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Virulence and Histopathology of *Vibrio anguillarum* like (VAL) Bacterium Isolated from Hatchery Produced Juveniles of *Lates calcarifer* (Bloch)

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Abstract

Hatchery produced Asian sea bass *Lates calcarifer* (Bloch) juveniles (120 days post hatch) maintained in a fish hatchery, Central Institute of Brackishwater Aquaculture (CIBA), were used in the present study. Diseased and moribund fish showed haemorrhages at the bases of dorsal, pectoral and anal fins. The affected fish stopped taking food. Bacteria isolated from the aseptic kidney samples drawn from the moribund fish belonged to the genus *Vibrio* as evidenced through selective growth on TCBS agar. Colony morphology, growth and the biochemical characteristics suggested that the bacterium was *Vibrio anguillarum*-like (VAL). This VAL bacterium, injected at varying doses in *Liza macrolepis* and *Oreochromis mossambicus* revealed LD_{50} end points of $10^{4.17}$ and $10^{5.47}$ CFU, respectively. Exposure of *L. calcarifer* juveniles to graded levels of virulent bacterium via injection and bath showed that a cell density of $10^{4.5}$ CFU/fish and $10^{6.3}$ CFU per ml, respectively, can kill half of the exposed population. Investigations carried out on the histopathology of the infected fish indicated severe necrotic degenerative changes in the gills with mild to extensive lamellar fusion and hyperplasia of naturally infected seabass juveniles, while that of the experimentally infected fish showed no discernible changes. Natural infection showed wide spread chronic histopathological manifestations while, the experimental infection accounted for acute phase pathogenesis – related manifestations.

Introduction

Disease due to Vibriosis has been a major cause of concern in brackishwater and marine aquaculture systems. Development and expansion of aquacultural activities have resulted in the enhancement of vibriosis associated fish kills. Annual losses due to this disease in Japan alone, were

reported to have accounted for £11 million (Smith 1988). *Vibrio* spp was responsible for a phenomenal 67.8% of the bacterial diseases of gilt-head seabream, collected from south-western Spain during 1990-1996 (Balebona et al. 1998). Vibriosis is a serious problem in farmed seabass (*L. calcarifer*) in Singapore (Cheong et al. 1983), Thailand (Chinabut and Danayadol 1983) and Australia (Glazebrook and Campbell 1986). First success in India, of induced maturation and breeding of the Asian seabass was achieved by the Central Institute of Brackishwater Aquaculture in 1997. Since then, the species has attracted much attention of the coastal aquaculturists in India. Identification of potential pathogens of farmed seabass is of immense importance. Isolation, identification, characterization, virulence and histopathology of infection of *Vibrio* spp. isolated from farm produced Asian seabass (*L. calcarifer*) are discussed in this paper. Tilapia and mullet are among the important brackishwater species as forage and food fish. Hence, susceptibility of these species to varying doses of exposure to VAL bacterium was tested and histopathologies of experimental and natural infection in *L. calcarifer* are discussed in the present paper.

Materials and Methods

Fish

Juveniles (120-150 days post hatch) of *L. calcarifer* produced by CIBA and maintained in filtered and aerated sea water (30-32 ppt) at the experimental station of CIBA, Muttukadu, Chennai, were used in the present study for the isolation of the bacterium. *L. macrolepis* (10 to 12 g) and *O. mossambicus* (12 to 15 g) were collected from the Muttukadu lagoon. The fish were maintained separately in 200 litre fiberglass tanks containing filtered and aerated seawater.

Bacterium

Infected moribund juveniles of sea bass, showing hemorrhagic body surface, were sacrificed and the kidney samples drawn aseptically were used for obtaining the bacterial cultures on Zobell's marine agar (ZMA) and TCBS agar.

Colony morphology and staining properties

Aseptic samples from the kidney plated on ZMA were used for studying the colony morphology and the selected colonies grown on TCBS agar were used for Gram staining, growth characterization and studying the biochemical properties of the isolate following standard bacteriological methods (Krieg and Holt 1984). Though five isolates were obtained, all of them showed identical colony, staining, motility and salt-loving characters. The isolates were thus termed *Vibrio anguillarum*-like (VAL).

Passage

These isolates, so characterized, were grown in saline tryptone soy broth (STSB), at a final salt concentration of 3% NaCl (w/v), for 24 to 36 hours at room temperature (33-34 °C). The cells were harvested by spinning at 10,000 rpm for 10 min and washing the pellet with sterile phosphate buffered saline, PBS (pH 7.2). Resuspension, centrifugation and washing were repeated (2x) to obtain a final bacterial suspension of 10^8 CFU/ml. The cell count was confirmed through spread plate assay. Each of the bacterial suspensions (0.1 ml having 10^7 cells) was injected to five juveniles of seabass. Injected fish were kept under observation for 7 to 10 days. Moribund fish were sacrificed for plating aseptic kidney samples on TCBS agar. A single type of the colony on TCBS was noticed and the bacterium so obtained was maintained for further investigations.

Bacterial challenge and LD_{50}

Five different dose regimes (10^3 to 10^8 CFU/animal) were used to arrive at lethal doses for 50% of the challenged fish. A preliminary trial to arrive at a rough LD_{50} dose was carried out and the results were used in arriving at the dose regimes. Bacterium grown in STSB as mentioned above was used in the challenge studies with *O. mossambicus, L. macrolepis* and *L. calcarifer* as the test animals for injection challenges. Juveniles of *L. calcarifer* from the hatchery of CIBA were challenged (immersion) with bath suspensions (10^4 to 10^8 CFU/ml) of bacterium. Twelve individuals (6 per replicate) per dose were challenged through intra-muscular injection or immersion. The fish were given intra-muscular injection (at the base of the dorsal fin) with 0.1 ml of the bacterium. Challenged animals were kept under observation for 7 to 10 days. Specific mortalities were recorded by plating the aseptic samples of kidney/blood of the moribund animals from the challenge trials on TCBS agar and showed the presence of yellow colonies. Samples from fish showing the presence of the yellow colonies on TCBS were considered positive and the related mortalities were counted as specific mortalities. Cumulative mortalities were used in computing the LD_{50} values following Reed and Muench (1938). A total of 60 juveniles of *L. calcarifer* with 20 fish each in immersion and injection challenge groups and 20 fish in control groups were used. Sublethal doses of 10^3 CFU/fish and 10^5 CFU/ml were used in the injection and immersion infection studies. A control group of 20 fish was used for comparing the histopathological manifestations due to experimental infection.

Histopathology

Infected and moribund juveniles, from both natural and experimental infections, of *L. calacrifer* were used for studying the histopathology of infection. Bacteria, isolated from moribund fish from challenge experiment, were grown in STSB and a suspension of 10^7 cells per ml was prepared as

explained above. Moribund fish from the experimentally infected groups were decapitated by giving a cerebral blow, belly slit and fixed in 10% buffered formalin. Two changes with fresh fixative were given at 24 and 48 h of initial fixation. Tissue samples from gills, liver, pro and mesonephros, heart and spleen were processed in graded levels of alcohol, cleared in xylene, impregnated and embedded in paraffin wax. Trimmed blocks of the embedded hard tissue (gills) were treated, overnight, with 0.5% trichloroacetic acid (TCA) as a decalcification process. Decalcified tissue was thoroughly washed in running water and sectioned (5 to 6 µm). Deparafinized sections were then rehydrated in graded alcohol, stained with H & E, dehydrated, cleared in xylene and mounted using DPX. Similar tissue preparations were made for the control group of fish, sampled simultaneously with that of experimental groups. Stained sections were observed under a compound microscope (Nikon) and the observations were recorded and photographed.

Results

Naturally, infected juveniles of *L. calcarifer* were sluggish with little or no escape refluxes and anorhectic. Most of the infected fish had hemorrhagic snout, fin bases and vent. However, distension of the abdomen was not consistent. All the primary cultures from the kidney and blood samples grown on ZMA were nonswarming colonies with serrated margins, while typical yellow colonies were observed on TCBS agar suggesting that the bacterium belonged to the genus *Vibrio*. Biochemical and growth characters of the isolate (Table 1) revealed that the bacterium was a VAL pathogen. The VAL bacterium is related to *V. anguillarum* in many of the characters and a standard reference isolate was not available for comparison hence, the isolate was termed *V. anguillarum*-like (VAL) bacterium. Antibiotic sensitivity tests conducted on the isolate revealed that the VAL bacterium was resistant to a variety of antibiotics such as penicillin, oxytetracyclin, bacitracin, ampicillin, tetracycline etc., and sensitive to furazolidon, norfloxacin, cotrimazine, chloramphenicol, and gentamycin.

Virulence of VAL bacterium was the highest in *L. macrolepis* followed by *L. calcarif*er and *O.mossambicus*. The LD_{50} values (Table 2) for the tested animals were $10^{4.17}$, $10^{4.5}$ and $10^{5.47}$, respectively. Immersion challenge of *L. calcarifer* revealed that the bacterium at a concentration of $10^{6.3}$ CFU per ml of the bath medium could cause mortality of 50% or more of the challenged fish. The bacterium was proved to be lethal to the juveniles at much lower concentration (LD_{50} = $10^{4.5}$°) when injected via intramuscular routes. These studies are indicative of the virulence of the pathogen in different fish species.

Examinations on the histopathology of different organs of naturally and experimentally infected *L. calcarifer* were carried out. The results indicate severe necrotic degenerative changes in the gills with mild to extensive lamellar fusion and hyperplasia of naturally infected seabass juveniles, while that of the experimentally infected fish showed no discernible changes

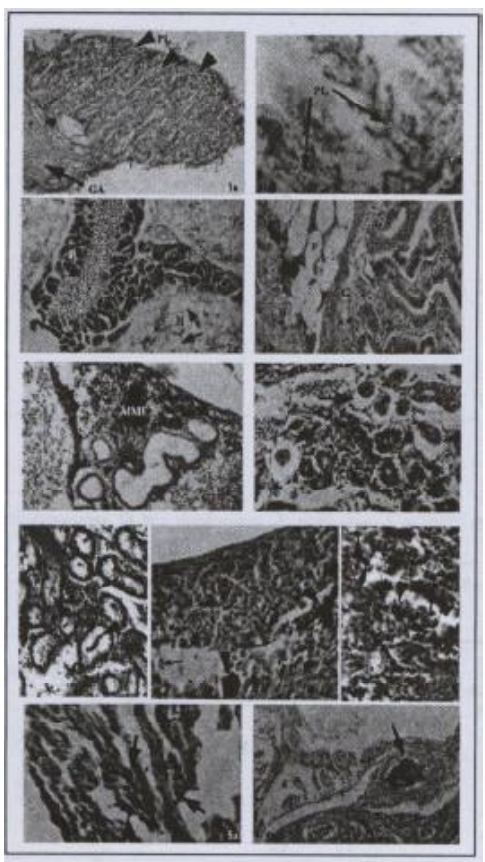
Table 1. Colony characters, growth conditions and biochemical properties of VAL bacterium isolated from *L. calcarifer*

Reactions for the species	VAL bacterium from *C. calcarifer*
1. Arginine dihydrolase	+
2. Lysine decarboxylase	-
3. Ornithine decarboxylase	-
4. Growth at 0 % NaCl	-
5. Growth at 3% NaCl	+
6. Growth at 8% NaCl	+
7. Growth at 10% NaCl	-
8. Growth at 0°C	
9. Growth at 20°C	+
10. Growth at 30°C	+
11. Growth at 35°C	+
12. Growth at 40°C	+
13. Citrate	+
14. Gelatinase	+
15. Glucose gas	-
16. Luminescence	-
17. NO_2	+
18. ONPG	+
19. Oxidase	+
20. O/F	+/+
21. Gram's staining	Gram negative
22. Swarming/motility	-/+
23. Urease	-
24. Voges-Proskauer	+
25. D-glucosamine	+
26. D-glucose	-
27. Lactose	-
28. Melibiose	-
29. Acid from Arbutin	-
30. Acid from Inositol	-
31. Acid from Mannitol	+
32. Acid from Salicin	-
33. Acid from Sucrose	+
34. Sensitivty to Amp icillin 10mcg	-
35. Sensitivity to O-129 150mcg	+
36. Tween 20	+
37. Tween 80	+
38. Chitin	+
39. L-Histidine	+
40. Glycerol	+
41. Lipase	-

Table 2. Fifty percent end point lethality (Reed and Muench 1938) in *L. calcarifer*, *L. macrolepis* and *O. mossambicus* challenged with VAL bacterium isolated from *L. calcarifer*

Bacterial Dose CFU/ml	*L. macrolepis*			*O. mossambicus*			*L. calcarifer*					
							Injection Challenge			Bath challenge		
	No. Chal-lenged	% Morta-lity	LD_{50}	No. Chal-lenged	% Morta-lity	LD_{50}	No. Chal-lenged	% Morta-lity	LD_{50}	No. Chal-lenged	% Morta-lity	LD_{50}
10^8	-	-	$10^{4.17}$	12 (11)	92	$10^{5.47}$	12	83	$10^{4.5}$	12	83	$10^{6.3}$
10^7	12 (12)	100		12 (8)	67		12	67		12	67	
10^6	12 (9)	75		12 (7)	58		12	58		12	33	
10^5	12 (8)	67		12 (6)	50		12	42		12	25	
10^4	12 (7)	58		12 (3)	25		12	42		12	0	
10^3	12 (3)	25		12 (12)	0		12	17		-	-	

(Figs. 1a and 1b). Necrotic changes in the hepatic tissue resulted in 'honey-comb' vacuolation in the naturally infected seabass juveniles while experimental infection did not produce any marked histopathological manifestations in the tissue (Figs. 2a and 2b). However, fatty degenerative changes in the pancreatic acinii were noticed in this case. Tissue sections from the pronephros (Fig. 3a) revealed infiltration of macrophages and aggregation of melano-macrophage centers (MMC) in both cases while, the meso and opisthonephric regions in the naturally infected fish showed highly degenerative tubular necrosis and hematopoietic hyperplasty which was not evident in the experimentally infected seabass (Figs. 3b and 3c). Depletion of the cells and oedema of the splenocytes were noticed in the spleen of the naturally infected *L. calcarifer* (Fig. 4a), as compared to thickened ellipsoids and proliferation of MMC in the experimental infection (Fig. 4b). Swelling of the endothelial macrophages of the ventricle (Fig. 5a) was evident from the tissue sections of naturally infected seabass, while, atrio-ventricular septum showed the presence of bacterial colony enmeshed in fibroblast inflammatory cells (Fig. 5b) in the experimentally infected fish.



Figs. 1-5. Various histopathological manifestations in the vital organs of naturally and experimentally infected *L. calcarifer*. Hematoxylene and Eosin stained.
1a: Extensive lamellar hyperplasty resulting in complete fusion primary lamellae (arrow heads primary lamellae) in naturally infected sea bass, 20 x; 1b: Limited alteration in the gill architecture (arrows PL) following experimental infection with VAL bacterium, 20 x; 2a: Natural infection by VAL bacterium resulting in honey-comb vacuolation of the hepatocytes (arrows) pancreatic tissue (P) is apparently normal, 40 x; 2b: Fatty degeneration of the pancreatic acinii, normal looking hepatocytes (H) and adjoining gut tissue (G), 40 x; 3a: Pronephric tissues of naturally infected seabass show aggregation of MMC with extensive tubular degeneration, 40 x; 3b and c: reflect on the oedamatous tubular degeneration (arrows), hyperaemia (thin arrows) and sloughing in the tubular lumen of experimentally infected sea bass, 40 x; 4a: Spleen of the naturally infected seabass showed pyknotic splenocytes (thin arrows), MMC aggregates (arrow heads) and depletion of splenocytes (thick arrow), 40 x; 4b: Spleen of the experimentally infected sea bass show similar manifestations, 40x; 5a: Ventricular myocardial macrophages show swelling (arrow) in naturally infected sea bass; while, 40 x; 5b shows enmeshed bacterial colony in the septum between the atrium (A) and the ventricle (V), 20 x.

Discussion

Presence of ubiquitous bacteria belonging to the genus Vibrio, *Vibrio anguillarum* in particular, in wild and cultured fish species has been recorded by several workers (Cisar and Fryer 1969; Levine et al. 1972; Hastein and Holt 1972; McCarthy 1976; Devesa et al. *1*984*;* Fouz et al. 1995; Balebona et al. 1998). The isolation of VAL bacterium from the Asian seabass speaks on the ability of the bacterial genus in infecting a range of hosts. The disease was noticed during the late larval stages of 25 to 60 days post hatch (dph). Biochemical and genotypic differences between *V. anguillarum* strains isolated from different hosts in various parts of the world led to many proposals for new groups (Roberts 1978). The VAL bacterium isolated from the Asian sea bass showed a little deviation in its biochemical characters from those defined for *V. anguillarum* (Larsen et al. 1994). Though the aim of the present investigation was to study the virulence and pathogenicity of the isolate, thorough characterization of the isolate and its serotyping can help in understanding the epidemiological importance of the species. Similar relatedness of vibrio isolates has been reported by Santos et al. (1997), who recorded *V. anguillarum* related bacterium from turbot and cod. Some of the *Vibrio* spp. phenotypically related to *Vibrio anguillarum* were termed VAR organisms by Myhr et al. (1991). Riquelme, Hayashida, Toranzo, Vilches, and Chavez, (1995) working on the *V. anguillarum*-related (VAR) strain of bacteria in causing epizootics in *Argopecten purpuratus* larvae, opined that the scallop larvae suffered due to this bacteria even at low temperatures. Larsen et al. (1994) showed that out of a total of 517 *V. anguillarum* strains isolated from diseased fish, 4.3% were nontypable. Thus, the isolate obtained in the present study could be considered as one among the *V. anguillarum* – like pathogens.

The VAL bacterium was found to resist the action of several antibiotics suggesting on a probable exposure of the bacterium to some of the tested antibiotics. Pathogenic bacteria have been known to develop drug related resistance (Roberts 1978) and R-factor plasmid responsible for multiple antibiotic resistance (Aoki et al. 1981). Resistance of *Vibrio* spp. isolated from fish farms in northeast Spain to oxalinic acid and nitrofurantoin was attributed to the previous use of these drugs in those farms (Toranzo et al. 1993). Vibrio isolates from diseased shrimp were resistant to oxytetracyclin, the more frequently used antibiotic in aquaculture systems of Thailand (Chanratchakool et al. 1995; Ruangpan and Kitao 1991).

Higher lethality due to injection challenge was due to the direct access of the bacterium to the circulatory system. Considering that immersion could result in a very low percent of the bacterial population gaining entry into the fish (Tatner and Horne 1993), it can be said that the bacterium could cause potential threats to the cultured seabass. *V. anguillarum* related (VAR) strain, isolated from turbot and cod was equally virulent in trout, recording LD_{50} ranging from 8.4×10^3 to 7×10^5 cells (Santos et al. 1997). *V. anguillarum* isolated from turbot, cultured in northwestern Spain, was virulent in both turbot and rainbow trout (LD_{50} of 7×10^4 and 8×10^2, respectively). Similar studies by Balebona et al. (1998) showed that a vibrio isolate from gilt head sea

bream was highly virulent registering LD_{50} values ranging from 10^4 to 10^8 CFU/g body weight. They reported that 62.5% of the *vibrio* strains isolated from the gilthead sea bream were highly virulent. Our results show that the VAL bacterium can become a potential threat to the hatchery production of the Asian seabass directly or through mullet and tilapia.

Juveniles of *L. calcarifer* from naturally infected groups showed typical clinical signs of V*ibriosis* such as hemorrhagic scale pockets and fin bases. However, few of the experimentally infected fish showed microhemorrhages on the snout. Experimentally infected mullet showed that the isolate could cause severe necrotic changes at the site of injection. Several species of *Vibrio* are known to produce extracellular proteases, esterases and hemolysins that assist bacterial invasion, colonization and pathogenesis (Sedano et al. 1996). Pathological manifestations in the vital organs of the experimentally infected seabass that showed oedematous separation of the tubules and necrotic degeneration in splenic tissue are comparable to those recorded by Diggles et al. (2000). Loss of appetite and hemorrhages are some of the common features of *Vibriosis*. Pathological manifestations in the gills of the naturally infected seabass were much more severe than those observed in the experimentally infected fish followed by the mullet which, was infected by injection challenge. External necrotic changes were more pronounced in the mullet as a result of injection infection. These results are clear indications of probable infection by the VAL bacterium through invasion of the mucosal barriers in the seabass. Excessive lamellar fusion, hyperplasty of the oropharyngeal epithelium and highly degenerative oedamatous tubular necrosis in naturally infected moribund seabass are the consequences of invasion by the VAL bacterium through theses routes.

Hemorrhagic necrosis of tubular interstitial cells of the kidney in the experimentally infected mullet and seabass was contrastingly different from the pathological changes observed in the naturally infected seabass, which showed highly depleted tubular inerstitium and extensively sloughed tubular lumen. Horne et al. (1977) and Fouz et al. (1995) recorded similar *Vibrio* associated pathological changes in the kidney and liver of turbot (*Scophpthalmus maximus*). Oedema of the kidney tubules, depletion of cells in the tubular interstitium and hyperaemia were the major histopathological manifestations in the kidney of the naturally and experimentally infected seabass. Ransom and co-workers reported similar results with *V. anguillarum* showing the capacity of invading, proliferating and destroying the kidney of the host (Ransom et al. 1984). It was clear from the observations that the natural infection resulted in wide spread chronic histopathological manifestations in the vital organs while; the experimental infection accounted for acute phase pathogenesis-related manifestations.

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