JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 189-197

Antibiotic Sensitivity of Different Bacterial Pathogens Isolated from Diseased Freshwater Prawn, *Macrobrachium rosenbergii*

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(Received: 05 September 2009; accepted: 24 October 2009)

Studies on diseases in freshwater prawn, *Macrobrachium rosenbergii* have been conducted to find out the pathogens associated with disease and their characteristic and antimicrobial sensitivity against different antibiotics. Based on the result of the study a variety of bacteria have been found among which four genera *Vibrio, Pseudomonas* and *Aeromonas* are found to be predominant and these are the probable pathogens of *Macrobrachium rosenbergii*. These bacterial strains are identified based on colony morphology on respective selective media, gram staining and biochemical test. Their *in-vitro* pathogenicity was studied and all the isolated are found to be pathogen as they showed a strong binding affinity in congo-red plate except *Vibrio anguillarum* (02) and haemolysed the blood cell except *Pseudomonas anguilliseptica*. These strains were examined for their resistance to twenty-two selective antibiotics. All the species are found to highly susceptible to ofloxacin, ciprofloxacin, and erythromycin-15. *Vibrio* species are found to be resistance to tetracycline and oxytetracycline where as all other strains are found to be susceptible to these antibiotics.

Key words: Freshwater prawn, Antibiotics, pathogens, *Macrobrachium rosenbergii*.

Fresh water prawn farming is gaining its importance since past few years as one of the lucrative enterprises among different agro-aquaculture farming due to its unlimited demand and high market price. However, this aquaculture industry has been facing serious threat due to microbial diseases caused by the opportunistic pathogens present in the culture system, which invades the immuno-suppressed host. Among the microorganisms causing serious losses, the best known are bacteria because of the devastating economic effects they have on affected farms. In a most extensive study by Miyamato *et al.* (1983) thirteen genera of bacteria including *Aeromonas, Pseudomonas* were associated with *Macrobrachium rosenbergii* larvae and *Vibrio* species are found to be the secondary invader as in white spot syndrome disease (Karunasagr *et al*. 1994; Leu *et al*. 1996; Charnvantchkool 1996;). According to Kanaujia *et al*. (1998) microbiological studies of *Macrobrachium malcolmsonii* indicated maximum bacterial count in larval rearing medium and bacterial flora

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comprising 65-72% *Bacillus*, 15-30% Pseudomonas, and 5-13% Kurthia.

The growing problem of diseases, which continue to threaten the production of prawn farming throughout the world, has focused on the aetiological agent of diseases occurred in cultured pond to avoid vast economic losses. Therefore, the present study aimed to identify and characterised the pathogens associated in diseased condition with regard to biochemical and physiological characteristics. The antimicrobial susceptibility test was also carried out to discern the effective antibiotic against a particular bacterial isolates. The *in-vitro* pathogenic capacity of these isolates was also reported to know the pathogenicity nature of the isolates.

MATERIAL AND METHODS

Prawns

Freshwater pawns (*Macrobrachium rosenbergii*) having disease symptoms like necrosis, black colouration of body and gill, red discolouration, loss of appendages, uropod and telson were taken into consideration. These were collected from the poly-cultured ponds of rural farmers.

Isolation of bacteria

The outer surface of prawn was cleaned by swabbing with rectified spirit in order to avoid external contamination. Materials were collected aseptically with the help of platinum loop from different organs in Nutrient broth (Hi-media Ltd. India) and incubated at 37°C for 24 h. Growth of bacteria was observed by noticing the turbidity. Colony morphology were recorded after incubation for 24 hrs days at 37°C on different selective media like TCBS for *Vibrio,* Pseudomonas Agar for *Pseudomonas*, Aeromonas Agar and R S Agar for *Aeromonas* . Cell morphology was studied in gram stained preparation from nutrient agar plate. Motility was studied using a drop of broth culture on a slide and observed under light microscope.

Biochemical studies

Biochemical tests were conducted as per the followed diagnostic scheme for identification of bacteria (Cowan 1993). The presence of catalase was tested with hydrogen peroxide and cytochrome oxidase was determined by oxidase discs (Hi-media Ltd. India).

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The presence of urase was detected by inoculating cultures in to urase broth followed by incubation. Indole formation, citrate utilization, MR test and VP tests were also performed. 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.

Tryptone Soya agar was used as the basal medium to test capability of 48 h cultures to degrade casein, gelatin, starch and cellulose. Gelatin liquefaction was tested with basal medium supplemented with 0.4% (w/v) gelatin on which acidic mercuric chloride was flooded. The ability to hydrolyse 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 positive result.

Antibiogram studies

Sensitivity to the following antibiotic was determined by the Kirby-Bauer zone of diffusion method (Bauer *et al*., 1966). Amikacin. Amoxycillion, Ampicillin, Bacitracin, Cefuroxime, Chlorotetracycline, Cloxacillin, Cephalexin, Cephalothin, Ciprofloxacin, Chloramphenicol, Flumequine, Gentamycin, Erythromycin, Penicillin G, Thrimethoprime, Nalidixic acid, Tetracycline, Ofloxacin, Neomycin, Nitrofurazone, Norflaxacin. Inhibition zone diameter was recorded after 48h incubation at 37°C.

***In vitro* virulence studies**

Presence of virulence factor in the isolates was ascertained by the Hemolytic test and Congo red binding assay. Hemolytic test was performed by supplementing basal medium with 5% sterile blood followed by incubation at 30ºC. The type of hemolysis and zone diameter was noted. 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.

RESULTS

The bacteria were isolated and identified based on colony morphology, growth and colour development in selective medium, gram staining and biochemical properties.

Colony morphology

Colony morphology of different isolates are studied on different selective media as described below:

On Pseudomonas Agar the colony of *P. aeruginosa* were large, convex, opaque, irregular, greyish white in colour and earthy smelling whereas the colony of *P. anguillaseptica* appeared large, convex, opaque and pale yellow colour in colour.

The colonies of *V. anguillarum* and *Vibrio* species are yellow in TCBS Agar while *V. Parahaemolyticus* is green in colour having raised centre. All are small, mucoid colonies.

The colonies of *A. hydrophilla* found to be round, convex, flattened and semi-translucent in nature on R-S medium where as in Aeromonas isolation medium they are appeared to be pinhead sized, round and green in colour.

Biochemical Properties

Pseudomonas aeruginosa was gram negative motile rod and utilised glucose, mannitol, arginine decarboxylase, oxidase, gelatinase, fluorescent pigment, and catalase. It did not utilise surcrose, D-xylose and starch.

Pseudomonas anguilliseptica was gram negative filamentous rod and was positive for citrate, arginine decarboxylase, gelatine hydrolysis, catalase and oxidase test where as it was negative for urase, nitrate reductase, sucrose, glucose, mannitol fermentation and O/F test.

From biochemical properties and gram staining it was revealed that *A. hydrophila* was gram negative slender rod and produced positive result for oxidase, catalase, VP, nitrate reductase, indole and did not utilise mannitol, lactose, sucrose and L-arabinose, lysine and arginine. All are found to degrade casein, gelatine and starch.

V. anguillarum (01) was gram negative comma shaped rod which produce oxidase, catalase, MR, arginine dehycarboxylase, nitrate reductase. They showed negative response to VP, ornithine and lysine decarboxylase.

V. anguillarum (02) was found different from *V. anguillarum* (01) in MR and VP.

Table 1. Biochemical characters of *Pseudomonas* sp

Test	P1	P2
Oxidase	+	+
Catlase	+	+
MR	+	+
urase	+	+
VP	-	-
O/F	O	O
Nitrate reductase	+	+
Arginine dehydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Acid from:		
D- xylose	+	+
Arabinose	-	-
Mannose	+	+
Galactose	+	+
Sucrose	-	-
Cellobiose	-	-
Mellibiose	+	+
Lactose	-	-
Salicin	-	-
Mannitol	+	+
Inositol	-	-
Production of:		
Amylase	+	+
Gelatinase	+	+
Lipase	+	+
Chitinase	+	+
Growth at		
4°C	-	-
20°C	+	+
30°C	+	+
37°C	+	+
42°C	+	+

P1- *Pseudomonas aeruginosa,* P2- *Pseudomonas angulliseptica*

Table 2. Biochemical characteristics of *Aeromonas* Isolates

Test	Organism		
	A1	A2	A3
Oxdase	+	+	+
Catalase	+	+	+
MR	-	-	+
VP	+	+	-
Indole	+	+	+
Urease	-	-	-
Nitate reductase	+	+	-
Sensitivityto Novobiocin	S	S	S
Arginine dehydrolase	+	+	+
Lysine decarboxylase	-	+	+
Ornithine decarboxylase	+	-	+
Production of:			
Amylase	+	+	+
Gelatinase	+	+	+
Lipase	+	+	+
Chitinase	+	+	+
Acid from:			
L-Arabinose	+	+	+
Socrose	+	-	-
Inositol	-	-	-
Mannitol	+	+	+
Lactose	+	-	+

V. angillarum (02) showed negative response to MR, ornithine and Lysine decarboxylase and positive response to VP, oxidase, catalase, nitrate reductase, arginine decarboxylase.

V. paraheamolyticus was gram negative rod showing positive to oxidase, catalase, MR, nitrate reductase, ornithine and lysine while it was negative to VP and arginine decarboxylase.

The *Vibrio* sp. had shown positive response to catalase, MR, VP, ornithine decarboxylase but had showed negative response to oxidase, nitrate reducatase, arginine and lysine decarboxylase. It was a gram negative rod.

Drug Sensitivity Test

Utilisation of disc diffusion method to determine drug sensitivity of pathogens revealed the following results.

Pseudomonas aeruginosa was found to be sensitive to cephalexin, co-trimaxozole, chloramphenicol, cephalothin, ciproflaxacin, ofloxacin, tetracycline, triomethoprime, nalidixic acid and norfloxacin while it was intermediate in response to cefuroxime and cloxacillin and were resistance to ampicillin, bacitracin, neomycin and penicillin-G. Out of twenty two antibiotics tested for the *Pseudomonas angulliseptica* ten were sensitive and eight were resistant and the remaining tested antibiotics were intermediate. However, erythromycin was seen to be highly sensitive among all the antibiotics.

Antibiogram table revealed that A1 strain of *A. hydrophila* was sensitive to twelve antibiotics and the remaining ten antibiotics were found either intermediate or resistant. The antibiotics oflaxacin showed highest sensitivity followed by co-trimaxozole and cephalaxin. A2 strain showed sensitivity to fourteen antibiotics and rest eight antibiotics were found either intermediate or resistant. The highest sensitivity was seen with oflaxacin followed by amoxycilin. Strain A3 showed sensitivity to amoxycilin, oflaxacin, cirpofloxacin followed by amikacin and tetracycline.

Vibrio paraheamolyticus was sensitive to twelve antibiotics and other were either intermediate or resistant. The zone of highest sensitivity was found in case of oflaxacin and erythromycin. *Vibrio anguillarum* (01) was resistant to nineteen antibiotics tested. Remaining antibiotic cephalaxin and chloro-tetracycline were intermediate and ciprofloxacin, tetracycline were highly sensitive where as *Vibrio anguillarum* (02) was resistant to cephalexin. Among the twenty-two antibiotics tested, six antibiotics were sensitive with ciprofloxacin showing highest zone of sensitivity. *Vibrio sp.* was sensitive to fourteen antibiotics and eight were either intermediate or resistant.

***In vitro* pathogenicity studies**

The *in-vitro* pathogenicity revealed that all the isolated showed positive response toward congo red binding assay except *Vibrio anguillarim* (02), where as it was found to heamolyse RBC of rabbit. Similarly, *Pseudomonas angulliseptica* showed negative response toward to haemolytic test and positive towards congo red test.

DISCUSSION

The isolation study revealed that the presence of fourteen bacterial isolates from four genera and some of the identified groups from shell region, hepatopancrease, gill, muscle tissue, pleapod of prawn. The different genera are

Table 3. Biochemical Characteristics of *Vibrio* Isolates

Test	Organisms			
	V1	V2	V3	V4
Oxidase	+	+	+	-
Catlase	+	+	+	+
MR	+	+	-	+
VP	-	-	+	+
O/F	F	F	F	F
Nitrate reductase	+	+	+	-
Arginine dehydrolase	-	-	+	+
Lysine decarboxylase	+	-	-	+
Ornithine decarboxylase	+	-	-	-
O/129 sensitivity	S	S	S	S
Acid from:				
D- xylose	-	-	-	-
Arabinose	+	+	+	+
Mannose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	-	-
Cellobiose	-	+	-	-
Mellibiose	-	-	-	-
Lactose	-	-	-	-
Salicin	-	-	-	-
Mannitol	+	+	+	+
Inositol	+	+	+	+
Production of:				
Amylase	+	+	+	+
Gelatinase	+	+	+	+
Lipase	+	+	+	+
Chitinase	+	+	+	+
NaCl tolerance	+	-	-	-
0%	+	+	+	-
1%	+	+	+	+
2%	+	+	+	+
4%	+	+	-	+
6%	-	-	-	-
7%	-	-	-	-
Growth on TCBS	G	Y	Y	Y

V1- *Vibrio paraheamolyticus*, V2- *Vibrio anguillarim (01)*,
V3- *Vibrio anguillarim (02)*, V4 - *Vibrio sp.*

Table 4. *In vitro* Pathogenicity of Different Isolates in Congo red binding Assay and Haemolytic test

Isolates	Congo red binding assay	Haemolytic test
Pseudomonas aeruginosa (P1)	+	+
Pseudomonas angulliseptica (P2)	+	-
Aeromonas hyrophilla (A1)	++	+
Aeromonas hyrophilla (A2)	++	+
Aeromonas hyrophilla (A3)	++	+
Vibrio parahaemolyticus(V1)	++	+
Vibrio anguillarum(01) (V2)	++	+
Vibrio anguillarum(02) (V3)	-	+
Vibrio sp. (V4)	+	+

Table 5. Antibiogram test of different isolates

Different antibiotics	Symbol	Disc Potency in mcg	R	I	S	P 1	P 2	A 1	A 2	A 3	V 1	V 2	V 3	V 4
Cephalexin	Cp	30	11	13-14	21	S	S	S	S	R	I	S	I	R
Co-Trimoxazole	Co	25	10	11-15	16	S	S	S	S	S	R	S	R	S
Chloramphenicol	C	30	12	13-17	18	S	I	I	S	S	S	S	R	R
Cefuroxine	Cu	30	14	15-17	18	I	R	R	R	I	R	R	R	R
Cephalothin	Ch	30	14	15-17	18	S	S	R	S	S	S	S	R	R
Ciprofloxacin	Cf	5	15	16-20	21	S	S	S	S	S	S	S	S	S
Chlorotetracyclin	Ct	30	14	15-18	21	S	I	S	S	S	S	S	I	I
Amoxycillin	An	30	13	14-17	18	S	R	S	S	S	R	S	R	R
Amikacin	Ak	30	14	15-16	17	S	S	S	R	S	S	S	R	S
Ampicillin	A	10	13	14-16	17	R	R	R	R	R	R	R	R	R
Bacitracin	B	8	9-12	13		R	R	R	R	R	R	R	R	R
Erythromycin-15	E-15	15		14-22	23	S	R	S	S	S	S	S	R	R
Naldixic Acid	Na	30	13	13	19	S	S	R	S	S	S	S	R	S
Norfloxacin	Nx	10	12	13-16	19	S	I	R	I	S	S	I	R	R
Neomycin	N	30	12	13-16	17	R	R	R	R	R	I	R	R	I
Gentamycin	G	10	12	13-14	15	S	S	S	S	I	S	S	R	R
Tetracycline	T	30	14	15-18	19	S	I	S	S	S	S	S	S	R
Trimethoprim	Tr	25	10	11-15	16	S	S	S	S	S	R	S	R	R
Ofloxacine	Of	2	12	13-15	16	S	S	S	S	S	S	S	S	S
Penicillin G	P	2	19	22-27	28	R	R	R	R	R	R	R	R	R
Cloxacillin	Cx	10	19		20	I	S	S	S	R	S	R	R	R

P1 - *Pseudomonas aeruginosa*, P2 - *Pseudomonas angulliseptica*, A1, A2 and A3 - *Aeromonas hydrophilla*, V1 - *Vibrio paraheamolyticus*, V2 - *Vibrio sp*., V3 - *Vibrio anguillaarum (01)*, V4 - *Vibrio anguillaarum (02)* R- Resistant, I- Intermediate, S-Sensitive.

Aeromonas, Pseudomonas, Vibrio, Staphylococcus, which are probable pathogens of prawn. Many workers included *A. hydrophilla* as a pathogen of prawn (Cook and Lofton, 1978). Kanaujia et *al.* (1998) reported the presence of *Pseudomonas* in hatchery reared post larvae of *Macrobrachium malcomsonii*. *Pseudomonas aeruginosa, Vibrio anguillarum* found to cause shell diseases in hatchery condition in *Macrobrachium rosenbergii* (Jaysree et *al.,* 2000). Anderson *et al.* (1989) reported eighteen genera of bacteria in the hatcheries of *Macrobrachium rosenbergii* in Malaysia, out of which *Aeromonas and Vibrio* encountered in high percentage. Bacteria belonging to genera *Aeromonas, Vibrio, and Pseudomonas* were isolated from black spot diseases in *Macrobrachium rosenbergii* (Lombardi and labao, 1991a, 1991b).

Vibrio species exist as normal flora in fish and shell-fish, but has also been recognized as an opportunistic pathogen in many marine animals (Austin & Austin 1993). Jayprakash *et al.* (2006) revealed an association of this genus with *M. rosenbergii*. The strains were motile, oxidase and catalase positive, Gram-negative, comma-shaped rods that reduced nitrate to nitrite, grew in TCBS agar medium and was sensitive to O/129, the vibriostatic agent (West & Colwell 1984).

The morphological features and the biochemical profile of the isolated strains (P1, P2) revealed that these were gram negative rods, catalase and oxidase positive, non spore forming, utilized glucose oxidatively and reduce nitrate to nitrite (Stainer *et al*. 1996) and produce a brown pigment called pyocyanin (Cowan and Steel, 1993). All the isolates showed typical biochemical characterization with slightly differences in their sugar utilization tests.

All the species of *Vibrio*, *Pseudomonas*, *Aeromonas* were found to be urease positive and the ability to hydrolyse urea has been proposed as a simple screening test to predict which strain is pathogenic (Kaysner *et al.,* 1994). From the in vitro pathogenicity study, it was found to be haemolytic and showed a positive response to the Congo red binding assay, which suggests that it is a pathogen, because the pathogenic strains are haemolytic in nature (Joseph *et al*., 1982). Jana (2000) studied the haemolytic characteristics of *V. parahaemolyticus* isolated from the Penaeus monodon. Further, the ability to hydrolyse gelatin, which represents another characteristic towards pathogenicity and this corroborates with our recent findings. Owing to the presence of protease enzymes, it can hydrolyse gelatin along with casein, elastin, collagen and haemoglobin (Jana 2000; Marhaul 2005).

Bauror *et al.* (1966) suggested that the choice of antibiotics to be tested should be depend on the type of practice of the laboratory and the local preference for a particular agent.

Multiple resistance is most common among *A. hydrophila* (Chang and Bolton, 1983; Motyl *et al.,* 1985; Ansary *et al.,* 1992) found that rimpaficin is found most sensitivity against *A. hydrophilla*. The present study shows that among the antibiotic tested against *A. hydrophilla* cophelaxin, co-trimazole, chloram phenicol, oflaxacin, Amikain, Erythromycin-15, Nalidixic acid, Tetracycline and trimethoprime are sensitive. Oflaxacin showed maximum sensitivity among all the antibiotics.

Drug sensitivity tests revealed that all the *Vibrio* isolates are highly sensitive to chloramphenicol which corroborates with Delan Pan *et al.* (1993) who reported that tetracycline and oxytetracyclne are less effective drugs for *Vibrio* species and found chloramphenicol to be most effective, but according to Devesa *et al.* (1985) oxytetracycline is intensively used is both hatcheries and grow-out condition and its effectiveness in treatment of vibiosis has been indicated. Further, from our investigation it was found that ciprofloxacin shows highest sensitivity against *V. anguillarum* and tetracycline was sensitivity to *V. parahaemolyticus.* Skyes and Mathew (1976) reported that *P. aeruginosa* was sensitive to β-lactin and resistant to carbecillin. Gaman *et al.* (1976) and Lowbury *et al.* (1969) reported that *P. aeruginosa* was resistant to carbencillin, penicillin. Our studies revealed that *P. aeruginosa* was mostly sensitive to tetracycline, chloramphenicol, co-trimaxozole and norfloxacin while resistance to ampicillin, neomycin and pencillin G. The resistivity of *Pseudomonas aeruginosa* to a number of antimicrobial agents is due to presence of aminoglycosidase-modifying enzymes (Carmeli *et al*. 1999).

CONCLUSION

The giant freshwater prawn has been widely domesticated in India for over a decade. Mortalities are encountered due to bacterial diseases in all its life stages, for which farmers have experienced substantial losses in the recent years. The detection and screening of pathogens and subsequent treatment is highly important. In this regard, this study has immense importance for the development of prawn culture in South east Asian countries.

ACKNOWLEDGMENTS

We would like to thank The Director and Aquatic Animal Health Division, Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Orissa for their kind permission to carry out the investigation and The Microbiology Department, Orissa University of Agriculture and Technology for their support. The authors are thankful to the rural farmers for their timely and cordially support in the collection of the sample.

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