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Antibiotic Resistance of *Vibrio* Bacteria Isolated From Mud Crab *Scylla Serrata* of Chakoria Coast, Bangladesh

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ABSTRACT

The mud crab *Scylla serrata* is an export fishery item in Bangladesh and the supply for export and for domestic consumption is mainly dependent on wild sources. The aim of the present study is to isolate and identify the *Vibrio* bacteria from wild mud crab and their surrounding waters from the Chakoria coast of Bangladesh and to investigate their antibiotic resistances. Live crabs were collected, washed and dissected into shells, gills, guts and muscle tissues and plated onto thiosulphate citrate bile salts sucrose (TCBS) agar media and tryptone soy agar (TSA). Based on the morphological, biochemical and physiological characteristics, in addition API 20E and 20NE test strip, the isolated species were *Vibrio alginolyticus*, *V. cholerae*, *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus* and *V. mimicus*. Among them *V. harveyi* was the dominantly isolated species. All the bacterial isolates showed sensitive to ciprofloxacin (91.90%), chloramphenicol (83.79%) and norfloxacin (83.78%) and resistant to ampicillin, erythromycin, kanamycin, nalidixic acid, neomycin, oxytetracycline, penicillin, streptomycin and tetracycline. Much variation was observed in bacterial populations among the tissues. A total of 82 bacterial strains consisting of 6 bacterial species from various tissues of wild mud crab in which highest numbers of bacteria were observed in the gills and lowest numbers in muscle tissue. The study is helpful to assess the safety of human food concern regarding the multi-resistant *Vibrio* bacteria found in edible mud crab.

Keywords: Wild mud crab, *Scylla serrata*, Antibiotic resistant.

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INTRODUCTION

Crabs of the genus *Scylla*, commonly referred as mud crabs, are commercially important crabs with an Indo-Pacific distribution. Recently the mud crab (*Scylla serrata*) has emerged as a potential aqua species in the coastal water of Bangladesh. Farming of mud crab is progressing rapidly due to a promising market and profitability. In recent times, the demand of seafood especially shrimp, lobster and live crab for export has been increasing quite considerably. In domestic market, *Scylla serrata* is much demand and fetches a good price compared to other crab species due to its excellent meat quality.

In Bangladesh, mud crabs are available in the coastal areas of Chakoria, Moheshkhali and Kutubdia Island in Cox's Bazar district and Khulna, Satkhira and Bagerhat in the Sundarbans regions. Two types of land based aquaculture are common in Bangladesh; fattening and grow-out of juvenile crabs capture from the wild. In Bangladesh, technology of mud crab fattening in the cage and pond was successfully developed by Zafar [1]. In 2011-2012 fiscal years, Bangladesh has earned 7.23 million US dollars indicating its importance in the foreign exchange earnings [2].

One of the major problems in aquaculture is disease [3]. The disease of mud crabs (*Scylla* spp.) have been reviewed recently [4]. A number of bacterial diseases such as shell diseases, filamentous bacterial diseases, luminescent bacterial disease etc. have also been reported in mud crabs [5]. The environment has a great influence on the bacterial flora of freshly caught crabs [6]. During handling, bacteria present on the body surface or in the intestine can be entered the crab meat and cause contamination [6]. Aquatic crustaceans always exercise large number of microbes into their body parts from water, sediments and food [6]. Najiah *et al.* [8] isolated 12 bacterial species from mud crab *Scylla serrata*. There are several reports on bacterial infection of the mud crab *Scylla serrata* and blue swimming crab *Portunus pelagicus* [6, 8, 9-10]. The use of antibiotics in aquaculture practices influenced the bacterial population in the culture environment [11]. Antibiotics and chemicals such as ampicillin, chloramphenicol, oxytetracycline, streptomycin, malachite green etc. are generally used in shrimp and fish culture farm [12]. As a result antibacterial residue could be present in culture environment. Presence of antibacterial residues in marine sediments and invertebrates was reported by Weston [13] and Brillantes *et al.* [14]. The increase demand of mud crab production and their value for export, attention should be given to their microbiological quality and concerning food safety for human consumption. The objective of this study was to isolate and identify the *Vibrio* bacteria potentially human pathogens and their sensitivity to various antibiotics.

Sample collection

The mud crab *Scylla serrata* were collected from Chakoria-Moheshkhali coastal area, Cox's Bazar coast, Bangladesh. Location of the study area and mud crab farms is shown in Figure 1. In total 40 alive mud crabs were used to isolate bacteria in this study. Samples were taken in March-April and July-August 2012, during the dry and rainy season respectively. Live

animals were brought to the Shrimp and Fish Disease Diagnosis Laboratory, Institute of Marine Sciences and Fisheries, University of Chittagong, Bangladesh in a plastic bucket. Samples were washed with 70% ethanol before being dissected.

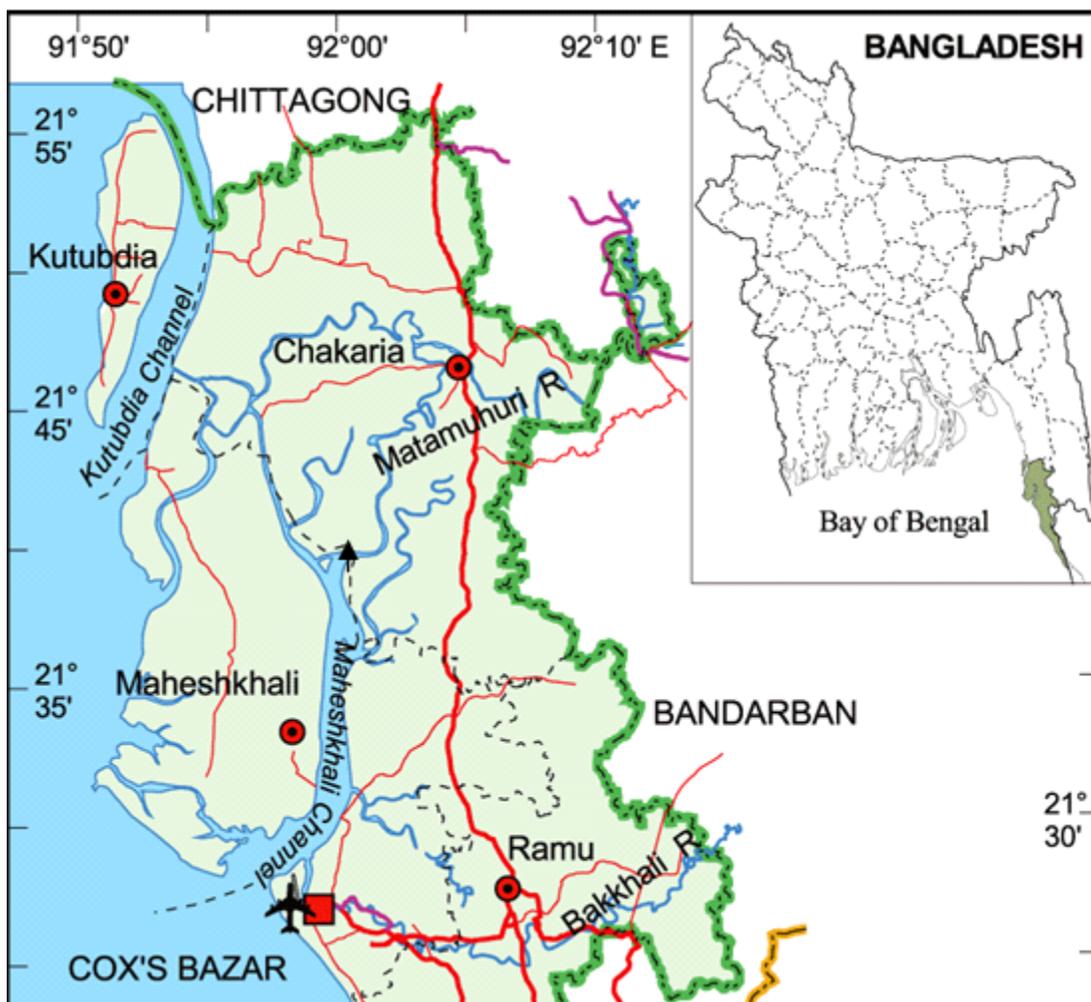


Figure 1: Location of the study area (→) of wild mud crabs where the samples were taken.

Isolation of *Vibrio* spp.

The carapace, muscles, gills and gut of each mud crab (1 g each) were separately taken aseptically from the mud crab and placed in individual sterilized test tubes and homogenized in 1 mL sterilized seawater using a sterile glass rod. Carapace was homogenized in a mortar and pestle. The homogenate was then serially diluted up to 10^3 in filtered seawater. One mL from each dilution (up to 10^{-3}) of the samples were enriched with alkaline peptone water (APW; Oxoid, England) and incubated at 30 °C for 24 h. The cultures obtained from the enrichment medium (APW), after 8 and 24 h of incubation, 0.1 mL of each dilution was plated onto the selective medium: thiosulphate citrate bile salts sucrose (TCBS, Difco, USA) agar [15]. After solidifying of the media the plates were inverted and placed in an incubator at 30 °C for 48 h.

From the TCBS, selected colonies were transferred again onto tryptone soy agar (TSA, Oxoid, England) and incubated at 30 °C for 24 h for purification of the bacterial strains.

Identification of *Vibrio* spp.

The identification of genus *Vibrio*, the following biochemical tests were studied according to Elliot *et al.* [15]. The capability of growth at 3% NaCl, the production of cytochrome oxidase was tested using oxidase reagent (Sigma, USA); the production of acid from glucose fermentation in kligler iron agar (KIA) was tested and the sensitivity to 150 µg vibriostatic discs (Oxoid, England) was tested. The identification of species was assayed with a taxonomic scheme (biochemical identification) of Alsina and Blanch [16-17] that, using six blocks of biochemical reactions, enables the exact classification of all known genospecies of *Vibrio*. The bacteria were examined to standard morphological, physiological and biochemical tube and plate tests according to the established procedures [16-18]. Each biochemical scheme was performed twice independently for all the strains, using different batches of standardized media. In addition API 20E and API 20NE (BioMérieux, France) test strips were also used for *Vibrio* identification. The strips were read and scored according to the kit directions. The code number obtained was used to find the probable *Vibrio* spp. in the code book.

Antibiotic sensitivity test

Thirteen different antibiotics were tested according to Kirby-Bauer disk diffusion method [20] by using Mueller Hinton agar media (Oxoid, England). Commercially available antibiotic disks obtained from Oxoid Ltd. (England) were used to test the susceptibility pattern. The antibiotic disks were Ampicillin (AMC), 25 µg; Chloramphenicol (C), 30 µg; Ciprofloxacin (CIP), 5 µg; Erythromycin (E), 15 µg; Kanamycin (K), 30 µg; Nalidixic acid (NA), 30 µg; Neomycin (N), 10 µg; Norfloxacin (NOR), 10 µg; Oxytetracycline (OT), 30 µg; Penicillin G (P), 10 units; Polymyxin B (PB), 300 units, Streptomycin (S), 10 µg and Tetracycline (TE), 30 µg.

RESULTS AND DISCUSSION

A total of 82 Gram negative bacterial strains were isolated from 40 wild mud crabs (300-400 g each) representing the six *Vibrio* bacterial species. Thirty five strains were isolated during the dry season (March-April) and 47 strains during the rainy season (July-August). Most bacterial species were isolated during the rainy season probably due to freshwater mixing with the coastal water. The distribution of the *Vibrio* bacterial strains during the dry and rainy seasons is presented in Table 1. The six major *Vibrio* species were *V. alginolyticus*, *V. cholera*, *V. fluvialis*, *V. harveyi*, *V. parahaemolyticus* and *V. mimicus*. Among them *V. harveyi* was the dominantly isolated species followed by *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus* and *V. cholerae*. The total plate count (TPC) of *Vibrio* like bacteria in various tissues of carapace, gills, muscles and guts of wild mud crabs and their adjacent water are shown in Table 2. The highest concentrations of bacteria occurred in the gills and the lowest in muscle tissue.

Table 1: Numbers and types of bacteria isolated during the dry (D) and rainy (R) seasons.

Vibrio Bacteria	Sampling season	Number of isolates
<i>V. alginolyticus</i>	D	5
	R	7
<i>V. cholerae</i>	D	3
	R	5
<i>V. fluvialis</i>	D	6
	R	9
<i>V. harveyi</i>	D	10
	R	12
<i>V. parahaemolyticus</i>	D	7
	R	8
<i>V. mimicus</i>	D	4
	R	6

Table 2: Average counts of Vibrio bacteria (cfu/g) in various tissues of wild mud crab.

Season	Carapace	Gills	Gut	Muscle	Water
March	1.1x10 ¹	1.8x10 ²	2.1x10 ³	0.8x10 ²	1.5x10 ¹
April	1.3x10 ¹	1.6x10 ²	2.5x10 ³	0.7x10 ²	2.1x10 ¹
July	3.5x10 ¹	2.5x10 ³	2.4x10 ⁴	1.0x10 ²	3.6x10 ¹
August	4.1x10 ¹	4.9x10 ³	2.6x10 ⁴	1.2x10 ²	3.8x10 ¹

All the *Vibrio* spp. grew in alkaline peptone water (APW) when incubated at 30 °C for 24 h with the only exception of *V. cholerae* that grew faster and was detectable even after 8 h. All microorganisms showed a significant growth in TCBS and in TSA after 24 h when incubated at 30 °C. Table 3 shows the different biochemical and physiological characteristics of various bacterial strains. Out of 82 bacterial strains a total of 8 different API 20E and API 20NE profiles was recorded during the study (10%). Three types of bacteria were identified by using this system. These are *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*. Marked heterogeneity was observed in the individual API profile. Two numbers of *V. harveyi* was identified by the API 20E system and this bacterium showed two different strain profile i.e. '4066105' and '4366105'. Three numbers of *V. alginolyticus* and *V. parahaemolyticus* were identified by the API 20NE system and this bacterium showed different strain profile i.e. 3044744, 3054344 and 7476444 for *V. alginolyticus*; '7036644' '7037345' and '7257644' for *V. parahaemolyticus*.

Members of the genus *Vibrio* are everywhere all over the world and are found associated with many marine and freshwater crustaceans including crabs. They also exist as normal flora in fish and shellfish but have also been recognized as opportunistic pathogens in many marine animals [21]. The present study showed that six species of *Vibrio* are associated with carapace, gill, muscle and gut tissues of wild mud crab *Scylla serrata*. Faghri *et al.* [22] isolated *V. cholerae* and *V. parahaemolyticus* from the shell, gut and gill tissues of cold wateredible crab. Najiah *et al.* [7] isolated *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* from various tissues of mud crab in Malaysia. Members of the genus *Vibrio* had also found in healthy blue crabs [23].

Table 3: Different biochemical and physiological characteristics of various bacterial strains.

Tests	<i>V. alginolyticus</i>	<i>V. cholerae</i>	<i>V. fluvialis</i>	<i>V. harveyi</i>	<i>V. parahaemolyticus</i>	<i>V. mimicus</i>
Number of strains tested	9	8	15	20	12	10
Gram stain	-	-	-	-	-	-
Growth on TCBS	Y	Y	G	Y	G	G
Motility	+	+	+	+	+	+
Oxidase reduction	+	+	+	+	+	+
Catalase reduction	+	ND	+	+	+	ND
Arginine dihydrolase	-	-	+	-	-	-
Lysine decarboxylase	+	+	-	+	+	+
Ornithine decarboxylase	+	+	-	+	+	+
Voges Proskauer	+	V	-	-	-	-
ONPG	-	+	+	V	-	+
Indole production	+		+	+	ND	+
Nitrate reduction	+	+	+	+	+	+
Citrate utilization	V	V	+	+	V	+
Gelatinase	+	+	+	+	+	+
Esculin hydrolysis	+	-	+	-	ND	-
Sensitivity to O/129 (10 µg)	R	S	R	S	R	R
Sensitivity to O/129 (150 µg)	S	S	S	S	S	S
Growth at 4 °C	ng	ng	ng	ng	ng	ND
Growth at 42 °C	g	g	V	g	g	g
Growth in NaCl (w/v)						
0%	ng	g	ng	ng	ng	g
3%	g	g	g	g	g	g
6%	g	ng	g	g	g	g
8%	g	ng	V	ng	g	ng
10%	g	ng	ng	ng	ng	ng
Acid production from						
Arabinose	-	-	+	-	+	-
D-Cellobiose	-	-	V	+	-	-
Galactose	+	+	ND	+	+	+
Lactose	-	-	-	-	-	+
D-Mannitol	+	+	+	+	+	+
Sorbitol	+	-	ND	+	ND	V
Sucrose	+	+	+	+	-	-
Urease	+	-	-	-	V	-

+ = 90-100% strains positive; - = 0-10% strains positive; F = fermentative; G = green; Y = yellow; S= susceptible; R=resistant, ND= no data available; g=growth; ng=not growth; V=variable (11-89% strains positive)

Luminous *V. harveyi* was found in gill, shell and muscle tissues of healthy blue swimming crab

Portunus pelagicus [9]. *V. harveyi*, *V. holisae*, *V. fisheri*, *V. fluvialis*, *V. ordalii*, *V. anguillarum* and *V. mimicus* was also isolated from farmed mud crab meat in India [5]. The results of the present study strongly agree with the results of the above mentioned researcher.

The enumerated *Vibrio* species in the gill tissues of the mud crabs were higher than those on the surrounding water. The results of the present study are closely related to that of Faghri *et al.* [22], who reported concentration of *Vibrio* bacteria in TCBS media were <50 and 1×10^3 cfu/g in muscle and gill respectively. In a quantitative study, Faghri *et al.* [22], found that *V. cholerae* reached a concentration of 5×10^3 cfu/g in gill tissue compared to a maximal concentration of 5×100 cfu/mL in the water which is closely related to the present findings. International Commission of Microbiological Specification for Foods (ICMSF, 1998) recommended that counts of chilled/frozen crab meat should be below 10^5 cfu/g and the maximum counts of *V. parahaemolyticus* should be 1×10^2 . The results of the present study indicated that any setback in icing after collecting the crabs will favor the multiplication of these *Vibrio* bacteria.

Antibiotic susceptibility

A total of 74 isolates were examined for their resistance to fifteen different antibiotics. The susceptibility to 13 different antibiotics were used to test 9 strains of *V. alginolyticus*, 8 *V. cholerae*, 15 *V. fluvialis*, 20 *V. harveyi*, 10 *V. mimicus* and 12 *V. parahaemolyticus* isolated from the wild mud crab *Scylla serrata*. The susceptibility results of various *Vibrio* spp. are shown in Table 4. Antibiotic sensitivity studies revealed that all the bacterial isolates were sensitive to chloramphenicol, ciprofloxacin and norfloxacin and resistant to ampicillin and penicillin G (Table 4).

In the present study, antibiotic sensitivity tests indicate that all the six isolates of *Vibrio* spp. were over 90% resistant to ampicillin and more than 80% of the isolates were sensitive to ciprofloxacin, chloramphenicol and norfloxacin. In the present study, 77.8%, 89%, 22.22% and 77.77% of the *V. alginolyticus* showed sensitivity to chloramphenicol, ciprofloxacin, erythromycin and norfloxacin respectively. Vaseeharan *et al.* [24] reported that 40% of the 5 isolates from *Penaeus monodon* larvae were susceptible to chloramphenicol, ciprofloxacin and erythromycin.

During the present investigation, *V. harveyi* was highly susceptible to ciprofloxacin (90%), followed by norfloxacin (80%), chloramphenicol (75%), erythromycin (46.66%), nalidixic acid (20%) and oxytetracycline (15%). Talpur *et al.* [8] reported that *V. harveyi* (n=180) isolated from blue swimming crab *Portunus pelagicus* from Malaysia was highly susceptible to chloramphenicol (70.55%). *V. parahaemolyticus* was susceptible to chloramphenicol (75%), ciprofloxacin (83.33%) and norfloxacin (75%) and intermediary to erythromycin (66.66%), kanamycin (41.66%), oxytetracycline (75%), polymyxin B (58.33%), streptomycin (75%) and tetracycline (50%) (Table 4).

Table 4: Antibiotic susceptibility of various species of *Vibrio* (n=74). R=Resistance (1-5mm diameter), I=Intermediate (6-10 mm), S=Sensitive (11-18 mm).

Antibiotic (dose)	Response	Percentage (%)						
		<i>V. alginolyticus</i> (n=9)	<i>V. Cholerae</i> (n=8)	<i>V. fluvialis</i> (n=15)	<i>V. harveyi</i> (n=20)	<i>V. mimicus</i> (n=10)	<i>V. parahaemolyticus</i> (n=12)	Total (%)
Ampicillin (25 µg)	R	100.00	75.00	100.00	100.0	70.00	100.00	93.24
	I	00.00	25.00	00.00	0	30.00	00.00	06.76
	S	0.00	00.00	00.00	00.00	00.00	00.00	00.00
Chloramphenicol (30 µg)	R	00.00	00.00	00.00	05.00	00.00	00.00	01.35
	I	22.20	00.00	13.33	20.00	00.00	25.00	14.86
	S	77.80	100.00	86.66	75.00	100.00	75.00	83.79
Ciprofloxacin (5 µg)	R	00.00	00.00	00.00	00.00	00.00	00.00	00.00
	I	11.00	00.00	06.66	10.00	00.00	16.66	08.10
	S	89.00	100.00	93.33	90.00	100.00	83.33	91.90
Erythromycin (15 µg)	R	22.22	00.00	13.33	10.00	0.00	00.00	8.10
	I	55.55	37.50	40.00	60.00	50.00	66.66	52.70
	S	22.22	62.50	46.66	30.00	50.00	33.33	39.19
Kenamycin (30 µg)	R	44.44	100.00	40.00	60.00	70.00	50.00	58.11
	I	55.55	00.00	53.33	35.00	30.00	41.66	37.83
	S	00.00	00.00	06.66	5.00	0.00	08.33	04.05
Nalidixic acid (30 µg)	R	33.33	00.00	40.00	35.00	40.00	25.00	31.08
	I	66.66	100.00	40.00	50.00	60.00	50.00	56.76
	S	00.00	00.00	20.00	15.00	0.00	25.00	12.16
Neomycin (10 µg)	R	55.56	62.50	60.00	50.00	30.00	75.00	55.41
	I	44.44	37.50	40.00	40.00	50.00	25.00	39.19
	S	00.00	00.00	00.00	10.00	20.00	00.00	05.40
Norfloxacin (10 µg)	R	00.00	00.00	00.00	05.00	00.00	00.00	01.35
	I	22.22	00.00	20.00	15.00	00.00	25.00	14.86
	S	77.77	100.00	80.00	80.00	100.00	75.00	83.78
Oxytetracycline (30 µg)	R	44.44	00.00	40.00	40.00	30.00	25.00	32.43
	I	55.56	37.50	60.00	45.00	70.00	75.00	56.76
	S	00.00	62.50	00.00	15.00	00.00	00.00	10.81
Penicilline G (10 units)	R	100.00	100.00	80.00	75.00	80.00	75.00	83.78
	I	00.00	00.00	20.00	20.00	20.00	25.00	16.22
	S	00.00	00.00	00.00	05.00	00.00	00.00	00.00
Polymyxin B (300 units)	R	33.33	00.00	26.67	55.00	40.00	41.67	36.49
	I	55.56	100.00	73.33	40.00	60.00	58.33	60.81
	S	11.11	00.00	00.00	05.00	00.00	00.00	02.70
Streptomycin (15 µg)	R	22.22	00.00	66.67	55.00	70.00	25.00	44.59
	I	77.78	100.00	33.33	35.00	10.00	75.00	50.00
	S	00.00	00.00	00.00	10.00	20.00	00.00	05.41
Tetracycline (30 µg)	R	44.44	12.50	20.00	25.00	30.00	25.00	25.67
	I	55.56	87.50	80.00	75.00	70.00	50.00	70.27
	S	00.00	00.00	00.00	00.00	00.00	25.00	04.05

The results of antibiotic susceptibility tests fairly matched with the study of Vaseeharan *et al.* [24] and Najiah *et al.* [8-9, 25]. The result of the present investigation shows that *Vibrio* spp. obtained from wild mud crabs were same antibiotic resistant than those from the *P. monodon* hatchery and culture ponds [24]. These findings also indicate that antibiotic-resistant *Vibrio* bacteria are widespread in Chakoria-Moheshkhali coastal area, Bangladesh. Different *Vibrio* species have been concerned in food borne illness consequential from eating seafood. These include *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *V. fluvialis*, and *V. alginolyticus* [26]. *Vibrio parahaemolyticus* is relatively common in warm-water areas and is a cause of gastroenteritis in coastal regions of India and in Japan and countries of Southeast Asia [27]. Disease due to *Vibrio* species is usually occurring where large populations of living *Vibrio* species are present on Seafoods, which are stored at high temperatures that permit the bacterial growth. The present study concluded that the wild mud crab *Scylla serrata* of Chakoria coastal area presents various antibiotic resistant *Vibrio* species and collection should be done from uncontaminated water and immediate icing to store them to avoid possible microbial contamination.

Conflict of Interest: The authors declare that there is no conflict of interest

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