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PREVALENCE, CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY OF *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM FISHES AND SHELLFISHES OF COASTAL REGIONS OF BANGLADESH

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Abstract

Occurrence of *Vibrio parahaemolyticus* in fishes and shellfishes of coastal regions of Bangladesh was investigated. Fish and shellfish samples were collected from three coastal areas, namely Kuakata, Chittagong and Cox's Bazar. Thirty five *V. parahaemolyticus* strains were isolated from 33 finfish and 6 shellfish samples where all the isolates were *tlh* positive which was species specific gene and no isolate had possessed the virulence gene encoding *tdh*. Overall prevalence rate of *V. parahaemolyticus* in fish sample was 45.45%; having 18.75% from Kuakata, 22.22% from Chittagong and 62.5% from Cox's Bazar. Fifty per cent shellfish were found to be positive for *V. parahaemolyticus*. Antibiotic susceptibility of the isolated strains was carried out against 11 antibiotics where the isolates were sensitive to the tested antibiotics except metronidazole (50 μg) and nalidixic acid (30 μg). Presence of this pathogenic organism in fish and shellfish could pose a serious threat to fish industry as well as human health hazard in Bangladesh.

Introduction

Vibrio parahaemolyticus, a halophilic bacterium, is a contributory agent of seafood-related gastroenteritis over the world which is now recognized as one of the most important food-borne pathogens, causing approximately half of food poisoning outbreaks in Asian countries like Korea, Taiwan, Japan, Vietnam, and other south-east Asian countries(1). It forms a part of the indigenous microflora of aquatic habitats at various salinities and is the major causative agents for some of the most serious diseases in fishes, shellfishes, shrimps and even human(2). Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively are well-known two major virulence factors for the pathogenesis of *V. parahaemolyticus*(3). Although most of the *V. parahaemolyticus* isolates from the environmental and seafood samples are *tdh* and *trh* negative, some investigations reported the presence of *tdh* or *trh* genes in the isolates from seafood products(3,4).

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As *V. parahaemolyticus* has affinity to saline water, upcoming climatic changes and subsequent increase in salinity with saline water intrusion will make this pathogen more available in inner Bangladesh coasts. This bacterium is often isolated from seafood including shrimp, crab, oyster and clam due to its halophilic characteristics. Other than shellfish, finfishes are also vulnerable to *V. parahaemolyticus* and frequently consumed by the inhabitants of these coastal areas[5]. Moreover, increasingly, there have been more reports of antibiotic resistance in *Vibrio* species. Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment, increasing the fatality rate in human[6]. But effective studies have not been carried out to spot *V. parahaemolyticus* in finfish from coastal regions of Bangladesh yet. Present study was thus carried out to investigate the prevalence and characterization of *V. parahaemolyticus* in fish and shellfish of coastal regions of Bangladesh.

Materials and Methods

A total of 33 different finfish and 6 shellfish with three replicates were collected from Kuakata sea beach, Chittagong and Cox's Bazar BFDC Fisheries market (Table 1) which are the most economically important species from coastal regions of Bangladesh.

Samples were collected during March to June, 2013. After collection, the samples were stored in the refrigerator at –20ºC and were processed within 24 hours. The muscle, gill and intestine were separated aseptically following the method of American Public Health Association (APHA) [7]. In case of shellfish only muscle sample was taken for microbial analysis. Then samples were homogenized with phosphate buffer solution (PBS) using homogenizer. One hundred µl of homogenized samples were spreaded on TCBS and CV plate and incubated for 18 - 24 hours at 37ºC. Green colonies from TCBS plate and violet colonies from CV plates were suspected for *V. parahaemolyticus*[8]. Suspected characteristic colonies subcultured on gelatin agar (GA) plates. After incubation, the colonies showing gelatinase activity were subcultured on TCBS and CV plate following patch inoculation technique for further confirmation. The presence of cytochrome oxidase was detected by Kovacs' oxidase test[9]. Colonies green on TCBS, violet on CV plate, gelatinase positive and cytochrome oxidase positive were then subjected to biochemical test[2]. Salt tolerance (0, 6.5 and 8% salt [w/v] with 1% peptone supplement) of isolates were also observed.

DNA was extracted from biochemically identified positive strains using heat treatment. Multiplex PCR amplification was performed according to Bej *et al*.[10]. PCR primer for *tlh* was F-tlh: 5'-AAAGCGGATTATGCAGAAGCACTG-3', R-tlh: 5'-GCTA CTTTCTAGC ATTTTCTCTGC -3' and PCR primer for *tdh* was F-tdh: 5'-GTAA AGGTCTCTGACTTT TGGAC-3', R-tdh: 5'-TGGAATAGAACCTTCATCTTCACC-3'. PCR amplification of the target DNA was carried out in a thermal cycler (BIO RAD, PTC-0200G, USA). The amplification of the target genes through PCR was determined by gel

electrophoresis in 1% agarose. During gel electeophoresis a 100 bp molecular weight marker was used as standards to compare the amplicon size of the PCR products. After migration for 2 hours at 90 volts, the gel was stained with ethidium bromide and photographed in an UV-transilluminator (Alpha Innotech, SA-1000, USA).

Table 1. List of fishes and shellfishes collected from Kuakata, Chittagong and Cox's Bazar.

Sl. No.	Fish and shellfish species collected from Kuakata	Fish species collected from Chittagong	Fish and shellfish species collected from Cox's Bazar
Name of fishes			
1	*Gastrophysus lunaris*	*Tenualosa ilisha*	*Tenualosa ilisha*
2	*Tenualosa ilisha*	*Megalaspis cordyla*	*Megalaspis cordyla*
3	*Secutor ruconius*	*Johnius belangeri*	*Johnius belangeri*
4	*Brachypleur novazeelandia*	*Rita rita*	*Polynemus paradiseus*
5	*Epinephelus megachir*	*Gudusia chapra*	*Rastrelliger kanagurta*
6	*Carangoides malabericus*	*Eleotris fusca*	*Dasyatis benenttii*
7	*Mugil corsula*	*Scoliodon sorrakowah*	*Sphyrna blochii*
8	*Panna microdon*	*Lepturacanthus savala*	*Glossogobius giuris*
9	*Eleutheronema tetradactylum*	*Argyrops spinifer*	
10	*Cynoglossus versicolor*		
11	*Mystus bleekeri*		
12	*Dasyatis zurgei*		
13	*Colisa fasciatus*		
14	*Sillago domina*		
15	*Eleotris butis*		
16	*Johnius dussumieri*		
Name of shellfishes			
1	*Matuta planipes*		*Penaeus monodon*
2	*Penulirus polyphagus*		
3	*Penaeus monodon*		
4	*Ocypoda ceratopthalma*		
5	*Penaeus indicus*		

Antibiotic susceptibility was carried out using the disc diffusion method. Procedures were based on the standardization of disc agar diffusion method of the National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests[11]. Isolates were inoculated on Mular Hinton Broth (Hi-Media, M173-500G, India) and incubated for 6 hours. The bacterial suspension was then inoculated onto the surface of the Muller-Hinton agar using sterile cotton swabs, which were then left to dry for 10 minutes. The antimicrobial sensitivity discs (Hi-Media, India) were applied on the agar

surface. The discs used in the study were Ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), nitrofurantoin (300 μg), oxolinic acid (20 μg), tetracycline (30 μg), metronidazole (50 μg), nalidixic acid (30) and ciprofloxacin (5 μg). The plates were incubated for 18 - 24 hrs at 37ºC and then the zone of inhibition was measured.

Results and Discussion

In recent years *Vibrio parahaemolyticus* has been emerged as a pandemic pathogen causing seafood related gastroenteritis worldwide and responsible for most of the diarrhoeal diseases occur in the third world country like Bangladesh and India[12]. In this study 35 *V. parahaemolyticus* strains were isolated from 35 finfish and 7 shellfish species based on phenotypic and PCR characterization (Table 2, Fig. 1).

Table 2. Phenotypic characterization of the isolated strains of *V. parahaemolyticus*.

Phenotypic characters		Properties of isolated strain	Properties of ATCC 43996 strain
Colonies on CV plate		Violet	Violet
Colonies on TCBS plate		Green	Green
Growth on GA plate with salt		Showed gelatinase activity	Showed gelatinase activity
Growth on GA plate without salt		No growth	No growth
Salt tolerance	0%	No growth	No growth
	3%	Growth	Growth
	8%	Growth	Growth
	10%	No growth	No growth

All the isolates were positive for *tlh* which is specific for *V. parahaemolyticus*. No toxigenic strain of *V. parahaemolyticus* was found in the present study (Fig. 1).

Among 16 fish species sampled from Kuakata, *V. parahaemolyticus* was found only in the muscle of *Mystus bleekeri* ($3.8 \pm 0.4 \times 10^3$ cfu/g), gill of *Secutor ruconius* ($1.6 \pm 0.9 \times 10^3$ cfu/g) and *Eleutheronema tetradactylum* ($1.1 \pm 0.8 \times 10^3$ cfu/g) (Table 3). Besides, off 5 shellfish species *V. parahaemolyticus* was detected in *Matuta planipes* and *Penaeus monodon*. No *V. parahaemolyticus* was observed in other fish and shellfish species collected from Kuakata.

Eighteen *V. parahaemolyticus* isolates were obtained from 18 fish and 6 shellfish species collected from Kuakata (Table 4). The highest numbers of isolate (9 isolates) were obtained from the muscle of fish and shellfish which was about 60% of total isolates and the lowest was gained from gill which was about 40% of total. No isolate was obtained from intestine of fish from Kuakata (Table 4).

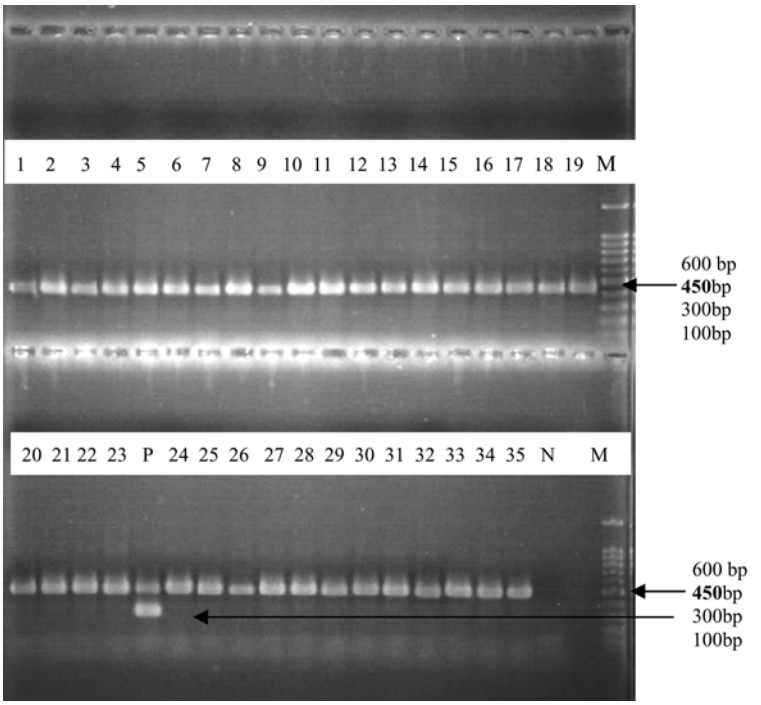

Fig. 1. PCR amplification of isolated strains from fish and shellfish showed *tlh* and *tdh* genes that is species-specific for *V. parahaemolyticus*. Lane P, positive control; Lane N, negative control, *Escherichia coli;* Lane M, 100 bp molecular weight marker; Lane 1-35, *V. parahaemolyticus* strains.

Table 3. Prevalence of *Vibrio parahaemolyticus* (cfu/g) (mean ± SEM) in the various organs of fish sampled from three coastal areas.

Coastal areas	Name of fish/Shellfish	Organs of fish		
		Muscle	Gill	Intestine
Kuakata	*Secutor ruconius*	ND	$1.6 \pm 0.9 \times 10^3$	ND
	Eleutheronema tetradactylum	ND	$1.1 \pm 0.8 \times 10^3$	ND
	Mystus bleekeri	$3.8 \pm 0.4 \times 10^3$	ND	ND
	Matuta planipes	$4.0 \pm 0.1 \times 10^2$	-	-
	Penaeus monodon	$4.9 \pm 0.04 \times 10^3$	-	-
Chittagong	*Tenualosa ilisha*	$3.5 \pm 0.6 \times 10^3$	ND	ND
	Gudusia chapra	$2.7 \pm 0.5 \times 10^3$	$1.4 \pm 0.2 \times 10^4$	$2.5 \pm 0.3 \times 10^4$
Cox's Bazar	*Tenualosa ilisha*	ND	$5.6 \pm 1.2 \times 10^2$	ND
	Polynemus paradiseus	$2.3 \pm 1.4 \times 10^2$	ND	ND
	Rastrelliger kanagurta	ND	$5.3 \pm 1.7 \times 10^2$	ND
	Dasyatis benenttii	$4.6 \pm 1.2 \times 10^2$	ND	ND
	Sphyrna blochii	ND	$1.3 \pm 0.8 \times 10^2$	ND

ND: Not detected.

Nine fish species collected from Chittagong, were analyzed in the present study where *V. parahaemolyticus* was found in all the organs of *Gudusia chapra* and in the muscle of *Tenualosa ilisha* (Table 3). On the other hand, no *V. parahaemolyticus* was detected in other fishes sampled from Chittagong. Eight isolates were obtained from 2 fish species sampled from Chittagong (Table 4).

Prevalence of *V. parahaemolyticus* was higher in fish sampled from Cox's Bazar. Of 8 fish species, *V. parahaemolyticus* was found in 5 species (Table 3). On the other hand, one shellfish species was analyzed where bacterial load was $3.0 \pm 0.5 \times 10^2$ cfu/g. In Cox's Bazar a total of 12 isolates were obtained from 8 fish species and one species of shellfish (Table 4).

Table 4. Isolates of *Vibrio parahaemolyticus* in the various organs of fish sampled from three coastal areas.

Coastal areas	Name of fish/Shellfish	Organs of fish			Total
		Muscle	Gill	Intestine	
Kuakata	*Secutor ruconius*	0	3	0	3
	Eleutheronema tetradactylum	0	3	0	3
	Mystus bleekeri	3	0	0	3
	Matuta planipes	3	-	-	3
	Penaeus monodon	3	-	-	3
	Total	**9**	**6**	**0**	**15**
Chittagong	*Tenualosa ilisha*	2	0	0	2
	Gudusia chapra	2	2	2	6
	Total	**4**	**2**	**2**	**8**
Cox's Bazar	*Tenualosa ilisha*	0	2	0	2
	Polynemus paradiseus	2	0	0	2
	Rastrelliger kanagurta	0	2	0	2
	Dasyatis benenttii	2	0	0	2
	Sphyrna blochii	0	2	0	2
	Penaeus monodon	2	-	-	2
	Total	**6**	**6**	**0**	**12**

In the current study, overall prevalence rate of *V. parahaemolyticus* in fish sample was 45.45% (15/33); having 18.75% (3/16) from Kuakata, 22.22% (2/9) from Chittagong and 62.5% (5/8) from Cox's Bazar. In a study, Sanjeev reported 35 to 55% prevalence in marine and brackish water fish in India[13]. Yang *et al.* revealed 19.0% prevalence of *V. parahaemolyticus* from seafood samples in China[14]. These studies support the findings of the present study. Previous study reported the incidence of *V. parahaemolyticus* highest in intestine, least in external surface and moderate in gills of the fish[15]. However, Natarajan

et al. showed that *V. parahaemolyticus* was quite high in the gills of planktivores [16]. In the present study similar numbers of isolated strains were obtained from muscle (11 isolates), gill (14 isolates) and intestine (2 isolates) of fish. However, prevalence of *V. parahaemolyticus* in the muscle of different fish was higher than gill and intestine which may be due to the suitability of the bacteria to adhere in muscle. From the muscle of shellfish 8 isolates were obtained.

Around 18% isolated strains of *V. parahaemolyticus* were resistant to amphicillin and all the strains were sensitive to the chloramphenicol (30 μg), nitrofurantoin (300 μg) ciprofloxacin (5 μg) and polymyxin B (300 μg) (Table 5). In case of other antibiotics, variable susceptibility was observed. Most of the isolates were sensitive to the tested antibiotics. In this study no resistance was observed for polymyxin B, ciprofloxacin and chloramphenicol. Similar result was observed in the shrimp industries of Cox's Bazar [17]. Oxytetracycline and erythromycin are commonly used antibiotics in Bangladesh and these drugs mainly defer plasmid-mediated resistance in aquatic bacteria [18]. In present study 10% of the isolates of *V. parahaemolyticus* showed resistance against oxytetracycline and ampicillin. Since FDA legalized oxytetracycline in addition to four more drugs for use in US aquaculture but it is essential to control their use in prescribed doses for safe use in Bangladesh [19]. The present study also recommends two more drugs - polymyxin B and ciprofloxacin that can be considered for controlling vibriosis, provided the drugs are discharged in appropriate doses once the target pathogens are identified.

Table 5. Percentage of resistance of *Vibrio parahaemolyticus* (n = 35) against 11 antibiotics.

Antibiotics	Number of resistant strains (%)
Ampicillin (10 μg)	6 (17.14)
Chloramphenicol (30 μg)	0 (0.0)
Erythromycin (15 μg)	9 (25.71)
Gentamicin (10 μg)	14 (40.0)
Nitrofurantoin (300 μg)	0 (0.0)
Oxolinic acid (20 μg)	17 (48.57)
Tetracycline (30 μg)	14 (40.0)
Metronidazole (50 μg)	23 (65.71)
Nalidixic acid (30 μg)	25 (71.43)
Ciprofloxacin (5 μg)	0 (0.0)
Polymyxin B (300 μg)	0 (0.0)

In spite of being a halophilic bacterium, *V. parahaemolyticus* prefers low salinity for optimal growth and is capable of spreading inland to freshwater, indicated by outbreaks of diarrhea caused by *V. parahaemolyticus* in Dhaka, Bangladesh and Kolkata, India [12]. The existence of *V. parahaemolyticus* in this region is indicated by past records for

Bangladesh, India and Thailand[3,12]. The high degree of divergence demonstrated by Bengal strains of *V. parahaemolyticus* is in agreement with many studies reporting similar results for other regions[1, 12].

Though no isolate was TDH (virulent gene for *V. parahaemolyticus*) positive in the present study, nevertheless, it may be expressed in future which would be a destructive situation for the fish industry as well as for the poor people along the coastal region in Bangladesh.

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