increasing as the infestation progressed. The circulation was also observed to be rather slow in the infested larvae as compared to that of the normal and healthy ones.

Filamentous bacteria were also found on the larvae infested with ciliates. These bacteria were seen attached to the eye of the host superficially (Pl. III, Fig. 3).

Remarks: The peritrichous ciliate observed in the present case was identified as belonging to the genus Zoothamnium sp. on the basis of description given by Couch (1978). Heavy infestations of Zoothamnium sp. have been reported to cause surface fouling in all life stages of cultured penaeid prawns (Villela et al., 1970; Overstreet, 1973; Johnson, 1974a; Feigenbaum, 1975; Lightner, 1975,1977, 1978a; Couch, 1978, Issac Rajendran et al., 1982; Santhakumari and Gopalan, 1980). Couch (1978) has observed only pairs and small colonies (3,4 trophonts) of Zoothamnium sp. attached to the body surfaces of larval (protozoea and mysis) brown shrimp. In the present case, colonies varying from 2 to 20 trophonts were seen attached to the eye and appendages of larvae (Pl. III, Fig. 3). Zoothamnium sp. is a free living ciliate (epicommensal) and not a true pathogen (Lightner, 1978a). The attachment stalk of Zoothamnium sp. does not penetrate the cuticle of the prawn. Death occurs when the effective respiratory surface of the gills is reduced by the presence of numerous colonies of Zoothamnium sp. and subsequently, the suffocation of the animals (Lightner, 1975). Death usually coincides with periods of low concentration of dissolved oxygen in the

water, which normally occurs on warm overcast days or following the decomposition of large algal blooms (Overstreet, 1978). Soni (1986) has observed mortalities of P. indicus associated with Zoothamnium sp. infestation in the cultured ponds. He noted low concentration of dissolved oxygen (2.36 ppm) in the pond water. Lightner(1975) pointed out that in normal conditions, when Zoothamnium sp. was absent, dissolved oxygen level of 2.6 ppm was not lethal as P. aztecus was seen surviving in the culture ponds where the dissolved oxygen level was as low as 1.0 ppm. In the present case, the mean dissolved oxygen level of the rearing medium was 2.9 ppm.

Kramer(1975) observed the behavioural pattern of the young brown shrimp in low oxygen medium and concluded that brown shrimp were able to detect and tolerate low levels of dissolved oxygen by becoming inactive. Slow rate of heart beat and reduced circulation of haemolymph observed in the larvae at present would indicate the larval response to hypoxia due to the reduction of respiratory surface by Zoothamnium infestation. Zoothamnium infestation, besides reducing the respiratory surface, also interferred with the moulting and feeding of larvae. Several specimens of protozoea III infested with Zoothamnium failed to moult to mysis I stage. In certain cases, duration of metamorphosis to the next stage was seen extending considerably as compared to the normal uninfested larvae. The observation of the gut content of the infested larvae of P. indicus showed the empty condition of the gut although adequate quantity of Chaetoceros was present in the rearing medium.

Johnson et al. (1973) reported successful control of Zoothamnium sp. on penaeid prawns reared in ponds by treating with formalin at 25 ppm. A lower concentration of formalin (15 ppm), potassium permanganate at 2 and 4 ppm, copper sulphate at 1 ppm and malachite green at 1 ppm were not found effective in controlling Zoothamnium colonies from the gills of prawn.

3.I. 2. DIATOM INFESTATION (NITZSCHIA CLOSTERIUM)

(Plate III, Figs. 4 to 6)

Host	: Mysis stage and postlarvae of Penaeus indicus.
Locality	: Narakkal Prawn Hatchery Laboratory.
Date of collection	: 14-1-1986, 20-1-1986, 26-1-1986, 30-1-1986 2-2-1986, 18-2-1986, 23-2-1986, 3-3-1986 & 24-3-1986.
Incidence	: Frequent.
Season	: January - March.
Environmental parameters of the rearing medium	: Temperature 29-33°C; salinity 31-34 ‰; pH 8.0-8.3; dissolved oxygen 4.0-5.0 ml/l; rearing medium appeared yeallow due to phytoplankton bloom, composed principally of Nitzschia closterium.
Material studied	: Several specimens of mysis (3.7-3.9mm) and postlarvae(4.5-5.0mm) of P. indicus collected on various days indicated above.
Clinical signs	: Larvae weak;expansion of the chromotophores in the eyestalk, cephalothoracic appendages abdominal segments, uropods and telson: body opaque.

Observation: At the Narakkal Prawn Hatchery Laboratory, algal culture for feeding the larvae of P. indicus was raised in the rectangular fibreglass tanks containing 1000 l of fresh seawater (30-34 ‰. salinity) filtered through a 50 micron mesh bolting cloth and fertilized with chemicals (sodium nitrate, 12 ppm; potassium orthophosphate, 3 ppm; sodium silicate, 6 ppm and EDTA disodium salt, 6 ppm). These tanks were kept in the shed having glass roof, where the intensity of sunlight varied from 20,000 to 1,20,000 lux during day time and the temperature of the seawater in the tank from 28° C - 35° C. Under these conditions, the diatoms present in the medium multiplied rapidly and developed into bloom within 16-24 hours. The mixed phytoplankton then raised was predominantly (75-90%) composed of Chaetoceros spp. The other diatoms found were Thalassiosira, Skeletonema, Nitzschia, Pleurosigma and Peridinium which contributed to 10-25% of the total population. In the rearing experiments carried out during January-March, 1986, the protozoeae were reared by feeding with the mixed phytoplankton cultured routinely in this way. However, as the larvae reached mysis stage, a bloom dominated by Nitzschia closterium was observed in the rearing medium. The water became dark yellow in colour and the bloom contained more than 90% of N. closterium. Mortaliy of larvae ranging from 75% to 100% was observed 10-24 hrs after the appearance of N. closeterium bloom in the rearing medium.

Dead, moribund and live larvae were observed under the microscope and it was found that large number of diatoms were attached to the eyes, appendages, abdominal segements and telson (Pl. III, Figs. 5 and 6). The

attachment was so profuse that some larvae appeared yellow. On closer examination it was revealed that the spines of the N. closterium were piercing and damaging the host tissue. When it was present in large number in the cephalothoracic region, it damaged the gill tissue. In certain cases it also damaged the eyes of the host. Haemocytic response of the host due to N. closterium infestation was observed in the eyes, appendages, abdomen and telson (Pl. III, Fig. 6). Histological sections of the gill region stained with Harri's haematoxylin and eosin showed the necrosis of gill tissue of the host. The other organs such as heptopancreas, heart and gut were, however, normal. These observations indicated that the death of the host occurred when the effective respiratory surface of the gills was reduced by the infestation of large number of N. closterium causing hypoxia to the host.

To confirm the above observation experiments were carried out on the rearing of mysis larvae of P. indicus fed principally with N. closterium. For this purpose N. closterium was cultured in 1000 ml of filtered fresh seawater fertilized with potassium nitrate, potossium orthophosphate, sodium silicate and EDTA disodium salt at the rate of 12 mg, 3 mg, 6 mg and 6 mg respectively. After 24 hrs, the culture medium along with N. closterium was filtered through Sartorius filter paper (0.45 um pore size) to obtain a concentration of 1 lakh cells per ml. The Sedgwick - Rafter slide was used to count N. closterium (McAlice, 1971) and necessary dilution was done as required to aviod overcrowding while counting.

Two sets of larval rearing expreiments with feeding on N. closterium were carried out (Table 3.1). Sterilized beakers of one litre capacity were used for the rearing experiments. 50 healthy mysis I were introduced into beakers containing 800 ml of filtered seawater. Besides aerating the medium, the environmental parameters such as temperature, salinity, pH and dissolved oxygen were adjusted to 28° C, 32 ‰, 8.1 and 4.5 ppm respectively. N. closterium was introduced into the rearing medium to give a concentration of 20,000- 30,000 cells per ml. In control, the larvae were fed with mixed phytoplankton. In experiment I, 100% mortality of the larvae was observed after 24 hrs of the inoculation of N. closterium (Table 3.1). The dead larvae from this experiment were examined under the microscope, and it was found that the stomach was empty, thus indicating that mortality would have occurred due to the combined effect of starvation and N. closterium infestation. To avoid the starvation effect on the larvae, the experiment II was carried out by feeding the larvae with N. closterium (20,000 - 30,000 cells/ml) and mixed phytoplankton (10,000 cells/ml). In this experiment, all the larvae died after 48 hrs and their stomach showed partially filled condition. Expansion of the chromatophores and opaqueness of the body were observed in test animals in both the experiments as in natural infestation. In the control, all larvae were healthy and active.

Remarks: The diatom observed in the present case was identified as Nitzschia closterium on the basis of the characters described by Subrahmanyan (1946). It is a free living and motile form, measuring 35 -

Table 3.1. Deatils of the experiments* carried out on rearing of mysis I of *P. indicus* fed with *N. closterium*

	Control	Experiment I	Control	Experiment II
Date of expt.	4-2-1986	4-2-1986	22-2-1986	22-2-1986
No. of larvae	50	50	50	50
Feeding regime	Mixed phytoplankton (10,000 cells/ml of of rearing medium)	*Nitzschia closterium* (20,000-30,000 cells/ml of rearing medium)	Mixed phytoplankton (10,000 cells/ml of rearing medium)	Mixed phytoplankton (10,000 cells/ml of rearing medium) + *N. closterium* (20,000-30,000 cells/ml of rearing medium)
Percentage of larval mortality				
a) after 24 hrs	8	100	0	48
b) after 48 hrs	-	-	0	100
Other observations	Larvae healthy and active; stomach full	Larvae infested with *N. closterium*; expansion of chromatophores in the eyestalk, appendages, abdomen, uropods and telson; body opaque and stomach empty.	Larvae Healthy and active; stomach full.	Larvae infested with *N. closterium*; expansion of chromatophores in the eyestalk, appendages, abdomen, uropods and telson; body opaque and stomach partially filled.

* The experiments were carried out in triplicates.

154 µm long and 3.5 - 7.0 µm broad, valves are spindle-shaped in the middle and ends are extended into beaks and curved in opposite directions (Pl. III, Fig. 4).

Mortalities of penaeid larvae associated with N. closterium have not been reported so far except by Kungvankij (1984). He (Kungvankij, 1984) observed high mortality of larvae of P. monodon particularly in the outdoor hatcheries due to the overbloom of Nitzschia sp. He further observed that it is an undesirable species to feed the larvae; attaches to the appedages; and interferes with moulting (Kungvankij, 1984). Lexan and Trang (personnel communication) observed high mortalities of mysis of P. monodon in the hatcheries at Vietnam whenever blooms of N. closterium were encountered. Rathesh (personnel communication) also recorded high mortality of reared larvae of P. merguiensis at Karwar when fed with N. closterium which damaged the gill tissue, interfered with moulting and feeding of the host. Diatoms such as Amphora sp., Nitzschia sp. and Acanthes sp. were found on and between the gill filaments of the white prawn, P. setiferus by Overstreet and Safford (1980) and these authors observed haemocytic response against these diatoms by the host as observed in the present case against N. closterium. N. closterium, thus, causes considerable damage to the soft tissues of P. indicus larvae and ultimately leads to their mortality in large numbers. The present case forms the first report from India on the mortalities of larvae of the P. indicus associated with N. closterium.

3.I. 3. PARASITIC INFECTION BY LEPTOMONAS SP.
(Plate IV, Figs. 1 to Plate V, Fig. 2)

Host	: Protozoea and mysis of P. indicus and P. semisulcatus.
Locality	: Narakkal Prawn Hatchery Laboratory and the experimental marine prawn hatchery at the Mandapam Regional Centre of C.M.F.R.I., Mandapam Camp.
Date of collection	: 13-1-1986, 15-1-1986, 10-2-1986, 21-2-1986 22-2-1986, 18-3-1986, 12-4-1986, 3-1-1987 26-1-1987, 8-2-1987, 14-2-1987, 15-4-1987 & 18-12-1987 at NPHL; 22-4-1987 at Mandapam.
Incidence	: Frequent.
Season	: Throughout the year.
Environmental parameters of the rearing medium	: Temperature 26-33°C; salinity 30-34 ‰; pH 8.0-8.2; dissolved oxygen 3.7-4.5 ml/l.
Material studied	: Protozoea I, II and III measuring 0.8-0.9mm, 1.4-1.5 mm, and 2.3-2.6 mm respectively and mysis I, II and III measuring 3.0-3.5 mm, 3.3-3.6 mm and 3.5-4.0 mm respectively of P. indicus collected at the Narakkal Prawn Hathery Laboratory and mysis I and II (3.0-3.3 mm and 3.4-3.5 respectively) of P. semisulcatus collected at Mandapam Regional Centre of C.M.F.R.I.
Clinicalsigns	:Larvae weak and/or inactive; body opaque.

Observations: This protozoan parasite was collected from live, moribund and dead protozoea and mysis larvae of P. indicus and P. semisulcatus. It was not encountered in the naupliar and postlarval stages. It was tentatively assigned to the genus Leptomonas on the basis of the description given by Couch(1978). The parasite was found to invade both the external and internal body parts. Externally it was seen in the appendages, eye stalks, eyes and rostrum (Pl. IV, Fig. 1). The parasite showed polymorphism in its life cycle. The whole infected mysis were stained with Harri's haematoxylin and eosin, dehydrated and mounted on cavity slides with DPX mount to study the different forms of the parasite found inside the host (Pl. IV, Fig. 2). Three forms, namely pyriform (Pl. IV, Fig. 3), oval form (Pl. IV, Fig. 4) and cyst (spherical in structure) (Pl. IV, Figs. 5 and 6) were observed inside the body of the host. The size of the parasite was different in different forms. A straight flagellum was observed at the pointed end of the pyriform. At the base of the flagellum, a pointed projection was present. The size of this form was ranging from 18 to 28 um in length and 16 µm width at the broadest region. It had a compact nucleus measuring 4-7 µm in diameter at the pointed end. The flagellum and the pointed projection were absent in the oval form. Its size ranged from 15 to 23 µm antero-posteriorly and 15 µm laterally at the widest region. A nucleus was present at narrow end of the organism. The cyst was spherical in structure and existed in two different sizes ranging from 9 to 15 µm and 4 to 8 µm in diameter. The cytoplasm of all the forms ranged from clear to opaque and contained various inclusions. Histological sections of infected mysis stained with Harri's haematoxylin and eosin showed the

presence of these parasites in the vital organs such as hepatopancreas, alimentary canal and abdominal muscle (Pl. V, Figs. 1 and 2).

Remarks: Laramore and Barkate (1979) reported an unspeciated amoeba associated with mortalities of the protozoeal stages of P. vannamei and P. stylirostris in the hatchery. The present parasite was found to be different from amoeba described by Laramore and Barkate (1979) in morphology, size, position of the nucleus and polymorphism, but agreed with the description of Leptomonas given by Couch (1978). Leptomonas is reported to be a parasite in the gut of insects such as house flies, some other invertebrates and reptiles. Couch (1978, 1983) reported first on the mass mortalities of protozoea and mysis of penaeid prawns caused by Leptomonas. The present case is the second report on Leptomonas infection causing mortalities to the larvae of penaeid prawns.

Leptomonas invades the appendages including the eye stalk and eyes. It shows polymorphism in its life cycle. The parasite does not affect the healthy larvae, but it attacks the larvae that become weak. Couch (1978) suggested that the Leptomonas parasite is a secondary invader of a weakened host, possibly from encysted forms which may exist in the hindgut of the host. The pathogenic mechanism of the parasite on the host is not clearly known except for the mechanical damage to the host tissue (Couch, 1978). Although the parasite was seen in the histological sections of the hepatopancreas, midgut and abdominal muscle of infected mysis, no appreciable changes in the cellular structures of the organ was observed.

However further detailed studies on the patho-physiological aspects are necessary to understand effect of the parasite on the host. The treatment of the rearing medium with 10 -15 ppm formalin has shown to control the parasite.

3.I. 4. PARASITIC DINOFLAGELLATE INFECTION
(Plate V, Figs. 3 to 5)

Host	: Nauplius of *P. indicus*.
Locality	: Narakkal Prawn Hatchery Laboratory.
Date of collection	: 7-3-1986, 8-3-1986 and 13-10-1986.
Incidence	: Moderate.
Season	: October-November/March-April.
Environmental parameters of the rearing medium	: Temperature 25-29°C; salinity 32-34‰; pH 8.0-8.1; dissolved oxygen 4.5-5.0 ml/l.
Material studied	: 100 larvae of naupliar stage measuring 0.3-0.32 mm collected at Narakkal Prawn Hatchery Laboratory.
Clinical signs	: Larvae inactive, not sensitive to light and exhibit a tendency to settle at the bottom of the rearing tank.

Observation: The examination of the dead, moribund and live nauplius larvae obtianed from the spawning of the unilateral eye stalk ablated P. indicus in March-April and again in October-November'86 revealed the presence of a large number of spheroid organisms inside the larvae (Pl. V, Fig3), and motile forms in the rearing medium. Three forms were observed. Form I was spherical, measuring 8-12/µm in diameter, and with one to eight nuclei (2 µm in diameter) (Pl. V, Fig. 4) This form occurred in large numbers inside the host and found to fill the entire body of the larvae invading the soft tissue. When the form I was disturbed, smaller forms were liberated (Pl. V, Fig. 5). These constituted the second form and were non-motile, nucleated bodies of various sizes measuring 3 to 5 um. The third forms were motile, fast swimming flagellated cells. This form was found in the rearing medium and not inside the host. The structure and measurements of these different forms agree with the uninucleate, binucleate and plasmodial forms of Hematodinium described by Couch and Martin (1979).

Remarks: Chatton (1910) gave the first detailed account of parasitic dinoflagellate on copepods and later (Chatton, 1920) dealt with the peridinian dinoflagellate parasities on aquatic animals. Hematodinium perezi was reported as a pathogen in portunid crabs and blue crabs (Chatton and Poisson, 1930: Newman and Johnson, 1975). McCauley (1962) observed the dinoflagellate parasite Amallocystis capillosus on the rostrum of the shrimp Pasiphae pacifica, while Cachon (1968) recorded the parasitic dinoflagellate Chytriodinium parasiticum on the eggs of a penaeid prawn (species not

mentioned by the author). An unspeciated peridinian parasite was reported to be parasitic on eggs of Pandalus borealis by Stickney (1978). This parasite was found to attack the host by penetrating deeply into the egg cytoplasm through its stalk and to feed by osmosis, gradually absorbing the cytoplasm. Although detailed observation on the feeding of the present dinoflagellate parasite on the nauplius of P. indicus was not made, the pattern of infection implies that this parasite also probably feeds by osmosis.

The infected nauplius larvae of P. indicus were inactive and not sensitive to light. These observation agree with those of Couch (unpublished) an Hematodinium which causes fatal disease with no external signs except lethargy or weakness in the crab Callinectes sapidus. Couch and Martin (1979) described three stages, namely, uninucleate cells, binucleate cells and plasmodia of Hematodinium in the tissues of C. sapidus, but in the present case, only two forms were observed inside the host and the flagellate form only in the rearing medium. Couch and Martin (1979) also did not report any flagellated stages of Hematodinium in the host tissues.

The parasite observed in the present case was lethal to nauplius of P. indicus because of its ability to proliferate extensively and replace vital tissue as in the case of Hematodinium in the blue crab (Couch and Martin, 1979). The present case forms the first report of dinoflagellate parasite causing mortality of nauplii of penaeid prawns from India.

3. I. 5. APPENDAGE NECROSIS
(Plate V, Fig. 6 to Plate VIII, Fig. 6)

Host	: Protozoea, mysis and postlarvae of *P. indicus*.
Locality	: Narakkal Prawn Hatchery Laboratory and Kovalam Prawn Hatchery Laboratory, Madras.
Date of collection	: 21-3-1986, 18-4-1986, 14-3-1987, 28-3-1987 (Narakkal) & 14-8-1986 (Kovalam).
Incidence	: Moderate.
Season	: March and April.
Environmental parameters of the rearing medium	: Temperature 31-35°C; salinity 32-34 ‰; pH 8.1-8.3; dissolved oxygen 4.1-4.8 ml/l.
Material studied	: Several specimens of protozoea I (0.8-0.9mm), protozoea II(1.4- 1.5mm) and protozoea III(2.4-2.7mm); mysis I(3.0-3.5mm), mysis II(3.3-3.6mm) and mysis III (3.4-4.0mm)and postlarva I(4.5-5.0mm)of *P. indicus*.
Clinical signs	: Expansion of chromatophores, opaqueness of the body, and blackening and degeneration of cephalothoracic appendages, uropods and telson.

Observations: This disease syndrome was encountered both at the Kovalam prawn hatchery and at Narakkal. At the former hatchery it was recorded during the course of one of the seed production runs carried out in August, 1986 on P. indicus. Spawner was obtained from the wild and kept in the hatchery for spawning following the routine procedures. The viable eggs liberated by the spawner were reared further and the hatched out nauplii were stocked in two one tonne capacity rearing tanks containing filtered seawater at a stocking density of 75,000 nauplii/tonne. The rearing pools were managed as described by Silas et al. (1985). The protozoea and mysis larvae were fed with mixed phytoplankton dominatd by Chaetoceros sp. and Skeletonema sp., while the postlarvae, with the artificial feed (NPCL/117). Sudden mortality accounting for more than 30% was observed in one of the tanks as the larvae developed to mysis III and postlarva I stages. The infected larvae became opaque. The dead, moribund and live larvae were examined under the microscope and it was revealed that the chromatophores were expanded and uropods ulcerated with broken setae, telson swollen and blackened (Pl. V, Fig. 6; Pl. VI, Fig. 1). Small rod, motile bacteria were observed inside the infected parts. Fungal hyphae were not observed. These signs were also observed in the adult P. indicus and the lobster (Panulirus homarus) which were being reared in the laboratory (Pl. VI, Figs. 2 and 3). However, it was seen that adults usually recovered from this condition after the affected prawn or the lobster moulted.

Morphological, biological, physiological and biochemical characters of the isolates isolated from the infected larvae of P. indicus, adult prawn and lobster were studied. The characters of the isolates are given in Table 3.2. These isolates were tentatively identified as Vibrio alginolyticus (Pl. VI, Fig. 4) based on the biochemical and physiological characters given in the Bergey's Manual of Systematic Bacteriology (Kriez and Holt, 1984).

The pathogenicity of V. alginolyticus was tested on mysis of P. indicus. The mysis larvae were reared in the rearing medium inoculated with V. alginolyticus at the concentrations of 28 x 10^5 and 28 x 10^7 cells/ml of the rearing medium in 500 ml capacity beakers. The results obtained are given in Table 3.3. The mysis larvae treated with V. alginolyticus at a concentration of 28 x 10^5 cells/ml of the rearing medium in the first experiment showed a mortality of 13.3% and 20% respectively at the end of 24 hrs and 48 hrs of the experiment. At higher concentration of the bacterium the mortality rates increased. V. alginolyticus was not able to develop black lesion on the uropod and telson as observed in nature, but black lesion was observed at the junction of third and fourth abdominal segments of the experimental animals (Pl. VI, Fig. 5).

Histological sections of hepatopancreas, muscle and gut of the naturally infected and experimentally infected larvae stained with haematoxylin and eosin showed certain structural changes in these organs. Hepatopancreas was the most affected organ. Extensive vacuolation was observed in the hepatopancreatic epithelial cells and the vacuoles were

Table 3.2. Characeristics of the isolates isolated from diseased mysis larva and adult *P. indicus* and lobster (*Palinurus homarus*)

Characeristics	Mysis stage	Audlt prawn	Adult lobster
Swarming	+	+	+
Gram-stain	-	-	-
Motility	+	+	+
Growth at 42 °C	+	+	+
NaCl tolerance			
0%	-	-	-
3%	+	+	+
6%	+	+	+
8%	+	+	+
10%	+	+	+
Growth on MacConkey agar	+	+	+
TCBS agar	Yellow colour colony	Yellow colour colony	Yellow colour colony
Sentitivity to O/129	+	+	+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Nitrate reduction	+	+	+
Voges-Proskauer reaction	+	+	+
Catalase	+	+	+
Oxidase	+	+	+

Contd...

Table 3.2 contd.

Hydrogen sulphide production	-	-	-
TSI	A/A	A/A	A/A
Indole production	+	+	+
Cholera red reaction	-	-	-
OF test	F	F	F
Gas from glucose	-	-	-
Amylase	+	+	+
Gelatinase	+	+	+
Lipase	+	+	+
Chitinase	+	+	+
Casein digestion	+	+	+
Acid from			
Arabinose	-	-	-
Dextrin	+	+	+
Fructose	+	+	+
Glucose	+	+	+
Glycogen	+	+	+
Lactose	-	-	-
Maltose	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
Sucrose	+	+	+

Table 3.3. Pathogenicity experiments of *V. alginolyticus* on mysis of *Penaeus indicus*

Experiment	Type of Experimental system	Larval stage used	No. of larvae used	No.of bacterial cells/ml of rearing medium	Accumalated % mortality of larvae at the end of		% of larvae w. black lesion on abdominal segment
					24 hrs	48 hrs	
Control	Filtered sea water + mixed phytoplankton	mysis I	10 x 3	Nil	Nil	Nil	Nil
Treatment I	Filtered sea water + mixed phytoplankton + *V. alginolyticus*	mysis I	10 x 3	28×10^5	13.3	20	37.5
Treatment II	Filtered sea water + mixed phytoplankton + *V.alginolyticus*	mysis I	10 x 3	28×10^7	23.2	36.6	78.9

filled with eosinophilic materials (hyaline degeneration) (Pl. VI, Fig. 6) which were not seen in the noraml hepatopancreas. The tubular epithelium of some tubules was completely destroyed (Pl. VII, Fig. 1).

Next to hepatopancreas, abdominal muscle showed impairment due to V. alginolyticus. Muscle fibres were separated and haemocyte infiltration was observed between the muscle bundles. Pyknotic and karyorhexic nuclei were seen. The sections of blackened area of the host revealed the melanisation of exoskeleton and the underneath muscle (Pl. VII, Fig. 2). Bacteria and haemocytes were also observed in this blackened area (Pl. VII, Fig. 3). The highly infected muscle fibres became black while the modertely infected ones were brown in colour.

The epithelium of the gut was edematous. The epithelial cells were highly vacuolated and vacuoles, as in the hepatopancreas of infected larvae, contained eosinophilic material (Pl. VI,Fig. 6). No significant pathological changes were observed in the heart and gill.

In the larval rearing experiments carried out at Narakkal Prawn Hatchery Laboratory, healthy nauplii obtained from the spawning of unilateral eye stalk abalated female P. indicus were reared in the larval rearing tanks. Mortality of larvae (protozoea and mysis) was observed during March and April. Live and moribund larvae were examined under the microscope and it was revealed the expansion of chromatophores, opaqueness of the body and blackening and degeneration of the appendages as seen in

the V. alginolyticus infection of mysis and postlarvae of P. indicus at the Kovalam Prawn Hatchery (Pl. VII, Figs. 4 to 6; Pl. VIII, Fig. 1).

Histopathological studies carriedout on naturally infected mysis of P. indicus revealed the moderate to marked destruction in the hepatopancreas, gut, (Pl. VIII, Figs. 3 to 5) and muscle (Pl. VIII, Fig. 5), and melanisation of muscle fibres and haemocytes in the infected regions (Pl. VIII, Fig. 6).

These pathogens were isolated from the infected parts of the host. The morphological, biological, physiological and biochemical characteristics of this bacterium were found to be different from those of V. alginolyticus and hence tentatively identified as Vibrio sp., only to take up detailed investigation subsequently.

Remarks: Diseases of the exoskeleton of Decapod Crustacea have been variosuly referred to as spot disease, brownspot, blackspot, spotted disease or shell disease. Rosen(1970) has extensively reviewed the shell diseases of Decapod Crustacea. Ulceration and melanisation of the uropods have been observed in tank reared Macrobrachium rosenbergii and crayfish (Burns et al., 1979; Amborski et al., 1976). Amborski et al. (1976) identified several genera of bacteria and implicated them as the causative pathogen for ulcerative lesions in the crayfish. Chitinoclastic bacteria such as Beneckea and Vibrio were isolated from the shell lesion of M. rosenbergii, penaeid prawn P. setiferus and blue crab Callinectes sapidus (Cook and Lofton, 1973; Delves -Broughton and Poupard, 1976).

Several factors have been suggested for the manifestation of the brown spot disease. These include bacterial species which produce extracelluar lipases, proteases (Cipriani et al., 1980) and chitinases (Delves-Broughton and Poupard, 1976; Sindermann, 1977; Cipriani et al., 1980), fungi (Dugan et al., 1975; Burns et al., 1979; Johnson, 1980), mechanical trauma (Delves - Broughton and Poupard, 1976; Sindermann, 1977), precipitating chemicals (Nimmo et al., 1977; Johnson, 1980), nitrogenous waste products (Johnson, 1980), nutritional defficiencies and developmental abnormalities which result in damage to the epicuticular layer of the exoskeleton (Fisher et al., 1976). The biochemical analysis carried out on V. alginolyticus (Table 3.2) showed its ability to produce lipase, protease and chitinase which may cause the necrotic lesion on the appendages as observed by Cook and Lofton (1973), Delves- Broughton and Poupard (1976) and Sindermann (1977).

The dark brown to black colouration observed in the affected parts of the exoskeleton is due to melanin formation which generally indicates the host response to injuries, pathogens or parasites. The presence of small rod shaped bacteria in the melanised body parts of the larvae of P. indicus and the successful isolation suggests involvement in developing the ulcers in the uropod and telson. Pylant (1980) initiated the infection process of brown spot disease with V. alginolyticus through an injured integument in the adult prawn P. setiferus, but in the present case, black lesion was produced in the pathogenecity experiment without any injury because of the tender nature of the exoskeleton of the larvae.

The epithelial cells of hepatopancreatic tubules were vacuolated and vacuoles contained eosinophilic materials. This reaction has been described as hyaline degeneration (Runnells et al., 1960). Hyaline degeneration has not been reported so far in the prawn. Microscopically it appeared as smooth, homogeneous and deep pink in colour in eosin stained material. The cause and pathological significance of hyaline degeneration are not known. Boyd (1970) believed that hyalinisation was an end stage of many degenerative process. The presence of bacteria in the hepatopancreatic tubules and muscles, degeneration of some hepatopancreatic tubules, hyalinisation in the hepatopatopancreas and gut, and melanin formation in the muscle indicate the strucutural impairment of these organs due to V. alginolyticus infection, which would naturally interfere with the normal functioning of the host.

Vibrio sp. isolated from the infected protozoea and mysis of P. indicus at the Narakkal Prawn Hatchery Laboratory showed differnces in most of the morphological, biological, physiological and biochemical characters from V. alginolyticus and other described species of Vibrio. Thus, the appendage necrosis disease described in the larvae of P. indicus from Kovalam Prawn Hatchery and from Narkkal belonged to two different species of Vibrio. Of these the Vibrio sp. isolated from the larvae at Narakkal appeared to be the most virulent form as it produced higher mortality rates than that of V. alginolyticus infecting larvae of P. indicus at the Kovalam hachery.

3. II. ABNORMALITIES

3. II. 1. HETEROMORPHIC EYE
(Plate ix, Figs. 1 and 2)

Host	: Postlarva of *P. indicus*.
Locality	: Narakkal Prawn Hatchery Laboratory.
Date of collection	: 20-12-1985.
Incidence	: Rare.
Environmental parameters of the rearing medium	: Temperature 26°C; salinity 31 ‰; pH 8.1;dissolved oxygen, 4.6 ml/l.
Material studied	: One specimen of postlarvae III (4 mm total length) collected from the rearing experiment carried out in December 1985 at the Prawn Hatchery Laboratory, Narakkal.
Clinical signs	: Swimming in zigzag manner.

Observations: During the course of the larval rearing experiment conducted in December, 1985 one postlarva was seen swimming in zig-zag manner. The specimen was collected from the rearing tank with the aid of scoop net and examined under the microscope. External infection or infestation by any pathogen or parasite was not observed. The external morphological features of the postlarva were also similar to the normal postlarva of corresponding stage. On closer scrutiny, however, it was revealed that the left ophthalmopod and cornea differed from its counterpart on the right side in certain characters and size. The differences noticed in the structure of the left eye stalk and cornea are illustrated in Pl. IX, Figs. 1 and 2. The right eye stalk of the postlarva was normal with the usual eye stalk segments and the pigmented compound eye, but the left eye showed abnormality in its development.

The measurements of the heteromorphic left eye and the normal right eye (given in bracket) are as follows : total length of the ophthalmopod, 0.28 mm (0.25 mm); width of the eye stalk at the midregion 0.06 mm (0.14 mm); width of the basal segment, 0.02 mm (0.02 mm); diameter of the compound eye, 0.07 mm (0.13 mm) and diameter of eye pigment spot, 0.04 mm (0.09 mm). This measurements indicated that the length of the left eye was slightly longer, the eye stalk thinner and the cornea ill developed as compared to the normal right eye. However, the feeding behaviour and moulting were normal.

Remarks: Structural abnormalities in the organs of decapod crustaceans have been observed by several workers (Matsumoto, 1955; Suseelan, 1967). Abnormality in the form of heteromorphosis is relatively a rare phenomenon in decapods (Bliss, 1960). Kulkarni et al. (1979) have described heteromorphosis in the eye structure in Parapenaeopsis stylifera. Heteromorphosis of the eye was successfully induced artificially in Palaemon and a few other decapods by Herbst (1910). He opined that heteromorphosis was produced only when the optic ganglion was severed along with the eye. Studying the effects of denervation of the first two pairs of pleopods of Asellus aquaticus, Needham (1949, 1950) concluded that "local tissues are important (both) for quality and for the quantitative aspects of regeneration while the peripheral nerve supply affects only the quantitative aspects". Following these authors, the present case of abnormality of the eye may be considered as a quantitative defect since there is no change in external characteristics of the eye and would have resulted due to impaired development or growth mechanism of the nervous system.

3. II. 2. ABNORMAL EGGS AND DEFORMED NAUPLII
(Plate II, Fig. 1 and Plate IX, Figs. 3 to 5)

Host	: Egg and nauplius of *P. indicus*.
Locality	: Narakkal Prawn Hatchery Laboratory.
Date of collection	: 20-12-1985, 28-12-1985, 30-12-1985, 15-3-1986, 16-3-1986, 14-5-1986, 15-5-1986, 8-11-1986, 10-12-1986, 23-12-1986, 6-4-1987, 13-11-1987, 14-12-1987 & 5-3-1988.
Incidence	: Frequent.
Season	: November - December and March -May.
Environmental parameters of the rearing medium	: Temperature 26-33°C; salinity 31-34 ‰.;pH 8.0-8.3; dissolved oxygen 4.0-5.0 ml/l; ammonia, 10-18 µg/l.
Material studied	: Several abnormal eggs measuring 0.24-0.26mm in diameter and nauplii measuring 0.29-0.31 mm in total length of *P. indicus* collected from the prawn hatchery labouratory at Narakkal during November - December and March - May along with the water samples from the spawning and rearing tanks.
Clinical signs	: Abnormal nauplii with broken setae and unequal limbs; eggs with undifferentiated mass and asymmetrical embryo inside the egg.

Observations: Adult P. indicus were collected from the perennial prawn culture fields at Idavanakadu (Vypeen Island) for breeding experiment. They were acclimatized to laboratory conditions by maintaining them in one tonne capacity fibre glass tank with seawater. After 24 hrs of acclimatization in the laboratory, unilateral eye stalk abalation was performed on selected healthy and active females to accelerate the maturation of gonads following the method described by Muthu and Laxminarayana (1979,1981). The eye stalk abalated females were transferred carefully into the maturation pool along with a few males in the ratio of 4:1. The prawns were fed with clam meat. After 3 - 4 days of eye stalk abalation, the females with fully mature ovary were removed from the maturation pool and transferred into spawning tanks (200 l capacity) in the evening. Generally, the spawning took place in the night between 2200 hrs and 0200 hrs. The following morning, the water in the spawning tank was examined for the presence of eggs. The eggs were examined under the microscope for their development and viability.

During the period of investigation (Dec. 1985 - Mar. 1988) several types or varieties of eggs with normal and abnormal developments were observed. On the basis of the morphological features and pattern of development described by AQUACOP (1977) and Primavera and Posadas (1981) these eggs were grouped as follows:

Group A: normal fertilized eggs which were spherical and with continuous external membrane; generally free of bacterial and other growth; dark-green

colour; embryonic membrane distinct; symmetrical naupliar structure clearly seen inside the egg. These eggs produced healthy nauplii (Pl. II, Fig. 1).

Group B: fertilized eggs, but showing delayed or abnormal development; external membrane continuous; asymmetrical naupliar structure seen inside the egg. These eggs produced abnormal nauplii with broken setae and unequal limbs (Pl. IX, Figs. 3 and 5).

Group C: fertilized eggs with undifferentiated embryonic mass; the embryonic mass gradually degenerated in the course of time; rod, motile bacteria seen inside the egg (Pl. IX, Figs. 3 and 4).

Group D: fertilized eggs but the cytoplasm divided into large and small irregular formations; bacteria seen inside the egg (Pl. IX, Figs. 3 and 4).

Group E: unfertilized eggs, differentiated by orange colour; cell division was not observed and the embryonic membrane did not separate from the egg membrane; small rod motile bacteria seen inside the egg (Pl. IX, Fig. 3).

The hatching rate was more than 70% in the A type egg while it was less than 10% in the B type. C, D and E types did not hatch due to impaired development.

Water samples from the spawning tank and the abnormal eggs and deformed nauplii were analysed for the bacterial population. Abnormal eggs

and deformed nauplii were washed in sterile seawater and homogenised together and then diluted with sterile seawater.

Morphological, physiological and biochemical characters of the bacterial isolates from the rearing medium, abnormal eggs and deformed nauplii analysed in this study are given in Table 3.4. Among the bacterial population isolated, the dominant onces belonged to the genus Vibrio. Among the Vibrio isolates, the principle constituent species was tentatively identified as V. alginolyticus. Other isolates belonged to the genera such as Alcaligenes, Pseudomonas, Flavobacterium and Moraxella.

Remarks: Poor quality of penaeid prawn eggs and the abnormal development in controlled spawning and rearing have been reported by several workers (AQUACOP, 1977; Primavera and Posadas, 1981; Tseng and Cheng, 1981; Primavera, 1985). AQUACOP (1977) classified the eggs of P. monodon into four types, namely, type 1 eggs which were unfertilised and characterised by several unequal big cells; type 2 eggs with fragmented internal membrane; type 3 eggs exhibiting abnormal asymmetrical embryo and type 4 eggs showing normal development with symmetrical embryo. Primavera and Posadas (1981) studying different types of eggs in P. monodon, however, classified them into five types: A1 - fertilized eggs undergoing normal development with bilaterally symmetrical nauplius inside the egg; A2 - fertilized eggs with abnormal development ; B - unfertilized eggs with irregular cytoplasmic formations; C- unfertilized eggs with undifferentiated mass of egg cytoplasm and D - unfertilized eggs with

Table 3.4. Characteristics of bacteria isolated from abnormal eggs, deformed nauplii and from the rearing medium

Characters analysed	Isolates				
	Vibrio	Pseudomonas	Alcaligenes	Flavobacterium	Moraxella
Gram - stain	-	-	-	-	-
Motility	+	+	+	+	-
Oxidase	+	+	+	+	+
Glucose (fermentation)	+	-	+	+	+
Glucose (oxidative)	+	+	-	+	-
Lactose	-	+/-	-	+/-	-
Sucrose	+/-	+/-	-	-	-
Pigment	-	Fluorecent green	-	Yellow, orange red	-
Requirement of NaCl	+	-	-	-	-
Growth in TCBS agar	+	-	-	-	-
MacConkey agar	+	-	-	-	-
Sensitive to pencilin	-	+	+	+	+
O/129	+	-	-	-	-

gradual degeneration of egg cytoplasm due to bacterial invasion. The present observation agrees generally with the descriptions of abnormal egg types by Primavera and Posadas (1981), except that the types B,C and D eggs were noted by them as unfertilized, whereas in the present case, only one type of unfertilized egg was observed. Tseng and Cheng (1981) observed that partial spawnings of P. semisulcatus produced poor eggs with irregular cytoplasmic formation and autolysis. Similarly Primavera and Posadas (1981) noted that eye stalk ablated pond reared females of P. monodon when used on spawner source, produced poor quality eggs, whereas wild spawners or eye stalk abalated wild females gave the highest proportion of viable eggs. AQUACOP (1977) also reported about the incidence of unfertilized eggs and abnormal nauplii when the qualtity of brood stock was poor. Although the prawns used for breeding in the present study were active and in healthy condition, they were subject to eye stalk abalation to induce maturity. Following the observation of Primavera and Posadas (1981) one of the reasons for the incidence of abnormal eggs and deformed nauplii might be due to this factor.

In the present study, when the temperature prevailed at 26°C - 33°C and salinity at 31 - 34 ‰. during breeding and spawning experiments, total heterotrophic bacteria associated with abnormal eggs and deformed nauplii and in the water collected from the tanks were estimated to range from 4.5×10^6 to 6.72×10^9 / gram and 3.6×10^4 to 5.4×10^6 / ml respectively. Whereas the bacterial population of the eggs undergoing normal development and rearing medium was found to be relatively less,

ranging from 3.2 x 10^3 to 5.4 x 10^4 /gram and from 2.9 x 10^3 to 4.8 x 10^4 / ml respectively. Different species of bacteria were found to be associated with abnormal eggs and nauplii. The bacterial isolates were identified as Alcaligenes, Pseudomonas, Flavobacterium, Moraxella and Vibrio. Vibrio represented dominant species among the total population of the heterotrophic bacteria. Singh (1985,1986) estimated the heterotrophic bacteria assoicated with eggs of P. indicus reared at the Regional Shrimp Hatchery, Azhikode near Cochin as ranging from 1.3 x 10^4 to 8.72 x 10^7 / gram and observed that 80 to 100% hatching rate was achieved in this condition. He further observed that when the heterotrophic bacterial population was found more than 4.5 x 10^6 / gram of egg, the hatching rate of the eggs was high. In the present study, however, the eggs failed to hatch out when the total heterotrophic bacteria exceeded more than 4.5 x 10^6 / gram of egg and 3.6 x 10^4 / ml in the rearing medium. The reason for this situation might be due to dominance of Vibrio species in bacterial population associated with abnormal eggs and deformed nauplii. Singh (1987) observed less than 10% of Vibrio in the total heterotrophic bacteria of the eggs and more than 50% in mysis stage. He also noted poor survival rate of mysis stage larvae and postlarvae due to the dominance of Vibrio sp. Thus the dominance of Vibrio in the egg and the rearing medium could interfere with the normal development of the eggs and nauplii. It is worth mentioning here that Gunther and Catena (1980) have reported that V. parahaemolyticus and V. alginolyticus invade the body cavity of Artemia and metabolise all the tissue within a few hours. The eggs of P. indicus might therefore form the substratum for vibrios

(chitinoclastic bacteria) as observed by Singh (1986) and might proliferate very rapidly in high temperature and invade into the eggs. The abnormal eggs and deformed nauplii observed in the present study might also thus be due to the invasion of Vibrio into the egg from the water in which they were reared, besides the quality of the brood stock prawns. However, further experimental studies on pathogenicity of vibrios on the eggs of prawn are needed to confirm these observations.

Although there is not much information on the affect of bacteria present in the rearing medium and in the reared eggs and larvae, it is possible that these organisms were present at certain levels of concentration and in certain conditions of rearing, might affect the development and survival of eggs and larvae. Because, it is now known that certain bacteria like Vibrio elaborates extracellular chitinase, amylase, gelatinase, proteases and esterases, rendering it capable of degrading a wide variety of tissues (Ulitzur, 1974), it is also that higher temperature (above 31°C) is not an optimum condition for the development of larvae (Muthu, 1982) and hence their rearing at such high temperature would weaken the larvae making them easily susceptible for Vibrio invasion (Singh, 1986). Ulitzur (1974) has pointed out that vibrios isolated from seawater have very short generation time (12 - 14 minutes) at higher temperatures. In sub - tropical and tropical areas where temperature often increases beyond the desirable limit, particularly in prawns, the role of Vibrio sp. as pathogen of prawn is found to enhance considerably (Couch, 1978).

GENERAL REMARKS

During the present survey, two kinds of abnormalities and five types of diseases were encountered in the larvae and postlarvae of P. indicus and P. semisulcatus. It is noteworthy that no instances of diseases were observed in the larval and postlarval population in the wild and all the cases came from the hatcheries. However, this is not surprising as it may be possible that wild stocks are not likely to be exposed to pathogens and therefore be at less risk of disease. On the other hand in the hatcheries where the larvae are raised under controlled conditions, often at a high stoking rate, and when inadequate management practices in which adequate husbandry practices decline, aeration and water quality become insufficient, overfeeding occurs, increased incidence of diseases is more likely to occur. Thus most of the diseases recorded and described relates to those encountered within the hatchery.

Among the diseases encountered in the present survey, N. closterium infestation, Leptomonas infection and Vibrio infections caused high mortality in the larval and postlarval stages of penaeid prawns. Mortalities of penaeid larvae associated with N. closterium has not been reported in detail so far. In the present study, mortality of mysis and postlarvae of P. indicus due to N. closterium infestation was observed frequently during January - March. This is the first report of N. closterium infestation on mysis and postlarval stages of P. indicus from India.

Protozoan infection is another serious problem in the hatchery. Leptomonas parasites are not harmful when the larvae are healthy, but these parasites invade into the host and destroy the host tissue when the larvae become weak.

Vibrio infection has been implicated as a major cause of mortality in larval, postlarval and juvenile penaeid prawns (Sindermann, 1971b, 1974; Lightner, 1975, 1977, 1985; Takahashi et al., 1984, 1985). Shigueno (1975) observed two different types of Vibrio infection in the larvae of P. japonicus. In the present study also two species of Vibrio,namely V. alginolyticus and Vibrio sp. have been encountered. Of these the latter isolate appeared to be more virulent than the former, as revealed from the mortalities caused to larval population. The morphological, physiological and biochemical characteristics of this isolate are found to be different from the known vibrios in the literature. In view of these, this bacterium is taken up for detailed investigation and the results are presented in following chapters.

CHAPTER 4

AEROBIC HETEROTROPHIC BACTERIA ASSOCIATED WITH EGGS, LARVAE AND POSTLARVAE OF *PENAEUS INDICUS*

INTRODUCTION

Bacteria, first discovered by Antony van Leeuwenhoek, are among the most widely distributed forms of life. They are found in air, water, soil and internal and external regions of the animals and plants. *Aeromonas*, *Edwardsiella*, *Myxobacteria*, *Pseudomonas* and *Vibrio* are ubiquitous in the aquatic environment (Gilmour *et al.*,1976; Sakata *et al.*,1980; Kaper *et al.*,1981) and usually found on the body surface or in the intestinal lumen of fish and shellfish. All these bacteria, which are facultative pathogens, may produce epizootic outbreaks under environmental or physiological stress. Recently, Austin and Austin(1987) summarised the bacterial flora of freshwater and marine fish farms, and observed that as many as 37 bacterial taxa were encountered in the former and 40 in the latter ecosystems. These authors also reviewed the qualitative and quantitative data available on the bacterial flora of fish. As compared to this, information on the normal bacterial flora of prawn culture farms and culturable prawns appears to be limited. Vanderzant *et al.* (1971) studied the microbial flora of pond-reared brown shrimp *Penaeus aztecus* in relation to the environment. They found the dominance of coryneform bacteria and *Vibrio* in the prawn, and coryneforms and species of *Flavobacterium*, *Moraxella* and *Bacillus* in the pond water. Bacterial flora

in digestive tract of the penaeid prawns such as P. setiferus and P. japonicus were studied by Hood et al.(1971) and Yasuda and Kitao (1980) respectively. Vibrio sp. was found to be predominant bacterium in the freshly harvested, pond-reared prawns P. stylirostris, P. vannamei and P. setiferus (Christopher et al.,1978).

In India, most of the works on the bacterial flora of prawns are related to spoilage. However, Shaikmahamud and Mahar (1956) estimated the bacterial flora of Parapenaeopsis stylifera and observed 40 strains from different parts of its body. The bacterial load of freshly caught prawns P. indicus, Metapenaeus dobsoni and M. affinis caught from the sea off Cochin was estimated as ranging from 9.3×10^3 to 2.3×10^5 organisms per gram of prawn (Karthiyani and Iyer,1975).

There is a paucity of information concerning the normal bacterial population of penaeid larvae and postlarvae in relation to their environment. Singh (1986) has studied the heterotrophic bacteria associated with larvae and postlarvae of P. indicus. Vibrio and Aeromonas have been found to be the preponderant genera associated with larvae of Macrobrachium rosenbergii (Colorni, 1985). Before discussing the precise nature of Vibrio infection on the larvae and postlarvae, it is felt desirable that the normal bacterial flora encountered in the hatcheries and on the reared population are assessed so as to obtain a greater insight into the bacterial disease problem. It is in this context, the present work is carried out and the results are discussed.

MATERIAL AND METHODS

The eggs, larvae and postlarvae of P. indicus used for the estimation of bacterial population were obtained from the hatchery operation carried out at Narakkal during December'86 and January and February'87. Viable, developing eggs and healthy live larvae and postlarvae collected from the rearing tanks, were washed in sterile seawater and transferred to a sterile screw capped bottle containing sterile seawater. Water samples from the larval rearing tank and from phytoplankton culture tank were also collected in sterile bottles (250 ml capacity) for estimation of total aerobic heterotrophic bacteria and physicochemical parameters. The sample of the compounded feed used for feeding the postlarvae was taken in dry sterile screw capped bottle. These samples were kept in an ice box (4° C) and transported to the laboratory.

Estimation of physico-chemical parameters

The methods employed for estimating the pH, dissolved oxygen, temperature and salinity of the rearing medium are given in the chapter 2 on material and methods.

Bacteriological analysis

Processing of the sample

The sample of egg/larva/postlarva along with sterile sea water was poured into sterile bolting silk cloth kept on a sterile glass funnel and the water was allowed to drain off and the water adhering to the filtered

egg/larval/postlarval material was removed by means of sterile blotting paper. The egg/larval/postlarval material thus obtained was weighed aseptically and transferred to sterile tissue homogeniser along with 1 ml of sterile seawater where they were fully ground. Sterile suspension medium (9 ml) was prepared using aged seawater for egg, larva, postlarva, and their rearing medium, and 1% NaCl in distilled water as diluent for feed, and autoclaved. All the samples were serially diluted upto 10^5 using this diluent.

Plating procedures

ZoBell's 2116e agar of the following composition was used for the isolation of heterotrophic bacteria.

Bacto-peptone (Difco)	5.0 g
Yeast extract (Difco)	2.5 g
Ferric phosphate	0.1 g
Bacto-agar (Difco)	15.0 g
Aged and filtered seawater	1000.0 ml
pH 7.4 - 7.6	

In the present study, pour plate technique was followed for estimating the total heterotrophic bacterial flora present in the rearing medium and associated with eggs, larvae and postlarvae of P. indicus. One ml aliquot of inoculum was introduced into each sterile petri dish from 10^{-1} to 10^{-5} dilutions. About 15-20 ml of the sterile medium (40°C) was poured into each petri dish and mixed thoroughly by rotating the plates clockwise and

anticlockwise for 4-5 times, and allowed to solidify. The plates were incubated in an inverted position at 29°C for 72-96 hours. After incubation period, the plates were examined for bacterial growth, the plates showing 30- 300 colonies were selected. Counts were made and expressed as number of colonies per ml of water and per gram of solid samples (wet wt.). All the estimations were made in triplicate.

Isolation, identification and maintenance

The methods employed for isolation, identification and maintenance of bacterial isolates are same as those described in chapter 2.

RESULTS AND DISCUSSION

The estimated numbers of aerobic heterotrophic bacterial population (THB) in the rearing medium and on the eggs, larval and postlarval stages were found to vary in the samples collected during different months (Table 4.1). In the eggs, it fluctuated between 68 x 10^2 and 54 x 10^3, the lowest count being recorded in December and the highest in February. In the nauplius larva, the values ranged from 10.2 x 10^3 in December to 64 x 10^3 in February. In the protozoeal stage, total number of heterotrophic bacteria varied between 10.8 x 10^4 and 25.4 x 10^4 cells/gram. In the mysis stage, number of the heterotrophic bacteria was estimated between 5.25 x 10^5 and 6.1 x 10^5 cells/gram. In the postlarval stage, the bacterial population was found to vary from 7.1 x 10^5 to 20.3 x 10^5. The water collected from phytoplankton culture tank showed the heterotrophic bacterial flora ranging from 43 x 10^2 to 38 x 10^3 cells/ml.

Table 4.1. Number of aerobic heterotrophic bacteria* associated with eggs, larvae and postlarvae of P. indicus and in the rearing medium during the experiments carried out in December '86, and January and February '87

	Egg**	R.M.***	Nauplius	R.M	Protozoea	R.M.	P.W.	Mysis	R.M.	P.W.	Postlarva	R.M.	A.F.
Dec.'86													
Heterotrophic bacteria	68×10^2	29×10^2	10.2×10^3	85×10^2	10.8×10^4	33×10^3	55×10^2	52.5×10^4	40.4×10^3	43×10^2	15.1×10^5	42×10^3	36×10^5
Hatching rate	92%		-		-			-			-		
Survival rate	-		85%		75%			62%			53%		
Jan.'87													
Heterotrophic bacteria	32×10^3	48×10^2	24×10^3	38.8×10^3	11.3×10^4	45×10^3	73×10^2	61×10^4	66×10^3	98×10^2	7.1×10^5	73×10^3	28×10^5
Hatching rate	86%		-		-			-			-		
Survival rate	-		80%		68%			60%			49%		
Feb.'87													
Heterotrophic bacteria	54×10^3	92×10^2	64×10^3	18×10^3	25.4×10^4	54×10^3	36×10^3	54.2×10^4	75.2×10^3	38×10^3	20.3×10^5	61×10^3	38×10^5
Hatching rate	85%		-		-			-			-		
Survival rate	-		83%		70%			66%			63%		

* Average counts from triplicate plates of ZoBell's agar at 30°C for 72 hours
** No. of bacteria per gram of egg/ larva/ postlarva/artificial feed (A.F.)
*** No. of bacteria per ml of rearing medium/ water from phytoplankton tank
R.M. Rearing medium, P.W. Phytoplankton water.

In the feed, the bacterial population was varied between 28 x 10^5 and 38 x 10^5 cells/gm. The data on the total heterotrophic bacteria on the egg, larval and postlarval stages revealed a gradual increase of the population from the egg to postlarva. This observation differs from those of Yasuda and Kitao (1980) and Singh (1986) on the larvae and postlarvae of Penaeus japonicus and P. indicus respectively. Yasuda and Kitao (1980) observed an increase of bacterial population from egg to mysis stage and thereafter the population reduced as the larva transformed to postlarval stage. Singh (1986), however, noted first the increase of bacterial population from egg to protozoea and then reduction in the mysis stage, and again increase of bacterial population in the postlarval stage.

In the present study, the lowest bacterial population was recorded in the eggs of P. indicus. This is natural to expect as the eggs liberated from the prawns would be free of bacteria. However, as eggs come into contact with the rearing medium on their release, the bacteria present in the water get attached and proliferate on the surface of the eggs. In addition, the non-viable eggs present in the tank, would also enhance the development of bacterial population. As the rearing process continues, the bacterial population present in the rearing medium and associated with the larval stages, and those derived from the feeding material add up to the population to harbour higher bacterial population in the mysis and postlarvae. Austin and Allen (1982) have reported a similar increase of bacterial population in the rearing of Artemia from egg to adult stage.

The bacterial population estimated in the rearing medium were relatively lower than that on the eggs and larvae (Table 4.1). This might be due to the fact that the surface of the eggs and larvae provides an ideal micro-environment for bacterial growth (Stevenson,1978).

It is interesting to note that the total bacterial number associated with the eggs and larvae estimated in the present study were lower than that reported previously. Singh (1986) who studied the bacterial population of the larval samples and the rearing medium at the Regional Shrimp Hatchery, Azhikhode, where the prawn seed production is being carried out by the Japanese method in which the larvae were fed with tissue particles prepared from the muscle of Oratosquilla nepa, found increase in the number of Gram - negative bacteria on protozoeal stage onwards and reached the highest number in postlarval stage. This increased load of bacterial population might be due to the addition of tissue particles into the rearing medium which could increase the organic matter in the rearing medium facilitating the growth of bacteria as reported by Rheinheimer (1980). In the present case, the larvae were fed with mixed phytoplankton dominated by Skeletonema costatum and Chaetoceros affinis. The addition of mixed phytoplankton into the larval rearing medium might supress the bacterial flora especially Vibrio in the water and larvae as observed by various workers (Lugas, 1955; Sieburth, 1959; Jorgensen and Nielson, 1961; Jolley and Jones, 1974; Bell et al.,1979; Kogure et al., 1979, 1980). Kogure et al. (1979, 1980) reported that S. costatum suppressed the growth of Vibrio and Pseudomonas in the culture medium. The present observation

on the relatively lower population of heterotrophic bacteria associated with the larvae and in the rearing medium supports this view.

The total heterotrophic bacteria associated with the eggs, larvae, postlarvae and in the rearing medium during December '86, January and February '87 was fluctuating widely. Such fluctuation of bacterial flora in the marine fish farm was observed by Yoshimizu et al. (1976) and Austin (1982). Quantitative investigation on the THB in the marine fish farms showed that there was a seasonal fluctuation in the number of aerobic heterotrophic bacteria with minimum and maximum in winter and summer respectively (Austin, 1982; Yoshimizu et al., 1976). The quantitative fluctuation observed at present in the bacterial count on the eggs, larvae and rearing medium during December '86, and January and February '87 probably related to the temperature of the rearing medium. In December '86 and January '87 temperature of the rearing medium was between 27°C and 30.5°C. However, in February higher temperature at 29° C - 32°C prevailed when the larval rearing was undertaken (Table 4.2). This increased temperature might have facilitated the multiplication of bacterial population at a higher rate. Besides, localised favourable conditions such as nutrient availability in the rearing medium would have also contributed to the greater population of the bacteria. It is also observed that some strains of Vibrio have very short generation time at higher temperature (Ulitzur, 1974). Karthiyani and Iyer (1975) found that the Vibrio population in freshly caught wild prawn increased from 5% to 30% in summer.

Table 4.2. Environmental parameters of the rearing medium in which the larvae and postlarvae of Penaeus indicus were raised during December '86, January and February '87

Months	Environmental parameters of the rearing medium			
	Temperature (°C)	pH	Salinity (‰)	Dissolved oxygen (ml/l)
December '86	27 - 29	8.0 - 8.2	30 - 32	4.0 - 5.0
January '87	27 - 30.5	8.0 - 8.2	30 - 32	4.5 - 5.0
February '86	29 - 32	8.1 - 8.3	31 - 33	4.0 - 5.0

From the bacterial isolates isolated from the eggs/larvae/ postlarvae, and rearing medium during December '86, January and February '87, 100 isolates in each of the months (total 300 isolates), composing of 50 isolates from eggs, larvae and postlarvae, and 50 from rearing medium were selected for identification. Bacterial strains were identified upto generic level (Table 4.3). Most of the isolates belonged to Gram - negative rods as observed by Singh (1986). It might be mentioned here that generally Gram - negative rods abound in sea water (ZoBell and Upham, 1944; Pfister and Burkholder, 1965; Baumann et al., 1972) and marine invertebrates (Sochard et al., 1979). Vibrio was found to be the dominant (28-32%) taxa in the larvae followed by Pseudomonas (20-24%), Alcaligenes (10-14%), Aeromonas (6- 10%) and Flavobacterium (6-10%). In the rearing medium, however, Alcaligenes (20-34%) was predominant followed by Vibrio (16-20%), Flavobacterium (6-16%) and others. Based on the difference between the generic composition of bacteria in zooplankton and seawater samples, Simidu et al. (1971) observed that Vibrio and Aeromonas constituted the common indigenous marine bacteria, often closely associating with certain marine organisms. Further, Huq et al. (1983) reported about the ability of Vibrio to attach to copepods, whereas strains of Pseudomonas and Escherichia coli did not adhere to them. The dominance of Vibrio sp. on the larvae and postlarvae of P. indicus observed at present agrees with the certain observations of Singh (1986) on P. indicus, Yasuda and Kitao (1980) on P. japonicus and Colorni (1985) on Macrobrachium rosenbergii.

Table 4.3 Percentage composition of bacterial taxa recorded from the egg/ larva/ postlarva of P. indicus and rearing medium.

Months	Percentage composition															
	Aeromonas		Alcaligenes		Cytophaga		Flavobacterium		Moraxella		Pseudomonas		Vibrio		Unidentified	
	E/L/P	Water	E/L/P	Water	E/L/P	Water	E/L/P	Water	E/L/P	Water	E/L/P	Water	E/L/P	Water	E/L/P	Water
Dec.'86	10	12	12	34	4	0	8	6	8	6	20	14	28	16	10	12
Jan.'87	6	14	14	20	0	10	10	14	6	6	24	10	30	18	10	8
Feb.'87	10	10	10	22	4	6	6	16	6	4	20	14	32	20	12	8

E/L/P - Eggs/Larvae/Postlarvae sample

When Vibrio population was 10.4% and 12.24% of the total heterotrophic bacterial population in the egg and rearing medium respectively, Singh (1986) observed 91.06% of hatching rate of the eggs of P. indicus. As the larvae grew to postlarvae, Vibrio population increased to 89.39% in the postlarvae and 67.06% in the rearing medium, and the survival rate was reduced to 29.98% from egg to postlarval stage (Singh, 1986). In present case, Vibrio population associated with eggs, larvae and postlarvae, and in the rearing medium had never exceeded more than 32% of the total heterotrophic bacteria. In this condition, more than 50% survival rate was achieved in the rearing experiments from egg to postlarvae. Yasuda and Kitao (1980) and Singh (1986) observed abundant Pseudomonas population in the gut of healthy cultured and wild adult prawns P. japonicus and P. indicus. Further they also noted poor growth of the prawn when Aeromonas and Vibrio were dominant in the gut. These observations indicate that species of Vibrio are harmful to prawn when present in large quantity.

CHAPTER 5

PATHOGENIC VIBRIOS

In the previous section, it is shown that among the various taxa of bacterial flora associated with the eggs, larvae and postlarvae of P. indicus, Vibrio constitutes the dominant group. Infact, vibrios are emerging as the most important pathogenic bacteria among fishes and shellfishes. Due to the heavy economic losses accounted by the diseases caused by this group considerable interest resulting in the description of several species and better understanding of their taxonomy and pathological significance of the group has been enfolded. This chapter endeavours to briefly summarise the important features of the various species of Vibrio, particularly those causing diseases in fish and shellfish. The genus Vibrio contains organisms which are small, straight or curved, motile rods. The taxonomy of Vibrio is still in a considerable state of flux. Twenty species of Vibrio have been described in the 1984 edition of Bergey's Manual of Systematic Bacteriology. (Kriez and Holt, 1984). Table 5.1 lists these species as well as the other isolates assigned to the genus Vibrio. Colwell and Grimes (1984) have listed eight species of Vibrio namely, V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae, V. damsela, V. ordalii, V. parahaemolyticus and V. vulnificus as fish pathogens. Besides these several unspeciated vibrios have also been found to be pathogenic to fishes and shellfishes by various workers (Tubiash et al., 1970; Harrell et al., 1976; Muroga et al., 1976a,b, 1979; Elston et al., 1982; Takahashi et al., 1984, 1985; Bruno et al., 1986).

Table 5.1. Species of *Vibrio* with their habitats

Species	Pathogenic to	Habitat
Vibrio alginolyticus	Man, fish, prawn, crab, lobster and *Aretemia*	Marine
V. anguillarum	Fish	Marine, brackish water and freshwater.
V. campbellii	Not stated	Marine
V. cholerae	Man	Marine and freshwater
V. cholerae (non-01)	Fish and prawn	Marine and freshwater
V. carchariae	Fish	Marine
V. costicola	Not stated	Hypersaline environment
V. damsela	Fish	Marine
V. fischeri	Not stated	Marine
V. fluvialis biovars I & II	Man	Marine and fresihwater
V. gazogenes	Not stated	Marine
V. harveyi	Not stated	Marine
V. logei	Not stated	Marine
V. marinus	Not stated	Marine
V. metschnikovii	Man	Marine and freshwater
V. natriegens	Not stated	Marine
V. nereis	Not stated	Marine

Table 5.1 contd.

Species	Pathogenic to	Habitat
V. nigripulchritudo	Not stated	Marine
V. ordalii	Fish	Marine and freshwater
V. parahaemolyticus	Man, fish, prawn, crab snail and *Artemia*	Marine
V. pelagius biovars I & II	Not stated	Marine
V. proteolyticus	Not stated	Marine and freshwater
V. salmonicida	Fish	Marine
V. splendidus biovars I & II	Not stated	Marine
V. vulnificus	Man and Fish	Marine
Vibrio sp.(Tubiash *et al.*, 1970)	Bivalve molluscs	Marine
Vibrio sp.(Cook and Lofton, 1973)	Crab and prawn	Marine
Vibrio sp.(Harell *et al.*,1976)	Fish	Marine
Vibrio sp.(Muroga *et al.*,1976a)	Fish	Marine and brackishwater
Vibrio sp.(BML 79-078) (Bowser *et al.*,1981)	Lobster	Marine

Table 5.1. Contd..

Species	Pathogenic to	Habitat
Vibrio sp.(25-1),(25-2) and *Vibrio* sp. (26-1) (Elston *et al.*, 1982)	Oyster and Clam	Marine
Vibrio sp.(Takahashi *et al.*, 1984)	Prawn	Marine
Vibrio sp.(Takahashi *et al.*, 1985)	Prawn	Marine
Vibrio sp.(Bruno *et al.*, 1986)	Fish	Marine

V. alginolyticus was formerly classified as V. parahaemolyticus biotype 2. It abounds in the marine and estuarine environments. It exibits swarming growth on the surface of complex media, produces a positive Voges-Proskauer reaction, tolerates 10% (W/V) NaCl and grows at temperature upto 42°C. It ferments sucrose and decarboxylates lysine and ornithine. It is considered as a weak pathogen of stressed fish or an oppurtunistic invader of already damaged tissues, although it has been associated with ulcer disease (Akazawa, 1968), mortalities of sea-bream (Iwata et al., 1978; Coloroni et al., 1981) and prawns (Lightner and Lewis, 1975) and "red spot" disease in mullet (Burke and Rodgers, 1981). Leong and Fontaine (1979) observed significant mortality with gross signs similar to those observed in actual bacterial infections when V. alginolyticus was injected into P. setiferus. V. alginolyticus has been found to be pathogenic to Artemia at the higher concentration (Gunther and Catena, 1980).

V. anguillarum is primarily recognised as a fish pathogen and is generally associated with vibriosis in fish. It is found both in the marine and freshwater environments and usually isolated from diseased fish. It decarboxylates arginine and tolerates 6% (W/V)NaCl, gives positive result for Voges - Proskauer reaction and ferments sucrose. Strain to strain variations occur in the results of methyl red, Voges - Proskauer test, salt tolerance, temperature tolerance and haemolysis of horse blood. Vibriosis of V. anguillarum etiology has been reported in over 42 species of fish including ayu, eel, cod, pike, brown trout, flounder, stripped bass and salmon from all over the world (Colwell and Grimes, 1984). V. anguillarum has not

been reported as crustacean pathogen. Leong and Fontaine (1979) observed the LD_{50} values of V. anguillarum for P. setiferus and compared its virulence with that of other vibrios. It has been found to be pathogenic to juvenile lobster when injected intramuscularly (Bowser et al., 1981). The high survival percentage of Artemia nauplii exposed to V. anguillarum showed that this bacterium was not pathogenic to Artemia nauplii (Gunther and Catena, 1980).

V. carchariae has been isolated from a dead sandbar shark (Carcharhinus plumbeus) (Grimes et al., 1984a). Subsequently it has also been isolated from the lemon shark Negaprion brevirostris and from the trematodes infesting the skin of lemon sharks (Grimes et al., 1984b). It is a swarming Vibrio which exhibits mixed flagellation. It is sensitive to 150 µg of the vibriostatic agent, O/129. It grows in 3 - 8% (W/V) NaCl but not in 10% . V. carchariae has been proved lethal for spiny dog fish when injected intraperitoneally (Grimes et al., 1985). The histological examinations of the internal organ of the lemon shark revealed active disease processes in the spleen and liver (Grimes et al., 1985).

V. cholerae (non - 01) has been isolated from the diseased ayu in the River Amano, Japan (Muroga et al., 1979). It is morphologically and biochemically very similar to V. cholerae but does not agglutinate in either Ogawa or Inaba antisera . Recently, V. cholerae (non-01) has been isolated from the eyeballs and haemolymph of Penaeus orientalis with epizootic locally called "blind disease" (Zheng, 1986a,b). It has been proved

a highly virulent pathogen to ayu, eel and prawn (Yamanoi et al., 1980; Zheng, 1986a,b).

V. damsela has been isolated from the damselfish off the coast of Southern California (Love et al., 1981). It is Gram-negative and weakly motile rod. It grows in 1 to 6% (W/V) NaCl and produces gas during fermentation of glucose and other selected sugars. It has been associated with skin ulcer in damselfish Chromis punctipinnis (Love et al., 1981) and it has caused rapid death to spiny dog fish (Squalus acanthias in experimental condition (Grimes et al., 1985).

V. ordalii was formerly classified as V. anguillarum biotype II, and subsequently it has been treated as a new species of the genus Vibrio (Schiewe, 1983; Schiewe et al., 1981). It grows at 15 to 22°C and in 0.5 to 3% (W/V) NaCl. It associates with vibriosis in salmonid fish in the Pacific North East and in Japan. It colonizes in skeletal muscle, cardiac muscle, gill and the gastrointestinal tract of salmonids.

V. parahaemolyticus was placed in the genus Vibrio by Shewan and Veron (1974). It is the most thoroughly studied Vibrio sp. next to V. cholere. It grows at 42°C and 8% (W/V) NaCl but not in 10% . It does not ferment sucrose. It has been frequently isolated from both healthy and diseased finfish and shellfish throughout the world (Krantz et al., 1969; Vanderzant et al., 1970 ; Sizemore et al., 1975; Tubiash et al., 1975; Brinkley et al., 1976; Lhuillier, 1977; Qadri and Zuberi, 1977; Lall et al.,

1979; Franca et al., 1980; Nair et al., 1980), but the role of V. parahaemolyticus as a fish pathogen is controversial eventhough several reports are available (Krantz et al., 1969; Vanderzant et al., 1970; Lightner, 1977; Leong and Fontaine, 1979). Vanderzant et al. (1970) have reported that addition of 3% inoculam (24 hrs cultured in Brain Heart Infusion broth) of V. parahaemolyticus to an aquarium caused the death of brown prawn P. aztecus in a few hours. V. parahaemolyticus has been found to be pathogenic for white prawn P. setiferus (Leong and Fontaine, 1979), crab (Krantz et al., 1969), snail, Biomphalaria glabrata (Ducklow et al., 1980) and Artemia nauplii (Gunther and Catena 1980).

Recently V. salmonicida has been added to the list of Vibrio species pathogenic for fish (Egidius et al., 1986; Wiik and Egidius, 1986). It causes cold water vibriosis (Hitra disease) in Atlantic salmon Salmo salar. The histological studies showed the severe necrosis of internal organs such as kidney, alimentary canal, gills and spleen (Egidius et al., 1986; Bruno et al., 1986).

V. vulnificus, also called as lactose fermenting Vibrio, resembles V. alginolyticus and V. parahaemolyticus in many respects. V. vulnificus biogroup 2 has been found to be pathogenic to eels (Tison et al., 1982). It grows at 20 to 30°C, but not at 5 or 42 °C, and in 0.5 to 5% (W/V) NaCl. It has caused high mortality in eels experimentally (Austin and Austin, 1987). Harell et al. (1976) isolated a Vibrio sp. (1669) from the Pacific salmon in Puget Sound and found it to be pathogenic and responsible for

vibriosis in salmon. It produced acid from mannitol, glucose and sucrose and was sensitive to the vibriostatic agent, O/129.

A new species of Vibrio has been isolated from the diseased eel (Muroga et al., 1976a). The physiological, biochemical and serological characteristics of this organism have been studied in detail (Muroga et al., 1976b; Nishibuchi et al., 1979; Nishibuchi and Muroga, 1980). This organism has been proved to be pathogenic to eel by inoculation experiments (Muroga et al., 1976b). It grows at 18 - 39 °C and in 1-4% (W/V) NaCl. It gives negative to Voges-Proskauer test and ornithine decarboxylation test and positive to methyl red test, lysine decarboxylation test. It ferments glucose. Recently a bacterium associated with low level mortalities of farmed Atlantic salmon Salmo salar has been isolated and placed in the genus Vibrio (Bruno et al., 1986). It grows at 5 to 20 °C but not at 30 °C. It gives negative to arginine dihydrolase, lysine decarboxylase ornithine decarboxylase tests. It is sensitive to the vibriostatic compound , O/129.

Tubiash et al. in 1970 isolated and identified a Vibrio sp. associated with bacillary necrosis of larvae and juveniles of bivalve molluscs. It grows in 3% (W/V) NaCl and at 25 °C but not in 8% (W/V) and at 42 °C. It ferments glucose, sucrose and maltose. Similarly three isolates of vibrios, namely, Vibrio sp. (25 - 1), Vibrio sp. (25 - 2) and Vibrio sp. (26 - 1) isolated from the cultured oysters Crassostrea virginica and Ostrea edulis, and clam Mercenaria mercenaria (Elston et al., 1982), are found to be sensitive to the vibriostatic agent, O/129. They grow at 25 - 30 °C and

produce acid from cellobiose, dextrin, fructose, glucose, glycerol, glycogen, maltose, mannitol, mannose, starch and trehalose.

A Vibrio - like organism has been isolated from moribund juvenile American lobster Homarus americanus and designated as Vibrio sp. (BML 79 - 078) (Bowser et al., 1981). It is sensitive to vibriostatic compound, O/129 and novobiocin, shows poor growth in 7 - 10% (W/V) NaCl, ferments glucose and produces acid from sucrose. Koch's postulates have been satisfied for this organism (Bowser et al., 1981). Cook and Lofton (1973) isolated 3 isolates of Vibrio from the necrotic lesion on the crab Callinectes sapidus and prawn P. setiferus. These isolates required salt to grow and showed negative reaction in Voges-Proskauer test. While two of the these isolates fermented sucrose, none fermented lactose; found to be sensitive to the vibriostatic compound, O/129.

A bacterium has been isolated from the midgut gland of diseased postlarvae of Kuruma prawn P. japonicus and identified as Vibrio sp. (Takahashi et al., 1984). It grows at 15 - 37 °C and in 0.5 - 5% (W/V) NaCl but not in 6% . It ferments glucose in Hugh - Leifson medium. It gives positive reaction to Voges - Proskauer test and citrate test and negative to arginine hydrolytic test and swarming test. It is sensitive to vibriostatic compound, O/129. This organism has been proved to be pathogenic to Kuruma prawn by inoculation experiments (Takahashi et al., 1984). Recently Takahashi et al. (1985) isolated another species of Vibrio from the heart, lymphoid organ and muscle of the diseased Kuruma prawn

P. japonicus. It grows at 10 - 30 °C and in 1- 4% (W/V) NaCl but not in 5%. It gives positive oxidase and catalase reactions and utilises glucose fermentatively in Hugh - Leifson's medium. It is sensitive to the vibriostatic compound, O/129 and novobiocin. This bacterium has been proved to be pathogenic to Kuruma prawn by infectivity trials (Takahashi et al., 1985).

Since the description of V. anguillarum in 1893, several species of Vibrio inhabiting the marine, brackiswater and freshwater regimes have been described (Table 5.1). While some of these are non- pathogenic, some cause diseases in invertebrate and vertebrate animals including humans. Among the pathogenic bacteria reported to date, eight species of Vibrio have been recognised as pathogens of fishes (Colwell and Grimes, 1984). However, the taxonomical status of some of the species, for example, the bacterial isolates with the specific epithets of anguillarum is still controversial. Similarly, there is controversy on the role of some of the bacteria such as V. parahaemolyticus as fish pathogen, and certain workers believe that this species does not constitute a fish pathogen.

Seven species of Vibrio have been described as pathogen to crustacea (Table 5.1). Among these V. alginolyticus, V. cholerae (non-01), V. parahaemolyticus, Vibrio sp. (Cook and Lofton, 1973), Vibrio sp. (Takahashi et al., 1984) and Vibrio sp.(Takahashi et al., 1985) are found to cause diseases in prawns. Although the realisation of the role of these bacteria in bringing forth the diseases in fishes, particularly in the context of rapid expansion of their aquaculture and the development of improved technologies

in diagnosis and isolation have resulted in better understanding of their pathobiology, information avialable on the pathogens causing diseases in penaeid prawns is still incomplete. And much research works needs to be undertaken in this field not only to understand the basic aspect of the biology and ecology, but also to understand appreciable control measures.

CHAPTER 6

STUDIES ON THE TAXONOMY OF A NEW ISOLATE OF *VIBRIO* ISOLATED FROM THE DISEASED LARVAE OF *PENAEUS INDICUS*

INTRODUCTION

As mentioned earlier, *Vibrio alginolyticus* and *Vibrio* sp. were found to cause appendage necrosis in the larvae and the postlarvae of *Penaeus indicus* reared in the hatcheries at Narakkal and Kovalam. Of these two species, *Vibrio* sp. isolated from the larvae of *P. indicus* at the former centre, was found to be different from the other vibrios in several characters as revealed in the preliminary investigation. It was also observed that between these species, *Vibrio* sp. was more virulent causing relatively higher mortality to the larvae in the hatcheries. This concerns with detailed study on the new isolate of *Vibrio* in the aspects of its morphological, biological, physiological and biochemical characters and on the basis of these characters, the taxonomic status of the new isolate of *Vibrio* is discussed.

MATERIAL AND METHODS

Source of bacterial strains

Ten isolates of *Vibrio* were isolated from the diseased mysis of *Penaeus indicus* and from the rearing medium at the Prawn Hatchery Laboratory, Narakkal. *V. parahaemolyticus* (untypable), *V. parahaemolyticus* (O6 - K46) and *V. alginolyticus* were isolated from the prawns showing

`soft-shell´ syndrome collected from the grow-out ponds attached to the hatchery. These isolates were identified and strains of V. parahaemolyticus were serotyped at the National Institute of Cholera and Enteric diseases, Calcutta. The methods followed for isolation, purification, preservation and identification of the bacterial isolates were similar to those described in chapter 2. V. anguillarum (Mb 493) was obtained from Prof. John L. Fryer, Dept. of Microbiology, Oregon State University, Oregon, U.S.A. V. parahaemolyticus (untypable), V. parahaemolyticus (O6 - K46), V. alginolyticus and V. anguillarum were used as reference strains to compare their characters with that of the present isolates isolated from the diseased mysis larvae. The media used for isolation, source and laboratory code for the bacterial strains are given in Table 6.1.

The present isolate was sent to taxonomic experts in India and abroad. The isolate was transported in screw cap bottles (5ml capacity) containing seawater nutrient agar. After stab inoculation of the organism, the bottles were tightly sealed and sent by Air mail to Dr. G.Balakrish Nair, Reasearch Officer, National Institute Cholera and Enteric Diseases, Calcutta; Prof. Donald H. Lewis, Texas A & M university, U.S.A.; Prof. John L. Fryer, Department of Microbiology, Oregon State Univesity, U.S.A.; Prof. Rita R. Colwell, Department of Microbiology, University of Maryland, U.S.A,; Dr.James D.Oliver, University of North Carolina, U.S.A.; Dr. R. Sakazaki, National Institute of Health , Tokyo , Japan; and Dr. Jim J. Farmer, Vibrio Refernce Laboratory, Division of Bacterial Diseases, Centres for Diseases Control, Georgia, U.S.A.

S.No.	Bacterial strain	Laboratory code	Source	Medium used for isolation
1.	Present isolate	M1	Diseased mysis larvae of *P. indicus*	TCBS
2.	,,	M2	,,	TCBS
3.	,,	M3	,,	TCBS
4.	,,	M4	,,	Alkaline peptone water
5.	,,	M5	,,	ZoBell's agar
6.	,,	M6	,,	,,
7.	,,	M7	,,	,,
8.	,,	W1	Larval rearing medium	TCBS
9.	,,	W2	,,	TCBS
10.	,,	W3	,,	ZoBell's agar
11.	*V. alginolyticus*	SO1	`Soft' prawn	,,
12.	*V. anguillarum* (Mb 493)	SA1	Fish, Dept. of Microbiology Oregon State University	Not known
13.	*V. parahaemolyticus*(O6-K46)	SP1	Soft prawn	ZoBell's agar
14.	*V. parahaemolyticus* (untypable)	SP2	Soft prawn	ZoBell's agar

Serology

Preparation of antigen: Bacterial isolate isolated from diseased larvae of P. indicus at Narakkal Prawn Hatchery Laboratory, was grown on TCBS agar medium (Hi-Media) for antigen preparation. After incubation for 24 hours, the bacterial cells were harvested in sterilised saline water and heated at 115 °C for 15 minutes. The antigenic substance was packed by centrifugation at 3500 rpm for 15 minutes and washed 3 times in normal saline water. Prior to injection the antigenic substances were tested for sterility. The final concentration of the antigen was adjusted to 1.0 OD at 530 nm.

Selection and care of experimental fish: From a fresh catch of Oreochromis mossambicus from the grow-out ponds at Narakkal, twenty five specimens ranging from 175 to 226 gm in weight and 20 to 25 cm in total length were randomly selected. These were grouped further into five sets, each with five specimens and placed in one tonne capacity fibre glass tank containing filtered pond water and provided with aeration. Five specimens of O. mossambicus from the catch were tested for naturally occurring antibodies against the new isolate of Vibrio. The hydrological parameters recorded during the experimental period were: temperature, 26 - 31°C; salinity, 5 -10 ‰ ; pH 7.6 - 8.0. Four groups of fish were maintained totally and one group was treated as control. The fish were fed with groundnut oil cake and cooked rice.

Inoculation of fish: Each test fish was inoculated intramuscularly with 0.1 ml of antigenic material of the present isolate immediately posterior to the first dorsal fin. Five injections each at an interval of 72 hours was given. One week following the fifth injection, 0.1 ml booster injection was administered. The control fish were injected with normal saline water.

Collection of blood samples: One week after the booster injection, blood samples were collected. Prior to the collection of blood, all the fishes were anaesthetized in a solution of 0.1% chlorbutol. Animals were bled by cardiac puncture directly through operculum with the help of hypodermic syringe. 1.5 - 2.0 ml of blood was collected in centrifuge tube, allowed to clot and centrifuged at 3000 rpm for 10 minutes. Serum was collected and stored at -15 °C.

Agglutination and titer determination procedures: Agglutination titers were determined using two fold serial dilution procedure (Kolmer et al., 1951) and slide agglutination technique. Cell suspensions used for agglutination tests were prepared as stated in preparation of antigen.

RESULTS

The ten isolates isolated from the diseased larvae and the rearing medium showed almost the same reaction for different characterization tests. The various characteristics of the present isolates studied are summarised in Tables 6.2, 6.3, 6.4 and 6.5.

Table 6.2 Biological characteristics of isolates and reference strains.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	S01	SA1
Pellicle on broth	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Swarming	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 5°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10°C	-	-	-	-	-	-	-	-	-	-	-	-	-	+
15°C	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+
30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	-
42°C	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Growth at pH 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	(+)

..... contd.

Table 6.2 contd.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	SO1	SA1
NaCl tolerance														
0%	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1%	-	-	-	-	-	-	-	-	-	-	-	-	-	+
2%	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+
3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8%	-	-	-	-	-	-	-	-	-	-	+	+	+	-
10%	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Growth on Pyronin G	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neutral red	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Crystal violet	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CLED Agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MacConey agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TCBS agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Teepol broth	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Susceptible to														
Bacitracin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cloxacillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nalidixic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrofurazone	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Oxytetracycline	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Olendomycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pencillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-

.... Contd.

Table 6.2 contd.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	SO1	SA1
Polymyxin B	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Strepromycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetracycline	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sensitivity to O/129	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O/129 sensitivity:														
5 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	+
10 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	+
50 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	+
100 µg	+	+	+	+	+	+	+	+	+	+	+	+	+	+
150 µg	+	+	+	+	+	+	+	+	+	+	+	+	+	+
300 µg	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ : Positive, - : Negative, (+) : Weak or delayed positive.

Colonial and cell morphology: Colonies developed on ZoBell's agar were circular, 3-6 mm in diameter, entire, smooth, slightly raised and cream coloured. This organism developed green coloured colonies on TCBS agar. Cells were small (2.0 - 2.5 x 0.7 - 1.0 μm), Gram-negative rods which were motile.

Biological characters: This organism was found to grow in the temperature range of 15 C to 37 °C, the optimum temperature for growth being 30 °C (Table 6.2). No growth was observed at 5 °C and above 42 °C. The isolate was cultured at different pH ranging from 3 to 10 and was found to grow well at pH 8.5. This organism was grown well in the peptone broth containing 3% (W/V) NaCl. However it failed to grow in the same medium without NaCl and in the medium with 8% (W/V) NaCl. Growth of this organism was observed on neutral red agar, crystal violet agar, CLED agar, MacConkey's agar, pyronin G agar and teepol broth. It was sensitive to chloramphenicol, polymyxin B and vibriostatic agent, O/129 and resistant to bacitracin, cloxacillin, nalidixic acid, oxytetracycline, olendomycin , penicillin, streptomycin and tetracycline. Minimum inhibitory concentration (MIC) of O/129 for the isolate was found to be 100 μg/ml of the medium (Table 6.2).

Biochemical properties: Lysine was decarboxylated in the Moller's medium, but neither arginine nor ornithine was decarboxylated. The organism reduced nitrate to nitrite and failed to deaminate phenylalanine. Voges - Proskauer reaction was negative and methyl red test was positive. Catalase

and oxidase were produced. The present isolate failed to produce hydrogen sulphide and urease in triple sugar iron agar and Christensen's medium respectively. Indole production and cholera red reaction were positive. The organism did not yield ammonia from seawater peptone and gluconate reduction was negative. The present isolate produced acid from glucose, but not that gas (Table 6.3). The organism also produced acid from the following carbohydrates: cellobiose, dextrin, ethanol, fructose, galactose, glycerol, glycogen, maltose, mannitol, mannose, salicin and trehalose, but not in adonitol, arabinose, dulcitol, inositol, inulin, lactose, melibiose, raffinose, rhamnose, sorbitol, sucrose and xylose (Table 6.4). Strach, gelatin, chitin and casein were digested and alginate was however not digested by the isolate. Aesculin and tributyrin were hydrolysed. Deoxyribonucleic acid was digested (Table 6.3)

Citrate was not utilized as a sole source of carbon in Simmon's citrate agar. The organism utilized the following compounds as a sole source of carbon and produced acid. alanine, cellobiose, dextrin, fructose, galactose, glycerol, mannitol, mannose and trehalose. However, the compounds such as adonitol, arabinose, dulcitol, ethanol, inositol, lactose, melibiose, phenol, phenylalanine, putrescine, raffinose, rhamnose, salicin, sodium acetate, sodium alginate, sodium citrate, sorbitol, sucrose, tyrosine and xylose were not utilised by this organism (Table 6.5).

The blood serum samples of five specimens of O. mossambicus collected from the pond were screened for naturally occurring antibodies

Table 6.3 Biochemical characteristics of isolates and reference strains.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	S01	SA1
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalnine deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges - Proskauer reaction	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Methyl red test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TSI	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	A/A	A/A
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cholera red reduction	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Gluconate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	+
OF test	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Gas production from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enzyme production:														
Alginase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+

....Contd.

Table 6.3 contd.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	SO1	SA1
Chitinase	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Gelatinase	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Lipase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein dgestion	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Tributyrin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DNAse	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Simon's citrate	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Amonia from peptone	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ : Positive, -: Negative, K/A : Alkaline slope and acid bud, A/A : Acid slope and acid bud
F : Fermentative.

Table 6.4. Carbohydrate utilisation of present isolates and reference strains.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	SO1	SA1
Acid from														
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	-	-	(+)	+
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Contd..

Table 6.4. Contd.....

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	S01	SA1
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ : Positive, - : Negative, (+) : Weak or delayed positive.

Table 6.5. Utilisation of organic compounds by the prsentation isolates reference strains.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	S01	SA1
Utilisation of a sole carbon source														
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Contd..

Table 6.5. Contd.

Characteristics	Present isolates										Reference strains			
	M1	M2	M3	M4	M5	M6	M7	W1	W2	W3	SP1	SP2	SO1	SA1
Putrescine	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium acetate	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Sodium alginate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ : Positive, - : Negative, (+) : Weak of delayed positive.

against the present isolate. There was no detectable agglutinin antibodies against the present organism. Following this, slide agglutination tests of antiserum raised in the fish with V. alginolyticus, V. anguillarum, V. parahaemolyticus, Aeromonas sp., Pseudomonas sp., Alcaligenes sp., Flavobacterium and the present isolate were carried out and the results are presented in Table 6.9. While V. alginolyticus, V. parahaemolyticus and the present isolate showed positive reaction in the slide agglutination test, all other species of bacteria tested gave negative reaction to the antiserum raised against the new isolate of Vibrio. The homologous titre of the serum ranged from 1 : 576 to 1 : 1152. V. alginolyticus and V. parahaemolyticus also gave titre values ranging from 1:104 to 1:256 and 1 : 120 to 1 : 352 respectively to the antiserum of the new isolate of Vibrio (Table 6.10).

The results obtained from the experts from India and abroad are given in Appendix 1.

Table 6.6. Characterics of the isolate from diseased mysis larvae of Penaeus indicus in comparision with those of genus Vibrio described by Bergey's manual (1984).

Characteristics	@ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Cytochromeoxidase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
O/129 Sensitivity																										
10 micro gram	-	-	+	+	-	+	-	+	-	-	+	-	V	-	+	-	-	-	-	+	+	-	+	+	+	+
150 micro gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swarming	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	+	-	-	-	-
Luminescence	-	-	-	-	-	V	-	+	-	-	-	V	+	-	-	-	-	-	-	-	-	-	+	-	-	-
Arginine dihydrolase	-	-	+	-	-	-	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-
Lysine decarboxylase	+	+	-	-	+	+	-	+	-	-	-	+	+	+	V	-	-	-	+	-	-	+	-	-	+	+
Ornithine decarboxylase	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-
Growth at 42°C	-	+	-	-	-	+	-	-	-	-	+	V	-	-	V	V	V	-	+	-	-	-	-	-	+	-
Growth in 0% NaCl	-	-	-	V	-	+	-	-	V	V	-	-	-	-	V	-	-	-	-	-	-	+	-	-	-	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	V	+	+	+	+	+	+	*	-	+	+	+	-	+	+	+	+	V	V	+	-
8% NaCl	-	+	V	-	V	-	+	+	V	V	-	V	*	-	V	V	V	-	+	V	V	+	-	-	-	-
10% NaCl	-	+	-	-	-	-	+	-	-	-	-	V	*	-	-	-	V	-	-	-	-	-	-	-	-	-
Voges - Proskauer reaction	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
Gas from glucose fermentation	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Contd..

Table 6.6. Contd..

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Fermentation to acid:																										
Arabinose	-	-	V	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-	V	-	-	-	-	-	-	-
Inositol	-	-	V	-	-	-	-	-	-	-	-	-	-	-	V	V	-	V	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	-	+	+
Mannose	+	+	+	-	-	V	+	+	+	+	+	+	+	+	V	-	-	-	+	V	V	+	+	-	-	+
Mannitol	+	+	-	-	V	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-
Sucrose	-	+	+	+	-	+	+	-	+	+	+	V	V	V	+	+	+	-	-	+	+	-	V	-	-	-
Enzyme production:																										
Alginase	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	+	+	-	V	-	-	-
Amylase	+	+	+	-	+	+	-	-	+	V	+	+	-	-	+	V	-	+	+	-	+	+	+	+	+	+
Chitinase	+	+	+	V	+	+	-	V	+	+	-	+	+	+	+	-	V	+	+	V	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	-	+	+	+	+	-	V	+	+	V	+	+	-	+	+	+	+	+	+
Lipase	+	+	+	-	+	+	V	+	+	+	+	+	V	+	+	+	-	+	+	+	+	+	+	+	+	+
Utilisation as sole source of carbon:																										
Cellobiose	+	-	V	-	-	-	-	+	V	-	+	+	+	-	-	V	-	+	-	-	-	-	+	V	+	+
Ethanol	+	V	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	V	+	V	-	-	-	-	-	-
Gluconate	+	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+	+	V	+	+	+	+	V	-	+	+
Putrecine	-	V	-	-	-	-	-	-	V	+	-	-	-	-	-	+	+	-	+	+	+	+	-	-	-	-
Sucrose	-	+	+	+	-	+	+	-	+	+	+	V	-	-	+	+	+	-	-	+	V	-	V	-	-	-
xylose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ : Positive, - : Negative, V : variable, * : not stated

1. Present isolate(Vibrio sp. 2448-88)
2. Vibrio alginolyticus

Contd..

6.6 Contd..

3. V. anquillarum I
4. V. anquillarum II
5. V. campbellii
6. V. cholerae
7. V. costicola
8. V. fischeri
9. V. fluvialis I
10. V. fluvialis II
11. V. gazogenes
12. V. harveyi
13. V. logei
14. V. marinus
15. V. metschnikovii
16. V. natriegens
17. V. nereis
18. V. nigripulchritudo
19. V. parahaemolyticus
20. V. pelagius I
21. V. pelagius II
22. V. proteolyticus
23. V. splendidus I
24. V. splendidus II
25. V. vulnificus I
26. V. vulnificus II

Table 6.7. Characterstics of isolates from diseased mysis larvae of Penaeus indicus in comparison with those of genus Vibrio described by various authors other than Bergey's manual (1984).

Characteristics	@ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Motility	+	+	+	+	*	*	+	+	+	+	+	+	+	+	+	*
Swarming colonies	-	+	-	-	-	*	*	*	-	*	*	*	*	-	-	*
Fermentative metabolism	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of:																
Catalase	+	+	+	+	+	V	+	+	+	*	+	+	+	+	+	*
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	+	-	*	+	*	-	-	*	-	-	-	-	-	-
H_2S	-	-	-	-	*	*	+	-	-	*	-	-	-	-	-	-
Indole	+	+	-	-	-	V	+	-	+	-	+	+	+	+	+	-
Lysine decarboxylase	+	+	-	-	*	-	*	+	+	-	+	-	+	+	-	-
Ornithine decarboxylase	-	+	-	-	*	-	*	-	-	-	-	-	+	+	-	-
Phenylalanine deaminase	-	-	-	-	*	*	*	-	-	*	-	-	-	*	-	*
Pigment	-	-	-	-	Red	-	-	*	*	*	-	-	-	*	-	-
Degradation of:																
Aesculin	+	*	*	-	*	*	*	*	*	*	*	*	*	*	*	*
Chitin	+	+	+	V	*	*	+	*	+	*	*	*	*	-	+	*
Gelatin	+	+	-	+	+	+	+	+	+	*	+	+	+	+	+	-
Lipids	+	+	-	-	*	+	+	*	*	*	+	+	+	+	+	*
Starch	+	+	+	-	+	+	+	+	+	*	*	*	*	+	+	*
Urea	-	+	+	-	*	*	*	-	-	*	-	-	-	-	-	-

Contd...

Table 6.7. Contd...

Characteristics	@ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Methyl red test	+	+	+	-	*	*	+	+	(+)	-	-	(+)	+	+	+	*
Nitrate reduction	+	+	+	V	+	*	+	+	+	*	+	+	+	+	+	*
Voges-Proskauer test	-	-	+	-	*	-	-	-	+	-	+	-	+	-	-	-
Sensitivity to O/129	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	*
Growth at 37°C	+	+	*	-	-	V	*	+	+	*	*	*	*	+	-	*
42°C	-	*	*	*	*	-	*	*	+	*	*	*	*	-	-	*
Growth in 0% NaCl	-	-	-	-	-	-	-	-	+	*	*	*	*	-	-	*
3% NaCl	+	*	*	*	+	+	*	+	+	+	*	*	*	+	+	*
6% NaCl	+	*	*	*	*	V	*	-	(+)	(+)	*	*	*	-	-	*
8% NaCl	-	+	-	-	*	-	*	-	-	(+)	*	*	*	-	-	*
10% NaCl	-	*	*	*	*	*	*	*	*	(+)	*	*	*	-	-	*
Utilisation of citrate	-	-	-	-	-	V	+	+	+	+	+	-	+	+	+	+
Acid from:																
Adonitol	-	*	*	*	*	*	*	*	-	*	-	-	-	-	*	-
Arabinose	-	-	-	-	-	*	*	+	-	*	-	-	-	-	-	-
Cellobiose	+	*	*	*	*	*	*	+	-	*	+	+	+	+	+	-
Dextrin	+	*	*	*	+	*	*	+	(+)	*	+	+	+	+	+	*
Dulcitol	-	*	*	*	*	*	*	-	-	*	-	-	-	-	-	-

Contd...

Table 6.7. Contd..

Characteristics	@ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ethanol	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Fructose	+	*	*	*	*	*	*	+	+	*	+	+	+	*	+	*
Galactose	+	*	*	*	*	*	*	+	+	*	−	+	−	+	+	+
Glucose	+	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	*	*	*	*	*	+	−	(+)	*	+	+	+	+	+	(+)
Glycogn	+	*	*	*	*	*	*	+	(+)	*	+	+	+	+	+	−
Inositol	−	−	−	−	*	*	*	−	−	*	−	−	−	−	−	−
Inulin	−	*	*	*	*	*	*	−	−	*	−	−	−	*	*	−
Lactose	−	−	−	−	*	−	−	−	(+)	*	−	+	−	(+)	+	−
Maltose	+	*	+	+	+	+	*	+	+	*	+	+	+	+	+	+
Mannitol	+	*	−	+	*	*	*	−	(+)	*	+	+	+	+	−	+
Mannose	+	+	+	*	*	*	*	+	−	*	+	+	+	+	+	+
Melibiose	−	*	*	*	*	*	*	*	−	*	*	*	*	+	(+)	−
Raffinose	−	*	*	*	*	*	*	−	−	*	−	−	−	−	−	−
Rhamnose	−	*	*	*	*	*	*	−	−	*	−	−	−	−	−	−
Salicin	+	+	+	*	*	*	*	+	−	*	−	−	+	+	−	−
Sorbitol	−	*	*	*	*	*	*	−	−	*	−	−	−	+	−	(+)
Sucrose	−	+	−	+	*	+	+	−	+	*	+	−	+	−	+	−
Trehalose	+	*	*	*	*	*	*	+	(+)	*	+	+	+	+	+	+
xylose	−	*	*	*	*	−	*	−	−	*	−	−	−	−	−	−

+ : Positive, − : Negative, (+) : weak or delayed positive, V : variable, * :Not stated.

@

1. Present isolate.

2,3 and 4 Vibrio species described by Austin and Austin (1987).

contd..

6.7 contd....

5. *V. psychroerythrus* described Aoust and Kushner (1972).

6. *Vibrio* sp. described by Cook and Lofton (1973).

7. *Vibrio* sp. described by Tubiash *et al*. (1970).

8. *Vibrio* sp. described by Muroga *et al*. (1976a).

9. Non-cholera *Vibrio* described by Muroga *et al*. (1979)

10. *Vibrio* sp. described by Bowser *et al*. (1981).

11, 12 and 13 *Vibrio* species described by Elston *et al*. (1982).

14. *Vibrio* sp. described by Takahashi *et al*. (1984).

15. *Vibrio* sp. described by Takahashi *et al*. (1985).

16. *Vibrio* sp. (NCMB 2245) described by Bruno *et al*. (1986).

DISCUSSION

The present isolate, on the basis of its morphological, physiological and biochemical characters, is found to belong to the genus Vibrio (Table 6.2, 6.3, 6.4 and 6.5). A comparison of the various characteristics of the present isolate with those of the different species of Vibrio described in the literature is given in Tables 6.6 and 6.7. This indicates that the present organism differs considerably from those described earlier. However, it shows close resemblance to Vibrio sp. (Takahashi et al., 1984), Vibrio sp. (Cook and Lofton, 1973), V. vulnificus II, Vibrio sp. (Muroga et al., 1976a), V. carchariae, V. campbellii, Vibrio sp. 25 - 1 and Vibrio sp. 26 - 1 (Elston et al., 1982) (Table 6.8).

The present isolate resembles Vibrio sp. isolated from the diseased postlarvae of P. japonicus by Takahashi et al.(1984). However, the present isolate differs from this species in citrate utilisation , ammonia production, chitin digestion , ornithine decarboxylation, growth in 6% (W/V) NaCl and acid production from lactose, melibiose and sorbitol (Table 6.7).

Vibrio sp. described by Cook and Lofton (1973) shows 85.71% resemblance to the present organism (Table 6.8), but certain characters such as hydrogen sulphide production, citrate utilisation and acid production from sucrose give opposite reaction with the present isolate (Table 6.7). V. vulnificus biogroup II has been associated with the mortality of cultured eels (Tison et al., 1982). This species can be differentiated from the present isolate by its negative reaction in indole production, acid formation from

Table 6.8. Percentage similarities* between the present isolate and currently described vibrios

S.No.	Species of *Vibrio*	% similarity
1.	*Vibrio* sp. (Takahashi *et al.*, 1984)	86.21
2.	*Vibrio* sp.(Cook and Lofton,1973)	85.71
3.	*V. vulnificus* II	85.29
4.	*Vibrio* sp. (Muroga *et al.*, 1976a)	83.33
5.	*V. carchariae*	82.61
6.	*V. campbellii*	82.35
7.	*Vibrio* sp. (25-1) (Elston *et al.*, 1982)	80.00
8.	*Vibrio* sp. (26-1) (Elston *et al.*, 1982)	80.00
9.	*V. marinus*	79.41
10.	*V. nigripulchritudo*	79.41
11.	*V. parahaemolyticus*	79.41
12.	*V. splendidus* II	79.41
13.	*V. vulnificus* I	79.41
14.	*V. logei*	76.67
15.	*V. fisheri*	76.47
16.	*Vibrio* sp. (Takahashi *et al.*, 1985)	75.86
17.	*V. harveyi*	73.53
18.	*V. fluvialis* I	73.53
19.	*V. damsela*	70.83

Table 6.8. Contd..

S.No.	Species of *Vibrio*	% similarity
20.	*V. splendidus* I	70.59
21.	*Vibrio* sp. (25-2) (Elston. *et al.*, 1982)	70.00
22.	*V. alginolyticus*	67.65
23.	*V. fluvialis*	67.65
24.	*V. pelagius* II	67.65
25.	*V. cholerae*	64.71
26.	*V. anguillarum*	64.71
27.	*V. ordalii*	63.64
28.	*Vibrio* sp. (Bruno *et al.*, 1986)	62.50
29.	*V. costicola*	61.77
30.	*V. gazogenes*	61.77
31.	*V. natriegenes*	61.77
32.	*Vibrio* sp. (Tubiash *et al.*, 1970)	61.11
33.	Non-cholera *Vibrio* (Muroga *et al.*, 1979)	59.26
34.	*V. pelagius* I	58.82
35.	*V. nereis*	52.94
36.	*V. metschnikovii*	52.94
37.	*V. proteolyticus*	51.73
38.	*Vibrio* sp. (Bowser *et al.*, 1981)	36.36

*Similarity percentage was calculated by the formula given in the ninth edition of fundamentals of microbiology by M. Frobisher, R.D. Hinsdill, K.T. Crabtree and C.R. Goodheart (1974).

Table 6.9 Slide agglutination test of antiserum raised in *Oreochromis mossambicus*.

Bacterial strain	Antiserum against *Vibrio* sp. 2448-88
Vibrio sp. 2448-88	+
V. alginolyticus	+
V. parahaemolyticus	+
V. anguillarum	-
Aeromonas sp.	-
Pseudomonas	-
Alcaligenes	-
Flavobacterium sp.	-

Table 6.10. Agglutination titer * of antiserum raised in *Oreochromis mossambicus* when tested with antigen of *Vibrio* sp. 2448-88, *V. alginolyticus* and *V. parahaemolyticus*.

Antigen	Agglutination titer			
	Control	1	2	3
Vibrio sp. 2448-88	0	1 : 1024	1 : 576	1 : 1152
V. alginolyticus	0	1 : 176	1 : 104	1 : 256
V. parahaemolyticus	0	1 : 256	1 : 120	1 : 352

*Based on five trials.

mannitol and ethanol and growth in 6% (W/V) NaCl. Further, V. vulnificus II is known as lactose fermenting Vibrio, but the present isolate does not ferment lactose. Dr. R. Sakazaki (Personal communication) who analysed the present isolate found close resemblance to V. vulnificus, but the above mentioned negative points observed in the present study disagree with Dr.Sakazaki's statement.

Muroga et al. (1976a) have isolated a pathogenic Vibrio from diseased eel. The present isolate shows close resemblance to this Vibrio (83.33%) except in characters such as indole production, cholera red test, citrate utilisation, growth in 6% (W/V) NaCl and acid formation from arabinose, mannitol and glycerol; for these characters the present isolate shows opposite reaction (Table 6.7).

V. carchariae has been found to be pathogenic to sharks (Grimes et al., 1984b). It shows 82.61% similarity to the present isolate. However, unlike the present isolate, V. carchariae grows well in 8% (W/V) NaCl, decarboxylates ornithine and produces acid from sucrose.

Species of Vibrio (Vibrio sp. 25 - 1 and Vibrio sp. 26 - 1) which have been isolated and characterised by Elston et al. (1982) also resemble the present isolate in many respects such as lysine decarboxylation and acid formation from cellobiose, dextrin, frutose, glycerol, glycogen, maltose, mannitol, mannose and trehalose. These vibrios, however, differ from the present isolate in Voges - Proskauer test, citrate utilisation and acid

formation from sucrose. Although V. marinus exhibits 79.41% resemblance to the present isolate, it differs in many respects such as failure of growth in 35 °C and 6% (W/V) NaCl, negative reaction in starch digestion and acid production in cellobiose, trehalose and mannitol.

The antibodies raised in fish against the present isolate were not specific for the present isoalte. This might be due to the formation of antibodies common for vibrios in fish.

The present isolate was referred to Dr.G.Balakrish Nair, Prof. John L.Fryer, Dr. Jim J. Farmer and Dr. James D. Oliver for comparing its characteristics with the reference to vibrios avaiable in their laboratories and to confirm the observations made by the candidate as well as to obtain their opinions to give specific status for the present isolate. API 20E multiple test was carried out by Dr. G. Balakrish Nair and Prof. John L. Fryer to identify the organism (Appendix 1). They found that the new isolate of Vibrio differs from the currently described vibrios as observed in the present study.

Computer analysis based on serological type, biochemical tests and antibiogram carried out on the present organism by Dr. Jim J. Farmer showed close resemblance of the present isolate to V. vulnificus (Appendix 1). However, colistin sensitive and negative reaction string test observed by Dr. Farmer and positive reaction indole production, acid formation from mannitol and ethanol and growth in 6% (W/V) NaCl observed in the present

study disagree to classify the present isolate as V. vulnificus.

The statement received from Dr. James D. Oliver , University of North Carolina regarding the present isolate is given below:
"The culture should be grown in broth and agar, and checked for bioluminescence, assuming it is negative then your culture is very similar to V. proteolyticus (formerly referred to as Aeromonas hydrophila sub sp. proteolytica). It does differ from V. proteolyticus, however in the arginine dihydrolase reaction, that should be retested, and you might also check the cellobiose reaction (V. proteolyticus is negative). If your culture is arginine dihydrolase negative, then this may be a new species. I have compared your data with that for all vibrios I have literature on, and it does appear different".

Accordingly, tests of luminescence, arginine dihydrolase and acid production from cellobiose were carried out and negative reaction in luminescence and arginine dihydrolase and positive reaction in acid production from cellobiose were observed.

Another species of Vibrio which closely approximates to the present isolate is V. campbellii. It has been characterized by Baumann et al. (1971). It shows 82.35% resemblance to V. campbellii. DNA-DNA hybridisation test carried out (Appendix 1) by Dr. Jim J. Farmer supports close resemblance of the new isolate of Vibrio to V. campbellii. However, Vibrio sp. at hand can be differentiated from V. campbellii on the

characters of growth in 8% (W/V) NaCl, utilisation of citrate, gluconate and tyrosine, and acid formation from salicin, galactose and glycerol. Further there is no report of V. campbellii affecting fish or shellfish.

Taking into consideration the opinions expressed by above experts from India and abroad and the detailed discussion on the comparison of the character between the present Vibrio and those described earlier, it could be concluded that the present isolate from the larvae of P. indicus differs appreciably from the known vibrios. The most diagnostic characters of the present isolate are : fermentative, motile, Gram - negative rod; it produces catalase, oxidase, indole and lysine decarboxylase, but not hydrogen sulphide, arginine dihydrolase, ornithine decarboxylase, phenylalanine deaminase or urease are produced. Gelatin, DNA, lipids, starch and aesculin are degraded , and sensitive to vibriostatic agent, O / 129. The API 20E profile for the present isolate is

+ - + - - - - + + - + + + - - - - - + - +

However the present study could be concluded that the above mentioned properties suggest the present isolate as a member of the genus Vibrio, but these properties disagree with those of known Vibrio in the literature . The present isolate was deposited in Vibrio Reference Laboratory, Division of Bacterial Diseases, Centres for Diseases Control, Atlanta, Georgia, U.S.A. and coded as 2448-88.

CHAPTER 7

EXPERIMENTAL PATHOGENICITY OF VIBRIO SP. 2488-88 AND RELATED SPECIES ON THE LARVAE AND POST LARVAE OF PENAEUS INDICUS, P. MONODON AND P. SEMISULCATUS.

INTRODUCTION

Vibriosis, a bacterial infection caused by the species of genus Vibrio, has been considered to be the most important infectious disease in the wild finfish and shellfish as well as those cultivated in different culture systems. In Japan, the production loss of the Kuruma prawn Penaeus japonicus, due to vibriosis has been estimated at about 30.8 tonnes per annum (Sano and Fukuda, 1987). Of the twenty species of Vibrio (Kriez and Holt, 1984), Colwell and Grimes (1984) listed eight species as fish pathogens while Austin and Austin (1987) described seven species namely V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae, V. damsela, V. ordalii and V. vulnificus to bring forth vibriosis in fishes. Besides these, vibriosis of marine organisms due to other Vibrio sp. has been reported by several authors (Muroga et al., 1979; Yamanoi et al., 1980; Bowser et al., 1981; Elston et al., 1982; Takahashi et al., 1984,1985; Bruno et al., 1986). Several species of Vibrio which otherwise form the normal microbial flora of marine organisms, have also been demonstrated in the laboratory as causative organisms of vibriosis (Vanderzant et al., 1970; Lewis, 1973b; Lightner and Lewis, 1975).

Although fish and shellfish diseases caused by primary bacterial

etiology have been reported in certain finfishes and shellfishes, majority of the causes are of secondary etiology associated with wounds and environmental stress. In such situation, the determination of the relationship between the host and the pathogen assumes great importance. To study this aspect and the effect of pathogen at different levels of concentration and condition, the pathogens are experimentally used to challenge the host. The German microbiologist Robert Koch set up criteria now known as Koch's postulates in order to relate the presence of a specific organism to a specific disease. Introduction of pathogen into the host is accomplished by several ways, such as addition of pathogen from a pure culture into the medium in which the host is reared, injection of pathogen into the host's body, feeding the pathogen incorporated diet to the host and introduction of the pathogen through an artificial wound. Thus, the bacterial isolates obtained from diseased fishes and shellfishes were administered into the healthy ones to produce disease and death in the experimental animal to confirm Koch's postulates in many laboratories in differnt parts of the world (Vanderzant et al., 1970; Lewis, 1973b; Lightner and Lewis, 1975; Takahashi et al., 1984,1985). The most important observations among them are given below.

V. anginolyticus though not recognised as a fish pathogen, was found to be pathogenic to shrimp when administered through intramuscular injection (Lightner and Lewis, 1975). It was further observed that the addition of bacterial isolates in the food given to the shrimp proved to be an unsuccessful means of infection, but inoculation by the intramuscular

injection was found to be the most reliable method of ascertaining the pathogenicity of bacterial isolates (Lightner and Lewis, 1975). Leong and Fontaine (1979) observed siginificant mortalities with gross clinincal signs similar to those observed in actual bacterial infections when V. alginolyticus was injected into P. setiferus. In the case of V. anguillarum which is occasionally involved in the vibriosis of prawn, Leong and Fontaine (1979) found the LC_{50} value for P. setiferus and compared its virulence with other Vibrio species. V. anguillarum was found to be pathogenic when injected into juvenile American lobster Homarus americanus held at 20 C (Bowser et al., 1981). V. carchariae was proved lethal for spiny dogfish injected intraperitoneally (Grimes et al., 1984a). It however, did not produce clinical disease when injected into lemon shark (Negaprion brevirostris). V. cholerae was also demonstrated to be a highly virulent fish pathogen when the ayu were immersed in 1.26×10^4 cells/ml (Yamanoi et al., 1980). V. damsela caused rapid death when injected into spiny dogfish Squalis acanthias (Grimes et al., 1984a), whereas it did not affect the lemon shark (Grimes et al., 1985). Vanderzant et al. (1970) reported that addition of 3% inoculum (24 hr culture in BHI broth) of V. parahaemolyticus to the medium in which brown shrimp was reared, brought forth its death within a few hours. This bacterium was also found to be pathogenic for white shrimp (Leong and Fontaine, 1979) and a snail (Ducklow, 1980). V. vulnificus caused 80% mortality in eels when 4.85×10^8 bacterial cells were injected to experimental animals intramuscularly (Austin and Austin, 1987). A bacterium of Vibrio sp. isolated from the midgut gland of diseased postlarvae of P. japonicus was proved to be pathogenic to Kuruma prawn by

inoculation experiments (Takahashi et al., 1984). In another case, Takahashi et al. (1985) have isolated Vibrio sp. from the diseased adult Kuruma prawn with the clinical signs of cloudiness of muscle, brown spot of gill and lymphoid organs which were reproduced experimentally in the healthy prawn by intramuscular injection. Necrotic lesions have been reproduced in crab Callinectes sapidus by inoculating Vibrio sp. into the mechanically damaged exoskeleton (Cook and Lofton, 1973). In India, vibriosis due to V. anguillarum has been reported in finfishes and shellfishes (Mahadevan et al., 1978; Pillai, 1982).

Although, there has been appreciable progress on the study of pathogenicity mechanism of certain species of Vibrio, particularly V. anguillarum, there is not much work from India. To understand the pathogenicity of the new isolate of Vibrio and to obtain a better insight on the role of this bacterium as a pathogen to the larvae and postlarvae of penaeid prawns, experimental inducement of disease on the larvae and postlarvae of P. indicus, P. monodon and P. semisulcatus and on the adult P. indicus was carried out. Further, the pathogenicity of Vibrio sp. 2448-88 was compared with that of V. alginolyticus and V. parahaemolyticus on the larvae and postlarvae of P. indicus. The results of these experimental studies are presented in this section.

The material and methods employed in the pathogenicity experiments are described in detail in chapter 2. The statistical analysis followed for calcualting the LC_{50} values of Vibrio for larval and postlarval stages of

prawn is also given in the chapter 2.

RESULTS

Pathogenicity of *Vibrio* sp. 2448-88 on larvae, postlarvae and adult *P. indicus*.

The accumulated percentage mortality of nauplius, protozoea, mysis, postlarvae and adult *P. indicus* in the immersion method of infection at different time intervals are given in Tables 7.1 to 7.5 respectively. The highest concentration of *Vibrio* sp. 2448-88 (35×10^8 cells/ml of the rearing medium, hereafter expressed as cells /ml) caused cent percent mortality in nauplius, protozoea and mysis after 12, 60 and 72 hours of exposure respectively. The concentrations of the new isolate of *Vibrio* at 35×10^4 and 35×10^5 cells / ml did not cause mortality in nauplius, protozoea, mysis and postlarva as in the control whereas the higher concentrations at 35×10^6 and 35×10^7 cells/ml caused significant mortality in the larval stages of *P. indicus* (Tables 7.1 to 7.3). Total mortality of postlarva I and postlarva X was not observed in any of the concentrations of *Vibrio* sp. 2448-88 tested in the study, although the highest concentration of 35×10^8 cells/ml produced 38.89 % mortality in the postlarva I after 48 hrs of immersion.

Fifty percent mortality was observed in the postlarva X of *P. indicius* when the rearing medium was treated with 3 ml of sterile filtrate of the new isolate of *Vibrio*, but the media treated with 1 ml and 2 ml of filtrate did not cause any mortality.

Table 7.1 Accumulated percentage mortality of nauplius stage of *Penaeus indicus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of the rearing medium	Accumulated (%) mortality at hrs of post-inoculation		
	12	24	32
Control	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00
35×10^6	20.00	25.33	40.00
35×10^7	49.33	62.67	65.33
35×10^8	100.00	100.00	100.00

Table 7.2 Accumulated percentage mortality of protozoeal stage of Penaeus indicus exposed to different concentrations of Vibrio sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation						
	12	24	36	48	60	72	80
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^6	0.00	15.00	15.00	18.33	30.00	36.67	43.33
35×10^7	15.00	41.67	51.67	65.00	66.67	75.00	81.66
35×10^8	50.00	70.00	86.67	93.33	100.00	100.00	100.00

Table 7.3 Accumulated percentage mortality of mysis stage of *Penaeus indicus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	0.00
35×10^6	0.00	13.33	20.00	23.33	23.33	33.33
35×10^7	0.00	20.00	30.00	43.33	50.00	76.67
35×10^8	13.33	40.00	60.00	63.33	83.33	100.00

Table 7.4 Accumulated percentage mortality of postlarva I of *Penaeus indicus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation									
	12	24	36	48	60	72	84	96	108	120
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^8	0.00	11.11	27.78	38.89	38.89	38.89	38.89	38.89	38.89	38.89

Table 7.5 Accumulated percentage mortality of adult Penaeus indicus injected with different concentrations of Vibrio sp. 2448-88 at different time intervals.

No. of bacterial cells/animal	Accumulated (%) mortality at hrs of post-inoculation			
	6	12	18	24
Control	0.00	0.00	0.00	0.00
70×10^5	0.00	0.00	20.00	20.00
70×10^6	40.00	80.00	80.00	80.00
70×10^7	80.00	100.00	100.00	100.00
70×10^8	100.00	100.00	100.00	100.00
70×10^9	100.00	100.00	100.00	100.00

In the adult, 70 x 10^8 and 70 x 10^7 viable cells of Vibrio sp. 2448-88/animal caused 100% mortality within 6 and 12 hrs of post- inoculation respectively when the animals were injected intramuscularly whereas the lower concentrations of 70 x 10^6 and 70 x 10^5 cells/animal registered 80 and 20 % mortality after 12 and 18 hours. Vibrio sp. 2448-88 did not affect the adult prawn when the bacterium was given to the animal through oral route.

The LC_{50} values of Vibrio sp. 2448-88 for nauplius, protozoea, mysis and adult prawn were determined at different time intervals (Table 7.6). For nauplius, LC_{50} value was found to be 7.7 x 10^7 cells/ml after 24 hrs of exposure; for protozoea at 7.03 x 10^8, 1.87 x 10^8, 7.4 x 10^7 and 5.23 x 10^7 cells/ml respectively after 24, 48, 72 and 80 hrs of immersion, and for mysis at 1.51 x 10^{10}, 8.06 x 10^8 and 8.09 x 10^7 cells/ml. The LD_{50} value for the adult P. indicus was seen at 2.18 x 10^7 cells/ animal after 24 hours of injection. This value, in fact was the lowest concentration recorded among the LC_{50} values of Vibrio sp. 2448-88 recorded for the various stages. This might be due to the injection of bacterium intramuscularly in the case of the adult whereas in the larval stages, immersion method was followed to infect the host.

Pathogenicity of Vibrio sp. 2448-88 on the larvae and postlarvae of P. monodon

Tables 7.7 to 7.11 show the accumulated percentage mortality of nauplius, protozoea, mysis, postlarva III and postlarva X of P. monodon at

Table 7.6 LC_{50} values* of *Vibrio* sp. 2448-88 for nauplius, protozoea and mysis larval stages and for adult of *Penaeus indicus* at different time intervals

Larval stage	LC_{50} values at different time intervals (time in hours)		
	24	48	72
Nauplius	7.7×10^7	-	-
	$(3.1 \times 10^6 \ldots 1.2 \times 10^9)$**		
Protozoea	7.03×10^8	1.87×10^8	7.4×10^7
	$(3.9 \times 10^8 \ldots 1.4 \times 10^9)$	$(1.2 \times 10^8 \ldots 2.8 \times 10^8)$	$(4.3 \times 10^7 \ldots 1.1 \times 10^8)$
Mysis	1.51×10^{10}	8.06×10^8	8.09×10^7
	$(2.2 \times 10^9 \ldots 2.5 \times 10^{11})$	$(2.4 \times 10^8 \ldots 6.7 \times 10^9)$	$(3.9 \times 10^7 \ldots 1.4 \times 10^8)$
Postlarvae I to V	ND	ND	ND
Postlarvae to V	ND	ND	ND
Adult	$2.18 \times 10^{7***}$		
	$(6.8 \times 10^7 \ldots 5.7 \times 10^8)$	-	-

* No of bacterial cells per ml of rearing medium.
** Confidence level of LC_{50}.
*** LD_{50} value.
ND Not determined as the mortality was not observed in more than one concentration.

Table 7.7. Accumulated percentage mortality of nauplius stage of *Penaeus monodon* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ of rearing medium	Accumulated (%) mortality at hrs of post-inoculation	
	12	24
Control	0.00	0.00
35×10^4	0.00	0 00
35×10^5	20.00	46.00
35×10^6	64.00	80.00
35×10^7	84.00	100.00
35×10^8	100.00	100.00

Table 7.8. Accumulated percentage mortality of protozoeal stage of *Penaeus monodon* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	3.11
35×10^4	0.00	0.00	0.00	0.00	18.83	23.33
35×10^5	11.67	33.33	40.00	43.33	46.67	56.67
35×10^6	18.33	45.00	58.33	71.67	80.00	88.89
35×10^7	35.00	68.33	75.00	86.67	91.67	100.00
35×10^8	55.00	100.00	100.00	100.00	100.00	100.00

Table 7.9. Accumulated percentage mortality of mysis stage of *Penaeus monodon* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	13.33	23.33	23.33
35×10^6	0.00	13.33	20.00	23.33	40.00	67.69
35×10^7	23.33	43.33	50.00	53.33	66.67	93.33
35×10^8	40.00	66.67	83.33	100.00	100.00	100.00

Table 7.10. Accumulated percentage mortality of postlarva III of Penaeus monodon exposed to different concentrations of Vibrio sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation									
	12	24	36	48	60	72	84	96	108	120
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	13.33	13.33	13.33	26.67	26.67	26.67	33.33	40.00
35×10^6	0.00	13.33	13.33	20.00	20.00	26.67	26.67	33.33	46.67	66.67
35×10^7	0.00	20.00	26.67	40.00	40.00	60.00	66.67	66.67	80.00	80.00
35×10^8	13.33	26.67	40.00	53.33	53.33	86.67	100.00	100.00	100.00	100.00

Table 7.11. Accumulated percentage mortality of postlarva of *Penaeus monodon* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation									
	12	24	36	48	60	72	84	96	108	120
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33
35×10^7	0.00	0.00	0.00	0.00	0.00	16.67	16.67	25.00	33.33	33.33
35×10^8	0.00	25.00	25.00	33.33	33.33	33.33	41.67	41.67	41.67	41.67

different concentrations of Vibrio sp. 2448-88 at different time intervals. The concentration of 35 x 10^8 cells/ml caused 100% mortality in nauplius,protozoea, mysis and postlarva III of P. monodon after 12, 24, 48 and 84 hrs of exposure respectively. Experimental infection at the lowest concentration of 35 x 10^4 cells/ml could not establish the disease in nauplius, mysis and postlarvae, but it caused 23.33% mortality in protozoeal population after 72 hrs of immersion (Table 7.8). This pathogen also produced 100% mortality in nauplius and protozoea of P. monodon at the concentration of 35 x 10^7 cells/ml whereas the same concentration could produce only 62.67 and 75% mortality in nauplius and protozoea of P. indicus respectively. Immersion of postlarva III of P. monodon in a suspension containing 35 x 10^8 cells/ml brought forth 100% mortality. However, the same concentration recorded only 38.89% mortality in the postlarva I of P. indicus; on the other hand this concentration failed to produce 100% mortality of postlarva X of P. monodon. But experimental exposure to 120 hrs at this concentration caused 41.67% mortality in the postlarva X of P. monodon (Table 7.11).

The LC_{50} values of the new isolate of Vibrio for the nauplius was seen at 4.81 x 10^6 cells/ml after 24 hrs of inoculation (Table 7.12). For protozoeal stage, the LC_{50} values recorded were at 2.56 x 10^7, 5.82 x 10^6 and 2.6 x 10^6 cells/ml respectively at 24, 48 and 72 hrs of exposure. As the larvae grew to mysis stage, the LC_{50} values were observed at relatively higher concentrations of 8.35 x 10^8, 2.02 x 10^8 and 1.31 x 10^7 cells/ml at 24, 48 and 72 hrs (Table 7.12). The LC_{50} values for postlarva III

Table 7.12. LC_{50} values* of Vibrio sp. 2448-88 for nauplius, protozoea, mysis and postlarval stages of Penaeus monodon at different time intervals.

Larval stage	LC_{50} values at different time intervals (time in hours)				
	24	48	72	96	120
Nauplius	4.81×10^6 ($2.7 \times 10^6 \ldots 7.5 \times 10^6$)**	-	-	-	-
Protozoea	2.56×10^7 ($7.5 \times 10^6 \ldots 7.1 \times 10^7$)	5.82×10^6 ($1.7 \times 10^6 \ldots 1.2 \times 10^7$)	2.6×10^6 ($1.0 \times 10^6 \ldots 4.6 \times 10^6$)	-	-
Mysis	8.35×10^8 ($3.5 \times 10^8 \ldots 2.9 \times 10^9$)	2.02×10^8 ($9.9 \times 10^7 \ldots 3.8 \times 10^8$)	1.31×10^7 ($4.5 \times 10^6 \ldots 3.6 \times 10^7$)	-	-
PostlarvaIII-VIII	8.24×10^{11} ($3.9 \times 10^9 \ldots 7.1 \times 10^{12}$)	2.06×10^9 ($3.5 \times 10^8 \ldots 1.3 \times 10^{12}$)	9.81×10^7 ($2.5 \times 10^7 \ldots 3.7 \times 10^8$)	8.61×10^7 ($1.7 \times 10^7 \ldots 9.6 \times 10^9$)	9.29×10^6 ($7.6 \times 10^6 \ldots 3.2 \times 10^7$)
Postlarvae - V	ND	ND	ND	ND	5.33×10^9 ($7.7 \times 10^8 \ldots 1.4 \times 10^{10}$)

* No. of bacterial cells per ml of rearing medium.
** Confidence level of LC_{50}
ND Not determined as the mortality was not observed in more than one concentration.

determined at 24, 48, 72, 96 and 120 hrs of experimentation were at 8.24 x 10^{11}, 2.06 x 10^{9}, 9.8 x 10^{7}, 8.61 x 10^{7} and 9.29 x 10^{6} cells/ml respectively. In the case of postlarva X, the LC_{50} value was determined only after 120 hrs of immersion and was found at 5.33 x 10^{9} cells/ml.

Pathogenicity of Vibrio sp. 2448-88 on the larvae and postlarvae of P. semisulcatus

The accumulated percentage mortality of nauplius, protozoea, mysis, postlarva I and postlarva V of P. semisulcatus due to the experimental infection by Vibrio sp. 2448-88 at different concentrations of bacterial cells at different time intervals are given in Tables 7.13 to 7.17. At the concentrations of 35 x 10^{8} cells/ml, 100% mortality of nauplius, protozoea, mysis and postlarva was observed after 12, 36, 48 and 60 hrs of water borne exposure. But no mortality in the larval and postlarval stages of P. semisulcatus was recorded at 35 x 10^{4} cells/ml as observed in P. indicus and P. monodon. In the naupliar stage, the concentrations of 35 x 10^{5}, 35 x 10^{6}, 35 x 10^{7} and 35 x 10^{8} cells/ml produced 15, 46.67, 70.67 and 100% mortality respectively after 24 hrs of exposure. The rearing medium treated with 35 x 10^{5}, 35 x 10^{6}, 35 x 10^{7} and 35 x 10^{8} cells of Vibrio sp. 2448-88/ml respectively caused 18.33, 61.67, 88.33 and 100% mortality of protozoea after 80 hrs of treatment. In mysis larvae, however, 40.0, 86.67 and 100% mortality was observed at 35 x 10^{6}, 35 x 10^{7} and 35 x 10^{8} cells/ml after 72 hrs immersion. The concentrations of 35 x 10^{6}, 35 x 10^{7} and 35 x 10^{8} cells/ml respectively produced 38.89, 72.22 and 100% mortality in postlarva I and 46.67, 60 and 80% mortality in postlarva V

Table 7.13 Accumulated percentage mortality of nauplius stage of *Penaeus semisulcatus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation	
	12	24
Control	0.00	0.00
35×10^4	0.00	0.00
35×10^5	10.00	15.00
35×10^6	29.33	46.67
35×10^7	54.67	70.67
35×10^8	100.00	100.00

Table 7.14. Accumulated percentage mortality of protozoeal stage of *Penaeus semisulcatus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No.ofbacterialcells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation						
	12	24	36	48	60	72	80
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	10.00	13.33	13.33	18.33
35×10^6	0.00	30.00	50.00	53.33	56.67	58.33	61.67
35×10^7	0.00	51.78	66.67	81.70	81.70	85.00	88.33
35×10^8	0.00	80.00	100.00	100.00	100.00	100.00	100.00

Table 7.15. Accumulated percentage mortality of mysis stage of Penaeus semisulcatus exposed to different concentrations of Vibrio sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	0.00
35×10^6	0.00	6.67	6.67	16.67	16.67	40.00
35×10^7	6.67	33.33	33.33	50.00	63.33	86.67
35×10^8	46.67	53.33	76.67	100.00	100.0 0	100.00

Table 7.16. Accumulated percentage mortality of postlarva I of *Penaeus semisulcatus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation							
	12	24	36	48	60	72	84	96
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	11.11	11.11	16.67	16.67	16.67	22.22	27.78
35×10^6	0.00	11.11	22.22	22.22	27.78	33.33	38.89	38.89
35×10^7	11.11	22.22	40.00	61.11	61.11	66.67	66.67	72.22
35×10^8	16.67	27.78	55.56	88.88	100.00	100.00	100.00	100.00

Table 7.17. Accumulated percentage mortality of postlarva V of *Penaeus semisulcatus* exposed to different concentrations of *Vibrio* sp. 2448-88 at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation							
	12	24	36	48	60	72	84	96
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35×10^5	0.00	0.00	0.00	0.00	0.00	13.33	20.00	20.00
35×10^6	0.00	13.33	13.33	20.00	26.67	33.33	33.33	46.67
35×10^7	6.67	26.67	33.33	33.33	40.00	43.33	46.67	60.00
35×10^8	20.00	40.00	40.00	46.67	53.33	60.00	66.67	80.00

after 96 hrs of inoculation of the rearing medium.

LC_{50} values for nauplius, protozoea, mysis and postlarva obtained at different time intervals are given in Table 7.18. LC_{50} value for nauplius larvae was found to be 4.84 x 10^7 cells/ml after 24 hrs of exposure. This value was found to be relatively lower than the values for the nauplii of P. indicus (7.7 x 10^7) but higher than that recorded for nauplii of P. monodon (4.81 x 10^6). For protozoea thev LC_{50} values recorded at the end of 24, 48 and 72 hrs of exposures were at 2.35 x 10^8, 3.87 x 10^7 and 3.06 x 10^7 cells/ml. In the case of mysis, the LC_{50} values estimated were 2.2 x 10^9,2.25 x 10^8 and 5.38 x 10^7 cells/ml after 24, 48 and 72 hrs of immersion. The LC_{50} values recorded for postlarva I to IV were 4.82 x 10^{10}, 1.97 x 10^8 and 1.09 x 10^8, and for postlarva V to IX the values were 1.21 x 10^{10} , 5.32 x 10^9 and 5.08 x 10^8 cells/ml respectively after 24, 48 and 72 hrs of exposure. The LC_{50} values of protozoea and mysis of P. semisulcatus were found to be intermediate between P. indicus and P. monodon.

Clinical signs due to Vibrio sp. 2448-88 infection in the larvae and postlarvae of P. indicus, P. monodon and P. semisulcatus

Expansion of chromatophores was observed in all the stages of P. indicus, P. monodon and P. semisulcatus due to vibriosis. In nauplius, the chromatophore expansion was seen in caudal region and appendages. In protozoea, mysis and postlarvae, the chromatophores were expanded at the base of eye stalk, rostrum, carapace, abodominal segments, cephalothoracic and abdominal appendages and telson. In severe case, the mysis and postlarvae became reddish in colour. Normally the larvae and postlarvae

Table 7.18. LC_{50} values* of Vibrio sp. 2448-88 for nauplius, protozoea, mysis and postlarval stages of Penaeus semisulcatus at different time intervals.

Larval stage	LC_{50} values at different time intervals (time in hours)			
	24	48	72	96
Nauplius	4.84×10^7	-	-	-
	$(2.4 \times 10^6 \ldots 4.4 \times 10^8)$**			
Protozoea	2.35×10^8	3.87×10^7	3.06×10^7	-
	$(1.1 \times 10^8 \ldots 4.6 \times 10^8)$	$(2.5 \times 10^7 \ldots 5.9 \times 10^7)$	$(1.9 \times 10^7 \ldots 4.8 \times 10^7)$	
Mysis	2.2×10^9	2.25×10^8	5.38×10^7	-
	$(8.6 \times 10^8 \ldots 1.5 \times 10^{10})$	$(1.3 \times 10^8 \ldots 3.8 \times 10^8)$	$(2.4 \times 10^7 \ldots 9.4 \times 10^7)$	
Postlarva I-IV	4.82×10^{10}	1.97×10^8	1.09×10^8	7.04×10^7
	$(2.9 \times 10^9 \ldots 4.5 \times 10^{11})$	$(6.5 \times 10^7 \ldots 5.0 \times 10^8)$	$(4.0 \times 10^7 \ldots 2.3 \times 10^8)$	$(1.8 \times 10^7 \ldots 1.6 \times 10^8)$
Postlarvae V-IX	1.21×10^{10}	5.32×10^9	5.08×10^8	6.3×10^7
	$(1.1 \times 10^9 \ldots 1.0 \times 10^{11})$	$(5.2 \times 10^8 \ldots 3.5 \times 10^{10})$	$(4.5 \times 10^7 \ldots 6.1 \times 10^9)$	$(4.7 \times 10^7 \ldots 3.5 \times 10^9)$

* No. of bacterial cells per ml of rearing medium.
** Confidence level of LC_{50}

are transparent. In diseased condition the larvae lose their transparency and become opaque. This sign was clearly seen in mysis and postlarvae just one hour before death.

The important symptom of the disease due to infection caused by Vibrio sp. 2448-88 was appendage necrosis in the larvae (Pl.X, Figs. 1 to 3). This was observed in 25-50% of the infected population, mostly in the tail region and rarely in cephalothoracic appendages (Pl.X, Figs. 2 and 3). Loss of setae in the telson was the first indication of appendage rot and it was followed by bending, twisting and gradual degeneration of appendages (Pl. X, Fig. 1).

As the nauplius larvae get infected, the swimming activity gets impaired. While the moderately infected larvae float in the rearing medium and swim occasionally, the heavily infected ones settle to the bottom and move only when disturbed. Similarly , the heavily infected protozoea larvae settle to the bottom upside down and beat their appendages vigorously. The infected mysis larvae after developing the signs such as expansion of the chromatophores and opaqueness of the body, never settle at the bottom, but swim at the surface water and show continous stretching and contracting of the tail portion. Further it is also observed that they swim vigorously, often dashing against the inner side of the container. The infected postlavae are seen to swim spirally in a vertical manner to the surface of the water and then to sink to the bottom. This swiming behavioural pattern is repeated till their death.

The moulting and development of larvae were very much affected when they were reared in the bacterial concentration of 35 x 10^7 cells/ml of the rearing medium. The larvae could not cast off the exoskeleton during moulting from the body; it remain attached to the body till their death (Pl. X, Fig. 4). This was observed frequently among the infected protozoea and mysis larvae of all three species of prawns.

The development of nauplius III to protozoea I, protozoea I to mysis I and mysis I to postlarva I were very much affected in the concentration of 35 x 10^7 cells/ml of the rearing medium. Although the metamorphosis of nauplius III to protozoea of P. indicus was not affected, only 40.91% of infected nauplius larvae metamorphosed to protozoea in the case of P. semisulcatus and none of the infected nauplius larvae of P. monodon metamorphosed to protozoea I. All the infected nauplius larvae took 4-6 hrs more to metamorphose to protozoea as compared to the duration in their normal development to protozoea. In the case of protozoea I of P. indicus, 36.36% of infected larvae metamorphosed to mysis I in 80 hrs, but none of the infected protozoea of P. monodon and P. semisulcatus metamorphosed to mysis I (Table 7.19). Similarly, only 14.29% of the infected mysis larvae of P. indicus metamorphosed to postlarva I, but none of the infected mysis of P. monodon and P. semisulcatus metamorphosed to postlarvae (Table 7.19).

Table 7.19. Percentage of metamorphosed larvae* of *P. indicus*, *P. monodon* and *P. semisulcatus* when reared in uninfected and infected medium with *Vibrio* sp. 2448-88 at concentration of 35×10^7 cells/ml

Larval stage	No. of larvae used	Percentage of metamorphosed larvae of *P. indicus*		*P. monodon*		*P. semisulcatus*	
		uninfected medium	infected medium	uninfected medium	infected medium	uninfected medium	infected medium
Nauplius III to protozoea I	75	100	100	100	0	100	40.91
Protozoea I to mysis I	60	100	36.36	100	0	100	0
Mysis I to postlarva I	30	100	14.29	100	0	100	0

* Based on three replicates.

Clinical signs observed in the infected adult P. indicus

Darkening of the dorsal portions of the cuticle is noticed. The pereiopods and the peleopods become red due to the expansion of chromatophores. A white patch is developed at the site of injection. This patch, subsequently gets blackened on the 2nd day and clearly seen on the 4th day. The behavioural pattern observed in the infected adult include reduced swimming activity, disorientation while swimming and often swimming on one side. In the advanced stage of infection, the prawns lay on their side and move their pleopods vigorously till their death.

Pathogenicity of V. alginolyticus on the larvae and postlarvae of P. indicus

V. alginolyticus at the concentrations of 37.8×10^6, 37.8×10^7 and 37.8×10^8 cells/ml caused 17.33, 64 and 94.67% mortality of nauplius stage following 32 hrs of exposure, but lower concentration of 37.8×10^4 and 37.8×10^5 cells/ml failed to produce any mortality (Table 7.20). The accumulated percentage mortality of protozoeal stage of P. indicus varied from 11.67 to 31.67% after 80 hrs of exporsure to different concentrations; the percentage mortality being higher in the greater concentrations (Table 7.21). Although the pattern of induction of disease in the mysis stage was similar to that of protozoeal stage; percentage mortality was observed generally to be higher, the highest recorded at 37.8×10^8 cells /ml after 72 hrs of exposure (Table 7.21 and 7.22). For protozoea and mysis, the LC_{50} values of this bacterium were respectively at 1.96×10^{11} and 1.39×10^{10} cells/ml after 48 hrs and 1.09×10^{11} and 8.71×10^9 cells/ml after 72 hrs of immersion (Table 7.23).

Table 7.20. Accumulated percentage mortality of nauplius stage of *Penaeus indicus* exposed to different concentrations of *Vibrio alginolyticus* at different time intervals.

No. bacterial cells/ ml of the rearing medium	Accumulated (%) mortality at hrs of post-inoculation		
	12	24	32
Control	0.00	0.00	0.00
37.8×10^4	0.00	0.00	0.00
37.8×10^5	0.00	0.00	0.00
37.8×10^6	0.00	0.00	17.33
37.8×10^7	6.67	17.33	64.00
37.8×10^8	42.67	78.67	94.67

Table 7.21. Accumulated percentage mortality of protozoeal stage of Penaeus indicus exposed to different concentrations of Vibrio alginolyticus at different time intervals.

No.of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation						
	12	24	36	48	60	72	80
Control	0.00	0.00	0.00	0.00	0.00	0.00	1.67
37.8×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
37.8×10^5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
37.8×10^6	0.00	0.00	0.00	6.67	6.67	8.33	11.67
37.8×10^7	0.00	0.00	0.00	8.33	10.00	10.00	26.67
37.8×10^8	15.00	16.67	26.67	26.67	26.67	28.33	31.67

Table 7.22. Accumulated percentage mortality of mysis stage of *Penaeus indicus* exposed to different concentrations of *Vibrio alginolyticus* at different time intervals.

No.of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	0.00
37.8×10^4	0.00	0.00	0.00	0.00	0.00	0.00
37.8×10^5	0.00	0.00	0.00	0.00	0.00	0.00
37.8×10^6	0.00	0.00	0.00	10.00	16.67	16.67
37.8×10^7	0.00	0.00	23.33	30.00	33.33	33.33
37.8×10^8	23.33	23.33	33.33	36.60	43.33	43.33

Table 7.23 LC_{50} values* of *Vibrio alginolyticus* for nauplius, protozoea and mysis of *Penaeus indicus* at different time intervals.

Larval stage	LC_{50} values at different time intervals (time in hours)		
	24	48	72
Nauplius	2.36×10^8 ($1.6 \times 10^8 \ldots 3.4 \times 10^8$)**	-	-
Protozoea	ND	1.96×10^{11} ($1.2 \times 10^{10} \ldots 1.1 \times 10^{13}$)	1.09×10^{11} ($1.1 \times 10^{10} \ldots 2.4 \times 10^{12}$)
Mysis	ND	1.39×10^{10} ($2.2 \times 10^9 \ldots 1.4 \times 10^{12}$)	8.71×10^9 ($1.3 \times 10^9 \ldots 1.6 \times 10^{11}$)
Postlarva I to V	ND	ND	ND
Postlarva × to ⅩV	ND	ND	ND

* No. of bacterial cells per ml of rearing medium.
** Confidence level of LC_{50}.
ND- Not determined as the mortality was not observed in more than one concentration.

Pathogenicity of V. parahaemolyticus on the larvae and postlarvae of P. indicus

The results obtained from the experimental pathogencity of V. parahaemolyticus on nauplius, protozoea and mysis of P. indicus are given in Tables 7.24 to 7.26. V. parahaemolyticus at 34.3 x 10^6, 34.3 x 10^7 and 34.3 x 10^8 cells/ml caused 6.67, 29.33 and 90.67% mortality in the nauplius after 32 hrs of immersion. The lower concentrations of 34.3 x 10^5 and 34.3 x 10^4 cells/ml however did not kill the host (Table 7.24). Similarly, no appreciable mortality of protozoea and mysis of P. indicus was recorded by exposure to different concentrations of V. parahaemolyticus at different time intervals (Tables 7.25 and 7.26). The LC_{50} value of V. parahaemolyticus determined for nauplius larvae at 24 hrs exposure was 4.08 x 10^8 cells/ml. In respect of protozoea and mysis larvae, values were found to be at 7.27 x 10^{10} and 5.66 x 10^{11} cells/ml after 72 hrs of exposure. The LC_{50} values for postlarval stage could not be determined.

Alcaligenes sp. was used as a positive control to assess the pathogenicity of virulence of Vibrio sp. 2448-88. No mortality was observed among the larvae and postlarvae of P. indicus, P. monodon and P. semisulcatus treated with Alcaligenes sp.

Confirmation of pathogenicity

The pathogenicity of new isolate of Vibrio, V. alginolyticus and V. parahaemolyticus was confirmed by satisfying Koch's postulates. Vibrio sp.

Table 7.24. Accumulated percentage mortality of nauplius stage of *Penaeus indicus* exposed to different concentrations of *Vibrio parahaemolyticus* at different time intervals.

No. bacterial cells/ ml of the rearing medium	Accumulated (%) mortality at hrs of post-inoculation		
	12	24	32
Control	0.00	0.00	0.00
34.3×10^4	0.00	0.00	0.00
34.3×10^5	0.00	0.00	0.00
34.3×10^6	0.00	0.00	6.67
34.3×10^7	0.00	14.67	29.33
34.3×10^8	50.67	80.00	90.67

Table 7.25. Accumulated percentage mortality of protozoeal stage of Penaeus indicus exposed to different concentrations of Vibrio parahaemolyticus at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation						
	12	24	36	48	60	72	80
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^6	0.00	0.00	0.00	0.00	0.00	5.00	11.67
34.3×10^7	0.00	0.00	0.00	0.00	0.00	10.00	23.33
34.3×10^8	6.67	11.67	11.67	11.67	13.33	15.00	30.00

Table 7.26. Accumulated percentage mortality of mysis stage of *Penaeus indicus* exposed to different concentrations of *Vibrio parahemolyticus* at different time intervals.

No. of bacterial cells/ ml of rearing medium	Accumulated (%) mortality at hrs of post-inoculation					
	12	24	36	48	60	72
Control	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^4	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^5	0.00	0.00	0.00	0.00	0.00	0.00
34.3×10^6	0.00	0.00	0.00	0.00	0.00	6.67
34.3×10^7	0.00	0.00	0.00	0.00	13.33	13.33
34.3×10^8	0.00	0.00	0.00	10.00	20.00	23.33

Table 7.27. LC_{50} values* of *Vibrio parahaemolyticus* for nauplius, protozoea and mysis of *Penaeus indicus* at different time intervals.

Larval stage	LC_{50} values at different time intervals (time in hours)		
	24	48	72
Nauplius	4.08×10^8 ($2.8 \times 10^8 \ldots 6.1 \times 10^8$) **	-	-
Protozoea	ND	ND	7.27×10^{10} ($2.6 \times 10^9 \ldots 9.5 \times 10^{11}$)
Mysis	ND	ND	5.66×10^{11} ($9.1 \times 10^9 \ldots 9.5 \times 10^{12}$)
Postlarva I-V	ND	ND	ND
Postlarva - V	ND	ND	ND

* No. of bacterial cells per ml of rearing medium.
** Confidence level of LC_{50}.
ND- Not determined as the mortality was not observed in more than one concentration.

2448-88 was isolated from moribund and dead animals subjected to experiments. Besides it was also isolated from the hepatopancreas, heart and muscle of experimentally infected adult P. indicus. V. alginolyticus and V. parahaemolyticus were also isolated from experimentally infected larvae of P. indicus. Neither Vibrio sp. 2448-88 nor V. alginolyticus or V. parahaemolyticus were isolated from the control groups. The characters of these reisolates resembled the original isolates.

DISCUSSION

In recent years, great emphasis has been placed upon understanding the pathogenicity of pathogens to elucidate the mode and mechanism of infection, nature of virulence and epizootiology. The information is also useful for vaccine development programmes. There has been considerable progress on the pathogenicity of some of the representatives of Vibrio, particularly V. anguillarum. However, most of these studies relate to fishes and there is only limited informations available on prawns.

In nature, it may be conceived that the prawns are continuously exposed to an aqueous suspension of microorganisms. The bacteria, which constitute part of the natural microflora, and those ingested along with the food continuously interact with the prawns, and under certain conditions the potential pathogenic bacteria prevail and ensue the disease cycle. Under experimental conditions, the disease is induced by different methods such as by immersing the host in the medium containing pathogen, oral administration through food, intramuscular injection and by introducing the

pathogen through the wounds or lesions. In the present study, the experimental infection of larvae and postlarvae of P. indicus, P. monodon and P. semisulcatus was essentially carried out by exposing them to different concentrations of Vibrio sp. 2448-88 , V. alginolyticus and V. parahaemolyticus in bath treatment. This mode of infection was selected, because intramuscular injection could not be administered to the larval and postlarval stages due to their small size and delicate nature. In the case of adult P. indicus, however, the pathogen (Vibrio sp. 2448-88) was introduced orally and intramuscularly. Several authors have successfully employed bath challenge (Croy and Amend, 1977; Schiewe and Hodgins, 1977; Harbell et al., 1979) to transmit the pathogen to the host. In the case of fishes, the entry of pathogen into the host in this method was found to be either through the gill or through the lateral line system. Alexander et al. (1981) in separate experiments studied the mode of entry of Escherichia coli in the trout through anterior (head and gill) part of the fish and posterior (body and lateral line) region, and concluded that the gill, and not the lateral line was the main portal of entry. Hjeltness et al. (1987) found the entrance of Vibrio sp. in Atlantic salmon through the gills.

Although, the precise site of entry of Vibrio into the larvae and postlarvae studied at present could not be conclusively determined, it is possible that the main route of transmission might involve attachment of the pathogen to the delicate exoskeleton of the larvae and thence penetration to the host tissue. The exoskeleton of the larvae, during the developmental stage, is soft and thin and is cast off frequently within short

intervals of a day or two. Gacutan et al.(1979) experimenting on the larval rearing of P. monodon observed the occurrence of Ephelota infection in earlier stages of the penaeid prawn through the relatively soft exoskeleton of the larvae.

The oral transmission of Vibrio sp.2448-88 by feeding the already injected prawn meat to the adult P. indicus failed to establish the infection. Approximately 14 x 10^{11} viable cells given in the feed daily for three days did not cause any mortality of the test prawns. This indicates the inability of Vibrio sp. 2448-88 to produce infection. This observation agrees with those of Lightner and Lewis (1975) who found that the addition of bacterial isolates in the food offered to the juveniles of P. aztecus, P. setiferus and P. duorarum to be an unsuccessful means of infection. Similar observations were also reported by Baudin-Laurencin and Tang- trongpiros (1980) in the trout (Salmo gairdneri) , when the fish was administered with 6.7 x 10^{10} viable cells of Vibrio orally in the diet daily for 5 days could kill only five fish out of 142 experimented with. The failure of Vibrio sp. 2448-88 to produce infection when administered orally to the adult P. indicus might be due to the inability of the pathogen to establish in adequate numbers in the complex gut environment of the host. Besides it is also possible that the prawn is naturally endowed with certain amount of resistance to bacterial infection as they inhabit in an environment surrounded by bacterial flora including Vibrio and their gut harbours a host of microorganisms.

In contrast to the results of experiments on oral transmission of Vibrio, infectivity experiments through intramuscular injection showed the capability of Vibrio sp. 2448-88 to cause infection at varying degrees at different concentrations. 70 x 10^5 cells of Vibrio/animal when injected intramuscularly to the adult P. indicus, the mortality percentage after 18 hrs was only 20% . The rate of mortality was found to increase as the level of concentration of Vibrio was increased, and 100% mortality of prawn was registered after 12 hrs when 70 x 10^7 cells of Vibrio sp.2448-88 were administered intramuscularly.

From the results of the various experiments on the transmission of Vibrio sp. 2448-88 conducted during the study, it may be concluded that the main portal of entry of the pathogen in the larval and postlarval stages of P. indicus, P. monodon and P. semisulcatus is through the exoskeleton of the larvae and in the adult P. indicus, it is by means of penetration of tissue at the site of lesions or wounds. The pathogen is not able to establish the infection when taken orally.

Although experimental infections have been achieved by exposing the larvae and postlarvae to water-borne suspension of the pathogen and by intramuscular injection in the adult prawn, this may or may not be representative of a disease in the natural environment. To find out whether the new isolate of Vibrio is instrinsically pathogenic to the larvae/postlarvae and adult prawns, experiments were conducted simultaneously with comparable doses of Alicaligenes sp. and Vibrio sp. While the former

bacterium failed to cause infection or establish the disease or to kill the host at 41.2 x 10^8 cells/ml. Vibrio sp. 2448-88 challenged with dose at 35 x 10^6 was found sufficient to establish the infection and at 10^8 cells/ml produced 100% mortality of the larvae of P. indicus, P. monodon and P. semisulcatus. Similarly the Vibrio sp. 2448-88 at the concentration level of 70 x 10^6 cells/ animal injected into the adult P. indicus produced symptoms of infection and subsequently mortality of the test prawns after 12 hrs. These results thus proved that Vibrio sp. is instrinsically pathogenic to larvae of all the three species of penaeid prawns tested at present as well as to the adult P. indicus.

The Vibrio sp. 2448-88 was also observed to affect the larval development of all the three species of prawn. In the case of P. indicus, the metamorphosis of the nauplius to protozoeal stage was not affected when the larvae were reared in the rearing medium containing 35 x 10^7 cells/ml. However only 36.4% of protozoea transformed to mysis stage and only 14.3% mysis to postlarvae when they were reared in the treated medium. The development of nauplius larvae of P. semisulcatus in the infected medium was affected to certain extent as only 41% of the larvae metamorphosed to protozoea. Thereafter, the development of the larvae was found to be completely arrested in the treated medium. The development of P. monodon larvae was observed to be most affected when reared in Vibrio infected medium as none of the larval stages metamorphosed to the succeeding stages.

The dosage mortality and time mortality data in the pathogenicity experiments with Vibrio sp. 2448-88 showed that the pathogenic nature of the bacterium depend on the concentration of dose, the period of exposure of the host and age of the prawn. In the nauplius stage of P. indicus , exposure of the larvae to 35 x 10^8 cells/ml for 24 hrs was found to cause 100% mortality of larvae. As the development proceeds and the nauplius metamorphoses to protozoeal stage, this concentration level could cause only 70% of mortality after 24 hrs of exposure. For the mysis stage, the same concentration (35 x 10^8 cells/ml) caused only 40% mortality after 24 hrs, and longer duration of exposure to this concentration for about 72 hrs was found necessary to produce 100% mortality. In the postlarval stage, Vibrio sp.2448- 88 at 35 x 10^8 cells/ml could produce only 38.89% after 120 hrs of exposure. In general, the new isolate of Vibrio at 10^8 cells/ml caused cent percent mortality to all the larval stages of P. indicus, P. monodon and P. semisulcatus.

The LC_{50} values of Vibrio sp. 2448-88 for the different larval stages of the three species of prawns also reflect variation in the susceptibility with the stage of development of the larvae and the level of dosage concentration. The early stages such as nauplius and protozoea had low LC_{50} values and the later developmental stages of mysis and postlarvae had relatively greater LC_{50} values, indicating that resistance to Vibrio sp. 2448-88 was progressively manifested by the larvae as they develop and grow to advanced stages. In the case of the adult P. indicus, Vibrio sp. 2448-88 produced 100% mortality within 12 hrs when injected intramuscularly

at the concentration of 70 x 10^7 cells/ prawn, but the pathogen did not cause any mortality at a lower dose of 70 x 10^5 cells/ml after 12 hrs (Table 7.5). The LD_{50} value for adult P. indicus was 2.18 x 10^7 cells/prawn. Such differeces or variations on the level of mortality and susceptibility correlated with the age, dosage, and time of exposure have been recorded by several workers (Lightner and Lewis, 1975; Leong and Fontaine, 1979; Lewis et al., 1982; Takahashi et al., 1984, 1985). Lewis et al. (1982) found that Pseudomonas piscicida and Flavobacterium sp. at 10^4 cells/ml produced aggregation of shrimp larvae in suspension and that the larvae exposed to 10^6 cells/ml or greater concentrations of Ps. piscicida, Aeromonas formicans, Flavobacterium sp. or Vibrio sp. died within 24 hrs of exposure. Gacutan et al. (1979b) reported decreased susceptibility to Ephelota infection in the larvae of P. monodon with age. They found that out of 100 infected larvae and postlarvae, 55 were protozoeal stage, 35 were in mysis stage and 10 were postlarvae, and concluded that the occurrence of infection in the early stages could be attributed to the relatively soft exoskeleton of the larvae. In natural and experimental epizootics in brown and white shrimp larvae, the protozoeal stages seemed to be the most susceptible, although occasionally infected larvae in the naupliar stage or the first mysis stage were also observed (Lightner, 1975). Lightner (1975) observed that P. setiferus seems to be resistant to the infection of Lagenidium from the mysis stage onwards. The rapid propagation of the larval necrosis caused by bacteria in zoea of penaeids and young stages of Macrobrachium showed that the age of the larvae is certainly an important factor in sensitivity to disease (AQUACOP, 1977). It

is likely that the increased tolerance exhibited by mysis and postlarval stages, as compared to nauplius and protozoea, may be related to the ontogenic development of defence mechanisms such as haemocytes, which may protect the animals from Vibrio infection. Besides the dosage level, and age of the prawns, factors such as crowding and temperature may also influence the susceptibility to infection. These aspects, however, were not studied at present.

A comparison of the data on LC_{50} values of Vibrio sp. 2448-88 for larval and postlarval stages of the three species studied at present also reveals the relative susceptibility, nature of tolerance to the pathogen by these species. Among the three species, the larvae and postlarvae of P. monodon appeared to be the most sensitive to Vibrio sp. as the larvae and postlarvae had the lowest LC_{50} values of Vibrio sp. 2448-88 (Table 7.12). The larvae of P. semisulcatus was found to be intermediate between P. indicus and P. monodon in sensitivity to the infection caused by Vibrio sp. 2448-88. It was evident that P. indicus was the most tolerable species to Vibrio sp. 2448-88 as the larval stages had the highest LC_{50} values (Table 7.6). P. monodon is the most sensitive to Vibrio sp. 2448-88 followed by P. semisulcatus and P. indicus is also evident in the pattern of development in the Vibrio infected medium mentioned earlier. It is worthwhile to note that among the three species, P. indicus was considered to be the relatively hardier species. Lightner et al. (1979) also observed similar difference in susceptibility to Fusarium solani infection among P. californiensis, P. stylirostris and P. vannamei.

The LC_{50} values of Vibrio sp. 2448-88 V. parahaemolyticus and V. alginolyticus against the larvae and postlarvae of P. indicus enable to indicate the degree of virulence of pathogens. In comparison, the lowest LC_{50} values were recorded by Vibrio sp. 2448-88 for different larval stages of P. indicus. This was followed by V. alginolyticus and V. parahaemolyticus which had the highest LC_{50} values (Tables 7.23 and 7.27). It is evident from the data that, of the three species of Vibrio , Vibrio sp. 2448-88 is the most virulent form.

Although the precise nature of pathogenic mechanism of Vibrio could not be explicitly elucidated, it is interesting to note that the sterile filtrate obtained from the 24 hrs broth culture of Vibrio sp. 2448-88 caused significant mortality to the postlarvae of P. indicus. This indicates the involvement of toxic factor or factors in the death of the host. A similar postulate was presented by Vanderzant et al. (1970 when they attempted to infect adult brown shrimp by addition of 3% inoculum (24 hrs culture in BHI broth) of V. parahaemolyticus directly to aquarium water. Further, the biochemical analyses carried out on the Vibrio sp. 2248-88 have shown its ability to produce proteases, lipase, gelatinase, amylase and chitinase which are capable of degrading a wide variety of tissues. More recently Inamura et al. (1984) and Kodama et al. (1984) reported that proteases were implicated with virulence. Further studies are essential to understand the pathogenic mechanism which involves complex interaction of exotoxin and/or endotoxin as well as the physiological and biochemical milieu of the host and its response to the pathogen. To sum up, it may be considered that

Vibrio sp. 2448-88 is an instrinsic pathogen to P. indicus and relatively more virulent to this species than its related taxon. The pathogen gains entry in the larval stages through the exoskeleton and fails to establish infection by the oral route. The pathogenic nature of Vibrio sp. 2448-88 depends on the age or the developmental stage of the larvae, being more infectious to early larval stages. Among the three species of penaeid prawns studied, P. monodon is the most sensitive to Vibrio sp. 2248-88 than P. semisulcatus and P. indicus, although the latter species is more tolerant. The pathogenic mechanism of the Vibrio sp. to the prawn appears to be by means of haemolysins and proteases.

CHAPTER 8

STUDIES ON HISTOPATHOLOGY OF VIBRIOSIS IN LARVAE AND ADULT *PENAEUS INDICUS* AND POSTLARVAE OF *PENAEUS MONODON*

INTRODUCTION

The first cellular investigations were carried out in the mid- nineteenth century (Virchow, 1858). Since then histopathological investigations of fixed body fluids or tissues form an important and powerful research area for facilitating proper diagnosis of the diseases, their effect on the various systems and in the understanding of the functional organisation of the affected organisms. It also forms one of the essential techniques for determination of the diseases when the infection is not heavy enough to be detected by the macroscopical means. A study on pathological changes occurring at the tissue and cellular levels helps considerably for clarifying the physiological functions of the host organisms. The degree of divergence from normal cell structure indicates the relative health of the host. When the changes are detrimental, they interfere with normal physiological functions, reproductive capability and survivability of the host organism. Thus a clear understanding of the disease is essential not only to diagnosis the case, but also cure the host organism to prevent the disease among the population.

Although the histopathology of vibriosis has been studied extensively in fishes and molluscs by different authors (Funahashi *et al.*, 1974; Harbell, 1976; Miyazaki and Kubota, 1977; Miyazaki *et al.*,1977; Miyazaki and Egusa,

1977; Ransom, 1979; Elston et al., 1981 Ransom et al., 1984; Bruno et al., 1986), similar studies on penaeid prawns are rather limited. Prior to 1973, documented disease studies of penaeid prawns were mainly concerned with parasitological aspects and contained very little information on histopathological aspects (Sprague, 1950, 1966; Iversen and Manning, 1959; Kruse, 1959; Aldrich, 1964; Iversen and Van Meter, 1964; Baxter et al., 1970). One exception was the histological study on spontaneous necrosis in muscles of brown shrimp Penaeus aztecus (Rigdon and Baxter, 1970). Histopathological investigations due to viral infection in penaeid prawns have been carried out by different workers (Sano et al., 1981, 1984; Lightner and Redman, 1981, 1985; Lighter et al., 1983; Anderson and Shariff, 1987) and include observations on extensive necrosis and atrophy of the hepatopancreas and nuclear hypertrophy accompanied by the presence of intranuclear inclusion bodies in the hepatopancreas of infected prawn. The necrosis of the mucosal epithelium and consequent haemocytic infiltration have been observed in P. stylirostris with a disease syndrome caused by Spirulina subsala (Lightner, 1978). Histopathological investigations have also been carried out on spontaneous necrosis in the muscles of P. aztecus (Rigdon and Baxter, 1970; Lakshmi et al., 1978), P. japonicus (Momoyama and Matsuzato, 1987) and Macrobrachium rosenbergii (Nash et al., 1987). The structural changes observed in the spontaneous muscular necrosis were classified into three stages namely necrosis of muscle fibres with fusion and cross splitting of myofibrils, diminution of necrotic muscle fibres and replacement of necrotic muscle fibers by connective tissue (Momoyama and Matsuzato, 1987).

Histopathological investigation of diseased penaeid prawns has been carried out for the first time in India by Soni (1986) who observed the thickening of cuticle and haemocytic infiltration and encapsulation in the lobes of tumor-like outgrowth on dorsal side of the carapace of P. indicus. Histological changes in the exoskeleton, heart, hepatopancreas, muscles, gut and gills of P. indicus showing soft-shell disease syndrome were studied by Soni(1986) and Ramesh (1988). Extensive histopathological studies on microsporidian disease causing severe damage to gonad and hepatopancreas of P. semisulcatus was also made by Soni (1986).

Histopathology of vibriosis in prawn has not been studied in detail except the work of Egusa et al. (1988). These authors reporting on the extensive necrosis of lymphoid organ caused by severe Vibrio infection in P. japonicus, observed multiple formation of melanized nodules in the lymphoid organ. Although no extensive necrotic lesions were found in other organs such as heart, gills, hepatopancreas, gonads and abdominal musculature, small melanised nodules were frequently observed in these organs. In the present study, an attempt was made to study the histopathological changes due to Vibrio sp. 2448-88 in different vital organs of the larvae and adult P. indicus and postlarvae of P. monodon.

The material and methods employed in the histopathological studies are described in detailed in chapter 2.

OBSERVATIONS

Hepatopancreas

In the normal uninfected P. indicus, hepatopancreas forms a large compact, paired glandular mass occupying much of the cephalothoracic cavity. Generally the colour of the hepatopancreas is brown to orange red. However it varies considerably in the individual of the same species with different maturity and moulting stages. It is ensheathed by a thin connective tissue membrane. It consists of numerous blindly ending tubules (Pl. XI, Fig. 1) which are lined by simple columnar epithelial cells (Pl. XI, Fig. 2). Each of the tubules is connected to secondary ductules which, in turn, join the primary duct of the respective side. The primary ducts open into the gut at the junction between the pyloric stomach and the midgut. Each hepatopancreatic tubule has a lumen in the centre (Pl. XI,Fig. 2). The epithelial lining of the tubules, except at the distal blind end, is only a single cell layer thick. Individual tubules are loosely held together by basophilic connective tissue strands (Pl. XI, Fig. 1). Wandering cells are present in the connective tissue and blood space between the hepatopancreatic tubules (Pl. XI, Fig. 1).

The tubular epithelium is composed of four cell types, namely the E-, R-, F- and B- cells (Pl.XI, Fig. 2) lying on a thin basophilic basement membrane. The E-cells (Pl. XI, Fig. 2) about 25 µm tall and 5 µm wide, occupy the distal tip of each tubule and have proximal nuclei, 4-5 µm in diameter, and conspicuous nucleolar bodies. No brush border can be

discerned along the luminal margin of the cells. The R-cells (Pl. XI, Fig.2) measuring on average 60 µm by 10 µm, are the most abundant cell type and occur throughout the hepatopancreatic tissues. They are characteristically multivacuolate. The F-cells (Pl. XI, Fig.2) are basophilic in nature. The F-cells measure 50-90 µm in height and are distributed in the middle region of the tubules, interspersed between the R-and B-cells. The B-cells (Pl. XI, Fig.2) found in the middle portions of the tubules, are up to 70 µm in over all height and contain a single large ovoid vacuole measuring 50 µm in maximum diameter.

The histological structure of the hepatopancreas of larvae and postlarvae is similar to that of the adult in all aspects except in the number and size of the tubules.

The hepatopancreas of heavily infected adult prawns showed extensive vacuolation in all four cell types of tubular epithelium (Pl. XI, Fig.3). The tubules coalesced together due to rupture of connective tissues forming irregularly shaped structures. The lumen and intertubular spaces were not discernible (Pl. XI, Fig.4). The connective tissue was thickened especially at the junction of connective tissue strands (Pl. XI, Fig. 5). All the four cell types were necrotic in some tubules (Pl. XI, Fig.6). Tubules without secretory cells could be observed (Pl. XII, Fig.1). The excessive vacuolation observed in the infected tubules was due to the accumulation of fat. The frozen sections stained with Oil red O or Sudan black B confirmed the accumulation of fat in entire tubule

(Pl. XII, Figs. 2 and 3). In comparison, the normal tubules showed fat materials only at the peripheral region of the tubules. Most of the secretory cells in the tubules lost their chromatic character and became acidophilic (Pl. XII, Fig.4). Signet cells were observed in the peripheral tubules of the hepatopancreas (Pl. XII, Fig.5). Moderate to marked haemocytic infiltration was observed in some tubules (Pl. XII, Fig.6). Tissue remnants with disintegrated nuclei were seen in the tubular lumen rendering it a woolly appearance (Pl. XIII, Fig.1). E- and F- cells were highly vacuolated (PL. XIII Figs. 2 and 3). Wandering cells found in the blood spaces were migrated towards the lumen of the tubule (Pl. XIII, Fig.4). The hepatopancreas of moderately infected animals showed unusual vacuolation in the tubule with reduction in the size of E- and F- cells. However, the tubules were intact and the lumen in the tubule was discernible (Pl. XIII, Fig.5).

The hepatopancreas of infected protozoea and mysis of P. indicus and postlarvae of P. monodon showed similar changes as observed in the hepatopancreas of infected adult prawn such as extensive vacuolation, disappearance of lumen and intertubular space, necrosis of tubular epithelial cells and appearance of acidophilic cells (Pl. XXIII, Figs. 1, 4 and 5 ; Pl. XXV, Fig. 2).

The occurrence of bacteria in the hepatopancreas of the host was demonstrated. Bacteria were observed in the tubular lumen and periphery of the hepatopancreas and connective tissue (Pl. XIII, Fig. 6; Pl. XIV, Figs. 1-

3). Bacterial colonies were also observed in the larval and postlarval hepatopancreas (Pl. XXIII, Fig. 6; Pl. XXIV, Fig. 1; Pl. XXV, Fig. 3).

Heart

The heart lies immediately dorsal and slightly caudal to the hepatopancreas. It is a sac-like, contractile structure with thin walls composed of cross-striated muscle fibres and enclosed by a thin pericardium (Pl. XIV, Fig.4). In the infected adult P. indicus, the pericardium was thickened and highly vacuolated (Pl. XIV, Figs. 5 and 6; Pl. XV, Fig. 1). Pyknotic nuclei were observed in the epithelial cells of pericardial membrane (Pl. XIV, Fig. 5). Focal colonial localisation of bacteria was observed in cardiac tissue (Pl. XV, Figs. 2 and 3) which revealed the necrosis of myocardium with pyknotic nuclei. The myocardium was edematous (Pl. XV, Fig. 4). The endothelial cells showed swelling and vacuolation (Pl. XV, Fig. 5). Accumulation of haemocytes were seen around the bacteria exhibiting phagocytosis. In larvae, the myocardium was edematous and necrotic (Pl. XXIII, Figs. 3 and 4).

Haematopoietic tissue

In adult P. indicus, the haematopoietic tissue is located on dorsoposterior side of the hepatopancreas as observed in the lobster, Homarus americanus (Johnson et al., 1981) and P. orientalis (Oka, 1969). Circulating cells are derived from the haematopoietic tissue (Johnson et al., 1981). In P. indicus, the haematopoietic tissue stained with haematoxylin and eosin contains deeply, moderately or lightly stained

stem cells (Pl. XV, Fig. 6; Pl. XVI, Fig. 1). In the infected animals, the haematopoietic tissue was necrotic (Pl. XVI, Figs. 2 and 5); karyorhectic and pyknotic nuclei were observed in the haematopoietic tissue (Pl. XVI, Figs. 3 and 4). The cells were reduced in number and those present appeared fobby (Pl. XVI, Fig. 3). Most of the haematopoietic tissue in the infected animals became vacuolated (Pl. XVI, Fig. 4). The rod-shaped bacteria were observed around and inside the haematopoietic tissue (Pl. XVI, Fig. 6). Haematopoietic tissue could not be located in the normal larvae of P. indicus and postlarvae of P. monodon.

Gill

The principal respiratory organ of the prawn consists of paired gills enclosed in branchial chambers on either side of the cephalothorax. The structure of the gill is dendrobranchiate (Barnes, 1963). Each lamella of the gills consists of a single layer of epithelial cells covered by a thin cuticle (Pl. XVII, Fig. 1). It contains a blood-filled cavity (lamellar sinus) which connects the afferent and efferent blood vessels in the central axis of the gill with an outer lamellar sinus running round the outside edge of the lamella. Other cells, including pillar cells, nephrocytes and axons are also present. The gill lamellae of infected adult prawn were edematous with haemocytic infiltration (Pl. XVII, Figs. 2 and 3). The nuclei of the shrunken lamellar cells were pyknotic. The outer surface of the gill epithelium was irregularly arranged and showed multiple projections. Fusion of one lamella with neighbouring lamellae was observed (Pl. XVII, Fig. 4). Some lamellar cells

showed extensive vacuolation (Pl. XVII, Fig. 5). In larvae and postlarvae, the gill lamellae were edematous and necrotic. In certain cases, the gill lamellae were highly vacuolated as observed in the adult (Pl. XXIV, Fig.2). Besides these changes, emphysema or distention of outer lamellar sinuses of postlarval gill lamellae was observed (Pl. XXV, Fig. 5).

Muscle

Prawn locomotory muscle is striated and presents a histologic appearance that is similar to that of vertebrate striated muscle (Pl. XVII, Fig.6). At the site where Vibrio sp. 2448-88was injected into the muscle of adult P. indicus, a white "column" visible through the exoskeleton, was formed. This "column" assumed a yellowish-brown colour by 72 hrs and completely black by 96 hrs (Pl. XVIII, Fig.1) . The site of injection in the muscle tissue was necrotic and characterised by infiltration of haemocytes and fibrocytes (Pl. XVIII, Figs. 2 and 3). Many of these infiltrating cells had pyknotic nuclei (Pl. XVIII, Fig.2). The site of injection was encircled by several layers of haemocytes and fibroblast and browning of the muscle fibres was also observed (Pl. XVIII, Fig.3). The necrotic muscle fibres were encircled by several layers of haemocytes, giving it a typical appearance of granuloma (Pl. XVIII, Figs. 4 and 5). Among the infiltrated haemocytes and necrotic muscle fibres, brown nodules were observed (Pl. XVIII, Fig. 6). Muscle fibres were separated from each other and haemocytic infiltration was observed in the intermuscular bundles (Pl. XIX, Fig. 1). Usual cross striations were lost in the muscle fibres and showed `moth - eaten´ appearence (Pl. XIX, Fig. 2). The necrotic muscle fibres were

replaced by connective tissue in certain regions of the abdominal muscle of infected animals (Pl. XIX, Fig. 3). Colonization of bacterial organisms was observed in the muscle (Pl. XIX, Figs. 4 and 5).

In the infected larvae, the muscle fibres were separated and haemocytic infiltration was observed between the muscle fibres. The muscle tissue was edematous, vacuolated and necrotic with pyknotic myonuclei (Pl. XXIV, Fig. 3; Pl. XXV, Fig. 6). The exoskeleton of infected larvae showed extensive accumulation of melanin pigment and the muscle fibres beneath the melanised exoskeleton also appeared brownish due to melanisation (Pl. XXIV, Fig. 4). Bacterial clusters morphologically indistinguishable from Vibrio sp. were observed in melanised area where haemocytic infiltration was also observed. Some haemocytes also appeared black due to melanisation.

Digestive tract

According to Roberts (1966), the digestive tract in prawn is composed of three divisions: 1) the foregut, which includes the mouth, oesophagus, stomach and associated glands; 2) the midgut and hepatopancreas and 3) hind gut. The cardiac stomach is a a large sac- like chamber with muscular walls and a folded lining (Pl. XIX, Fig. 6). The epithelium is overlain by cuticular layer. The pyloric stomach has complex folded walls strengthened by plates and chitinized hairs (setae) which form filtering apparatus (Pl. XX, Figs. 1 and 2). The midgut is straight tube lacking a cuticular lining. The musculature of the midgut is made up of longitudinal muscle fibres and

circular musculature (Pl. XX, Fig.3). The hindgut possesses similar structure of the midgut and has a cuticular lining (Pl. XX, Fig. 4).

In the infected adult prawn, the chitinous epithelial layer of the foregut was necrotic and the necrotic epithelial cells were sloughed into the lumen of stomach (Pl. XX, Fig.5). The foregut epithelial cells were edematous. The epithelial cells of foregut had cytoplasmic vacuolation at their apical region (Pl. XX, Fig. 6). Fat bodies were dislocated and aggregated in certain places of epithelial layer (Pl. XXI, Fig. 1). The nuclei in the epithelial cells of the foregut appeared pyknotic. The median ridge of filtering apparatus of pyloric stomach appeared swollen in moderately infected prawns (Pl. XXI, Figs. 2 and 3) and bifurcated in heavily infected ones (Pl. XXI, Fig.4). The epithelial cells in the chitinous plate were disorganised and contained cytoplasmic vacuoles at their apical region (Pl. XXI, Fig. 3). The setal secreting cells were degenerated and setae were detached and thrown into the supra- ampullary ridge (Pl. XXI, Fig. 4). The mucus epithelium of the midgut was necrotic with pyknotic and karyorhectic nuclei (Pl. XXI, Fig. 5). Haemocytic infiltration was observed in sub-mucosa and muscularis layers of the midgut (Pl. XXI, 6).

The cuticular and epithelial layers of hindgut were necrotic The epithelial cells of the hindgut fused together to form a hyalinized cytoplasm which sloughed into the lumen of the hindgut (Pl. XXII, Figs. 1 and 2). The sloughed off tissue gave the appearance of a copious catarrhal exuduate

containing tissue debris (Pl. XXII, Fig. 2). The epithelial cells in the lumen exhibited signet-ring appearance (Pl. XXII, Fig. 3) containing innumerable bacteria. The connective tissue of the hindgut contained a thin band of circular muscle, located just below the epithelial layer; was wavy in appearance, edematous and was completely degenerated in some region (Pl. XXII, Fig. 2). Bacterial colonies were seen in these regions as well as in the space between the connective tissue and the longitudinal muscle bundle (Pl. XXII, Figs. 4 and 6). The muscularis layers of the hindgut were edematous with ground glass appearance or hyalinization (Pl. XXII, Fig. 5).

In the infected larvae and postlarvae, the foregut epithelial cells were highly vacuolated, desquamated and sloughed into the lumen (Pl. XXIII, Fig. 2; Pl. XXIV, Figs. 5 and 6). The chitinous epithelial layer was necrotic and showed presence of bacterial organisms (Pl. XXV, Fig. 1). The epithelium of pyloric stomach showed extensive disquamation. The mucosal and muscularis layers were degenerated and infiltrated by bacterial organisms that were morphologically indistinguishable from Vibrio sp. Setae and chitinous plate of filtering apparatus were markedly degenerated whereas supra-ampullary ridge was filled with bacterial organisms (Pl. XXV, Fig. 1). The midgut and hindgut epithelium was either edematous and highly vacuolated or necrotic. Lumen was filled with the bacterial colonies (Pl. XXV, Fig. 4).

DISCUSSION

Vibriosis of fish and shellfish has been described by various workers. The previous works on the vibriosis of prawn have been restricted to the identification and pathogenicity of causative organisms (Vanderzant et al., 1970; Lewis, 1973; Lightner and Lewis, 1975; Leong and Fontaine, 1979; Takahashi et al., 1984,1985). Information on the histopathology of vibriosis in prawn is still lacking. Under the present investigation, histopathological studies were carried out in experimentally infected adult P. indicus apart from the protozoea and mysis of P. indicus and postlarva of P. monodon in order to get a clear picture on affected organs by Vibrio sp. 2448-88. The foregoing observations and comparison on the histological characteristics of the organs such as hepatopancreas, heart, haematopoietic tissue, gill, muscle and digestive tract of the uninfected larvae, postlarvae and adult prawn and those infected by Vibrio sp. 2448-88 reveal moderate to marked destruction of most of the vital organs due to the infection of the new isolate of Vibrio.

The excessive accumulation of fat, as observed in the hepatopancreas of the infected animals of the present study, has also been described in the stomach, style sac, digestive gland and intestine of Vibrio infected larvae of American oyster Crassostrea virginica by Elston et al. (1981). Similarly, Bowser et al. (1981) have observed high vacuolation in the hepatopancreas of Vibrio infected American lobster Homarus americanus as in the present study, but they have not explained the nature of vacuoles. The

exact mechanism responsible for excessive accumulation of fat in infected prawn is not known. However, Smith et al. (1972) have suggested that excessive accumulation of fat in the metabolically active cells under diseased conditions in the vertebrates may be due to 1) bacterial toxin interference with co-factors essential to oxidation and utilisation of fatty acids, 2) decreased phospholipid synthesis caused by lipotrope as in the case of methionine deficiency resulting in esterification of diglycerides to triglycerides and 3) reduced protein synthesis resulting in decreased lipoprotein synthesis and subsequent cytoplasmic deposition of lipid. On the basis of these views it may be opined that the toxins liberated by the colonies of Vibrio sp. 2448-88 might have interfered with lipotrophic factors and phosphorylation, and led to the accumulation of lipid in the hepatopancreas. Thus, the present study indicated that high degree vacuolation (excessive accumulation of lipid) of the hepatopancreas may be indicative of abnormal metabolic activities of the organ and an important pathological change in the Vibrio infected prawns.

The acidophilic cytoplasm in the dead cells is believed to be largely due to the loss of proteins from the cytoplasm and nucleus (Smith et al., 1972). Acidophilic cytoplasm observed in the hepatopancreas of infected prawn might have been caused by Vibrio sp. 2448-88 Similarly, acidophilic cytoplasm due to necrosis of hepatopancreatic cells under dietary stress has been observed in the hepatopancreas of H. americanus (Rosenmark et al., 1980).

Pyknotic nuclei observed in the hepatopancreas of infected prawn indicated the death of the cells. The pyknosis of the nuclei and acidophilic cytoplasm observed in the present study support the above observation of interruption in the normal metabolic activity of hepatopancreas.

Lightner and Lewis (1975) and Lightner (1977) have observed reduction in the haemocyte number and prolongation of coagulation in Vibrio infected P. aztecus, P. duorarum and P. setiferus. Necrosis of haematopoietic tissue is very common in vibriosis of fish (Anderson and Conroy, 1970; Ribelin and Migaki, 1975; Harbell et al., 1979; Ransom et al., 1984; Miyazaki, 1987). Inhibition in the differentiation of stem cells of haematopoietic tissue has been observed in H. americanus infected with Aerococcus viridans var. homari (Johnson et al., 1981). Harbell et al.(1979) and Ransom et al. (1984) have observed reduction in the number of red and white blood cells and haemoglobin in the blood of salmon associated with vibriosis. Leuocytosis and anaemia have been reported in Pseudopleuronectes americanus with chronic vibriosis (Watkins et al., 1981). The extensive necrosis and the presence of melanised nodules have been noticed in lymphoid organ of Vibrio infected kuruma prawn, P. japonicus (Egusa et al., 1988). The present observation on the necrosis of haematopoietic tissue due to the infection of Vibrio sp. 2448-88 in P. indicus as similar to the above observations. The exact mechanism of Vibrio sp. 2448-88 on the destruction of haematopoietic tissue is not known. However, Fuller et al. (1977), have reported a leucocytolytic factor (leucocidin) from Ameromonas salmonicida, responsible for the destruction of

haematopoietic tissue in Salmo gairdneri. Further studies similar to those of Fuller et al. (1977) are warranted in Vibrio sp. 2448-88.

Cardiac necrosis is most common in fishes infected with aeromonads and vibrios (Roberts, 1978) and is characterised by edema and vacuolation of the myofibrils. The myocardial necrosis was clearly evidenced by the pyknosis of nuclei in the heart of infected adult P. indicus. It is thus deduced that the focal colonial localization of bacteria in the heart might be associated with cardiac necrosis. Toxic substances released from the bacterial organisms might have interfered with the permeability of the capillary wall and caused cardiac edema in the present case. Boyd (1970) opined that chronic anaemia is apt to be associated with edema in mammals. Haematopoietic tissue necrosis observed in the present study might have resulted in the reduction of haemocytes in the circulation and hence might have been responsible for cardiac edema in prawn.

Pathological changes such as haemocytic infiltration, edema of lamellae, extensive vacuolation observed in gills of adult P. indicus and emphysema in the gills of infected postlarva of P. monodon indicate impairment of gills due to the infection of Vibrio sp. 2448-88.

Histopathological changes observed in the muscle of larvae and adult P. indicus infected with Vibrio sp.2448-88 related to phagocytosis, haemocytic infiltration and encapsulation, nodule formation and melanisation.

These observation were similar to those made by Fontaine and Lightner (1974) on the muscles of P. setiferus to which carmine particles were injected. According to Salt (1970), phagocytosis, nodule formation, haemocytic encapsulation and melanisation are all part of general haemocytic response of insect to injected material. The brown nodules, observed in the muscle of infected prawn in response to Vibrio sp. 2448-88 have been described in different names, such as `black cap', or `chitinoid nodules' (Pixell- Goodrich, 1928), brown or chitinoid bodies or cysts (Sindermann, 1971) and cysts (Ernst and Neff, 1979). Sindermann (1971) has described brown or `chitinoid' nodules or cysts in the gills as characterising later stages of a number of crustacean diseases. Fontaine and Lightner (1973) have described the development of brown nodules by haemocytes. According to them, nodules of necrotic tissues or foreign material had been encapsulated by several layers of fusiform haemocytes and had become melanized. In the present case, colonies of Vibrio sp. 2448-88 were encapsulated by several layers of fusiform haemocytes at the site of injection and brown nodules were observed among the haemocytes. The exact composition of brown substance observed in the present study is not known, however, Sindermann (1971) and Bang (1970) have referred to the brownish nodules or "brown bodies" found in decapod crustacea as chitin or chitinoid like material. Although no specific chemical analysis of this material has been carried out for penaeid prawn, Fontaine and Lightner (1974) believe it to be melanin, similar to that described in crayfish (Unestam and Nyland, 1972) and in insect (Salt, 1970). The present study indicates

that phagocytosis, haemocytic infiltration and encapsulation and melanin production were all part of haemocytic response to Vibrio sp. 2448-88 to protect the host from the infection caused by Vibrio sp. 2448-88.

The observation of phagocytosis in penaeid prawn has posed the question of recognition. Phagocytosis of foreign particles by mammalian leucocytes is often dependent upon the presence of recognition factors or opsonins in the serum (Guyton, 1976; Ganong, 1979). Similar serum dependent phagocytosis has been demonstrated in the invertebrates such as Crassostrea virginica (Tripp, 1966), Parachaeraps bicarinatus (Mckay and Jenkin, 1970), Aplysia california (Pauley et al., 1971) and Homarus americanus (Peterson and Stewart, 1974). Similar works are highly warranted to answer the question of recognition of foreign material by phagocytes in the prawn.

Vibrio sp. 2448-88 was colonised in hepatopancreas, heart, gills, muscle, haematopoietic tissue and mid gut and hind gut. Localisation of injected material in specific body regions has also been reported in H. americanus (Cornick and Stewart, 1968), Penaeus setiferus (Fontaine and Lightner, 1974) and Callinectes sapidus (Johnson, 1976; McCumber and Clem, 1977). In most species, the gills appear to be the most significant site for deposition, although the hepatopancreas, heart and body musculature may often also be involved, but in the present case, Vibrio sp. 2448-88 was localized in all above said organs. Several authors have reported a system of fixed phagocytic haemocytes in the

gills, hepatopancreas and other organs in crustaceans (Mayhard, 1960; Reade, 1968; Cornick and Stewart, 1968; Johnson et al., 1981). These fixed phagocytic haemocytes may be responsible for the localization of Vibrio sp. 2448-88 observed in the hepatopancreas (Pl. XIV, Fig. 2), heart (Pl. XV, Fig. 2) and muscle (Pl. XIX, Fig. 4).

From the foregoing discussions on the histopathological observation and in consideration of the results of the pathogenicity experiments, it may be inferred that when the virulent cells of Vibrio sp.2448-88 come across the host prawn, the organisms first attach themselves on the surface of the exoskeleton and cause damage to the exoskeleton by the activity of chitinase. Then the bacteria enter into the muscle. Although most of the cells might get killed or inactivated by haemocytic activity of the host (phagocytosis, haemocytic infiltration and encapsulation and melanisation), some cells might penetrate still interior of the host and proliferate there if they successfully escape from the surveillance system of the host defense mechanism. Upon proliferation, the excess organism are conveyed to heart, hepatopancreas and haematopoietic tissue through haemolymph (phagocytic haemocytes) and induce the systemic infection resulting in death of the host by interfering normal activities of hepatopancreas and causing myocardial necrosis and destruction of haematopoietic tissue.

CHAPTER 9

EVALUATION OF CERTAIN ANTIMICROBIAL AGENTS FOR CONTROLLING VIBRIOSIS

INTRODUCTION

Disease is one of the main factors limiting the survival, growth and production of farmed fishes and shellfishes. The control and/or prevention of disease transfer has received an equal importance as that of the diagnosis and determination of the disease in the stocked population. Disinfection of water, containers in which larvae and postlarvae are held and other implements, is necessary to overcome the disease problems in controlled conditions. The aim of successful disinfection is to destroy infectious agents in the water, implements and in the containers. Disinfectants used in fisheries should be aimed at destroying all types of pathogens including viruses, bacteria, fungi and protozoa. The term "Mariculture medicine" coined by Klontz (1970) to denote the medical aspect of mariculture, includes 1) recognition that disease does exist, 2) definition of the disease, 3) correction of the disease and 4) prevention of recurrence of the disease. Six basic approches such as adequate husbandry practices, use of genitically resistant strains, adequate balanced diet, use of vaccines, use of antimicrobial compounds and prevention of movement of infected stock have been recognised to control bacterial diseases in fishes. Valuable informations on different aspects of disease control means are avilable for fishes (Austin and Austin, 1987). However, similar studies on prevention and prophylactic measures for penaeid prawn diseases are limited. Although

four viral diseases have been reported in penaeid prawns (Lightner et al., 1983 a,b) difinite curative measures to control these diseases have not so far been developed. In the case of bacterial diseases drying, cleaning and disinfection of spawning, hatching, larval rearing and nursery tanks are found to considerably reduce bacterial infection (AQUACOP, 1977; Lightner, 1977; Lightner et al., 1980). Generally four methods of bacterial disinfection have been tested to reduce bacterial population in shellfish hatchery. These are ultraviolet treatment, addition of silver nitrate ($AgNo_3$) to culture water, ozone disinfection of contaminated culture medium and standard antiobiotic treatment (neomycin). Of these, the ultraviolet treatment is found to be effective to control bacterial population in the seawater (Blogostawski et al., 1978).

The use of antibiotic compounds to control bacterial diseases was studied as early as 1946 by Gutsell. Since then a range of antimicrobial compounds were tested. Thus Oppenheimer (1955) employing thirteen antibiotics to control marine bacteria has observed that penicillin, streptomycin and chloromycetin were the most effective in reducing the bacterial populations. However, the latter antibiotic was found to be toxic to fish and eggs. Monroe (1970) found that tetracycline hydrochloride, terramycin and nitrofurans were effective for controlling the pathogens of pompano, whereas penicillin, streptomycin and triple sulpha drugs proved ineffective. Further, bacterial kidney and furunculosis encountered in the adult spring chinook salmon were controlled by the complex of penicillin G procaine, dihydrostreptomycin sulphate and oxytetracycline HCl (De Cew,

1972). Struhsaker et al. (1973) reported that polymixin, penicillin and particularly erythromycin were effective in reducing the bacterial population and enhancing the larval survival of carangid fish.

Several reports are available on the use of antimicrobial compounds to control diseases of crustacean. Christiansen (1971) successfully reared the larvae of Hyas araneus to the megalopa larva in antibiotics (penicillin and streptomycin) treated seawater. When the antibiotics were not used, he (Christiansen, 1971) observed higher mortality of larvae.

Chan and Lawerence (1974) studied the effect of oxytetracycline HCl and olendomycin phosphate on the respiration of larvae and postlarvae of Penaeus aztecus and on the bacterial populations associated with larval and postlarval stages and found that the bacterial population was controlled by a mixture of 500 µg of oxytetracycline and 200 µg of olendomycin per ml of seawater without causing any damage to larval stages of P. aztecus. Delves - Broughton (1974) and Tareen (1982) reported that the broad spectrum antibiotic, furanace, which inhibits the growth of almost all pathogenic bacteria of crustacea when added directly to the water, was non-toxic to crustaceans. Similarly, Johnson and Holcomb (1975) observed that potassium dichromate besides maintaining the quality of water, could effectively control the bacterial diseases in prawns.

Lightner (1975, 1977, 1983, 1985) on the basis of a series of studies recommended terramycin at a rate of 360 mg/kg body weight/day

administerred with feed to control vibriosis in the penaeid prawn, P. setiferus and furacin for its larval stages by immersion method. Oral application of sulfisozole, nifurstyreic acid and chloramphenicol at appropriate levels of concentration and compounded with the formula feed was also found to be effective in saving the Vibrio inoculated prawn (Shigueno, 1975). Mixtures of malachite green oxalate and formalin were being used to cure the shell disease in prawns (Lightner, 1975). Similarly, hyamine and methyline blue were proved to be useful for the treatment of bacterial and fungal diseases in commerical prawns (Hanks, 1976).

Johnson (1976a) observed that nitrofurozone and oxytetracycline were not toxic to the larval stages of P. setiferus and recommended their use in prawn farming. AQUACOP (1977) reported that the antibacterial compounds such as streptomycin-bipenicillin, erythromycin phosphate, tetracycline chlorohydrate, sulphamethazinane and furanace were effective to cure appendage necorsis in larval stages of penaeid prawns. Better survival rates of zoea larvae of Cancer magister were recorded by Fisher and Nelson (1977) when the larvae were reared in the sea water treated with streptomycin and penicillin. Corllis et al. (1977), however, reported that oxytetracycline was effective only to preventing Vibrio alginolyticus infection in prawns at very high dosage levels and thus it was retained by prawns when the juvenile prawns were fed at 5000 mg and 10,000 mg per kg of feed (Corliss, 1979). A seawater soluble copper compoud commercially known as Cutrine Plus, was used to control the filamentous bacterium Leucothrix mucor (Lightner and Supplee, 1976).

Curative measures for fungal diseases in adult and larval stages have been suggested by several workers (Delves -Broughton, 1974; Hatai et al., 1974; Johnson, 1974b; Ruch and Bland, 1974; Lightner, 1975, 1977, 1983, 1985; Bland et al., 1976; AQUACOP, 1977; Schnick et al., 1979; Lio-Po et al., 1982; 1985; Johnson, 1983b). Lio-Po et al. (1982,1985) have studied in vitro effect of antifungal agents on Lagenidium sp. and Haliphthoros philippinensis isolated from infected larvae of P. monodon. Testing the twleve potential fungitoxic compounds on the growth and development of L. callinectes. Bland et al. (1976) found that malachite green and DS 9073 could effectively control the larval mycosis. Toxicity of furanace, malachite green and other fungicides against Lagenidium sp. and H. philippinensis on protozoea and mysis larvae of P. monodon. was studied by different workers (Gacutan and Llobrera, 1977; Lio-po et al., 1978, 1986; Gacutan et al., 1979a). Higher survival of weak larvae has been observed when exposed for six hours in 1.0 mg of furanace per litre of seawater. Besides malachite green, oxylate at 0.006 ppm (static) and Treflan (trifuralin, Elanco Products Co.) were also found to be useful in preventing Lagenidium and Sirolpidium epizootics in the culture system. Hatai et al. (1974) tested various fungicides such as acrinal, 2-amino-4- nitrophenol, amphotericin B, azalomycin F, benlate, benzalkonium chloride, blastin, celylpyridinium chloride, chloramine T, 5- chlorosalicylanilide, crystal violet, dehydroacetic acid, hyamine, malachite green, methylene blue, methyl violet and polymyxin B to control the black gill disease of P. japonicus caused by Fusarium sp., but found that none of them was effective for curing Fusarium disease.

Methods of chemotherapy for controlling protozoan infestation have been reported by several workers (Johnson et al., 1973; Johnson, 1974a,b, 1976b; Johnson and Holcomb, 1975; Overstreet, 1975; Overstreet and Whatley, 1975; Lightner, 1977; Schnick et al., 1979; Tareen, 1982). 25 ppm formalin and chloramine T, quinine bisulphate (both at 5 ppm) and particularly qunarcine hydrochloride (at 0.6 ppm) were found useful in the treatment of Zoothamnium sp. and Epistylis sp.

Overstreet and Whatley (1975) tested eight drugs against microsporidiosis caused by Nosema michaelis in Callinectes sapidus and reported that crabs fed with buquinolate could reudce the incidence rates rather than total control.

The black death disease due to ascorbic acid deficiency in prawns was controlled by providing appropriate feed having 2000-3000 mg of vitamin C per kilogram of feed (Deshimaru and Kuroki, 1976; Lightner et al., 1979a: Magarelli et al., 1979) or by feeding a supplement of fresh algae to the affected prawns (Lightner, 1977).

In India, studies on the control of diseases of aquatic organisms especially the freshwater fishes have received appreciable attention (Khan, 1939, 1944; Tripathi 1954, 1957; Hora and Pillai, 1962; Gopalakrishnan, 1963, 1964, 1968, Ghosh and Pal, 1969; Pal and Ghosh, 1975; Srivastava, 1975; Ghosh, 1978, Mandaloi, 1982; Seenappa and Manohar, 1982; Seenappa et al., 1982; Srivastava, 1982). However, in respect of penaeid prawn particularly

larve and postlarvae the available information is entirely scanty. Pandian (1982) reared the larvae and postlarvae of P. indicus. and M. dobsoni in the medium treated with tetracycline and acriflavin separately and reported that 1-3 ppm tetracycline treatment was not harmful to the larval stages of prawn, but acriflavin caused larval mortality. To fill the gap in our knowledge and in consideration of the fact that detailed comparative study of the various antimicrobial compounds against the pathogens of crustacea is lacking, the present study to evaluate the suitable antibacterial agent against vibriosis was taken up and the results presented.

MATERIAL AND METHODS

In-vitro antibiotics sensitivity testing procedure

Elevan antimicrobial compounds were tested against the marine vibrios in the first investigation to find out their sensitivity towards the antiobiotics. The antibiotic sensitivity test was carried out by the method of Bauer-Kirby (1966). The antibiotic discs were obtained from Hi-Media and Span Pharmaceutical Company, India. The antibiotic discs used in the investigation are given in Table 9.1.

Marine vibrios that were tested for their sensitivity against antibiotics were cultured in seawater nutrient agar of the following composition: Bacto-peptone (Difco) 1% ; beef extract (Difco) 0.3% and Bacto-agar (Difco) 1.5%. The medium was prepared with filtered aged seawater of salinity 30-34‰. The medium was autoclaved at 115°C for 15 minutes and allowed to cool. Approximately 15 ml of the cold sterile medium was poured to each

Table 9.1. Antimicrobial agents tested *in-vitro* for antimicrobial activity against *Vibrio* sp. 2448-88, *V. parahaemolyticus* and *V. alginolyticus*

Antimicrobial Agent	Make	Symbol	Strength/disc
Bacitracin	Hi-Media	B	10 units
Chloramphenicol	Span	C	30 µg
Cloxacillin	Hi-Media	Cx	1 µg
Nalidixic acid	Hi-Media	NA	30 µg
Nitrofurazone	Prepared with sprinkling powder	N	30 µg
Oleandomycin	Hi-Media	OL	15 µg
Oxytetracycline HCl	Hi-Meida	O	30 µg
Pencillin	Hi-Media	P	10 units
Polymixin B	Hi-Media	PB	300 units
Streptomycin	Hi-Media	S	10 µg
Tetracycline	Hi-Media	T	30 µg

sterilised petri dish and allowed to solidify. After the medium solidified, the plates were dried for 30 minutes in an incubator (35- 37 °C) to remove excess moisture from the surface.

The test organism was transferred from the stock into a glass test tube containing 5 ml of sterile seawater nutrient broth with the help of a wire loop. The inoculated broth was incubated at 30 °C for 10-12 hrs to obtain moderate turbidity which compared with that of barium chloride and sulfuric acid (0.5 ml of 1.175% barium chloride and 99.5 ml of 0.36 N sulfuric acid).

A sterile cotton swab was dipped into the broth culture inoculum. The cotton swab was then rotated pressing against the inside wall of the tube, above the fluid level to remove excess inoculum. The agar surface of the plate was inoculated by swabbing three times, turning plate by 60 in between swabbings. The lid of the petri dish was replaced and the plate was kept at room temperature for 5 to 10 minutes to dry the inoculum.

The sensitivity discs were removed from their respective vials with the help of sterilised forceps and the discs were carefully dropped onto the surface of the agar in the plates. The discs were placed sufficiently away from each other keeping a minimum of 1.5 cm distance to avoid overlapping of the zones of inhibition. Each disc was pressed down firmly on to surface of the agar plate, using a sterile needle. The plates were incubated upside down for 24 hrs at 30 °C.

The agar plates were examined after 24 hrs for circular clear area in the bacterial lawn around the antibiotic disc. The diameter of the zone of inhibition was measured. Each antibiotic, including the control was assayed with three replicates.

Discs with 30, 40, 60 and 90 microgram per disc of chloramphenicol, tetracycline or oxytetracycline were prepared to find out the maximum inhibition zone at minimum concentration of the antibiotic. The methods used for antibiotic sensitivity testing were followed for this investigation. The minimum concentration of antibiotic in the disc around which maximum zone of inhibition was observed, was considered as the minimum inhibitory concentration of that particular antibiotic.

Minimum Inhibitory Concentrations (MIC) of chloramphenicol, oxytetracycline and tetracycline against Vibrio sp. 2448-88.

The MIC of chloramphenicol, oxytetracycline HCl and tetracycline against Vibrio sp. 2448-88 were determined by tube dilution technique. The seawater peptone broth was prepared and autoclaved. The peptone broth was allowed to cool and varying concentrations of chloramphenicol (1, 5, 10 and 20 mg/l), oxytetracycline (1, 5, 10, 20, 50, 100, 150, 200 and 250 mg/l) and tetracycline (1, 5, 10, 20, 50, 100, 150, 200 and 250 mg/l) were added to the broth before pouring into the test tubes, and allowed to cool.

Vibrio sp. 2448-88 was inoculated into the tubes and tubes were incubated at 30 °C for 24 hrs. After 24 hrs the tubes were examined for bacterial growth in the broth. The growth of the organisam was measured by the method described in chapter 2. The minimum concentration of the antibiotic which inhibited bacterial growth was recorded as MIC of that particular antibiotic.

Effect of antibiotics on Vibrio sp. 2448-88 in sterile seawater

The effect of chloramphenicol and oxytetracycline on the growth of Vibrio sp. 2448-88 in the sterile seawater was studied. Three concentrations (5, 10 and 25 mg/l of the medium) of chloramphenicol, two concentrations of oxytetracycline (100 and 150 mg/l) and one combination of both antibiotics (2.5 mg + 50 mg/l) were tested. The antiniotics used were:

Antibiotic	Form	Source
Oxytetracycline HCl (Terramycin)	Pure, capsule	Pfizer
Chloramphenicol (Chloromycetin)	Pure, capsule	Parke-Davis

The seawater stored at the Marine Prawn Hatchery Laboratory, Narakkal was autoclaved. 400 ml of autoclaved seawater was placed in sterilised 500 ml capacity beaker with lid. Each antibiotic was weighed and dissolved into the autoclaved seawater. The experiments including control were carried out in three replicates.

The inoculum of Vibrio sp. 2448-88 for the experiment was prepared by the method given in chapter 7. From the bacterial suspension of 1.0 OD, one ml (1.4×10^{12} cells) was added and mixed thoroughly in the sterile antibiotic treated seawater. Samplings were done after 3rd, 6th, 12th, 18th and 24th hrs of incubation. The samples collected were used for serial dilutions and plating to estimate tha population of Vibrio sp. 2448-88 in the control and antibiotic treated sterile seawater.

Effect of antibiotics on larvae and postlarvae of Penaeus indicus

The toxicity of chloramphenicol and oxytetracycline HCl was tested by bathing the larvae and postlarvae of Penaeus indicus in antibiotic treated sea water for 24 hrs. The larvae and postlarvae were obtained from the Marine Prawn Harchery Laboratory, Narrakkal. The larval stages used in the experiment were:

Nauplius I	-	Nauplius IV
Protozoea I	-	Protozoea II
Protozoea II	-	Protozoea III
Protozoea III	-	Mysis I
Mysis I	-	Mysis II
Mysis II	-	Mysis III
Mysis III	-	Postlarva I
Postlarva I	-	Postlarva II

The larvae were acclimatised for one hour before starting the experiment in the rearing medium. The physical and chemical

characteristics of the rearing medium during the experiment were salinity 30-34‰ ; pH 7.9-8.1; temperature 26-30 °C and dissolved oxygen 4-5 ml/l.

Twenty larvae in naupliar stage, ten in protozoeal and mysis stages and 6 in postlarval stages were reared in 500 ml cleaned beakers containing 400 ml of culture medium. Ample aeration was provided throughout the experiment. After an hour of acclimatisation, pre-weighted antibiotics were thorougly dissolved in the water. Three replicates for each concentration including control were carried out. The concentrations used were:

Chloramphenicol : 5 mg, 10 mg and 25 mg/l

Oxtetracycline : 100 mg, 150 mg and 200 mg/l

Control : Without antibiotics.

The feeding of larvae and postlarvae were done by the methods mentioned in chapter 7.

The percentage survival of larvae and postlarvae was determined after 24 hrs. The larvae which did not respond to mechanical stimultation, those showing reduced swimming activity and unable to feed were considered unfit for further studies. The percentage survival of larvae in the presence of antibiotic was calculated after substracting the number of debilitated and dying larvae. Microscopic examination was also made to observe the gut condition of larvae and postlarvae.

Effect of chloramphenicol on Vibrio sp. 2448-88 associated with larvae of Penaeus indicus

The seawater was collected and filtered through 0.2 µm pore Sartorius filter paper. Four hundred protozoeae and mysis each in four litres of filtered seawater were reared separately in 5 litre capacity beakers. The inoculum of Vibrio sp.2448-88 was prepared as mentioned in Chapter 7. The inoculum was added into the sea water and mixed thoroughly and the bacterial cells in the rearing media of mysis and protozoea were adjusted to 35 x 10^8 cells/ml of the filtered rearing medium for mysis and 35 x 10^7 for protozoea.

After 24 hrs, the weak larvae with signs of expansion of chromatophore, opaqueness of the body and inability to move, were collected and treated with chloramphenicol at the concentrations of 10 mg and 25 mg per litre. The treatment was carried out for 24 hours and larval samples (10 larvae/sample) were collected at 0, 3rd, 6th, 12th, 18th and 24th hrs of the experiment to estimate the population of Vibrio sp. 2448-88 associated with protozoeae and mysis. Two trials were carried out. TCBS agar plates were used to estimate Vibrio sp. 2448-88 associated with the larvae.

Optimum exposure time to chloramphenicol for protozoea and mysis to recover from vibriosis

This experiment was carried out simultaneously with the above experiment. The infected protozoea and mysis larvae (300 each) were

collected and separately treated with 10 ppm and 25 ppm of chloramphenicol (150 protozoea/mysis in each concentration). The protozoea/mysis (10/sample) were collected after 0, 3, 6, 12, 18, and 24 hrs of exposure to chloramphenicol from each of the concentrations and reared further for 96 hrs in 500 ml beakers containing fresh seawater. The larvae that survived at the end of 24, 48, 72 and finally at 96 hrs were counted to determine the optimum time required for protozoea and mysis to recover from vibriosis. Two trials were conducted.

Effect of chloramphenicol and oxytetracycline on mixed population of Chaetoceros sp. and Skeletonema sp.

Five transparent white bottom, rectangular perspex tanks of 5 litre capacity were used for the experiment. The seawater was collected and fertilized with chemicals as described in Chapter 3. The physico-chemical characteristics of the culture medium were as follows: salinity 30-34 ‰; pH 7.9-8.3; temperature 29-31 °C and dissolved oxygen 4-5 ml/l. A known quantity of chloramphenicol and oxytetracycline were dissolved in culture media, to obtain concentration of 10 and 25 mg of chloramphenicol per litre of the medium and 100 and 200 mg of oxytetracycline per litre. Three replicates in each concentration including control were carried out. The mixed phytoplankton (Chaetoceros sp. and Skeletonema sp.) of uniform density (initial density in all runs 20.6×10^2 cells/ml) was placed in all tanks. The counting of phytoplankton was carried out with Sedgwick-Rafter slide. Population counts of the diatom were taken after 2, 4, 6 and 8 hrs exposures and recorded.

RESULTS

The sensitivity of V. alginolyticus, Vibrio sp. 2448-88 and V. parahaemolyticus against the eleven antibiotics tested is summarised in Table 9.2. All these vibrios showed sensitivity to chloramphenicol; they were however resistant to other antibiotics except nalidixic acid and polymixin B to which V. parahaemolyticus and Vibrio sp. 2448-88 respectively showed relatively less sensitivity. The diameter of inhibitory zone was found to increase as the antibiotic concentration per disc increased against Vibrio sp 2448-88 indicating the relatively high bactericidal effect of chloramphenicol on Vibrio sp. 2448-88 (Table 9.3).

The minimum inhibitory concentrations of chloramphenicol, oxytetracycline HCl and tetracycline against Vibrio sp. 2448-88 is summarised in Table 9.4. The growth of Vibrio sp. 2448-88 was inhibited at 5 mg of chloramphenicol per litre of the medium, while the bacterium showed appreciable growth in oxytetracycline HCl concentrations upto 50 mg/l. As the concentration of the antibiotic increased to 100 mg/l, poor growth was observed and it was inhibited at 150 mg/l. In tetracycline treated seawater nutrient agar, Vibrio sp. 2448-88 grew well up to 100 mg/l of the medium poor growth was observed at 150 mg/l and completely inhibited at the concentreations higher than 200 mg/l of medium.

The results of the experiment on the growth of Vibrio sp. 2448- 88 in antibiotic treated sea water at different time intervals are summarised in

Table 9.2. Sensitivity of *Vibrio alginolyticus* *V. parahaemolyticus* and *Vibrio* sp. 2448-88, against the different antimicrobial agents

Antimicrobial Agents	*Vibrio alginolyticus*	*Vibrio parahaemolyticus*	*Vibrio* sp. 2448-88.
Bacitracin	R	R	R
Chloramphenicol	S	S	S
Cloxacillin	R	R	R
Nalidixic acid	R	I	R
Nitrofurazone	R	R	R
Oleandomycin	R	R	R
Oxytetracycline HCl	R	R	R
Pencillin	R	R	R
Polymixin B	R	R	I
Streptomycin	R	R	R
Tetracycline	R	R	R

R- Resistant, I- Intermediate, S- Sensitive.

Table 9.3. Diameter (mm) of inhibition zone at different con centrations of chloramphenical, oxytetracycline and tetracycline against *Vibrio* sp. 2448-88

Antibiotics	Concentration in microgram per disc			
	30	40	60	90
Chloramphenicol	21	24	26	31
Oxytetracycline	8	10	12	14
Tetracycline	No zone	No zone	7	12

Table 9.4. Minimum inhibitory concentrations of antibiotic against *Vibrio* sp. 2448-88 tested by tube dilution technique.

Antibiotics	Concentration (ppm)								
	1	5	10	20	50	100	150	200	250
Chloramphenicol	+	-	-	-	ND	ND	ND	ND	ND
Oxytetracycline HCl	+	+	+	+	+	(+)	-	-	-
Tetracycline	+	+	+	+	+	+	(+)	-	-

+ : Growth of *Vibrio* sp. 2448-88, - : No grwoth, ND: not done, (+) : Poor Growth.

table 9.5. The two lower concentrations (5 ppm and 10 ppm) of chloramphenicol were not able to reduce the population of Vibrio sp. 2448-88 completely, whereas the highest concentration (25 ppm) of chloramphenicol reduced to 0 cells/ml after 24 hours of incubation (Table 9.5; Pl. XXVI, Fig. 1). In oxytetracycline treated seawater, the bacterial population was reduced to 21000 and 3650 cells/ml of water in the concentrations of 100 ppm and 150 ppm respectively after 18 hours of incubation (Pl. XXVII, Fig. 1). The combination of chloramphenicol and oxytelracycline at the rate of 2.5 and 50 ppm reduced the population of Vibrio sp. 2448-88 to 35500 after 18 hours of incubation. The bacterial population in the absence of antibiotics was increased to a level of 40 x 10^{12}/ml of seawater at the end of 24 hrs of the experiment.

Percentage survival of nauplius I through postlarva I of P. indicus exposed for 24 hours at different levels of chloramphenicol and oxyteracycline is given in Tables 9.6 and 9.7 respectively. High mortality of protozoea I was observed when the larvae were exposed to 25 ppm of chloramphenicol. In oxytetracycline, however appreciable mortality of nauplius I (91.63%) and protozoea I (63.3%) were recorded only at a concentration of 200 ppm. In 25 ppm of chloramphenicol and 200 ppm of oxytetracycline, the nauplii and early protozoea were very weak and their development was very slow. Nauplii with broken setae and appendage deformities and protozoea I with empty stomach were encountered when the larvae were exposed to 200 ppm of oxytetracycline. However, no adverse effects of both antibiotics on the physical development and moulting of

Table 9.5. : Plate count estimates of number of Vibrio sp. 2448-88 cells* per ml antibiotic treated seawater at different time intervals.

Antibiotics	Number of viable bacterial cells/ml					
	0 hr	3 hr	6 hr	12 hr	18 hr	24 hr
Control	35×10^8	112.5×10^9	118×10^{10}	184.5×10^{11}	173.5×10^{12}	40×10^{12}
Chloramphenicol						
5 ppm	35×10^8	45×10^5	33.5×10^5	39.5×10^3	2420	166000
10 ppm	35×10^8	113×10^4	38×10^4	106.5×10^2	140	79000
25 ppm	35×10^8	28×10^3	17×10^3	2000	30	0
Oxytetracycline HCl						
100 ppm	35×10^8	54×10^6	61×10^5	74.5×10^4	21000	230000
150 ppm	35×10^8	40×10^5	79.5×10^4	147×10^3	3650	184000
Chloramphenicol, 2.5 ppm + oxytetracycline HCl 50 ppm	35×10^8	59.5×10^5	76×10^5	47.5×10^4	35500	222000

* Based on 3 replicates.

PLATE XXVI

Fig.1. Estimated Vibrio sp. 2448-88 population in the inoculated sterile seawater treated with different concentrations of chloramphenicol.

PLATE XXVI, Fig. 1

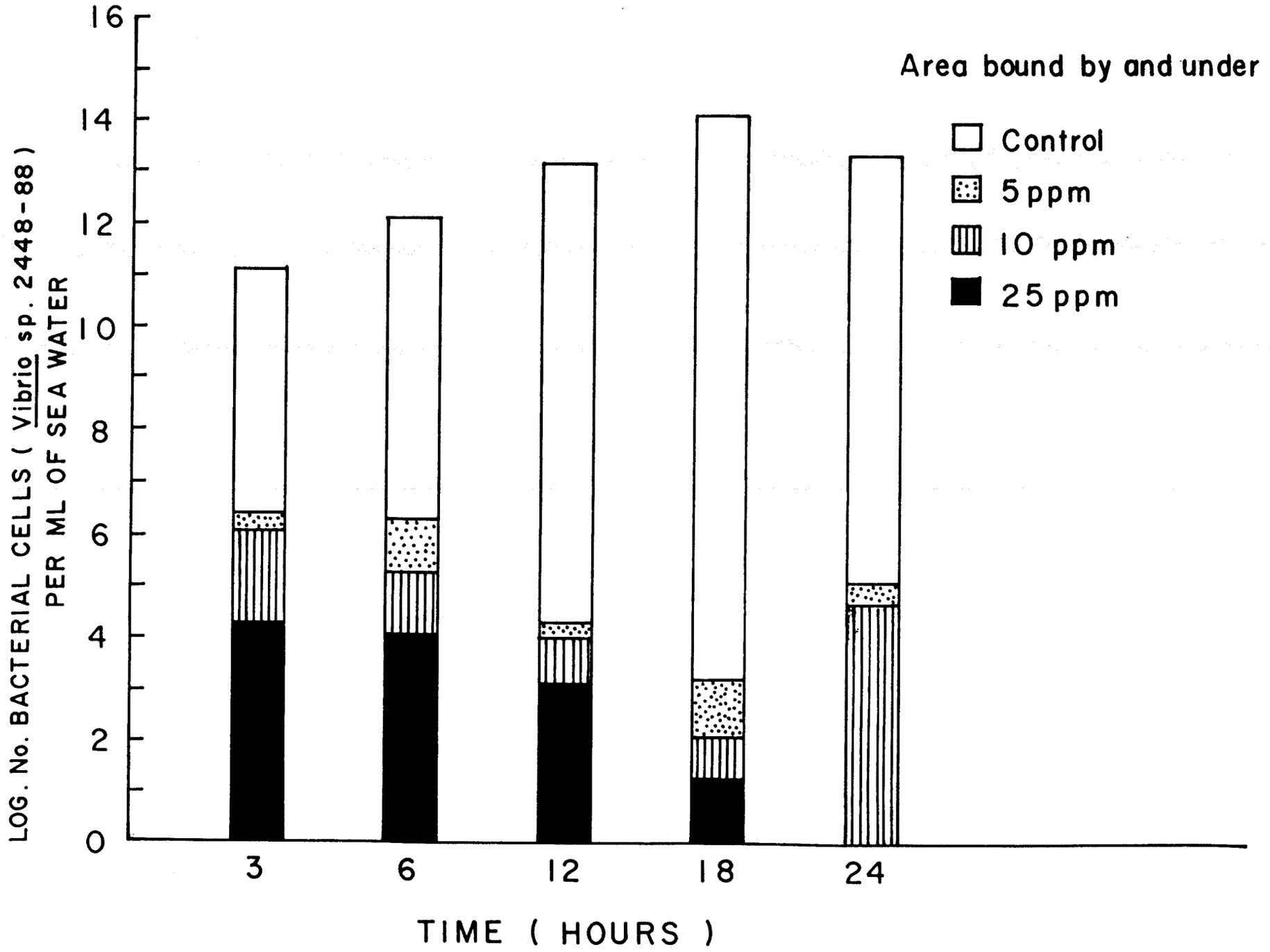

PLATE XXVII

Fig.1. Estimated Vibrio sp. 2448-88 population in the inoculated sterile seawater treated with different concentrations of oxytetracyline.

PLATE XXVII, Fig. 1

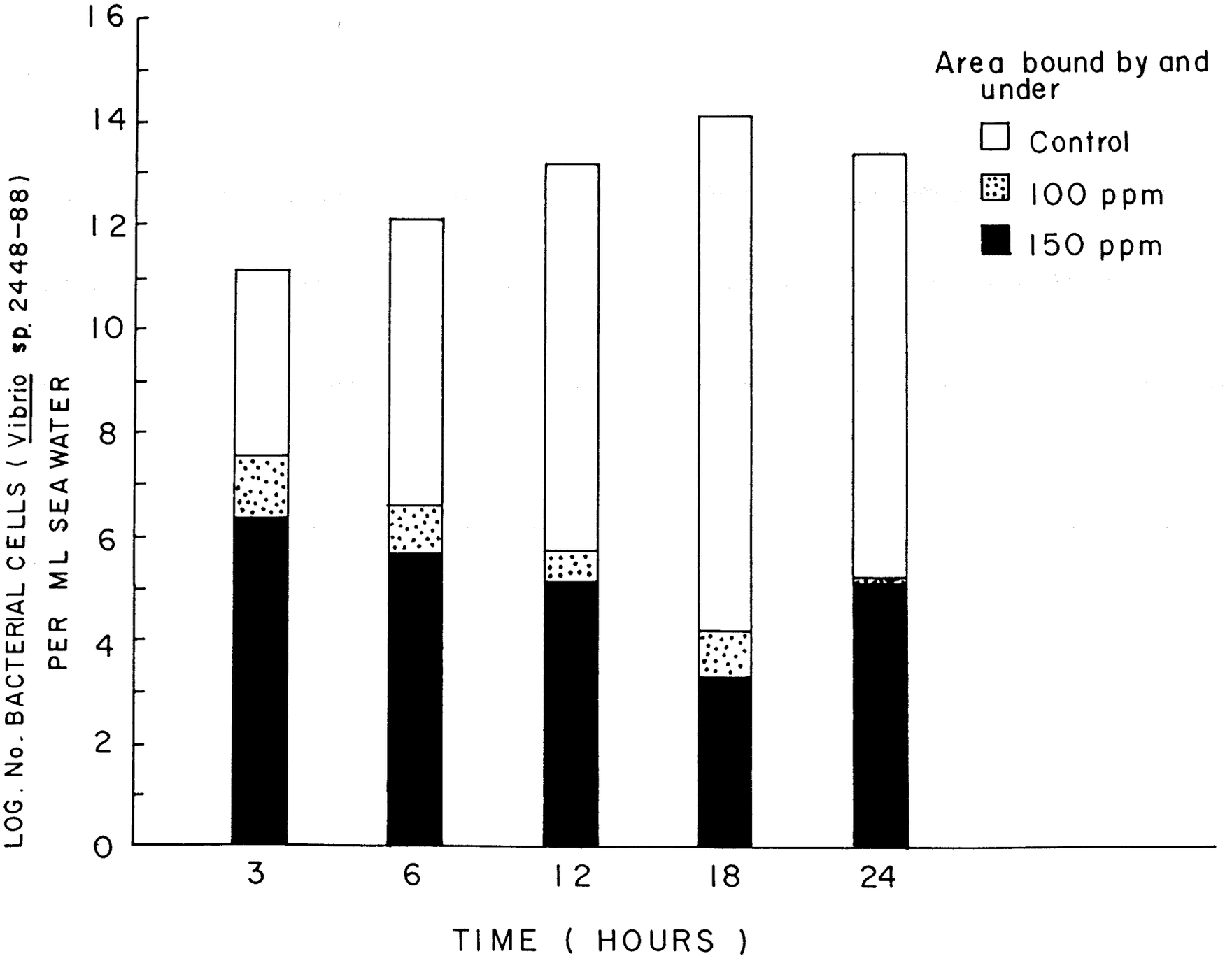

Table 9.6. Percentage of survival of larvae and postlarvae of Penaeus indicus exposed for 24 hours in bath treatment against different concentrations of choloramphenicol

Larval stage	Percentage of survival (3 trials)			
Concentrations (ppm)	0	5	10	25
Nauplius	98.33	95.00	85.00	80.00
Protozoea I	100.00	96.67	80.00	36.67
Protozoea II	100.00	100.00	96.67	90.00
Protozoea III	100.00	100.00	100.00	100.00
Mysis I	100.00	100.00	96.67	100.00
Mysis II	100.00	100.00	96.67	80.00
Mysis III	100.00	100.00	93.33	86.67
Postlarva I	100.00	100.00	100.00	100.00

Table 9.7. Percentage of survival of larvae and postlarvae of Penaeus indicus exposed for 24 hours in bath treatment against different concentrations of oxytetracycline HCl

Larval stage	Percentage of survival (3 trials)			
Concentrations (ppm)	0	100	150	200
Nauplius	98.33	81.67	21.67	8.33
Protozoea I	100.00	86.67	50.00	36.67
Protozoea II	100.00	90.00	66.67	53.33
Protozoea III	100.00	100.00	100.00	100.00
Mysis I	100.00	100.00	100.00	100.00
Mysis II	100.00	93.33	90.00	80.00
Mysis III	100.00	100.00	100.00	100.00
Postlarva I	100.00	100.00	100.00	100.00

protozoea II through postlarvae I were observed. Similarly no abnormal behavioural pattern was observed in the larvae exposed to the antibiotics.

Effect of chloramphenicol on the population of Vibrio sp. 2448-88 associated with protozoea and mysis was tested and the results are given in Table 9.8. Chloramphenicol at the concentration of 25 ppm reduced the population of Vibrio sp. 2448-88 from 1.5×10^7 to 7.4×10^2 and from 1.46×10^8 to 7.7×10^2 cells/g of larvae in protozoea and mysis respectively after 24 hours of incubation (Pl. XXVIII, Fig.1 and Pl. XXIX, Fig. 1.), but this concentration could not remove all Vibrio sp. 2448-88 from the larvae as in the case of seawater (Table 9.5). In the absence of antibiotic, Vibrio sp. 2448-88 population associated with protozoea and mysis increased to a level of 1.5×10^{10} cell/g and 6.7×10^{10} cells/g of larvae respectively after 24 hours of incubation.

The experimentally infected protozoea and mysis of Penaeus indicus by Vibrio sp. 2448-88 were exposed to 10 ppm and 25 ppm of chloramphenicol at different time intervals to find out the optimum exposure time to control the infection caused by Vibrio sp. 2448-88. The optimum exporsure time for protozoea to control the infection appeared to be 3 hrs in 25 ppm (survival 90%) and 6 hrs in 10 ppm (survival 80%) and for mysis 6 hrs in 25 ppm (survival 100%) and 18 hrs in 10 ppm (survival 100%) (Table 9.9).

Table 9.8. Estimated average *Vibrio* sp. 2448 - 88 population (cells/gm of larvae)* associated with protozoea and mysis of *Penaeus indicus* at different levels of concentration of chloramphenicol

Time (hrs)	Control		Chloramphenicol - bath treatment			
	Without antibiotics		10 ppm		25 ppm	
	Protozoea	Mysis	Protozoea	Mysis	Protozoea	Mysis
0	1.5×10^{7}	1.46×10^{8}	1.5×10^{7}	1.46×10^{8}	1.5×10^{7}	1.46×10^{8}
3	2.7×10^{7}	2.10×10^{8}	1.1×10^{5}	1.00×10^{6}	3.2×10^{4}	3.30×10^{4}
6	1.1×10^{7}	2.60×10^{9}	2.9×10^{4}	2.30×10^{4}	9.0×10^{3}	2.00×10^{4}
12	4.4×10^{9}	2.70×10^{9}	1.7×10^{4}	1.10×10^{5}	6.0×10^{3}	6.30×10^{4}
18	9.8×10^{9}	3.00×10^{10}	1.5×10^{4}	1.70×10^{5}	3.4×10^{3}	2.90×10^{4}
24	1.5×10^{10}	6.70×10^{10}	2.0×10^{5}	2.70×10^{5}	7.4×10^{2}	7.70×10^{2}

*Based on two trials

PLATE XXVIII

Fig.1. Estimated Vibrio sp. 2448-88 population associated with protozoea of P. indicus exposed to different concentrations of chloramphenicol at different time intervals.

PLATE XXVIII, Fig. 1

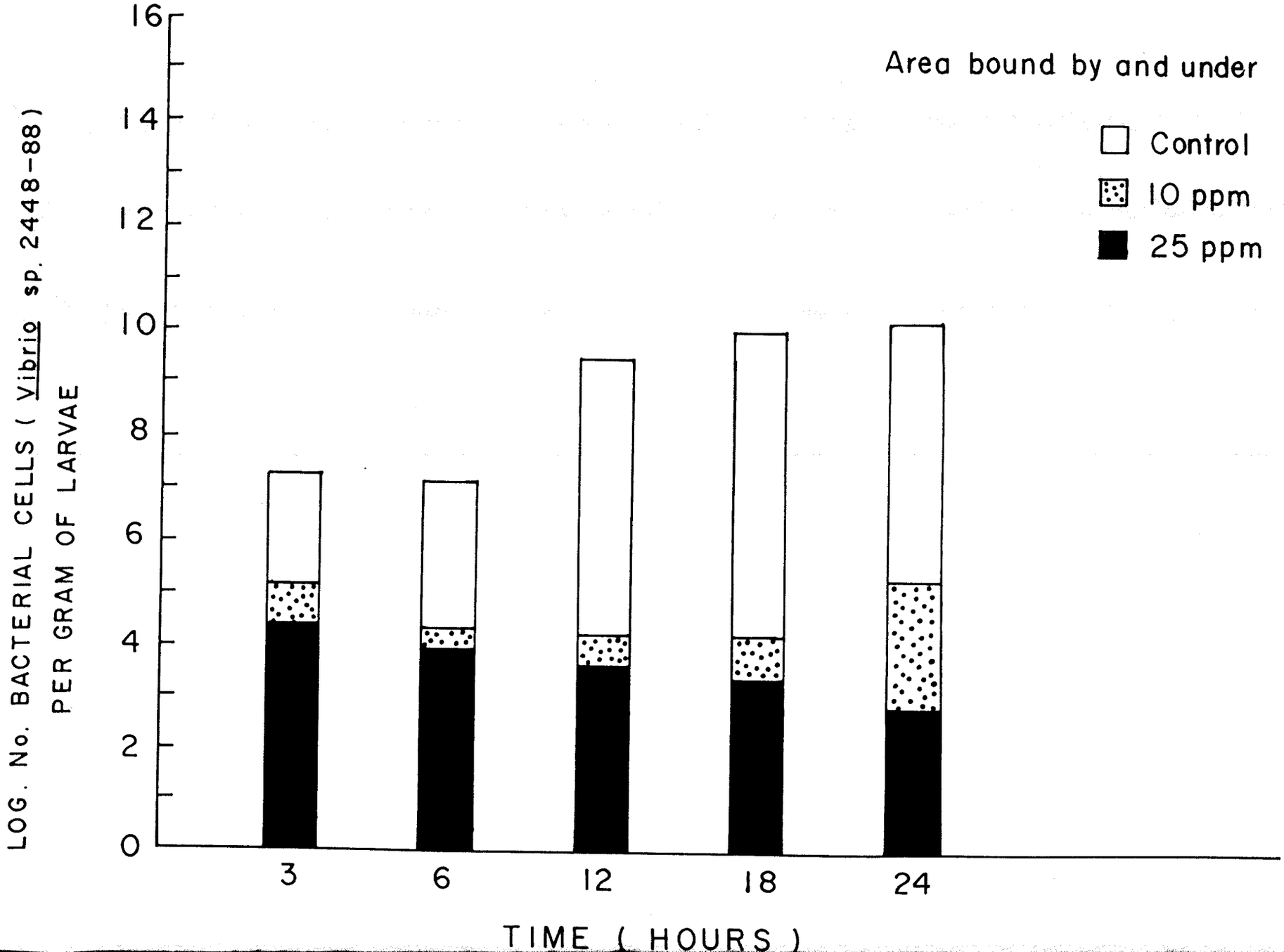

PLATE XXIX

Fig.1. Estimated Vibrio sp. 2448-88 population associated with mysis of P. indicus exposed to different concentrations of chloramphenicol at different time intervals.

PLATE XXIX, Fig. 1

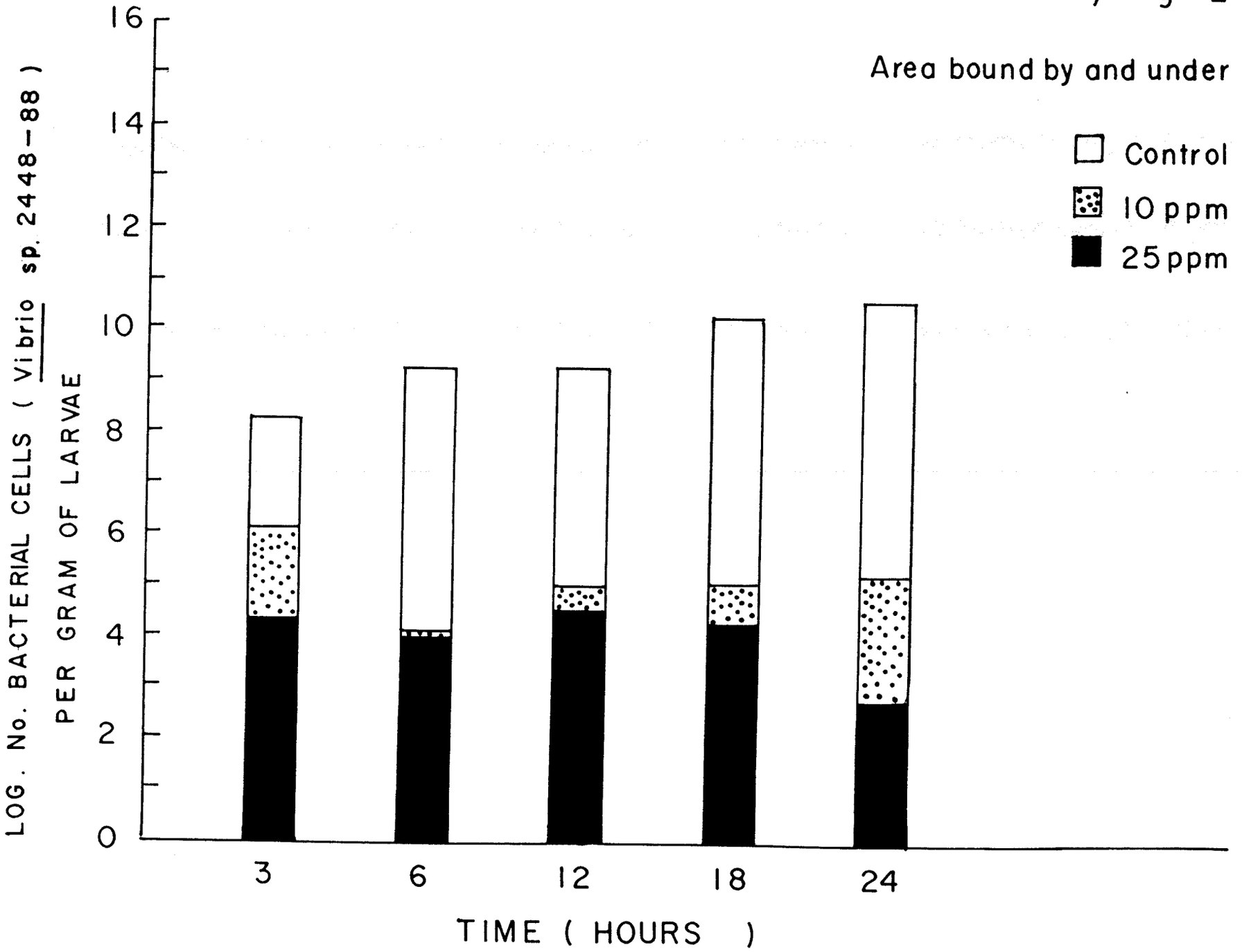

Table 9.9. Percentage survival* of *Vibrio* sp. 2448-88 infected protozoea and mysis of *Penaeus indicus* exposed for varied durations to 10 ppm and 25 ppm of chloramphenicol baths at different time intervals.

Exposure period (hrs)	Concentrations (ppm)	Protozoea				Mysis			
		Percentage survival at the end of (hr)							
		24	48	72	96	24	48	72	96
0	-	50	50	30	30	80	40	40	20
3	10	100	80	70	70	80	80	80	80
3	25	100	90	90	90	80	80	80	80
6	10	80	80	80	80	80	80	80	80
6	25	70	70	70	60	100	100	100	100
12	10	80	70	70	70	80	80	80	80
12	25	100	80	70	70	100	100	80	80
18	10	100	80	80	70	100	100	100	100
18	25	100	80	80	60	100	100	80	80
24	10	80	60	50	50	80	80	60	60
24	25	80	50	50	40	100	80	40	40
Percentage survival of infected larvae reared in infected medium		40	20	0	0	60	20	0	0

* Based on two trials.

The effect of antibiotics (chloramphenicol and oxytetracycline) in mixed populations of Chaetoceros sp. and Skeletonema sp. was tested and the results are given in Table 9.10. The mixed phytoplankton population declined to 68.3 x 10^2 and 32.5 x 10^2 cells/ml of the medium treated with chloramphenicol at the concentrations of 10 mg and 25mg/l of the medium respectively. Oxytetracycline caused adverse effect on mixed phytoplankton. It reduced the population of Chaetocerossp. and Skeletonema sp. to 4.3 x 10^2 cells/ml at the concentration of 200 mg/l of the medium after 8 hrs of incubation. In the absence of antibiotics, the population increased to 274.8 x 10^2 cells/ml of the medium.

A golden brown bloom of diatom was seen to develop in the water without antibiotics after 4 hrs of incubation where as no such bloom was seen in the water treated with the higher concentration of 25 ppm of chloramphenicol or 200 ppm of oxytetracycline. In the control long filaments of diatoms were observed, but in the antibiotic treated water, there were only a few strands of diatoms.

Table 9.10. Mean densities of Chaetoceros sp. and Skeletonema sp. populations (x 10^2 cells/ml) exposed to different levels of chloramphenicol and oxytetracycline HCl based on three trials.

Duration of exposure (hr)	Control without antibiotics	Chloramphenicol		Oxytetracycline HCl	
		10mg/l	25mg/l	100mg/l	200mg/l
0	20.6	20.6	20.6	20.6	20.6
2	112.8	62.0	71.4	69.3	27.6
4	163.2	70.8	68.5	34.9	21.5
6	259.8	43.5	27.5	13.5	6.0
8	274.8	68.3	32.5	17.0	4.3

DISCUSSION

The role of antimicrobial compounds in controlling the diseases in fishes and shellfishes, particularly in those cultured, is well recognised ever since the work of Gutsell (1946) on the potential sulphonamides for combating furunculosis. A wide range of antimicrobial compounds such as chloramphenicol, oxytetracycline, kanamycin, nifurprazine, oxolinicacid, sodium nifurstyrenate and flumequine are being used in the aquaculture. However as observed by Austin and Austin (1987), detailed comparative studies on different antimicrobial agents for facilitating selection of suitable compound or assessment of the value of one drug over the other are lacking. The various criteria which have to be considered to ensure safety and efficiency of antimicrobial compounds for use in controlling the diseases are : 1. the compound must kill only the pathogen quickly without being detrimental to the host, 2. it must not exacerbate the disease, 3. it must be relatively cheaper and available in the market, 4. it should be soluble in water and non-toxic to the animal at treatment level, 5. it must be absorbed in the active form and it should reach the pathogen, 6. it should be rapidly broken down and excreated from the animal after treatment, 7. it should have broad antibacterial and antifungal activity and 8. it should not interfere with the Nitrosamonas-Nitrobacter flora of biological gravel filters.

A series of eleven antimicrobial compounds such as bacitracin, chloramphenicol, cloxacillin, nalidixic acid, nitrofurazone, olendomycin,

oxytetracycline, penicillin, polymixin B, streptomycin and tetracycline were tested in-vitro against Vibrio sp. 2448-88 V. alginolyticus and V. parahaemolyticus to find out their sensitivity to these antimicrobial compounds. Of all these antimicrobial agents examined, only chloramphenicol was found to be the most effective, as all the three species of Vibrio were sentitive to this antibiotic; they were however resistant to other antinbiotics, except nalidixic acid and polymixin B to which V. parahaemolyticus and Vibrio sp. 2448- 88 respectively showed less sensitivity. This observation correlated to certain extent with the report by Lightner (1983) who found that bacitracin, oleandomycin, penicillin, polymixin B and streptomycin were ineffective to control Vibrio spp. isolated from cultured penaeid shrimp. The minimum inhibitory concentration of chloramphenicol, oxytetracycline and tetracyline required to arrest the growth of Vibrio sp. 2448-88 was, however, found to be different. Although oxytetracycline and tetracycline were resistant to Vibrio sp. 2448-88, these antibiotics were selected to determine the MIC values along with chloramphenicol as they are commonly used to control the bacterial diseases and for facilitating comparison with chloramphenicol. Although chloramphenicol was found to be the most effective in reducing the Vibrio sp. 2448-88 population with MIC value at 5 ppm, the effect of antibiotic when applied in seawater may be different as it is now known that the inhibiting properties of antibiotics in seawater is different from those of terrestrial or freshwater environment. In seawater, it depends on factors such as temperature, organic material, salinity and pH.

The results of the studies on the growth of Vibrio sp. 2448-88 in sterile seawater at different concentrations of chloramphenicol and oxytetracycline showed that chloramphenicol was bactericidal. It was found that the 25 ppm of chloramphenicol eliminated Vibrio sp. 2448-88 completely in the sterile seawater after 24 hrs of incubation, whereas oxytetracycline even at higher concentraction did not eliminate Vibrio sp. 2448-88 completely from the seawater. This observation agrees to some extent with that of Oppenheimer (1955) who reported that the most effective concentration of antibiotics to reduce the bacterial population in the seawater was 50 ppm, and that penicillin, streptomycin and chloromycetin appeared to be the most effective antibiotics in reducing the bacterial content of seawater. Besides, Marshall and Orr (1958) also observed that chloromycetin and streptomycin were found to be effective to control the bacterial population.

The healthy and uninfected larvae and postlarvae of P. indicus showed different responses to varying concentrations of chloramphenicol and oxytetracycline when they are exposed for 24 hrs in bath treatment. At 25 ppm concentration of chloramphenicol, 20% of nauplius 63% of protozoea I; 10% of protozoea II, 20% of mysis II and 13% of mysis III larvae sustained mortality. The other larval stages (Protozoea III, mysis I and postlarva I) were, however, not affected. In the case of oxytetracycline at 200 ppm concentration the percentage mortality observed was relatively higher, being 92% for nauplius, 63% for protozoea I, 47% for protozoea II and 20% for mysis I. Although the mortality rates observed for different developmental

stages were not consistent, it suggested that the concentration level above 25 ppm of chloramphenical and 200 ppm of oxytetracycline became toxic to the larvae after 24 hrs of exposure and that the higher concentration than this might not be safe to use for the larvae. Besides causing mortalities, the antibiotics also produced appendage deformities in the nauplius when they were treated with chloramphenicol and oxytetracycline at a concentration of 25 ppm or 200 ppm respectively. Further it was observed that the protozoeal and mysis stages were not feeding properly during the experiment even though adequate phytoplankton was provided initially in the rearing medium. Marshall and Orr (1958) reported similar observations in their study on the uses of antibiotics on the control of bacterial population in the seawater and on the influence of antibiotics on respiration, feeding and excretion of marine copepods. They reported that although chloramphenicol was the effective antibiotic used, some samples proved harmful to marine animals, and it sometimes inhibited the feeding of copepods. Although exact reasons for reduction in the feeding activity in the larvae in the presence of antibiotics in the present study could not be assigned, it might be due to, 1) the presence of chloramphenicol/ oxytetracycline could have inhibited the growth of phytoplankton in the rearing medium thereby reducing its population during the course of experiment, which in turn would have resulted in the non-availability of adequate quantity of phytoplankton for consumption by the larvae or 2) the antibiotics being bitter in taste could have rendered the phytoplankton unpalatable to the larvae. It is also probable that the combined effect of these factors would have resulted in the reduced feeding activity of the

larvae. That the growth and multiplication of the phytoplankton (Composed of chiefly Chaetoceros sp. and Skeletonema sp.) are affected by the presence of chloramphenicol and oxytetracycline is evident from the results of the experiment directed to evaluate the density of phytoplankton in antibiotic treated and untreated medium (Table 9.10). Chloramphenical at the level of 25 ppm and oxytetracycline at 200 ppm reduced phytoplankton population to 32.5 x 10^2 cells/ml and 4.3 x 10^2 cells/ml respectively whereas in the control it multiplied to 274.8 x 10^2 cells/ml.

The results of the experiments on the effect of different concentrations of chloramphenical and oxytetracycline on the healthy larvae and postlarvae of P. indicus showed that the nauplius and protozoea were more sensitive to chloramphenical and oxytetracycline at 25 ppm and 200 ppm respectively than the mysis and postlarval stages. It is possible that the early larval stages being delicate and fragile are more susceptible to antibiotics than the later stages. In nature it is observed that mysis and postlarvae show greater tolerance to changing environmental conditions than the early larval stages. Further Chan and Lawerence (1974) showed that the combination of oxytetracycline and olendomycine did not effect the respiration of mysis and postlarvae of P. aztecus but these antibiotics caused significant effect on the respiration of nauplius and protozoea at the level of 200 ppm of olendomycin and 500 ppm of oxytetracycline.

The exposure of Vibrio 2448-88 infected larvae for 24 hrs in the medium treated with 25 ppm chloramphenical showed clearly that it is

possible to regulate the bacterial population associated with the mysis larvae although the antibiotic could not eliminate completely the bacterial population. In the antibiotic treated medium, Vibrio sp. 2448-88 population associated with protozoea and mysis reduced to 7.4×10^2 and 7.7×10^2 respectively after 24 hrs of exposure. In the absence of antibiotics, the Vibrio sp. 2448-88 increased up to 1.5×10^{10} and 6.7×10^{10} in protozoea and mysis after 24 hrs. Chan and Lawerence (1974) observed the reduction of bacterial population associated with the larvae and postlarvae of P. aztecus to 0 cells on treatment with combination of oxytetracycline and olendomycin at 500 ppm and 200 ppm respectively.

From the foregoing, it is evident that chloramphenical at 25 ppm could control/reduce the Vibrio sp. 2448-88 population from the seawater as well as from the infection. However, beyond this level, they appear to be toxic to the larvae and early stages became more sensitive and susceptible to the antibiotic. This necessitates to find out a suitable exposure level for the application of chloramphenical so that the antibiotic is effectively applied to control the pathogen and at the sametime without causing damage to the larvae. It was observed that the protozoea larvae with appendage necrosis caused by Vibrio sp. 2448-88 could be recovered from the infection when they were immersed for 3 or 6 hrs continuously to 25 or 10 ppm chloramphenicol. In the case of mysis stage slightly longer duration of 6 or 18 hrs of immersion was found necessary. AQUACOP (1977) cured the appendage necrosis in larval stages of penaeid prawns by exposing them to streptomycin bipenicillin, erythromycin phosphate, tetracycline

chlorohydrate, sulphametha-zinane or furance.

To sum up, the results of the evaluation of eleven antimicrobial agents conducted at present have shown that chloramphenicol is highly effective against the disease caused by Vibrio sp. 2448-88 in the larvae and postlarvae of P. indicus. This antibiotic could control Vibrio sp. 2448-88 in the seawater peptone broth at a minimum inhibiting concentration of 5 ppm and is tolerated by even early stages such as nauplius and protozoea. Treatment of infected larvae for a period of 3 to 6 hrs in bath of chloramphenicol at 10 to 25 ppm for protozoea and for mysis is advised as safe level for treatment. It is also suggested that while treating the diseased larvae with chloramphenicol, optimum level of phytoplankton should be maintained as the antibiotic may inhibit the growth of phytoplankton. However, further research on aspect such as physiological changes in the larvae and postlarvae due to chloramphenicol, nutritional effect, growth development and moulting behaviour and on the effect of chloramphenicol on ammonifying bacteria in the rearing system is essential to obtain a better insight and on its wide use routinely in the large scale seed production system.

SUMMARY

1.The thesis presents the results of the studies carried out on certain diseases encountered in the larvae and postlarvae of penaeid prawns raised in the hatcheries at Cochin, Madras and Mandapam Camp during September 1985- April 1988.

2.In the preliminary survey carried out to understand the common diseases occurring in the penaeid larvae and postlarvae, seven cases of diseases and abnormalities were encountered. These included ciliate infestation, Nitzschia closterium infestation, parasitic protozoan infection, parasitic dinoflagellate infection, appendage necrosis, heteromorphic eye and abnormal eggs and deformed nauplii.

3.The clinical signs, seasonal occurrence and incidence of each of the above cases were provided along with the information on environmental factors such as salinity, dissolved oxygen, temperature and pH of the rearing medium. The identification of causative organism, characteristics of the diseases and its effect on host tissues due to infection or infestation in each of the seven cases were studied and discussed.

4.Ciliate infestation was encountered in protozoeal stages of Penaeus indicus. It was found to attach to the body of larvae and to

interfere with feeding and moulting, and finally it caused the death of the host.

5.Nitzschia closterium was seen to infest the mysis and postlarvae of P. indicus. Besides damaging the host tissue, it caused high mortality. Mortality of larvae was observed when mysis larvae were reared and fed with N. closterium.

6.A protozoan parasite, tentatively identified as Leptomonas, was observed to infect the protozoeal and mysis stages of P. indicus and P. semisulcatus. A dinoflagellate parasite similar to Hematodinium was found to cause mortalities in the nauplius stage of P. indicus.

7.Appendage necrosis was encountered in the larvae and postlarvae of P. indicus at Narakkal near Cochin and Kovalam near Madras. The causative organism, responsible for appendage necrosis of larvae and postlarvae of P. indicus from Kovalam, was tentatively identified as Vibrio alginolyticus. A bacterium, isolated from the infected larvae of P. indicus from Narakkal was found to be a new isolate of Vibrio as it differs from the currently described vibrios. The infection caused by the new isolate of Vibrio was selected and investigated in detail.

8.Heteromorphic eye recorded in the postlarva of P. indicus was described and its manifestation discussed. Abnormal eggs and deformed nauplii

of P. indicus were encountered during November- December and April-May. The abnormal eggs were grouped into 5 types namely type A (normal eggs), types B, C, D and E (abnormal eggs), and described in detail.

9.The total heterotrophic bacterial populations associated with egg, larval and postlarval stges of P. indicus showed a gradual increase of the population from the egg to postlarva. The total heterotrophic bacteria associated with eggs, larvae and postlarvae were found to fluctuate widely during December'86 and January- February'87.

10.Among the bacterial isolates, Vibrio was found to be dominant in eggs/larvae/postlarvae followed by Pseudomonas, Alcaligenes, Aeromonas and Flavobacterium. In the rearing medium, Alcaligenes was predominant followed by Vibrio, Flavobacterium and others.

11.A bacterium responsible for appendage necrosis was isolated and its taxonomy was studied. It was Gram-negative, fermentative and motile rod. It was sensitive to vibriostatic compound, 0/129. This bacterium was found to be a new isolate of Vibrio on the basis of its morphological, biological, physiological and biochemical characters and comparison of these characters with those described for other related vibrios. This new isolate of vibrio was deposited in Vibrio Referrence Laboratory, Centres for Disease Control, Georgia, U.S.A. and coded as Vibrio sp. 2448-88.

12. *Vibrio* sp. 2448-88 was found to be instrinsically pathogenic to larvae and postlarvae of *P. indicus*, *P. monodon* and *P. semisulcatus* and adult *P. indicus*.

13. The LC_{50} values of *Vibrio* sp. 2448-88 for nauplius, protozoea, mysis and postlarva of *P. indicus*, *P. monodon* and *P. semisulcatus* showed variation from species to species and the susceptibility of the larvae to the pathogen was found to depend on the stage of the development of larvae. The early stages such as nauplius and protozoea had low LC_{50} values, while the mysis and postlarvae had relatively greater LC_{50} values.

14. Approximately 14×10^{11} cells of *Vibrio* sp. 2448-88 given in the feed daily for three days to the adult *P. indicus* failed to infect the host, but 70×10^{7} cells/prawn when injected intramuscularly to the adult produced 100% mortality after 12 hrs of inoculation.

15. Based on the LC_{50} values of *Vibrio* sp. 2448-88 for larvae and postlarvae of three species of penaeid prawns studied, *P. indicus* was found to be the most tolerable to *Vibrio* sp. 2448-88. Larvae and postlarvae of *P. monodon* appeared to be the most sensitive to *Vibrio* sp. and those of *P. semisulcatus* intermediate between *P. indicus* and *P. monodon*. Further among *Vibrio* sp. 2448-88, *V. alginolyticus* and

V. parahaemolyticus, the former was found to be the most virulent form.

16. Histological observations on the organs such as hepatopancreas, heart, haematopoietic tissue, gills, muscle and gut of the uninfected larvae, postlarvae and adult prawn and those infected by Vibrio sp. 2448-88 revealed moderate to marked destruction of most of the vital organs. The excessive accumulation of fat in the tubules, cellular destruction, thickening of connective tissue and haemocytic infiltration were observed in the hepatopancreas of experimentally infected adult by Vibrio sp. 2448-88. The heart and gill became edematous and haematopoietic tissue was necrotic. The bacterial colonies were observed in the hepatopancreas, heart, muscle and gut of infected larvae and postlarvae and adult prawn.

17. The results of pathogenicity experiments and histo-pathological studies indicated the initial site of infection by Vibrio sp. 2448-88 was exoskeleton. The virulent cells of Vibrio sp. 2448-88 get attached to the exoskeleton, damages the exoskeleton by the activity of chitinase. Then the bacteria enter into the muscle and induce systemic infection and finally results in the death of the host.

18. Eleven antimicrobial agents were tested against Vibrio sp. 2448- 88, V. alginolyticus and V. parahaemolyticus to study their sensitivity. All the vibrios were sensitive to chloramphenicol, but resistant to other

antimicrobial agents except nalidixic acid and polimyxin B to which V. parahaemolyticus and Vibrio sp. 2448- 88 respectively showed relatively less sensitivity.

19. Minimum inhibitory concentrations of cholramphenicol, oxytetracycline and tetracycline for Vibrio sp. 2448-88 was found to be 5 mg/l, 150 mg/l and 200 mg/l respectively.

20. Chloramphenicol completely eliminated the population of Vibrio sp. 2448-88 when the bacterium was inoculated into sterile seawater treated with 25 ppm of chloramphenicol whereas oxytetracycline was unable to eliminate Vibrio sp. 2448-88 completely even at the level of 150 ppm.

21. The population of Vibrio sp. 2448-88 associated with infected protozoea and mysis was estimated after exposing the larvae for 3, 6, 12, 18 and 24 hrs to 10 ppm and 25 ppm of chloramphenicol. Chloramphenicol at the concentration of 25 ppm reduced the population of Vibrio sp. 2448-88 from 1.5×10^7 to 7.4×10^2 and 1.46×10^8 to 7.7×10^2 cells/g of larvae in protozoea and mysis respectively after 24 hours of exposure. Chloramphenicol and oxytetracycline caused mortality in nauplius, protozoea I and II, and inhibited the feeding activity of protozoea and mysis when the larvae were reared in the medium treated with 25 ppm of chloramphenicol or 200 ppm of oxytetracycline.

22.On the basis of the results of various experiments carried out on the evaluation of antimicrobial agents on Vibrio sp. 2448-88, it was concluded that chloramphenicol was the most effective among the antimicrobial compounds tested to control Vibrio sp. 2448-88 in the rearing medium and infection caused by this bacterium. In the case of infected protozoea 3 hrs bath treatment in 25 ppm or 6 hrs in 10 ppm of chloramphenicol and for mysis 6 hrs in 25 ppm or 18 hrs in 10 ppm were suggested to control the infection casused by Vibrio sp. 2448-88.

23.Chloramphenicol and oxytetracycline affect the growth of phytoplankton composed of Chaetoceros sp. and Skeletonema sp. when treated with 25 ppm of chloramphenicol or 200 ppm of oxytetracycline. Besides, feeding activity of the larvae was found to be reduced. It was therefore recommended that during bath treatment of infected larvae in chloramphenicol, optimum level of phytoplankton should be ensured.

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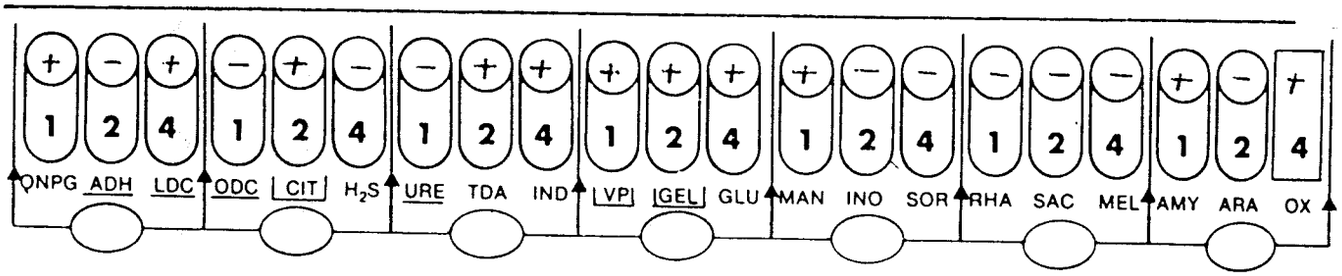
APPENDIX 1

The results of API 20E on the new isolate of Vibrio obtained from Dr.G. Balakrish Nair, Research Officer, N.I.C.E.D., Calcutta.

IDENTITY OF THE SIX ISOLATES FROM DISEASED PRAWNS

Your Strain No.	Our Lab. No.	Source of isolation	Identity	Serotype	Kanagawa phenomenon
S1	Not given	Diseased prawn	Unidentified marine Vibrio		
S2	Not given	- do -	Unidentified marine Vibrio		
S3	NICED 124	- do -	V. parahaemolyticus	06-K46	-
S4	NICED 125	- do -	V. parahaemolyticus	Untypable	-
S5	NICED 126	- do -	V. parahaemolyticus	Untypable	-
S6	NICED 127	- do -	V. parahaemolyticus	Untypable	-

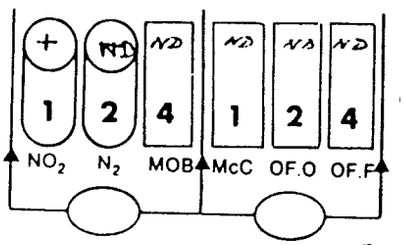


STRAIN No. S1

api 20 E

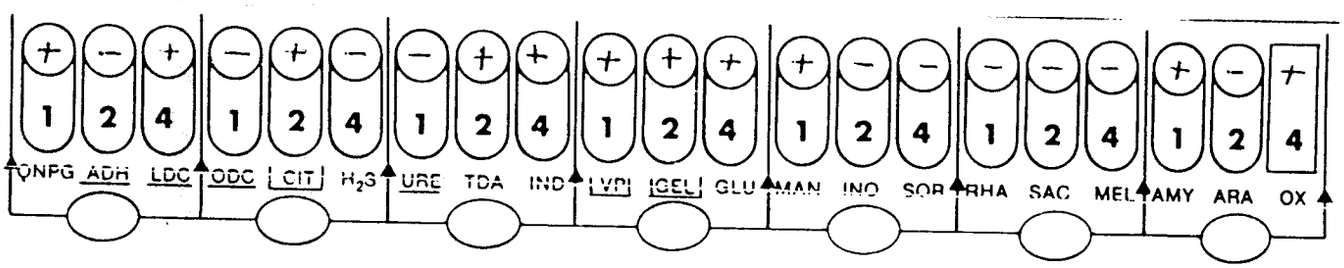
Identification : Unidentified Marine Vibrio						
	0	1	3	6	8	10
Growth on NaCl with final NaCl (%) concentration	-	-	+	+	-	-

REF. : CMFRI Strain — Patient : STRAIN No. S1
Date : 3/10/86 — Origine/Source : Diseased Prawn
Dr : G. B. NAIR — Service/Dept : MICROBIOLOGY - IV



ND = Not Done

41 0005 B

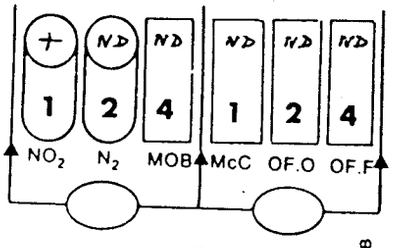


STRAIN No. S2

api 20 E

Identification : Unidentified Marine Vibrio						
	0	1	3	6	8	10
Growth on NaCl with final NaCl (%) conc	-	-	+	+	-	-

REF. : CMFRI Strain — Patient : STRAIN No. S2
Date : 3/10/86 — Origine/Source : Diseased Prawn
Dr : G. B. NAIR — Service/Dept : MICROBIOLOGY - IV



ND = Not Done

41 0005 B

The resutls of the biochemical and physiological tests of the new isolate of Vibrio carried out by Dr. Jim J. Farmer, CDC, Georgia, U.S.A.

AUG 09 1988

...e complete ... State health department laboratory
... an be accepted ... (CDC. Please check the first applicable
... men appropriate ... plete the statement with the *.
... pected to be of ... alth importance. Specimen is:

... from an outbreak.
- ☐ from uncommon or exotic disease.
- ☐ an isolate that cannot be identified, is atypical, shows multiple antibiotic resistance, or from a normally sterile site(s).
- (d) ☐ from a disease for which reliable diagnostic reagents or expertise are unavailable in State.
- 2. ☐ Ongoing collaborative CDC/State project.
- 3. ☐ Confirmation of results requested for quality assurance.

*Prior arrangement for testing has been made. Please bring to the attention of:

(Name) _____

Completed by: _____

Date: _____

STATE HEALTH DEPARTMENT LABORATORY ADDRESS:

STATE HEALTH DEPT. NO.: 51

DATE SENT TO CDC: Month Day

NAME AND ADDRESS OF PHYSICIAN OR ORGANIZATION:

A. Sait Sahul Hameed,
Senior Research Scholar,
C.M.F.R.I.,
P.O. Box No. 2704,
COCHIN - 682 031., INDIA.

2448-88

PATIENT IDENTIFICATION Hospital No.:

NAME: Last (18-37) First (38-47) Middle Initial

C 51

BIRTHDATE: (49-54) Month Day Year SEX: (55)

CLINICAL DIAGNOSIS: (56-57)

ASSOCIATED ILLNESS: (58-59)

DATE OF ONSET (Mo. Da. Yr.) (60-65)

FATAL? (66) ☐ YES ☐

(For CDC Use Only) CDC NUMBER

UNIT	FY (3-4)	NUMBER (5-10)	SUF (11)
27	88	024210	

DATE RECEIVED (12-17) Month Day Year: 04 14 88

REPORT OF LABORATORY INVESTIGATION
ENTERIC BACTERIOLOGY SECTION
ENTERIC DISEASES BRANCH

03 (12 13)

DATE REPORTED Mo. 06 (14 15) Da. 24 (16 17) Yr. 88 (18 19)

IDENTIFICATION OF BACTERIUM: See letter

EP (40 41)

SEROLOGICAL TYPE: O ... K ... H ... X 3 4 5 6 7 8

☐ SEROLOGY (S)
☐ PROFILE (A)
☐ PHAGE TYPE (P)
☐ BACTERIOCIN (B)

COMMENTS:

Frances W Brenn

J.J. Farmer III, Ph.D.
Chief, Enteric Identifica
Laboratories 404-639-3

EPI NUMBER: V29 30

REPORTED BY: F FB

2448-88 EN

04 (12-13)

BIOCHEMICAL TESTS: 0 = NEGATIVE; 1, 2, 3 ··· 7 INDICATE DAY REACTION BECAME POSITIVE; 8 = POSITIVE AFTER 7d; 9 = NO GROWTH OR CHANGE.

			24h	48h
40	0	INDOLE		NG
41	9	METHYL RED	NG	NG
42	9	VP	NG	NG
43	0	CITRATE (SIM.)	-	-
44	0	H_2S-TSI	N/A 5 Alk	
45	0	UREA	-	-
46	9	PHENYL-ALANINE	4h NG	
47	9	LYSINE	NG	NG
48	9	ARGININE	NG	NG
49	9	ORNITHINE	NG	NG
50	0	MOTILITY - 36C	-	-
51	0	GELATIN - 22C	-	-
52	0	KCN	-	-
53	0	MALONATE	-	-
54	9	D-Glucose-Acid	NG	
55	0	D-Glucose-Gas	-	-

		ACID FROM:	24h	48h
56	0	LACTOSE	-	-
57	0	SUCROSE	-	-
58	0	D-MANNITOL	-	-
59	0	DULCITOL	-	-
60	0	SALICIN	-	-
61	0	ADONITOL	-	-
62	0	I-INOSITOL	-	-
63	0	D-SORBITOL	-	-
64	0	L-ARABINOSE	-	-
65	0	RAFFINOSE	-	-
66	0	L-RHAMNOSE	-	-
67	0	MALTOSE	-	-
68	0	D-XYLOSE	-	-
69	1	TREHALOSE	+w	+w
70	0	CELLOBIOSE	-	-
71	0	α-CH_3-Glucoside	-	-

			24h	48h
72	0	ERYTHRITOL	-	-
73	0	ESCULIN	-	L
74	0	MELIBIOSE	-	-
75	0	D-ARABITOL	-	-
76	0	GLYCEROL	-	-
77	0	MUCATE	-	-
78	0	Tartrate (Jord.)	-	-
79	0	ACETATE	-	
80	1	LIPASE (Corn Oil)	+w	+w
81	1	DNA'ASE - 25C	+	
82	9	$NO_3^- \rightarrow NO_2^-$	NG	
83	1	Oxidase NG Kovacs +		
84	0	ONPG	w	
85	9	D-Glucose Fermented in OF Medium	NG	NG
86		GRAM STAIN		
87		YELLOW PIGMENT - 25C		

			24h
88		MacConkey-Growth	
89		CATALASE	
90		PECTATE	
91		MOTILITY -- 22C	
92	0	CITRATE (Chris.)	-
93	0	H_2S--PIA	-
94	1	DNA'ASE - 36C	+ w
95		D-MANNOSE	-
96		GELATIN Charcoal at 36C	-
97	0	GAS VOLUME	-

OTHER TESTS

STRING TEST: negative
BIOLUMINESCENCE: NEGA
VP (1% NaCl) Coblentz 2

TYR GAL -

94 20 20 (98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125)

EN (198 199)

ANTIBIOGRAM 05 (12-13)

40 41 42	43 44 45	46 47 48	49 50 51	52 53 54	55 56 57	58 59 60	61 62 63	64 65 66	67 68 69	70 71
11G X	CL10	NA30	SD250	GM10	S10	K30	TE30	C30	P10	AMI

73 74 75	76 77 78	79 80 81	82 83 84	85 86 87	88 89 90	91 92 93	94 95 96	97 98 99	100 101 102	103 104 105	106 107 108	109 110
CB100	CF30					M 15	12	21	23	17	15	18

112 113 114	115 116 117	118 119 120	121 122 123	124 125 126	127 128 129	130 131 132	133 134 135	136 137 138	139 140
22	25	6	6	6	10	10	9	17	EN

CDC 52.51B REV. 12-87
(Formerly 56.15A)

AUG 09 1988 MACH: This sheet contains results which are part of the final report; please mail them to sender.

88 024210

To KEYPUNCH: Please key-in results below as the 08 record

2448-88

The tests below were done in media which have added "marine cations" to enhance the growth of halophilic Vibrio species. One volume of sterile medium 1559 was added to 9 volumes of the standard biochemical test medium. (Medium 1559: 150g of NaCL, 51g $MgCl_2.6H_2O$, 3.7g of KCl and 912ml of H_2O. Autoclave 121C, 15 min.)

08 RECORD - RESULTS IN MEDIA WITH ADDED MARINE CATIONS
- TEMPERATURE OF INCUBATION: 25°C

0 8 (12-13)

BIOCHEMICAL TESTS: 0 = NEGATIVE; 1, 2, 3 ··· 7 INDICATE DAY REACTION BECAME POSITIVE; 8 = POSITIVE AFTER 7d; 9 = NO GROWTH OR CHANGE.

		24h	48h
40	2 INDOLE		+
41	METHYL RED		
42	0 VP		−
43	CITRATE (SIM.)		
44	H_2S—TSI		
45	UREA		
46	PHENYL-ALANINE		
47	1 LYSINE	+	
48	0 ARGININE	−	−
49	2 ORNITHINE	w	+
50	1 MOTILITY — 36C	+	+
51	GELATIN — 22C		
52	KCN		
53	MALONATE		
54	1 D-Glucose-Acid	+	
55	0 D-Glucose-Gas	−	−

4h — 4h — 4h

TYR + GAL +

		24h	48h
56	0 LACTOSE	−	−
57	0 SUCROSE	−	−
58	1 D-MANNITOL	+	
59	0 DULCITOL	−	−
60	0 SALICIN	−	−
61	0 ADONITOL	−	−
62	0 I-INOSITOL	−	−
63	0 D-SORBITOL	−	−
64	0 L-ARABINOSE	−	−
65	0 RAFFINOSE	−	−
66	0 L-RHAMNOSE	−	−
67	1 MALTOSE	+	
68	0 D-XYLOSE	−	−
69	1 TREHALOSE	+	
70	1 CELLOBIOSE	+	
71	0 α-CH_3-Glucoside	−	−

ACID FROM: M-6

		24h	48h
72	0 ERYTHRITOL	−	−
73	ESCULIN		
74	0 MELIBIOSE	−	−
75	0 D-ARABITOL	−	−
76	GLYCEROL		
77	MUCATE		
78	Tartrate (Jord.)		
79	ACETATE		
80	LIPASE (Corn Oil)		
81	DNA'ASE — 25C		
82	1 $NO_3^- \rightarrow NO_2^-$	+	
83	1 Oxidase	Kovacs +	
84	ONPG		
85	D-Glucose Fermented in OF Medium		
86	GRAM STAIN		
87	YELLOW PIGMENT — 25C		

88	MacConkey-Growth	
89	CATALASE	
90	PECTATE	
91	MOTILITY — 22C	
92	CITRATE (Chris.)	
93	H_2S—PIA	
94	DNA'ASE — 36C	
95	1 D-MANNOSE	
96	GELATIN Charcoal at 36C	
97	0 GAS VOLUME	−

OTHER TESTS

Galacturonate −

MC

98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	188	189	190	191	192	193	194	195	196	197	198	199
	1	0	0	0	0	1	1	1	1	1	1	1	3	0	0															X	2	5	M	C			E	N	

J.J. Farmer III
Chief, Vibrio and Enteric Phage Typing Laboratory
404 329-3331

(formerly 56.15A)

112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 198 199 200

AUG 09 1988

2448-88
88 024210

TO DASH: SEND COPY TO SENDER.

TO KEYPUNCH: PICK UP 06 RECORD.

0 6 BIOCHEMICAL TESTS: 0 = NEGATIVE; 1, 2, 3 ... 7 INDICATE DAY REACTION BECAME POSITIVE; 8 = POSITIVE AFTER 7d; 9 = NO GROWTH OR CHANGE.
(12-13)

1% NaCl, 36°C

No.	Code	Test	24h	48h
40		INDOLE H13+ 2		
41	0	METHYL RED 2		–
42		VP spec –		
43		CITRATE (SIM.)		
44		H_2S–TSI		
45		UREA		
46		PHENYL-ALANINE		
47	1	LYSINE (4h)	+	
48	0	ARGININE	–	–
49	0	ORNITHINE	–	–
50		MOTILITY – 36C		
51	8	GELATIN – 22C +7-19	–	–
52		KCN		
53		MALONATE		
54		D-Glucose-Acid		
55		D-Glucose-Gas		

ACID FROM:

No.	Code	Test	24h	48h
56		LACTOSE		
57		SUCROSE		
58		D-MANNITOL		
59		DULCITOL		
60		SALICIN		
61		ADONITOL		
62		I-INOSITOL		
63		D-SORBITOL		
64		L-ARABINOSE		
65		RAFFINOSE		
66		L-RHAMNOSE		
67		MALTOSE		
68		D-XYLOSE		
69		TREHALOSE		
70		CELLOBIOSE		
71		α-CH_3-Glucoside		

No.	Code	Test	24h	48h
72		ERYTHRITOL		
73	2	ESCULIN	–	+w
74		MELIBIOSE		
75		D-ARABITOL		
76		GLYCEROL		
77		MUCATE		
78		Tartrate (Jord.)		
79		ACETATE		
80		LIPASE (Corn Oil)		
81		DNA'ASE – 25C		
82	1	$NO_3^- \rightarrow NO_2^-$	+	
83	1	Oxidase + Kovacs		
84		ONPG		
85		D-Glucose Fermented in OF Medium		
86		GRAM STAIN		
87		YELLOW PIGMENT – 25C		

No.	Code	Test	24h
88		MacConkey-Growth	
89		CATALASE	
90		PECTATE	
91		MOTILITY – 22C	
92		CITRATE (Chris.)	
93		H_2S–PIA	
94		DNA'ASE – 36C	
95		D-MANNOSE	
96		GELATIN Charcoal at 36C	
97		GAS VOLUME	

OTHER TESTS

NaCl TOLERANCE

0% –	8% –
1% +	10% –
3.5% +	12% –
6% +w	

98: 0 — 111–119: 2 0 1 1 0 0 0 0 — 191–195: NACL — 198–200: E N 2

J.J. Farmer III
Chief, Vibrio and Enteric Phage Typing Laboratory
404 329 3331

The results of DNA-DNA hybridisation test of the new isolate of Vibrio carried out by Dr. Jim J. Farmer CDC, Georgia, U.S.A.

Vibrio Reference Strains

Vibrio 2448-88 April 1989

	RBR 60°						AVE	ΔTm 60°			AVE	RBR 75°			AVE
60. V. brio 2448-88	66.6	65.4	62.2	61.7	61.8	59.4		87.4°				45	45		
	60.2	61.5	61.9	58.7	60.0	59.3									
	59.0	57.7	56.0												
1 V. aestuarianus 9014-83	13	12					13								
2 V. alginolyticus 9065-79	32	34					33								
3 V. anguillarum 9063-79	12	13					13								
4 V. campbelli 9099-79	62	63	62	60			62	2.1°	1.8°		2.0°	56	51		54
5 V. carchariae 9800-84	40	40					40								
6 V. cholerae 9060-79	12	11					12								
7 V. costicola 9031-82	6	5					6								
8 V. damsella 2588-80	9	9					9								
9 V. diazotrophicus 9001-83	13	12					13								
10 V. fischeri 9064-79	10	9					10								
11 V. fluvialis 9555-78	15	14					15								
12 V. furnissii 9119-82	13	13					13								
13 V. gazogenes 2820-79	8	8					8								
14 V. harveyi 9098-79	48	49	48	46			48	6.6°	7.1°		6.9°				
15 V. hollisae 75-80	8	8					8								
16 V. logei 9033-82	10	10					10								
17 V. metschnikovii 9518-75	9	9					9								
18 V. mimicus 1721-77	15	13					14								
19 V. natriegens 9101-79	24	24					24								
20 V. nereis 9103-79	15	14					15								
21 V. nigripulchritudo 9104-79	12	12					12								
22 V. oedalii 9012-82	4	4					4								
23 V. orientalis 9588-84	15	13					14								
24 V. parahaemolyticus 9062-79	33	33					33								
25 V. pelagius (biv 1) 9105-79	no	DNA					—								
26 V. proteolyticus 9082-79	16	13					15								
27 V. splendidus (biv 1) 9030-82	17	16					17								
28 V. tubiashii 9012-81	21	17					19								
29 V. vulnificus 9107-79	21	22					22								
30 V. vulnificus (biv 2) 9576-84	16	14					15								
31 P. angustum 9093-79	12	10					11								
32 P. leiognathi 9094-79	11	11					11								
33 P. phosphoreum 9092-79	8	8					8								
— label only	0.9	1.4	0.0	0.5	0.5	0.9									
	0.6	0.4	0.6	0.9	0.6	0.4						0.4	0.8		

V. bivio 2448-88

	RBR 60°						AVE	ΔTm 60°				AVE	RBR 75°				AVE
34 A. caviae 9082-79	3	2					3										
35 A. hydrophila 9079-79	3	3					3										
36 A. media 9072-83	5	4					5										
37 A. salmonicida 9701-84	4	3					4										
38 A. sobria 9538-76	3	3					3										
39 Pl. shigelloides 9091-79	3	4					4										
40 E. coli K12	4	3					4										
41 P. multocida 9515-84	2	2					2										
42 Ch. violaceum 9114-79	no DNA						—										
43 EG 506 1398-82	7	7					7										
44 EG 509 9030-83	43	48					46										
45 EG 510 736-84	10	14					12										
46 EG 511 1612-79	7	6					7										
47 EG 512 9838-84	5	7					6										
48 EG 513	—						—										
49 EG 511 1611-79	6	6					6										
50	—						—										
51 V. 9010-86	—						—										
52 V. marinus 9022-82	3	2					3										
53 V. alginolyticus 9014-86	47	49					48										
54 V. mediterranei 2494-87	14	14					14										
55 V. bivio G522 2436-87	13	11					12										
56 " 2437-87	11	12					12										
57 EG 521 2520-86	51	50					51										
58 " 2521-86	44	47					46										
59 V. salmonicida 9060-88	7	8					8										
60 V. bivio 2448-88	100						100										