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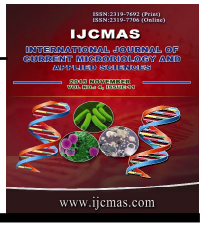

Original Research Article

Characterization and Serological Studies of *Vibrio anguillarum* Isolated from Diseased Freshwater Prawn, *Macrobrachium malcolmsonii*

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Keywords

Vibrio anguillarum, Macrobrachium malcolmsonii, biochemical, serological, pathogeneicity

A B S T R A C T

Vibrio anguillarum, an important pathogen infecting freshwater prawn, *Macrobrachium malcolmsonii* and causes severe mortality. Four strains of *Vibrio anguillarum* were isolated from gill, hepatopancreas, hemolymph and carapace of diseased freshwater prawn. The species were characterized based upon their biochemical and serological tests. On antibiotic assay, these strains were found to be highly susceptible to amikacin, ofloxacin, trimethoprim, cephalaxin, and erythomycin. *In vitro* pathogeneicity test showed all the strains are found to be positive to the Congo red binding assay and are hemolytic in nature. The strains VA1, VA5 and VA13 were virulent caused 100% mortality at $2x10^7$ CFUs/ml within seven days. Development of an immunological technique for detecting and screening of *Vibrio anguillarum* infection and its treatment is highly important in freshwater prawn aquaculture.

Introduction

Freshwater prawn is an important candidate species and has the high value item in the domestic market. It has taken a valuable status in production system in the country. Though the production has declined in the country due to risk of viral infection but small scale marginal farmers earn their income by incorporating in polyculture system. Besides giant freshwater prawn, Indian river prawn *Macrobrachium malcolmsonii* is widely being cultured by the farmers by collecting the seeds from the local rivers. However, the farmers face serious threat due to microbial diseases caused by opportunistic pathogens present in culture systems. Among the microorganisms causing serious losses, the best known are bacteria because of the devastating economic effects they have on affected farms. Bacteria, *Leucothrix* sp. and *Vibrio* sp. are regarded as the most damagers in culture systems causing severe mortality in prawns (Lavilla-Pitogo, 1995).

The genus *Vibrio* (Pacini, 1954), belongs to family *Vibrionaceae,* are gram-negative

facultative anaerobes, short to medium, comma-shaped rods found both in freshwater and marine ecosystems. Vibrios cause vibriosis, a serious threat to the aquaculture industry responsible for massive mortality of cultured fish as well as both marine and freshwater prawn worldwide (Lavillo-Pitogo *et al.,* 1998; Chen *et al.,* 2000). Among different species of *Vibrio, Vibrio anguillarum* was observed to be an important component of microflora in culture systems of prawn and shrimp. *Vibrio anguillarum* has been responsible for numerous epizootics among cultured and feral population of fish throughout the world (Hasten and Holt, 1972; Novotny, 1975).

The emerging disease problems, which continue to threaten the prawn farming throughout the world, have focused on the etiological causes of vibriosis. In the present investigation, we report the isolation *Vibrio anguillarum* from diseased freshwater prawns and characterize the pathogen on the basis of biochemical and sero-diagnosis tests. Further, *Macrobrachium malcolmsonii* species were challenged with the organism to manifest the disease in laboratory condition to determine the pathogenicity of this organism and antibiotic sensitivity of the pathogen was also carried out to discern the effective antibiotics against vibriosis.

Material and Methods

Indian river prawn

Freshwater river prawn, *Macrobrachium malcolmsonii* having disease symptoms like red discoloration, loss of appendages, black colouration of body and gill were taken for study. Healthy prawns (8–10 cm) weighing 10-15 g were acclimatized in FRP (Fibreglass Rainforced Plastic) tanks of capacity 500L for further experiment.

Isolation of Vibrios

Swabbing with rectified spirit (90% ethanol) to avoid contamination, the outer surface of infected prawn was cleaned. Samples were collected aseptically from different organs in nutrient broth (Himedia Ltd., India) and incubated at 37°C for 24 h. Growth of bacteria was observed by noticing changes in turbidity after 24 h. Colony morphology and colour was recorded after incubation for two days at 30°C on TCBS (Thiosulphate citrate bile sucrose) agar. Cell morphology was studied in gram-stained preparation from nutrient agar plate supplied with 1% (w/v) NaCl according to Hucker's modification methods (Barrow & Feltham 1993). Motility was studied using a drop of broth culture on a slide and observing under light microscopy.

Biochemical test

Biochemical properties were carried out following the diagnostic scheme for *Vibrio* species. The presence of catalase was tested with hydrogen peroxide and cytochrome oxidase was determined by oxidase discs (Himedia Ltd., India).

The presence of urease was detected by inoculating cultures into urease broth followed by incubation at 37°C. Indole formation, citrate utilization, MR test and VP test were also performed as per the standard protocol (Faddin, 1976). Nitrate reduction was determined by nitrate broth and carbohydrate usage was determined by triple sugar iron test and by using sugar discs in phenol red broth base (Faddin, 1976)

Tryptone soya agar was used as the basal medium to test capability of 48 h cultures to degrade casein, gelatin and starch. Gelatin liquefaction was tested with basal medium

supplemented with 0.4% (w/v) gelatin on which acidic mercuric chloride was flooded. The ability to hydrolyze casein was determined on basal medium containing 1% casein; the plates were examined for clear zone of hydrolysis in an otherwise opaque medium after flooding with acidic mercury chloride. Starch hydrolysis was determined on the same basal medium supplemented with 1% soluble starch followed by addition of iodine solution after incubation. Clear zones around the colonies indicated hydrolysis of starch. Cellulose decomposition was studied by supplementing basal medium with carboxy methylcellulose. Clear area around the colony flooded with 1% Congo red solution and HCl indicated a positive result.

Antibiogram studies

Antibiogram sensitivity was determined by the Kirby-Bauer zone of diffusion method (Bauer *et al.,* 1966). Amikacin, amoxycillion, ampicillin, bacitracin, cefuroxime, chlorotetracycline, cloxacillin, cephalaxin, cephalothin, ciprofloxacin, chloramphenicol, flumequine, gentamycin, erythromycin, penicillin G, thrimethoprime, nalidixic acid, tetracycline, ofloxacin, neomycin, norflaxacin and vibriostatic compound O/129 were used for the antibiotic sensitivity assay. Inhibition zone diameter was recorded after 48h incubation at 37ºC.

***In vitro* virulence studies**

The Hemolytic test and Congo red binding assay ascertained the virulence factor in the isolates. The hemolytic test was performed by supplementing the basal medium with 5% sterile blood followed by incubation at 37ºC. The type of hemolysis and zone diameter was noted (Su and Lee, 1997). The congo red binding assay was performed by adding 0.03% (w/v) Congo red to the basal medium. Deep red, raised colonies indicated the virulence of the isolate.

***In vivo* virulence study**

For immersion challenge prawns weighing 10-15 g were used. The prawns were divided into four groups with 20 in each group. Based on *in vitro* virulence bacterial inoculums (V13 strain) at concentrations of ($2x10^6$ CFUs/ml), ($2x10^7$ CFUs/ml) and ($2x10^8$ CFUs/ml) to first group, second group and third group were used, respectively. The fourth group was used as control. The conditions were maintained for 14 d. Koch's postulates were verified from the moribund prawns.

Sero-diagnosis test

Protein concentration of the isolates was determined by the colorimetric method using Biuret reagent (Bradford, 1976).

Preparation of antigen

The representative isolated cultured in TCBS agar was harvested by centrifugation at 10,000 x g for 10 min at 4ºC and a portion of the harvest was suspended in phosphate buffer saline (PBS, 0.01M., pH 7.2) and killed by heating at 56ºC for 1h. Another portion of the harvest was killed with 0.3% (w/v) formalin, washed twice and centrifuged and resuspended in PBS. This was then dialyzed against PBS using a dialysis membrane (Himedia Ltd., India) and resuspended in PBS. For sonicated antigen, a portion of the suspension of formalin killed bacterial cells was sonicated three times at 50 Hz for 10 min with a 5 min interval between the cycles (Artek Sonic Dismembrator, Model 150). The supernatant obtained after centrifugation was used as an unheated sonicated antigen.

Immunization and preparation of antiserum

One hundred fifty µl of formalin killed bacterial cells in Freund's Complete Adjuvant (FCA) was injected intramuscularly into the hind leg of the rabbits. The animals were given booster on 14^{th} and 28^{th} day of immunization with the same dose of emulsion of Freund's Incomplete Adjuvant (FIA) instead of FCA.

Antisera preparation

Blood from the rabbits was collected by ear vein puncture at 14 d and 28 d post-immunization. Serum was collected by centrifugation (1500 x g) of the clotted blood for 15 min and stored at -20ºC until further use.

Agglutination test

On a clean slide, a 10:1 dilution of formalin killed bacterial suspension was made. Then a 10:1 dilution of hyperimmune sera was added and the slide was gently rocked to mix the antibody with the bacterial suspension. Appearance of floccules or clumping of the bacterial antigen along with the clearing of solution within 5 min was judged as a positive result to facilitate the easier examination reaction antigens were stained with crystal violet dye.

Microtiter plate assay

The bacterial antigen prepared by formalin treatment of bacterial cells suspended in sterile PBS to an optical density of 0.8 at 543 nm. 50:1 of the sterile PBS was taken in each well of a microtiter plate. Serial doubling dilution of the serum were prepared by adding 50:1 dilution of serum to the first well, mixing and transferring 50:1 to the second well and continuing to the respective wells. A saline control was also set up to check for auto-agglutination of 50:1 of bacterial antigen was added to each well and plates were incubated overnight at room temperature in a moist chamber. Irregular settling of the bacterial aggregates at the bottom of the well was a positive control. Lack of agglutination was indicated by settling of the antigen in the form of a compact button at the base of the well. Agglutination titer was determined as the reciprocal of the highest dilution showing visible agglutination.

Dot-ELISA

Nitrocellulose paper strips of $5 \times 5mm^2$ were coated with 5:1 dilution of sonicated antigen. The coated NCP strips were dried at 65^oC for 2 h in an incubator. Then the strips were blocked in PBS containing 0.05% between 20 (PBS-T) and 5% skim milk powder at 37°C for 1 h. Strips were washed thrice in PBS-T and incubated with 1: 100 dilutions of hyper immune sera. The strips were again washed thrice with PBS-T. Then the strips were incubated with anti rabbit HRPO (Horse-radish peroxidase) conjugate (Genei, Bangalore, India) at 1: 2000 dilutions in PBS-T for 1 hour at 37°C. The strips were washed several times in PBS-T before putting them in a substrate solution (5 mg of 3,3 diaminobenzidine tetra hydro chloride (DAB), 10: 1 dilution of 30% of H_2O_2 and 5ml of 50mM Tris buffer (pH 7.6)) until the development of brown coloration. The strips were washed in running tap water to stop the reaction and then dried at room temperature before evaluation.

Indirect ELISA

An indirect enzyme linked immunosorbent assay (ELISA) was performed in order to

determine the concentration of antibody in the hyper immune sera and also to establish serological homogeneity among the isolates. Whole cell lysates (antigen) were diluted with coating buffer in the ratio 1: 20 dilution and were coated into the 96 well ELISA plate (Tarsons, India). The plate was sealed and incubated overnight at 4°C. The plates were then washed thrice with washing buffer (PBS-T) with 5 min interval between each washing. Then diluted blocking solution (200: 1) was added to each well to block the rest of the protein sites, and the plate was blocked at 37°C for 2hours. Then the plate was washed thrice with washing buffer (PBS-T) at 5 min intervals. Dilution serum (50: 1) was added per well using 3 wells per sample and the plate was incubated again at 37°C for one hour. The plate was then washed with PBS-T and subsequently a 50:1 dilution of goat anti rabbit Horse-radish peroxidase conjugate was added to the wells at 1: 5000 dilution as specified by the manufacturer. After incubation for one hour at 37°C and washing with PBS-T, a 100:1 dilution of the substrate solution was added to all wells and incubated at room temperature until colour development. The reaction was stopped by adding 1N H_2SO_4. Absorbance was recorded at 450 nm in an automated ELISA reader (Multiscan, MCC 340) against substrate control.

Results and Discussion

Identification of isolates

In TCBS agar, the strain formed small sized colonies after 24 h of incubation at 37ºC. The colonies were circular, mucoid having raised center and green in colour. From biochemical studies the isolates were identified as *Vibrio anguillarum.* They were motile, oxidase and catalase positive, gram-negative comma-shaped rods, which degraded D-glucose, reduced aminoacids like arginine and lysine. All were sensitive to the vibriostatic agent O/129 (150µg) (Table 1). The strain VA5 was positive to arginine whereas strain VA1 and VA13 was positive to Lysine decarboxylase. Most of the strains positive to indole except VA5, negative to VP except VA3. All strains were urease negative (Table 1).

Antibiotic sensitivity test

Among the twenty-one antibiotics tested, *Vibrio anguillarum* was found to be sensitive to thirteen antibiotics. The highest zone was found in the case of ciprofloxacin, cephalaxin, tetracycline and erythromycin (Table 2).

Virulence studies

In vitro pathogenicity test

The strains showed positive reaction towards Congo red binding assay. Strains VA1, VA3 and VA13 were also found to be highly ß- hemolytic type (Table 3).

In vivo pathogenicity test

Clinical signs and symptoms

The prawns infected with *Vibrio anguillarum* did not exhibit with any symptom up to 72h after that became gradually sluggish and rest into the bottom of the tanks. The exoskeleton became reddish in colour with black spots on the carapace. The infected prawns died within seven days. Dead and moribund prawns were dissected to note the gross internal organ changes. The gills became brownish and muscle became whitish. The hepatopancreas was pale white.

Infectivity study

The prawns challenged with 2x 10^6 CFUs/ml showed no mortalities during the study

period while 100% mortality was observed in 2x 10^7 CFUs/ml within seven days and within 4 days in 2x 10^8 CFUs/ml.

Serological diagnosis

Agglutination

Different agglutination tests like slide agglutination and colour agglutination showed that *Vibrio anguillarum* caused agglutination. Micro titer plate assay showed that the titer was 512 after day 14, increasing to 2048 after day 28.

Dot ELISA and Indirect ELISA

All the strains isolated positive for Dot-ELISA, which was revealed by brown colouration of the nitrocellulose paper strips (Table 4). The strain VA3 was weakly positive.

The isolates showed positive towards indirect ELISA, which was indicated by the increase of OD value at 450 nm as compared to that of control (Table 4).

Vibrio species exist as normal flora in fish and shellfish, but also has been recognized as opportunistic pathogen in many marine animals (Austin and Austin, 1993). The association of this genus with freshwater prawn reported by Jayprakash *et al.* (2006), Khuntia *et al.* (2008) and Behura *et al.* (2015).

The strains were motile, oxidase, and catalase positive, gram-negative, comma shaped rod, grew in TCBS agar medium and sensitivity to O/129 the vibriostatic agent (West and Colwell, 1984). During this investigation all the strains manifested typical biochemical characters with slight difference. *Vibrio anguillarum* strains were found to be urease negative. From the *in vitro* pathogenicity study, it was found to be hemolytic and showed positive response to the Congo red binding assay, which suggests that it is a pathogen, because the pathogenic strains are hemolytic in nature (Joseph *et al.*, 1982). Hemolysin production has been considered as one of the important virulence factor in bacteria including vibrios. Further, the strain was also able to hydrolyse gelatin, which adds another character towards pathogenicity and this corroborates with our one of the findings of *Vibrio parahaemolyticus* (Khuntia *et al.*, 2008). Due to the presence of protease enzymes it can hydrolyse gelatin along with other enzymesand haemoglobin as reported earlier workers in other vibrios (Jana, 2000; Khuntia, 2002; Marhual, 2005; Khuntia *et al.*, 2008).

Vibriosis has been experimentally induced in shrimps and freshwater prawn by either immersing prawn in water containing bacteria (Sung *et al.*, 1994; Hammed, 1995; Esteve and Herrera, 2000; Khuntia, 2002; Khuntia *et al.*, 2008; Marhual *et al.*, 2014) or injecting bacteria into muscle or hemolymph (Lee *et al.*, 1996). In the present study, to establish the *in vivo* pathogenicity, prawns were challenged by immersion. Prawns inoculated with *Vibrio anguillarum* at 2x10^7 CFUs/ml exhibited 100% mortality within 7 d, whereas 2xl0^6CFUs/ml showed no mortalities throughout the experiment period. Significant mortalities were recorded in *Macrobrachium rosenbergii* when challenged with 10^8 cells/ ml (Khuntia *et al.*, 2008). According to Martin *et al.* (2004), fifty percentage of mortality was recorded in case of *Sicyonia ingentis* at 10^5 CFUs/ml within seven days by immersion challenge, which is low as compared to our study. While working with the larvae and post larvae Oanh *et al.* (2001) found that 10^4–10^8 cells/ml caused significant mortality when they were challenged with *Vibrio* species.

Table.1 Biochemical characteristics of *Vibrio anguillarum* strains

Test	Organisms			
	VA1	VA3	VA5	VA13
Oxidase	+	+	+	+
Catlase	+	+	+	+
MR	+	-	+	+
Urase	-	-	-	-
VP	-	+	-	-
O/F	F	F	F	F
Indole	+	+	-	+
Arginine dehydrolase	-	-	+	-
Lysine decarboxylase	+	-	-	+
Ornithine decarboxylase	+	+	-	D
O/129 sensitivity	R	R	R	R
Mortility	+	+	+	+
Acid from:				
Arabinose	-	-	+	-
Trehalose	-	-	+	-
Glycerol	-	-	+	-
Sucrose	+/-	+	+	+
Sorbitol	-	-	+	-
Salicin	-	-	-	-
Mannitol	+	+	+	-
Hydrolysis				
Starch	+	+	-	-
Casein	+	+	-	+
Gelatin	+	+	+	+
TSI acid/alk	A/Alk	A/Alk	A	A/Alk
Gas	-	-	+	+
H_2S	-	-	-	+
Growth on TCBS	Y	Y	Y	Y

Table.2 Antibiogram test of different isolates

Different antibiotics	**Symbol**	**Disc Potency in mcg**	**R**	**I**	**S**	**V1**	**V3**	**V5**	**V13**
Cephalexin	Cp	30	11	13-14	21	S	S	S	S
Co-Trimoxazole	Co	25	10	11-15	16	S	S	S	S
Chloramphenicol	C	30	12	13-17	18	S	S	S	S
Cefuroxine	Cu	30	14	15-17	18	R	R	R	R
Cephalothin	Ch	30	14	15-17	18	R	R	R	R
Ciprofloxacin	Cf	5	15	16-20	21	S	S	S	S
Chlorotetracyclin	Ct	30	14	15-18	21	S	S	S	S
Amoxycillin	An	30	13	14-17	18	S	S	S	S
Amikacin	Ak	30	14	15-16	17	S	S	S	S
Ampicillin	A	10	13	14-16	17	R	R	R	R
Bacitracin	B	8	9-12	13		R	R	R	R
Erythromycin-15	E-15	15	03	14-22	23	S	S	S	S
Naldixic Acid	Na	30	13	13	19	S	S	S	S
Norfloxacin	Nx	10	12	13-16	19	I	I	I	I
Neomycin	N	30	12	13-16	17	R	R	R	R
Gentamycin	G	10	12	13-14	15	S	S	S	S
Tetracycline	T	30	14	15-18	19	S	S	S	S
Trimethoprim	Tr	25	10	11-15	16	S	S	S	S
Ofloxacin	Of	2	12	13-15	16	S	S	S	S
Penicillin G	P	2	19	22-27	28	R	R	R	R
Cloxacillin	Cx	10	19		20	R	R	R	R

Table.3 In vitro pathogenicity of different strains in Congo red binding assay and hemolytic test

Strains	Congo red binding assay	Hemolytic test
V A1	++	+ (9 mm)
VA 3	+	+ (6 mm)
VA 5	++	+ (9 mm)
V A13	++	+ (10 mm)

Table.4 Serological characteristics of different isolates

Strain	Agglutination test	ELISA	Dot-ELISA
V A1	+	+	++
V A3	+	+	++
VA 5	+	+	++
V A13	+	+	++

According to Lavilla-Pitogo et al.(1990) on post larvae of *Penaeus monodon*, a significant mortalities occurred upon exposure to 10^2 *Vibrio harveyi* cells/ ml within 48 hours. Any infection and mortality depend upon the infectious doses and pathogens have different infectious doses for different organisms depending upon their individual capacity to resist stress influenced by their nutritional and physiological factors, for which mortality rate varied from species to species. Vibriosis in prawn and shrimp is believed as a secondary infection influenced by factors such as stress, environmental failures and high number of potentially pathogenic bacteria (Chen, 1992; Nash *et al.,* 1992; Ruangapan *et al.,* 1995), but continuous exposure of shrimp post larvae to high numbers of *Vibrio* in the environment can easily cause primary vibriosis as the bacteria can multiply on the larval surface (Chen, 1993).

An enzyme-linked immunoassay based on monoclonal antibodies to *Vibrio vulnificus* and *Vibrio harveyi* was developed by Song *et al.* (1992). Similarly Khuntia *et al.* (2008) developed dot ELISA and indirect ELISA for *Vibrio parahaemolyticus*. In the present study, indirect ELISA was performed to cross check the results of agglutination and found that all the strains were positive in ELISA test, indicating the presence of common antigens. The Dot-ELISA method was more simple and rapid. It also ensured the confirmatory identification of the pathogen even when serum was diluted 100 times. The strains showed positive for agglutination and indirect ELISA also showed the equal response to Dot ELISA. Dot-ELISA is simple and therefore it can be recommended for field usage.

During the *in vivo* experiment, prawns become gradually sluggish in nature and fall into the bottom of the tank after 48h. The disease symptoms noticed in the present study after four days, which correlates with the symptoms of shell diseases as the black colouration has frequently recorded in fresh water prawn from many regions when it is associated with shell diseases (Delves-Broughton and Poupard, 1976; Tonguthai, 1993).

Oxytetracycline is intensively used in hatcheries and grow-out ponds and is effective in treatment of vibriosis (Devesa *et al.,* 1985). But De la Pana *et al.* (1993) reported that *Vibrios* are sensitive to

chloramphenicol rather than oxytetracycline. In the present study, we found that this species is highly sensitive to ciprofloxacin, cephalaxin, tetracycline and erythromycin.

The Indian river prawn has been widely domesticated in India for over two decade. Mortalities are encountered due to vibriosis in all its life stages including grow out culture system. The role of vibriosis in prawn aquaculture has become significant in recent years as farmers have experienced substantial losses. Development of rapid and accurate immunological techniques for detecting and screening of pathogens and subsequent treatment is highly important. To achieve this, the study has immense importance for the development of prawn culture in south east Asian countries.

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