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Pathogenicity and antibiotic susceptibility of *Vibrio* species isolated from the captive–reared tropical marine ornamental blue damsel fish, *Pomacentrus caeruleus* (Quoy and Gaimard, 1825)

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Microbiological assessment of vibriois infected blue damsel, *Pomacentrus caeruleus* reared in captivity in marine aquaria led to isolation of five distinct Vibrio species. Cultural, morphological and biochemical characteristics of these isolates identified them as *Vibrio alginolyticus* (29.4%), *V. vulnificus* (26.8%), *V. fluvialis* (15.3%), *V. pelagius* (9.1%) and *V. anguillarum* (19.4%). Predominant *Vibrio alginolyticus* and *V. vulnificus* were tested for pathogenicity and Koch postulate by experimentally infecting apparently healthy blue damsels, *P. caeruleus*. The lethal dose (LD_{50}) was 1.36×10^6 and 3.44×10^6 CFU/g fish (colony forming units) for *V. alginolyticus* and *V. vulnificus* respectively. All the vibrios were highly susceptible to the broad spectrum antibiotics Chloramphenicol (30 µg/disc), Erythromycin (15 µg/disc), Gentamycin (30 µg/disc) and Oxytetracycline (30 µg/disc).

[**Keywords**: Vibrios, Blue damsel fish, Pathogenicity, Antibiotic sensitivity]

Introduction

Damsel fishes grouped under the family Pomacentridae occupy the reef communities with about 350 species distributed all over the world[1,2] and 41 species were recorded from Indian waters[3]. Like other captive organisms, aquarium fish are vulnerable to a range of diseases. Incidence of microbial pathologies, mainly bacterial in origin are triggered by stress such as overcrowding, excessive noise, aggression from other fish, poor water quality and changes in temperature or water chemistry. In comparison to the food fish, the disease resistance situation for ornamental fish is very unfavorable[4]. Majority of bacterial fish pathogens are natural inhabitants of the aquatic environment and almost all are capable of living independently away from the fish host[5].

Vibriosis is one of the most serious bacterial diseases in cultured marine fish worldwide[6,7]. Species under the genus Vibrio of the family Vibrionaceae, are Gram-negative facultative anaerobes, short to medium, coma-shaped rods found in fresh, estuarine and marine ecosystems. Vibriosis is a serious threat to the aquaculture industry, and responsible for massive mortality of cultured finfish and shellfish worldwide[8,9]. Vibrios are dominant bacteria in seawater aquarium constituting 60% of total heterotrophic bacteria and are opportunistic pathogens[10]. Many disease problems of ornamental fish begin as external infections. If uncontrolled, the infections may become systemic, resulting in death of the fish. To control vibriosis and other bacterial diseases, antibiotics and chemotherapeutic agents are commonly used in aquaria and holding facilities.

The present study was carried out in the Marine Aquarium of the Central Marine Fisheries Research Institute, Vizhinjam, Kerala, India for a period of one year (November 2006 to October 2007). This is an attempt to characterize the most prevalent pathogenic *Vibrio* sp., their pathogenicity by experimentally infecting the apparently healthy group of fishes and to evaluate antibiotic sensitivity towards commonly used broad spectrum antibiotics.

Materials and Methods

Captive reared blue damsel fishes from Marine Aquarium of the Central Marine Fisheries Research Institute, Vizhinjam were used in the present study for the isolation of the bacterium. Fishes were maintained in 1 ton fiberglass tanks containing filtered and

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aerated sea water (30-32 ppt). They were fed with marine ornamental fish feed at the rate of 5% of body weight per day.

Bacteria were isolated directly from the external lesions, deep ulcers on the body surface, severe fin erosions and hemorrhages of infected blue damsels, *Pomacentrus caeruleus* on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar medium (Hi Media) supplemented with 2% NaCl. Plates were incubated at 30°C for 24 to 48 h. On the basis of morphological features, colonies were randomly picked and the isolates were purified by sub-culturing, inoculated on slants as pure culture and stored at 4°C for biochemical characterization and other tests. The biochemical tests were conducted as per the diagnostic scheme for *Vibrio* species given in the Bergey's Manual of Determinative Bacteriology[11].

Motility was evaluated by stab inoculating the 18 h old cultures into sterile motility medium in test tubes and observing after 24 h. Cytochrome oxidase was detected using sterile disc impregnated with N'N'N' tetramethyl diamine dihydrochloride and immediate appearance of purple colour was taken as a positive result. The presence of catalase was tested with hydrogen peroxide and appearance of effervescence within one minute was recorded as positive. Indole formation, citrate utilization, Hugh and Leifson's oxidation fermentation test, MR-VP test were also performed as per the standard protocol. Sodium chloride tolerance was tested ranging from 0% to 10%. Amino acid decarboxylase tests were carried out using decarboxylase broth base supplemented with 1.0% of corresponding test amino acid (Arginine, Lysine, and Ornithine) and incubating at 37ºC for 4 days. Color change to purple (alkaline) indicated a positive test whereas colour change to yellow (acid) indicated negative test for decarboxylation. For sugar fermentation, test sugar such as glucose, sucrose, lactose, arabinose, mannnose, mannitol, sorbitol, inositol and cellobiose @ 1.0% was added to Andrade peptone water. Durham's tube was placed and the test culture was inoculated and incubated at 37ºC for 24 h. Acid production and gas formation in Durham's tube were recorded.

The presence of urease was detected by inoculating cultures into urease broth, followed by incubation at 37ºC. Nitrate reduction was determined by nitrate broth and H_2S production using sterile lead acetate strips. Darkening of strips indicated the production of hydrogen sulphide. Production of gelatinase was tested with basal medium supplemented with 0.4% (w/v) gelatin on which acidic mercuric chloride was flooded and the zone of liquefaction was recorded. Starch hydrolysis was determined using starch agar and flooding the plates with Lugol's iodine solution and formation of clear zone around the inoculation indicated a positive test. Sensitivity to O/129 was performed using sterile discs of O/129 (2, 4–diamino 6, 7–diisopropyl pteridine phosphate) of 10 µg and 150 µg and the sensitivity was measured as area of inhibition of growth around the discs.

Antibiotic sensitivity pattern of the isolates was determined by the standard disc diffusion technique[12]. For this, 18 h old bacterial suspensions were swabbed on to the preset Muller Hinton Agar plates supplemented with 2% NaCl using sterile cotton swabs. Plates were then left to dry for 10 min before placing the antimicrobial sensitivity discs. Antibiotic impregnated discs (6 mm diameter, Hi Media) were placed aseptically on the seeded plates and incubated for 24 h at 30ºC. After incubation, the diameter of the zone of inhibition was measured and compared with zone diameter interpretative chart (Hi Media Ltd., Mumbai, India) to determine the sensitivity of the isolates towards the antibiotics. Antibiotic discs such as Ampicillin (25 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamycin (30 µg), Kanamycin (30 µg), Neomycin (10 µg), Novobiocin (30 µg), Oxytetracycline (30 µg), Streptomycin (10 µg) and Tetracycline (30 µg) were used.

For determining the virulence of *vibrio* sp. Isolated from captive reared blue damsel (*Pomacentrus caeruleus*), healthy blue damsel fishes were collected from Vizhinjam coast by using traps at depths ranging from 2 to 3 meters (Lat.9º'N – Long. 76º'E). They were acclimated for 10 days in 1 ton stocking tank at Marine aquarium. During acclimation, the ambient hydrological conditions viz., salinity: 33±2 ppt, temperature: 30±2ºC, *p*H: 7.8±0.5, dissolved oxygen: >5 ppm were recorded. Test was performed in 100 L aquaria. Each aquarium was filled with 75 L filtered seawater and introduced with 10 fishes with an average body weight of 3.0±0.5 g. Fishes were acclimated for 7 days before administration of live bacterial cells. They were fed with marine ornamental fish feed at the rate of 5% of body weight per day.

The test inocula were prepared as follows: The isolates were grown in nutrient broth at a final salt

concentration of 2% NaCl (w/v), for 18 h at 30±2°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min. The pellet was washed with normal saline (0.85%). Re-suspension, centrifugation and washing were repeated twice to obtain a final bacterial suspension of 10^7 CFU/mL. Suitable dilutions were made to obtain different doses of inocula and the viable cell count was confirmed by spread plate assay. Three different dose regimens (10^7 to 10^5 CFUs/fish) were used to derive the 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. Fishes were injected via intra-peritoneal route with 0.1 mL volume of live cells of *V. alginolyticus* or *V. vulnificus* strains. Control group of fishes were injected with 0.1 mL normal saline. The challenge experiments were done in triplicates. The fishes were then observed for a period of 5 days for recording survival, behavioral abnormalities or disease conditions. Dead and moribund fish were removed and subjected to standard bacteriological and pathological examination. Mortalities were considered to be caused by the organism only if it was recovered as dense pure culture growth from the internal organs of freshly dead or moribund fish. The median lethal dose required for 50% mortality (LD_{50}) was calculated using the probit method described by Miller and Tainter[13].

Results

A total of 14 bacterial isolates of different morphological characteristics were isolated from the infected and moribund fishes. All these isolates were Gram negative, motile, produced cytochrome oxidase and exhibited catalase activity. They were facultative anaerobes, capable of both fermentative and respiratory metabolism. Biochemical characterization distinguished 5 species as: *Vibrio alginolyticus* (29.4%), *V. vulnificus* (26.8%), *V. fluvialis* (15.3%), *V. pelagius* (9.1%) and *V. anguillarum* (19.4%). Results of biochemical characterization of bacterial isolates are presented in Table 1.

Vibrio vulnificus colonies were green on TCBS agar plates and the other identified *Vibrio* sp. formed yellow colonies. Difference in NaCl tolerance was also exhibited among various *Vibrio* strains. *Vibrio alginolyticus* and *V. fluvialis* tolerated up to 10% NaCl whereas *V. vulnificus*, *V. pelagius* and *V. anguillarum* tolerated up to 6%, 8% and 3% NaCl respectively. *Vibrio pelagius* had agrinine dehydrolase activity, while the rest of the *Vibrio* spp. gave negative results. All the strains produced acid from glucose and mannose. Arabinose and inositol were not utilized by the *Vibrio* strains characterized in this study. Gelatinase was produced by all the tested *Vibrios*. However starch was hydrolyzed only by V. alginolyticus. All the tested vibrios were sensitive to the vibriostatic agent, O/129 (150 µg). In the 10 µg/disc, *Vibrio alginolyticus* was resistant whereas all the others were sensitive (Table 1).

The inhibition zone in chloramphenicol and gentamycin against *V. alginolyticus* was 20 mm whereas moderate activity with 11 mm and 15 mm zones were noted in ampicillin and erythromycin. Weak activity for streptomycin and total resistance for neomycin were noted (Table 2). *Vibrio vulnificus* was resistant to ampicillin and kanamycin but moderately sensitive to neomycin, novobiocin and streptomycin. Oxytetracycline was highly effective against the virulent pathogenic strains of *V. alginolyticus* and *Vibrio vulnificus* to the extent of 30 mm and 28 mm dia zones respectively. Gentamycin was comparatively more effective against *V. fluvialis.* Erythromycin had no effect towards *V. pelagius* whereas neomycin had moderate activity. *Vibrio anguillarum* was sensitive to all the tested antibiotics as could be noted from Table 2.

Among the five identified *Vibrio* sp., the dominant *Vibrio alginolyticus* (29.4%) and *V. vulnificus* (26.8%) were selected for Koch Postulate test. Fishes challenged with live bacterial cells exhibited symptoms such as: lethargy, loss of balance, whirling movement and general weakness within 6 h of administering the bacteria. No mortality was observed in the control group. For *V. alginolyticus*, 100%, 80% and 20% mortality was obtained when challenged with 10^7, 10^6 and 10^5 CFUs/fish, respectively. The LD_{50} was 1.36×10^6 CFU/g fish. For *V. vulnificus*, the mortality was 90%, 60% and10% in 10^7, 10^6 and 10^5 CFUs/fish respectively and the LD_{50} was 3.44×10^6 CFU/g fish. The mortality was significantly higher ($p<0.01$) in the test groups than in the control. Moribund fish in the challenged groups exhibited the same signs as naturally infected fish, characterized by the presence of abdominal swelling, sloughing off scales, bilateral exophthalmia, and severe fin erosions.

Table 1—Biochemical characterization of isolated *Vibrio* sp. from diseased fishes

Biochemical tests	*V. alginolyticus*	*V. vulnificus*	*V. fluvialis*	*V. pelagius*	*V. anguillarum*
Growth on TCBS	Y	G	Y	Y	Y
Motility	+	+	+	+	+
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Indole	+	+	+	-	-
Citrate	+	+	+	-	-
O/F	F	F	F	F	F
NaCl tolerance (%)					
0	-	-	-	-	-
3	+	+	+	+	+
6	+	+	+	+	-
8	+	-	+	+	-
10	+	-	+	-	-
Arginine dehydrolase	-	-	-	+	-
Lysine decarboxylase	+	+	+	-	-
Ornithine decarboxylase	+	+	-	-	-
Methyl Red	+	+	+	+	+
Voges - Proskauer	-	-	-	-	-
Acid from Glucose	+	+	+	+	+
Sucrose	+	-	+	+	+
Lactose	-	-	+	-	-
Arabinose	-	-	-	-	-
Mannose	+	+	+	+	+
Mannitol	+	-	+	+	+
Sorbitol	+	-	-	-	-
Inositol	-	-	-	-	-
Cellobiose	+	+	+	-	+
Urease	-	+	+	-	+
Nitrate reductase	+	+	+	-	-
H_2S production	+	+	+	+	+
Production of gelatinase	+	+	+	+	+
Starch hydrolysis	+	-	-	-	-
O/129 Sensitivity (10 µg/disc)	R	S	S	S	S
O/129 Sensitivity (150 µg/disc)	S	S	S	S	S

Y: Yellow; G: Green; R: Resistant; S: sensitive; +: Positive reaction; - : Negative reaction

Table 2—Sensitivity of bacterial isolates to different antibiotics (data are the diameters of the antibacterial area in the plate, the unit is mm)

Antibiotics (µg/disc)	*V. alginolyticus*	*V. vulnificus*	*V. fluvialis*	*V. pelagius*	*V. anguillarum*
Ampicillin (25)	11	R	17	15	10
Chloramphenicol (30)	20	18	22	25	30
Erythromycin (15)	15	18	15	R	20
Gentamycin (30)	20	25	32	28	32
Kanamycin (30)	18	R	24	20	26
Neomycin (10)	R	13	R	14	10
Novobiocin (30)	22	16	24	30	27
Oxytetracycline (30)	30	28	25	20	34
Streptomycin (10)	10	12	15	16	12
Tetracycline (30)	25	23	16	24	20

R: resistant; weak: < 10 mm; moderate: 11 to 16 mm; strong: > 16 mm

Discussion

Vibrios are ubiquitous in marine and estuarine environments and are associated with fish and other poikilothermic animals. They exist as part of the normal microbiota and as primary or secondary pathogens[14]. Mortalities in finfish and shellfish have been associated with an increase in the *Vibrio* populations[15]. The Vibrio strains isolated and reported in the present investigations manifested typical biochemical characteristics of vibrios. They were motile, oxidase and catalase positive, Gram-negative that reduced nitrate to nitrite, grew in TCBS agar medium and was sensitive to the vibriostatic agent O/129 as reported by West and Colwell[16]. Balebona *et al*., isolated major pathogens such as *Vibrio* (67.8%), *Pseudomonas* (13.5%), *Photobacterium damsela* subsp. *piscicida* (6.7%), *Flexibacter* - like bacteria (4.8%), *Aeromonas* (0.5%) and Gram positive bacteria (6.7%) in intensive culture of gilt-head sea bream, *Sparus aurata* L. in Southwest Spain fish farms[17]. Among the 40 different Vibrio species recorded from wild and cultured fish, nine species viz., *Vibrio alginolyticus, V. anguillarum, V. damsela, V. harveyi, V. ordalii, V. pelagius, V. salmonicida, V. splendidus* and *V. vulnificus* were reported as pathogens infecting the marine fish[18,19].

Fishes infected by vibriosis could be diagnosed by their characteristic dark skin, pale gill, exophthalmia, hemorrhagic area near mouth and the base of the fins, corneal opacity and ulcers on the skin surface and accumulation of fluid in the peritoneal cavity[20]. Similar clinical signs noted in the present study confirmed the involvement of vibrios in the captive - reared blue damsels. Reports on vibriosis in captive reared marine ornamental fishes are scanty except that of *V. damsela* isolated from skin ulcers of the damselfish, *Chromis punctipinnis*[21].

Many epizootic outbreaks caused by vibriosis have been reported in cultured marine fishes like brown-spotted grouper *Epinephelus tauvina*, turbot, *Scophthalmus maximus* L., gilt head sea bream, *Sparus aurata* L., ayu, *Plecoglossus altivelis* causing mortalities and economic losses[22-25].

Among vibrios, *Vibrio alginolyticus* is considered as the major fish pathogen causing severe infections leading to massive mortality in various fish species throughout the world[26]. However, the virulence of *V. alginolyticus* to fish could not be firmly established because virulence vary from species to species, and in some cases even vary within the same fish species[27]. Moreover, the onset of vibriosis by *V. alginolyticus* is always associated with deteriorating culture conditions or physical damage of cultured fish; which lead to consider this as an opportunistic pathogen[28]. The results of the present study indicated a comparatively higher pathogenic influence by *V. alginolyticus* to the apparently healthy group of blue damsel, *P.caeruleus*. Fishes injected with *V. alginolyticus* succumbed to 50% mortality in 48 h whereas with *V. vulnificus*, it was 55 h. The LD_{50} determined by pathogenicity studies indicated that the mortality was dependent on density of cells indicating a dose dependency. The results also revealed that *Vibrio alginolyticus* and *V. vulnificus* were virulent to the blue damselfish and the bacteria could be re-isolated from moribund fish after bacterial challenge. In addition, gross signs such as dark skin, exophthalmia, hemorrhages and abdominal swelling as noted in the natural outbreaks could be noted in the experimental group of fishes. Thus, both the isolates fulfilled Koch's postulate.

Balebona *et al*., reported that *V. alginolyticus* was virulent to gilt-head sea bream with an LD_{50} ranging from 5.4×10^4 to 1.0×10^6 CFU/g fish[17]. The *Vibrio alginolyticus* isolated from cultured juvenile cobia *Rachycentron canadum* L. weighing 8 to10 g was virulent to cobia with an LD_{50} value of 3.28×10^4 CFU/g fish body weight[29]. Lee (1995) reported that *V. alginolyticus* was virulent to grouper with an LD_{50} value of 0.5×10^3 CFU/g fish body weight[30]. However, in the blue damselfish examined in the present study, the LD_{50} value for the intra-peritoneally injected *V. alginolyticus* was 1.36×10^6 CFU/g fish, which is higher compared to the LD_{50} value for grouper. The difference could be due to the status of virulence of the strain, susceptibility conditions of the host and the ambient environmental conditions.

The other pathogen, *Vibrio vulnificus* comprises virulent strains affecting humans, eels, tilapia and shrimp[31]. It is considered as a common bacteria causing severe vibriosis in eels that occurs as epizootics or outbreaks of high mortality and thus responsible for economic losses in intensive culture of European eel, *Anguilla Anguilla*[32]. The LD_{50} of *V. vulnificus* biotype 2 for eels ranged from 2.6×10^1 to 1.4×10^4 CFU/fish when intra-peritoneal route of inoculation was used[33]. In the present investigation, the LD_{50} of *V. vulnificus* to the blue damsels was 3.44×10^6 CFU/g fish, suggesting the possible low virulence status of the isolate.

In order to manage vibriosis, antibiotics and other chemotherapeutic agents are used prophylactics or therapeutics in fish farms and aquaria either as feed additives or as components in immersion baths. Antimicrobial compounds, including chloramphenicol, nitrofurazone, oxolinic acid, oxytetracycline and sulphamerazine have been proved to be useful in managing the bacterial fish diseases[14]. However, extensive use of antibiotics and other chemotherapeutic agents has resulted in an increase in drug-resistant bacteria in aquatic environments. The microbial biodiversity consisting of beneficial microbes will be affected apart from the normal micro flora of the fish[34]. It is essential to perform susceptibility tests, prior to using any antibiotics so as to reduce the indiscriminate use of antibiotics[35]. The results of susceptibility tests indicated that all the *Vibrio* species isolated were susceptible to broad spectrum antibiotics like chloramphenicol, gentamycin, novobiocin, oxytetracycline followed by streptomycin and tetracycline. The pathogenic strains of *V. alginolyticus* and *V. vulnificus* were more sensitive towards oxytetracycline which is commonly used to control bacterial diseases of fish. Considering the non-availability of published information, this is the first report on characterization, pathogenicity and antibiotic sensitivity of pathogenic strains of vibrios infecting the tropical captive reared marine ornamental blue damsel fishes.

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