ISOLATION AND CHARACTERIZATION OF *VIBRIO* SP PATHOGENIC TO *PENAEUS MONODON*

DISSERTATION SUBMITTED BY

KUM: M.B. DEEPA

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C E R T I F I C A T E

Certified that the dissertation entitled "Isolation and characterization of Vibrio sp pathogenic to Penaeus monodon" is a bonafide record of work done by **Kum.M.B.Deepa** under our guidance at the Central Marine Fisheries Research Institute during the tenure of her M.Sc. Mariculture Programme of 1994-96 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

Dr.A.P.Lipton,
Sr.Scientist,
CMFRI, Mandapam,
Major Advisor, Advisory Committee.

Dr.A.Regunathan,
O.I.C, CMFRI, Mandapam,
Member, Advisory Committee.

Shri.D.Kandasamy,
Sr.Scientist, CMFRI,Mandapam,
Member, Advisory Committee.

DECLARATION

I hereby declare that this thesis entitled **"Isolation and characterization of *Vibrio* sp pathogenic to *Penaeus monodon*"** is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin,
November, 1996.

Deepa MB
M.B.DEEPA

ABSTRACT

Vibriosis in shrimp culture causes about 80% mortality. In the present study, Vibrio isolated from diseased Penaeus monodon was characterized as Vibrio alginolyticus. The physical, cultural and biochemical investigations carried out in the study confirmed the isolate. Growth of V.alginolyticus in Zobell marine broth indicated no lag period, while the peak growth was observed at 18 hours. V.alginolyticus was found to cause mortality in healthy shrimps in artificial infection studies. One hundred percent mortality was noted at 5.7×10^8 cells, 5.7×10^7 cells, 5.7×10^6 cells per P.monodon. Reisolation studies indicated the presence of the same bacteria in haemolymph and other body tissues.

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सारांश

चिंगट संवर्धन में विब्रियोज़िस द्वारा करीब अस्सी प्रतिशत (80%) मृत्यु होता है। वर्तमान अध्ययन - विश्लेषण से यह सिद्ध हुआ है कि रोगग्रस्त या निम्न मोनोडोन से विमुक्त विब्रियों स्वभाव से विब्रियो अलजिनोलिटिकस था। इस हेतु से किए गए भौतिक संवर्धनिक एवं जीवरासायनिक अनुसंधान से यह विमुक्ति प्रमाणित किया गया है। लोबल समुद्री ब्रोत में अलजिनोलिटिकस की वृद्धि में विलंब नहीं है। पर 18 घंड में थोड़ा वृद्धि देखा है। स्वस्थ चांगट में मौत का कारण प्राय : विब्रियो अलजिनोलिटिकस माना जाता है , कृत्रिम संक्रमण अध्ययन की सहायता से। मृत्यु दर एक सौ प्रतिशत है। 5.7×10^{8}, 5.7×10^{7} और 5.7×10^{6} सेल पर थी सूचना देता है। वही बैकटीरिया (सुश्मापू) हीमोलिंक तथा अन्य शरीर ऊतकों में मौजूद है।

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PREFACE

Asia is recognised as the most important area for shrimp aquaculture since it produced about 90% of the cultured shrimp for the world market during the last three years. The industry, however has been facing serious threat as the cultured shrimp are being affected by diseases. Diseases result in mass mortalities and consequently the economic returns from shrimp farming.

Among the shrimps, Penaeus monodon culture has become very prominent in Asia during the last decade because of many favourable characteristics. Penaeus monodon exhibits the highest growth rate among all cultured penaeids. It is eurythermal, euryhaline and omnivorous rather than carnivorous and therefore requires a lower amount of cost of production. Moreover these shrimps also require simple culture facilities such as clay bottom ponds for grow out; hence, construction and maintenance costs are relatively low. No major problems were encountered under the traditional polyculture and extensive culture systems with the exemption of natural disasters and the presence of predators and competitors.

As the culture systems shifted to semi-intensive and intensive systems, stocking densities were raised to more than 20 individuals/m^2 and formulated feeds were used. Water quality and the general culture environment became harder to manage and the cultured species became more susceptible to diseases. As long as the culture conditions are optimal, P. monodon appears to tolerate light to moderate infections; however, poor management of the culture environment by the farmers has in many cases, caused disease outbreaks which were predisposed by stressors (such as poor water quality, deteriorating environmental conditions and unbalanced nutrition).

A combination of pathogenic and nonpathogenic factors are attributed to the mass mortality in culture systems. Serious disease of viral, bacterial, fungal, protozoan, rickettsial and unknown aetiologies have been reported. From economic view, the most important viral pathogen of P. monodon is Monodon-type baculovirus (MBV). This baculovirus affects all life stages and produces mass mortality when they are cultured at high densities or exposed to some other stressor. Infectious Hypodermal and Hematopoietic Necrosis virus (IHHNV) has also been found in cultured P. monodon. Postlarvae and juveniles

are considered particularly susceptible. Hepatopancreatic Parvo-like virus (HPV) is another viral pathogen of importance affecting P. monodon. A reo-like virus (REO) has also been found in P. monodon in Malaysia.

P. monodon suffer poor growth and perhaps mortality if exposed to excessive levels of toxins or if deprived of essential nutrients. The diseases and disease syndromes summarised by Baticados (1988) include Chronic soft shell syndrome, Red disease, Fatty filtration of the hepatopancreas, Blue disease, cramped tails, Hemocytic enteritis, heavy metal poisoning, Black gill disease, Muscle necrosis and Gas bubble disease.

Some of the previously mentioned diseases have uncertain aetiologies. In addition to these, at least five diseases of unknown aetiology have been reported to afflict P. monodon. These are red discolouration (Liao et al 1977), larval black spot syndrome, spongy tissue syndrome, one month mortality syndrome, and yellow head shrimp (Flegel et al 1992).

A number of bacterial diseases have been reported for P. monodon. The common ones are Vibriosis, Luminous

bacterial diseases, Epicommensal filamentous bacteria, Bacterial exoskeletal lesions, Necrosis of appendages and Bacteria induced hepatopancreatitis. Rickettsial diseases caused by unknown species are also reported. In many instances, the bacterial isolates were just screened for the purpose of application of suitable antibiotics. No detailed studies have been reported about the characterization of bacteria, establishing the pathogenicity as well as host - pathogen relationship. Considering the research needs in this area, the present work was undertaken with the following objectives:

1. To isolate the bacteria causing mortality among P. monodon in semi intensive culture conditions.
2. To characterize the isolate so as to determine the species of bacteria.
3. To artificially infect healthy shrimps with the isolated bacteria in order to establish the pathogenicity of bacteria and to understand the host-pathogen relationship.

INTRODUCTION

Bacteria play a major role in disease outbreaks of cultured giant tiger prawn, Penaeus monodon (Cheng 1989). Several species of the genus Vibrio were reported to cause infection in wild and cultured finfish and shellfish (Buchanan and Gibbons 1984). Colwell and Grimes (1984) listed eight Vibrio species as fish pathogens. Vibrio infection were reported to occur in penaeid shrimps (Vangerzant et al 1970; Johnson 1978, Lightner 1983, 1985, 1988; Takahashi et al 1984; Takahashi et al 1985; Baticados 1988, Anderson et al 1988, Nash, 1988,1990, Lin 1989, Boonyanatpalin 1990; Lavilla Pitogo; De la Pena et al 1993, Mohney et al 1994, Hameed 1994). Vibriosis caused by species of the genus Vibrio has been described as the most serious disease of penaeid shrimps (Egidius 1987).

Literature conerning the bacterial flora of penaeid shrimps appears to be limited. Vanderzant et al (1971) studied the microbial flora of pond - reared brown shrimp (P. aztecus) in relation to the environment and found coryniforms and species of Flavobacterium, Moraxella and

Bacillus dominant in pond water. Vibrios formed the normal microflora of pond and raceway reared shrimp (Vanderzant et al 1971, Lightner 1985). They were considered as opportunistic pathogens (Lightner, 1977) which constituted majority of bacteria associated with the gut, gills or cuticle of wild or cultured penaeid shrimp. Because of the relatively high population of Vibrio sp normally present in the shrimp's microflora, it is not surprising that many investigators have found species of Vibrio to be frequent and apparently opportunistic pathogens of penaeid shrimp. Furthermore, Yasuda and Kitao (1980) observed poor growth of the shrimp when vibrio populations were found dominant in the gut of P. japonicus. Hameed (1993) found Vibrio sp as the dominant taxa in eggs, larvae and post larvae of P.indicus followed by Pseudomonas, Alcaligenes, Aeromonas and Flavobacterium. It was also found that increased vibrio numbers in larval rearing tank water was one of the factors that reduced the survival rate of larvae and post-larvae of P.indicus. Investigations on bacterial flora in rearing water of shrimp farms by Ruangpan and Tabkaew (1994) indicated nine species of vibrios namely V.alginolyticus, V.anguillarum, V.fluvialis, V.harveyi, V.parahaemolyticus, V.splendidus, V.tubiashi and V.vulnificus were normally found throughout

the year. Variation of the flora in the water was $0.65x10^2$ to $1.22x10^4$ CFU/ml. Pathogenicity of this bacteria to shrimp was still unclear.

Investigations of the bacterial diseases of P.aztecus and P.setiferus revealed Vibrio, Aeromonas, Spirillum and Flavobacterium as possible pathogens (Glen et al 1980). A survey of the diseases of cultured P.monodon in Taiwan the major bacteria isolated from morbid shrimp included Vibrio sp, Pseudomonas sp and Flavobacterium sp (Lightner 1988). Yang et al (1995) also reported Vibrio, Aeromonas and Pseudomonas as major penaeid shrimp pathogens.

V.anguillarum, V.alginolyticus, V.parahaemolyticus, V.splendidus and V.harveyi have been described as pathogenic to penaeid shrimp (Vanderzant et al 1970, Lightner and Lewis 1975, Lightner 1983,1985,1988, Takahashi et al 1984,1985; Lavilla-Pitogo et al 1990, Vera et al 1992, De la pena et al 1993, Karunasagar et al 1994, Mohney et al 1994, Pizzaro and Alfaro 1994 and Hameed 1994). Research on vibrio bacteria associated with cultured tiger shrimp P.monodon was reviewed and discussed

by Ruangpan and Tubkaew (1994). Eleven species of vibrios including V.alginolyticus, V.anguillarum, V.cholerae Non01, V.damsela, V.tubiashi and V.vulnificus have been isolated from intensively cultured shrimps. Among these species, V.parahaemolytius was consistently predominant. The different species of vibrio pathogenic to shrimps are given in Table 1.

TABLE 1

PATHOGENIC VIBRIOS ISOLATED FROM SHRIMPS

Shrimp sp	Bacteria sp	References
P.brasiliensis P.notialis, P.schmitti, P.subtilis	V.anguillarum and V.alginolyticus	Bashirullah et al 1993
P.monodon	V.alginolyticus, V.parahaemolyticus, Pseudomonas sp	Anderson et al 1988
P.setiferus	V.alginolyticus, P.putrefaciens	Alfaro et al 1993
P.vannamei	V.parahaemolyticus, V.vulnificus, V.alginolyticus.	Mohney et al 1994

Lightner (1988) also reported that V.anguillarum, V.alginolyticus, V.parahaemolyticus were the main species isolated from morbid shrimp collected in 1986 from Taiwan. Investigating on disease problems in farmed penaeids in Italy, Giorgetti (1990) found that the most frequently isolated bacterium was V.anguillarum. Song et al (1990) reported that V.vulnificus may initiate a pathogenic effect in P.monodon.

Diseases of exoskeleton of decapod crustacean have been common and variously referred to as 'spot disease', 'brown spot', 'black spot', 'spotted disease', or 'shell disease'. These diseases were characterized by brown to black spots on the external carapace or cuticle of prawn. Chitinoclastic bacteria such as Beneckea and Vibrio were isolated from the shell lesion of Macrobrachium rosenbergii, the penaeid, P.setiferus and the blue crab, Callinectes sapidus (Cook and Lofton 1973; Delves - Broughton and Poupard, 1976).

Preliminary study on bacteria isolated from shrimp larvae infected with the 'bent shrimp disease' showed two types of bent shrimp disease causing Vibrios (Mariam et al 1987). Bell et al (1987) found that nearly all reported

cases of non-filamentous bacteria isolated from diseased shrimp were noted as motile, Gram negative, oxidase positive, fermentative rods. Usually these isolates were species of the genus Vibrio. Bacterial diseases caused by several species of Vibrio and the necrotizing hepatopancreatitis bacterium (NHPB) continue to be among the most troublesome and costly of the infectious diseases of shrimps (Lightner et al 1994).

Luminescence in shrimp (P.monodon) larvae followed by mass mortalities in hatcheries were also reported in several parts of Indonesia due to luminous V.albensis (Sunaryanto and Mariam 1987). V.harveyi also reported to cause luminous bacterial disease in P.monodon larvae (Pitogo, 1988). Abraham and Manley (1995) investigated that bacteria associated with melanized fissures of cultured P.indicus. Smooth, circular, cream coloured luminescent and non-luminescent colonies isolated from melanised fissures were identified as V.harveyi.

The loss in production of P.japanicus Bate due to 'vibriosis' was estimated at about 30.8 metric tonnes/year in Japan (Sano and Fukuda 1987). 70-80%

mortality penaeid larvae in hatchery due to vibrio infection particularly at the first stage upto the stage before mysis form also is reported (Ruangpan 1982). These studies suggest the importance of Vibrio sp being pathogenic to culturable species of shrimps throughout their life stages.

MATERIALS AND METHODS

I. Bacterial Strain:

The bacteria used in the present investigation was isolated from cultured P.monodon from Ramnad area. Initial isolation was carried out from the hepatopancreas of the diseased shrimp.

II Media:

1. Zobell Marine Agar:

Zobell marine agar from Hi-Media was used with the following composition:

Ingredients	g/l
Peptone	5.0
Yeast extract	1.0
Ferric chloride	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08

Strontium chloride	0.034
Boric acid	0.022
Sodium silcate	0.004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.0
Final P^H(at 25°C)	7.6±0.2

2. Zobell Marine Broth:

Zobell marine broth from Hi-media was used with the following composition.

Ingredients	g/l
Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
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Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
p^H(at 25°C)	7.6±0.2

3. TCBS agar:

TCBS agar from Hi-media was used with the following composition.

Ingredients	g/l
Yeast extract	5
Protease peptone	10
Sodium thisulphate	10
Sodium citrate	10
Oxbile	8
Sucrose	20
Sodium chloride	10
Ferric citrate	1
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15
Final p^H	8.6±0.2 at 25°C

III Shrimp Species:

Shrimp Penaeus monodon was used for the investigation. The P.monodon used for the experimental purpose was procured from Ms.Mas Aqua Hatchery, Marakayarpattinam. The shrimps were reared in the laboratory in one tonne FRP tanks. They were fed with pelletised feed ad libitum. Shrimps of average length and weight of 6.90 cm and 3.0 g respectively were used. Water exchange was carried out twice daily. Sea water for this purpose was taken from the Gulf of Mannar. The hydrological conditions of the sea water is given in Table 2.

TABLE 2

HYDROLOGICAL CONDITIONS OF SEAWATER USED FOR REARING EXPERIMENTAL SHRIMPS

Parameter	Range
Temperature (°C)	28.8 to 29.4
p^H	8.19 to 8.23
Dissolved Oxygen (ppm)	4.595 to 4.99
Salinity (ppt)	35.02 to 35.64
NH_4	Nil
H_2S	Nil

IV. Characterization of Bacterial Isolate:

Characterization experiments were conducted as per the methods given in Austin (1988).

I. Physical and cultural characteristics:

a) Growth on TCBS agar: Colonies were streaked on surface of TCBS agar plate and incubated for 24 hours at 37°C. Colour and diameter of the colonies were recorded.

b) Motility - Hanging drop method: A drop of liquid bacterial culture was placed in the centre of a 16 mm square No:1 cover-glass with the aid of a small (2 mm) inoculating loop. A small drop of water was placed at each corner of the cover glass. A cavity slide was inverted over the cover glass. The cover glass adhered to the slide and when the slide was inverted, the hanging drop was suspended in the well. This was observed under a microscope.

c) Gram's Staining: The slides were cleaned with soap, washed thoroughly with distilled water and dried with clean towel. Young culture was inoculated into

the middle of the slide, then fixed by slightly heating it over the flame. One drop of Crystal Violet was added for one minute and drained and washed with distilled water. Saffranin was added, drained after one minute and washed with distilled water. The slides were drained to dry then they were decolourised with ethanol for 20 seconds and Gram's Iodine was added. Then they were washed with distilled water, dried in air and observed under the microscope.

II. Biochemical characterization:

1) Acid from glucose, mannitol, inositol, sorbitol, rhamnose, saccharose.

One ml of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose were added into hundred ml of Basal medium* each. Media with different carbohydrate constituents were added into 30 ml screw cap tubes with durham's tubes. These tubes were then autoclaved for 15 minutes at 121°C. Two to three loopful of 18 to 24 h old broth cultures were inoculated into tubes and incubated for 24 to 48 h at 30°C in an incubator.

* The composition of the Basal medium:

Peptone	-	5g
Yeast extract	-	3g
0.2% bromocresol purple	-	10ml
Distilled water	-	1000ml
p^H	-	7±0.5

2) Hydrolysis of Arginine, Lysine and Ornithine:

The composition of basal medium, used to determine attack on aminoacide was as follows:

Peptone	-	5g
Yeast extract	-	3g
Glucose	-	1g
0.2% bromocresol purple	-	10ml
Distilled water	-	1000ml
p^H	-	7±0.5

Each 500ml of basal medium was poured into three different conical flasks. To that, 1mg of arginine, lysine and ornithine were added respectively into different beakers. 5ml of the different medium were taken in screw cap tubes and autoclaved. A loopful of 24 h old nutrient agar slant culture was

inoculated into the different tubes. Paraffin was added. The tubes were incubated for 48 h and observed.

3) Production of galactosidase: A large loopful of bacterial growth was emulsified in 0.25ml of physiological saline in a 10x75mm fermentation tube. A drop of toulene was added to each tube and shaken well. Tubes were allowed to stand for 5 minutes in 37°C water bath. 0.25ml of buffered 0.75 M ONPG solution was added to each tube and incubated again in 37°C water bath.

4. Citrate Utilisation: Vibrio isolate was inoculated into a tube of Simmons citrate agar using a needle to stab, then streaked on to the medium. This citrate media was incubated at 37°C for 48 h.

5. H_2S Production: The pure culture was inoculated by stabbing and streaking the triple sugar iron (TSI) slant. Incubated at 37°C for 18-24 h in an incubator and the results were recorded.

6. Indole Production: The pure culture of the isolate was inoculated into 5ml of tryptophane broth.

The tryptophane broth was incubated at 37°C for 24 h and mixed well. 0.2 to 0.3ml test reagent was added to the 24 h culture. The mixture was allowed to stand for 10 minutes after shaking well.

7. Oxidase reaction: A large loopful of culture from 18-24 h old Zobell marine agar slants were rubbed on the surface of the oxidase reactant disc containing 1% aqueous solution of NNN 'N' Tetra methyl paraphenylene diamine dihydrochloride

8. VP-reaction: Pure culture were inoculated into 5ml of buffered glucose peptone broth (glucose - 5g, K_2HPO_4 - 5g, Peptone-5g, distilled water-100ml and incubated for 48h at 37°C. 0.6 ml of Naphthol solution and 0.2 ml of KOH were added to 1ml of 48 h buffered glucose peptone broth culture in separate clean test tubes. The test tubes were shaken for mixing well and then it was allowed to stand for 2 to 3 h and resultant colour was noted.

V **Bacterial Growth Studies:**

For growth studies, Zobell marine broth was used. The bacteria was removed from the slants aseptically

and subcultured in 100ml flask containing 25ml of Zobell broth. After incubation for 12 h at room temperature, 0.1 ml of bacteria was taken and inoculated to the sterile Zobell broth in 500 ml flask. Samples from this flask were drawn periodically and counted on a Neubauer - Improved double ruling hemocytometer (Fein - Optick Blanken bierg, Germany). Periodically the samples from this flask were drawn and OD was measured at 660 nm in a EC made spectrophotometer. The values obtained were plotted on a semilog (4 cycles) and arithmetic graph paper respectively.

VI Studies on Infectivity of Vibrio sp on P.monodon:

P.monodon shrimp weighing 3.02±1.23 g and 6.91±1.49 cm (TL) were kept in glass aquaria of 15 l capacity and were acclimatized for 2 to 3 days. In each tank, 10 numbers were introduced. Water was well aerated and shrimps were fed ad libitum with pelletised feed. Unused food was removed after an hour of feeding.

The shrimps were given intramusular injection at the junction of 4th & 5th abdominal segments after

sterilization with alcohol. The desired dose of bacterial suspension was injected using a Dispovan insulin 1ml syringe (Plate 1). The control group animals were injected with 0.2% saline. After giving injections, the shrimps were introduced back into the aquarium tanks and observed. The experimental set up is given in Plate 2.

VII **Experiments on reisolation of bacteria from tissue and hemolymph:**

Bacteria was reisolated from the hepatopancreas and hemolymph of artificially infected shrimps immediately after their mortality.

With EDTA rinsed syringe, 0.01 ml of hemolymph was taken aseptically from the heart by inserting the sterile needle via coxa of the third walking leg. The hemolymph was serially diluted and plated on Zobell Marine agar media.

Bacteria was also reisolated aseptically from hepatopancreas and incubated overnight at 37°C to counter check the establishment of the pathogen in the shrimp's body.

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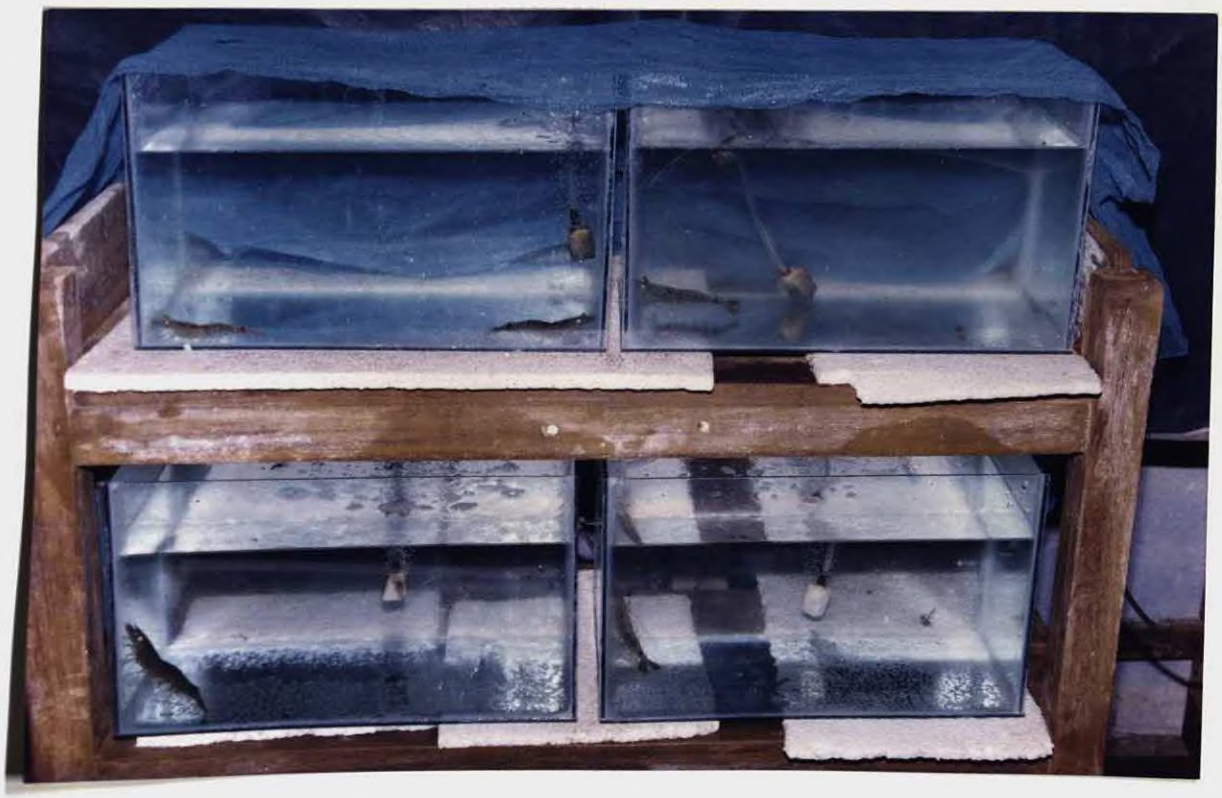
PLATE 1

PLATE SHOWING INJECTION OF LIVE BACTERIAL CELLS INTO EXPERIMENTAL SHRIMP, *P.monodon*

PLATE 2

PLATE SHOWING EXPERIMENTAL SET UP FOR ARTIFICIAL INFECTION STUDIES



RESULTS

The results of the present investigation indicated that the bacteria isolated from the hepatopancreas of infected shrimp being to Vibrio alginolyticus. The results of characterization studies are given in Table 3.

Physical and Cultural Characteristics:

The bacteria were motile, Gram negative rods. They gave yellow colonies of 2-5 mm diameter on TCBS agar.

Biochemical Characteristics:

The isolate gave positive results for acid from glucose, acid from mannitol, acid from saccharose, citrate utilisation, gelatin hydrolysis, Indole production, lysine decarboxylase, Ornithine decarboxylase, Oxidase test. The isolate gave negative results for the presence of acid from inositol, acid from sorbitol, acid from rhamnose, Arginine dehydrolase, Beta galacactosidase, H_2S production, and for VP reaction.

1. Acid from glucose, mannitol and saccharose:

Change in color of the medium to yellow was noted and recorded positive for acid production.

2. Citrate utilisation:

A distinct prussian blue colour was noted and was recorded as positive.

3. Gelatin hydrolysis

Clear zone around colony indicated the presence of gelatinase.

4. Indole production:

A dark red colour in the amyl alcohol layer on top of the culture gave positive indole test.

5. Lysine decarboxylase Ornithine decarboxylase:

Pink colour of the medium was observed and recorded as positive.

6. Oxidase:

Development of purple colour within 15 to 30 seconds was recorded as positive.

7. Acid from mannitol, sorbitol, rhamnose:

No change in colour of the medium to yellow and hence recorded negative for acid production.

8. Arginine dihydrolase:

Yellow colour of the medium was noted and recorded as negative.

9. Beta galactosidase:

Change of colour to yellow was not observed; recorded negative for presence of Beta-galactosidase.

10. H_2S production:

Black colour did not develop in the medium and hence recorded negative.

11. VP reaction:

No change in colour to pink or crimson within 2 to 3 hours and noted as negative.

TABLE 3

PHYSICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF *Vibrio alginolyticus*

I. Physical & Cultural Characteristics:	
Size and shape	rod
Motility	+
Gram's stain	-
Growth on TCBS agar	Yellow colonies(2 to 5mm dia)
II. Biochemical Characteristics	
1. Acid from glucose	+
2. Acid from mannitol	+
3. Acid from inositol	-
4. Acid from sorbitol	-
5. Acid from rhamnose	-
6. Acid from saccharose	+
7. Arginine dihydrolase	-
8. Beta galactosidase	-
9. Citrate utilisation	+
10. Gelatin hydrolysis	+
11. H_2S production	-
12. Indole production	+
13. Lysine decarboxylase	+
14. Ornithine decarboxylase	+
15. Oxidase test	+
16. VP reaction	-

II **Studies on growth of** *V.alginolyticus*

The growth pattern studies of *V.alginolyticus* by direct count as well as by OD measurements indicated that the peak growth occured at 18 hours and 24 hours respectively. At 18 h of culture in Zobell marine broth at room temperature, the cell number was 2.32×10^9 cells/ml. The results are given in table 4. Figure 1 which depicts the growth of *V.alginolyticus* indicated no lag phase. The death phase was found to start from 36 hours onwards as could be seen from figure 1.

The optical density measurements gave maximum OD of 0.330 at 24 hours in 660 nm. The results are given in Table 5. The pattern coincided with the growth curve as could be seen from figure 2.

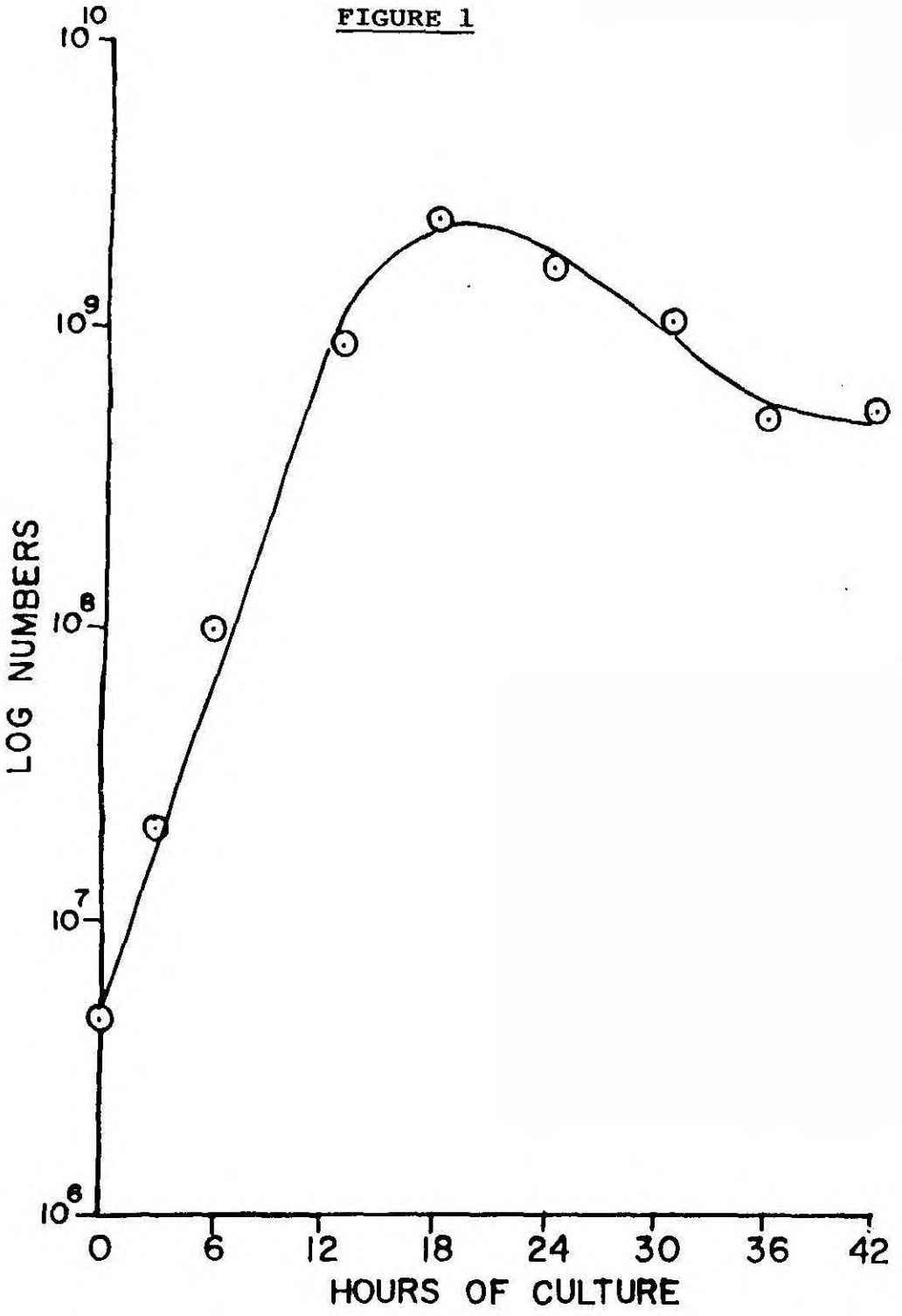
TABLE 4

GROWTH OF V.alginolyticus IN ZOBELL MARINE BROTH

HOURS OF CULTURE	MEAN NUMBER OF CELLS*
0	4.4×10^6
6	2.14×10^7
12	9.1×10^8
18	2.32×10^9
24	1.84×10^9
30	1.02×10^9
36	5.0×10^8
42	5.2×10^8

* Average of 3 replications

FIGURE 1



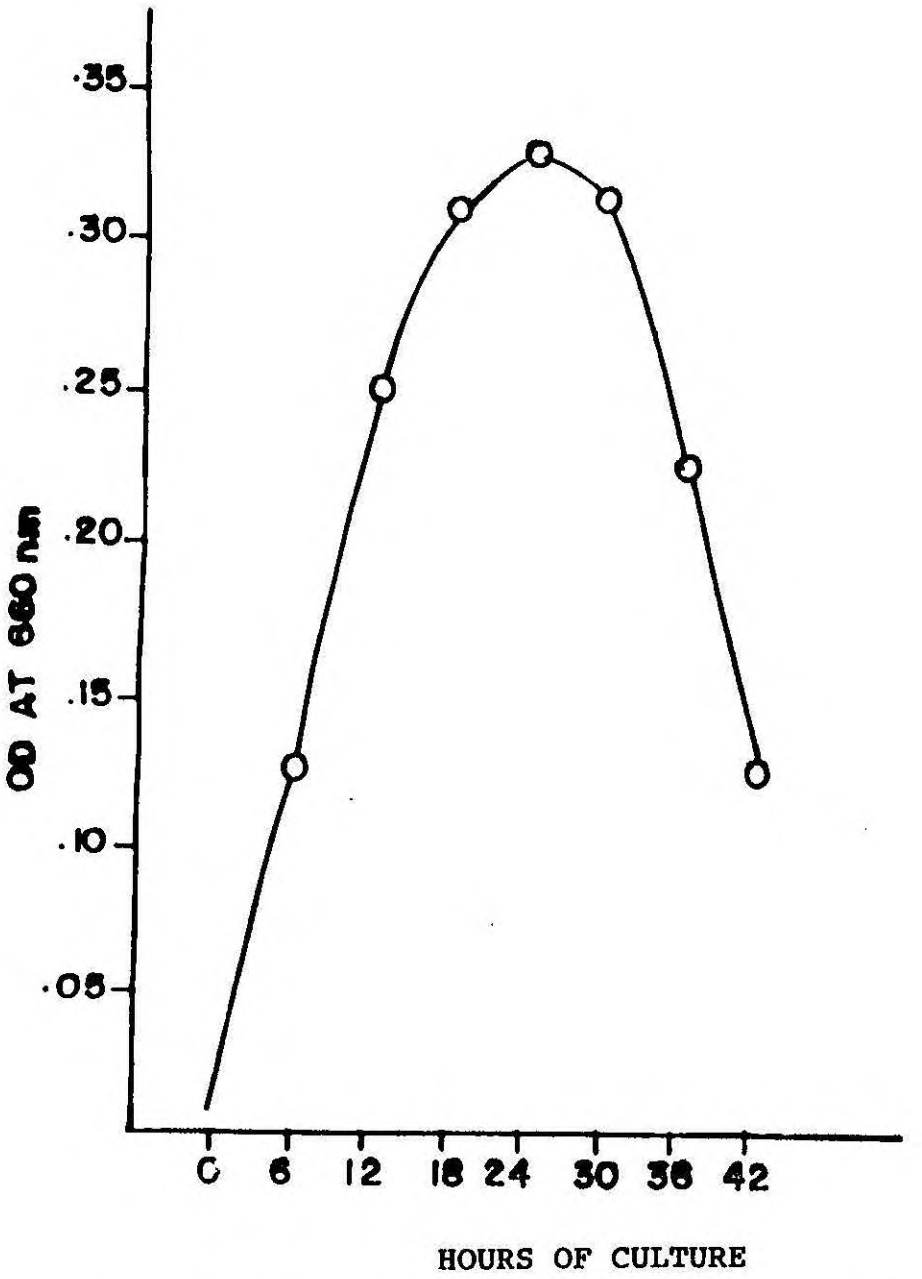
Growth of V.alginolyticus in Zobell Marine Broth at different time in hours. (X axis: Hours of culture, Y axis: Number of cells)

TABLE 5

OPTICAL DENSITY MEASUREMENTS OF GROWTH OF *V.alginolyticus* IN ZOBELL MARINE BROTH

HOURS OF CULTURE	OPTICAL DENSITY
0	.014
6	.128
12	.254
18	.310
24	.330
30	.315
36	.224
42	.120

FIGURE 2



Growth of V.alginolyticus at different time intervals measured by spectrophotometer. (X axis: Hours of culture, Y axis: OD of medium)

III. Studies on influence of V.alginolyticus on the survival of P.monodon.

100% mortality was observed in the first day, within 3 and 5 h of inoculation in shrimps which were administered with 5.7×10^8 cells and 5.7×10^7 cells respectively.

Abnormal behaviour of shrimps such as:

1. Erratic swimming
2. Jumping immediately after injection and
3. After a short time, lying still at the bottom of the experimental tank was noted.

The clinical symptoms were:

1. Increased pigmentation
2. Swollen hepatopancreas
3. Soft texture of musculature.

An inoculum of 5.7×10^6 cells/shrimp resulted in 100% mortality after 9 days of inoculation. Half of the animals died in the first day of inoculation, another 20% on second day and 10% each on 5th,6th and 9th day respectively.

Shrimps behaviour changes were the same as described earlier.

In the clinical symptoms, increased pigmentation was very prominent as days passed after inoculation. The other clinical symptoms were the same. The results are given in Table 6.

IV. Reisolation of bacteria from hepatopancreas and hemolymph

Observations on the results of reisolation of bacteria from artificially infected shrimps indicated that they belong to V.alginolyticus.

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TABLE 6

MORTALITY OF *P.monodon* ARTIFICIALLY INFECTED WITH *V.alginolyticus*

Dose-cells/animals	Mortality in Days										Total
	1	2	3	4	5	6	7	8	9	10	
5.7×10^8	10										10
5.7×10^7	10										10
5.7×10^6	5	2			1	1			1		10
Control (10 Nos.)	-	-	-	-	-	-	-	-	-	-	0

DISCUSSION

The physical, cultural and biochemical characterization of the isolated bacteria and the pathogenicity experiments indicated that the pathogen causing mortality in pond reared P.monodon belong to Vibrio alginolyticus.

V.alginolyticus was earlier recorded as a common pathogenic organism from diseased shrimp which exhibited bacterial septicemic symptoms (Lightner & Lewis 1975). The preliminary isolation of the bacteria used for all the experiments in the present investigation was from hepatopancreas of the diseased shrimp. Bashirullah et al 1993, who studied the diseases of penaeid shrimps of commercial interest (P.brasiliensis, P.notialis, P.schmitti, and P.subtilis) in the Eastern region of Venezuela found V.anguillarum and V.alginolyticus in gills and hepatopancreas. Hameed (1994) studied the occurence of shell disease in shrimps and lobster. Black lesion was observed on the abdominal appendages and telson of larvae and adult P.indicus and adult Panulirus homarus. Bacterial isolates belonging to the genus Vibrio especially V.alginolyticus were isolated. In

experimental transmission, V.alginolyticus was found to cause black lesions on the abdominal segments of larvae.

Mortalities attributable to V.alginolyticus infection was reported in 1972 and in 1973 in a commercial hatchery in the U.S. (Lightner, 1975). Both white and brown shrimp were affected, and mortalities approached 100%. Yang et al (1992) studied on the pathogenic bacterial isolation of epizootic brown spot of shell disease syndrome. The artificial infection tests and bacteriological identification showed that isolated organisms belong to V.pelagius and V.alginolyticus. Mohney et al (1994) investigating on the "sindroma gaviota", the massive epizootic of vibriosis in the gulf of Guayaquil, Ecuador found the predominant species isolated were V.parahaemolyticus, V.vulnificus and V.alginolyticus. V.parahemolyticus, which has been associated with human gastroenteritis in several countries, was found by Vanderzant et al (1970) to be lethal to experimental population of brown shrimp P.aztecus. Subsequent studies have shown that other vibrios (V.anquillarum and V.alginolyticus) can cause epizootics and mortalities of brown, pink (P.duorasum) and white shrimp (P.setiferus) (Lewis 1973, Lightner and Lewis 1975).

In characterization experiments V.alginolyticus was distinguished from V.anguillarum by negative reactions in beta galactosidase, arginine dihydrolase and positive reactions in lysine decarboxylase for V.alginolyticus and positive reactions in beta galactosidase, arginine dihydrolase and negative reactions for lysine decarboxylase for V.angullarum. V.alginolyticus was distinguished from V.parahaemolyticus by positive reactions for acid production from saccharose for the former and by negative reactions for the latter respectively.

Ishimaru et al (1995) isolated 6 similar strains as pathogens of cultured kuruma prawns, P.japonicus in Japan which had characteristics of the genus Vibrio. These organisms were distinguished by their positive results in tests for gelatinase and lipase activities and their negative reactions in tests for arginine dihydrolase and lysine and ornithine decarboxylase activities, production of acid from sucrose. De la pena et al (1993) isolated 75 strains of bacteria from diseased P.japonicus and examined for their morphological, biochemical and physiological characteristics. The bacterium was a Gram negative, motile and 0/129 (Vibriostatic compound) sensitive short rod. It did not decarboxylate arginine, lysine or ornithine, which was characterized as Vibrio sp (PJ)

V.alginolyticus was found to be lethal to healthy P.monodon juveniles at $5.7x10^8$, $5.7x10^7$ and $5.7x10^6$ cells/shrimp. $5.7x10^8$ and $5.7x10^7$ cells/shrimp caused mortality in 3 and 5 h after inoculation respectively. $5.7x10^6$ cells/shrimp caused 50% mortality in the first day of inoculation another 20% on second day and 10% each on fifth sixth and nineth day respectively. Vera et al (1992) on the experimental study of the virulence of 3 species of Vibrio bacteria in P.japonicus showed that V.alginolyticus was more virulent than V.parahaemolyticus and V.anguillarum in P.japonicus from 24 h to upper exposures. The V.alginolyticus doses of $4.3x10^2$ CFU/shrimp caused mortalities of 40% within 72 h while doses of $5x10^3$ and $5x10^5$ CFU/shrimp from V.parahaemolyticus and V.anguillarum provoked no mortality in the same period of time.

Esteve and Quijadar (1993) experimentally infected juveniles of P.brasiliensis with increasing numbers of V.anguillarum. Three techniques were assayed; immersion with and without incision, immersion after slight dehydration and inframuscular inoculation. The immersion without incision caused vibriosis in 100% of the individuals at bacterial concentrations of 10^5 and 10^6

cells/shrimp 88% mortality took place in 12 hours. Adding a factor of 10 registered a mortality of 100% in just 8 hours. Finally 10^8 cells/shrimp provoked total mortality within 2 hours.

Vibrio harveyi and Photobacterium phosohoreum were the causative organisums of luminous bacterial shrimp dieseases in shrimp hatcheris in Indonesia and other Asian countries. Pathogenicity tests demonstrated that several strains were pathogenic to P.monodon larvae at concentration of 10^3 cfu/ml (Prayitno and Latchford, 1995).

Jiravanich paisal et al (1994) studied diseased prawns with a hepatopancreatic infection of V.harveyi which showed bacterial invasions and multiplication in the tubular lumens. Two types of isolates (chitinase positive and negative were moderately pathogenic to prawns. Intramuscular injection of 10^5 to 10^6 cfu/prawn resulted in mortalities of 53-100% and chitinase negative isolate was more virulent than chitinase positive.

Hameed et al (1996) studied the susceptibility of larvae and post larvae of P.indicus, P.monodon and P.semisulcatus to Vibrio campbelli - like bacterium by

immersion challenge. Exposure to bacteria caused upto 100% mortality, depending upon bacterial dose and time of exposure. Based upon LD_{50} calculations, P.monodon was found to be most sensitive of the three shrimp species to the pathogen.

Song et al 1993 studied the isolation and characterization of Vibrio damsela from P.monodon in Taiwan. Affected shrimp display non specific signs, including poor growth rate, anorexia, rough shell and milky musculature. Takahashi et al 1991 studied the epizootic occured among cultured kuruma prawn in Japan. The typical symptoms of the diseased prawns were light brown colouring of the gills and swelling of the lymphoid organ. Hameed (1996) observed clinical symptoms such as lethargy, darkening of the dorsal portion of cuticle, opaqueness of body and reddening of periopods and pleopods in the infected adult shrimp with V.campbelli - like bacterium. A white patch developed at the site infection and it became blackened on the fourth day. The behavioural pattern included reduced swimming activity, disorientation during swimming and often swimming on one side. The symptoms observed in the present investigations corraborated with these earlier findings.

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