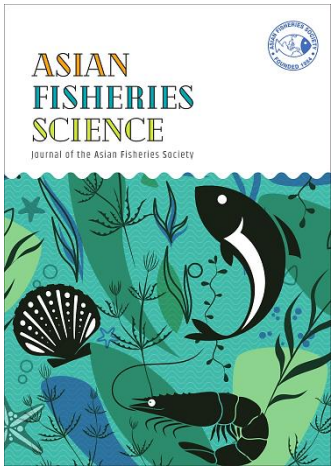

Diversity of *Vibrio* Species' and Their Antibiotic Resistance Patterns in Black Tiger Shrimp *Penaeus monodon* Fabricius, 1798 Cultured in South-West Region of Bangladesh

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Abstract

Shrimp aquaculture industry comprises 15 % of the internationally traded seafood products targeted for human consumption. Diseases of shrimp have caused severe economic losses to the aquaculture industry. Among pathogenic bacteria, several *Vibrio* species known as shrimp pathogens are also human pathogens. This study was conducted to identify the incidence of diversified *Vibrio* species in the shrimp *Penaeus monodon* Fabricius, 1798 farms in Bangladesh. Different *Vibrio* selective media were used for primary isolation and 16S rRNA gene amplicons were sequenced, followed by phylogenetic analysis. The *Vibrio* strains were also tested against twelve antibiotics. Two strains V32 and V38 showed 99.93 % similarity with *Vibrio azureus* whereas V9 and V33 showed 100 % identity with *Vibrio fluvialis*. Comparison of 16S rRNA gene sequence of V37 with sequences from GenBank identified the strain as *Vibrio xuii*. The phylogenetic tree indicates that V24, V26, V34 and V35 are closely related with *Vibrio parahaemolyticus*. The strains V28 and V31 were identified as *Vibrio vulnificus*. One strain of *Vibrio cholerae* was also identified. Moreover, V22 was identified as *Photobacterium damselae* subsp. *damselae*, and V25 as *Aeromonas hydrophila*. Twelve strains of *Vibrio* were resistant to amoxycillin, whereas all 16 bacterial isolates showed sensitivity to nitrofurantoin, sulphamethoxazole, chloramphenicol, ciprofloxacin and tetracycline. Multidrug resistance was observed in three isolates *viz*. V28, V32 and V35 which were identified as *V. vulnificus, V. azureus* and *V. parahaemolyticus*, respectively having MAR index of 0.25. These findings might have potential applications in controlling shrimp and human pathogenic vibrios in the farming regions.

Keywords: shrimp aquaculture, vibrios, 16S rRNA gene, antibiogram, amoxycillin

Introduction

Aquaculture is the significant production sector for high-protein aquatic animal food, especially shrimp products that have occupied a huge market share worldwide (Hasan, 2001). Due to high market demand, many countries of the world have been expanding their shrimp farming areas, and shifted from traditional to more intensive culture practices to maximise production. In 2017, it was reported that 60.5 % (5.5 million tons) of the total shrimp and prawn production were from aquaculture where *Penaeus monodon* Fabricius, 1798 accounted for 0.74 million tons (FAO, 2019). The export of shrimp and prawns in 2017 accounted for more than 4.4 million tons which share 6.72 % of the total exports (FAO, 2019). Bangladesh is among the top ten producers of marine crustacean aquaculture besides China, Vietnam, and Indonesia (FAO, 2018).

In an analysis by Darryl Jory, global shrimp production was expected to be 15 % less in 2015 than in 2011 due to severe disease occurrences in shrimp farms (Jory, 2014). Due to the intensification of the shrimp culture industry, emerging diseases outbreaks have increased and caused significant socio-economic impacts in affected areas. Losses due to the diseases are one of the major indicators of the current unsustainable system of shrimp farming (Hossain and Hasan, 2017).

Bacteria are commonly known to cause various endemic and epidemic diseases of shrimp (Lightner,

1996). Bacteria of the genus *Vibrio* are ubiquitous in the marine and estuarine ecosystems where shrimp grow naturally and are also farmed in the brackish water (Ruangpan and Kitao, 1991). Most of the bacteria that caused infections to shrimp are generally opportunistic pathogens causing disease whenever the shrimps are in stressed condition (Lightner, 1993). Among one hundred and thirty species of vibrios that have been described, twelve were classified as human pathogens that mostly causes food- or water-borne diseases. These include *Vibrio cholerae* that is the primary cause of diarrhoea, *Vibrio parahaemolyticus* causing seafood-borne gastroenteritis and *Vibrio vulnificus* responsible for 95 % of all deaths from seafood consumption (Thompson et al., 2006). These vibrios are the indigenous bacterial flora of shrimp and the culture water (Otta et al., 1999), but they are secondary or opportunistic pathogens that sometimes cause 100 % mortality of infected shrimp (Lightner, 1988).

In addition, some vibrios may cause disease of aquatic animals and as well as of human. In humans, vibrios usually cause wound infections and gastroenteritis. Austin (2010) has categorised *V. cholerae, V. parahaemolyticus* and *V. vulnificus* as higher risk organisms for zoonoses, whereas *Grimontia (=Vibrio) hollisae, Photobacterium (=Vibrio) damselae* subsp. *damselae*, *V. alginolyticus, V. harveyi, V. fluvialis* and *V. mimicus* have been classified as lower risk organisms.

Members of the genus *Vibrio* consist of a significant portion of the microbiota in shrimp farming environments. The high stocking density of shrimps and huge artificial feed supply support the proliferation of vibrios in these farming systems. As vibrios have been known to cause large-scale mortality and great economic losses in the aquaculture industry (Gopal et al., 2005), their control necessitates prophylactic and therapeutic use of antibiotics (Devi et al., 2009; Manjusha and Sarita, 2011). The excessive application of antibiotics has caused the threat of antimicrobial resistance (AMR) traits in vibrios (Sudha et al., 2014; Letchumanan et al., 2015). In addition, the use of antibiotics in shrimp farming is very well-known, but due to ignorance, little is done to change the practice at the farm. Moreover, cultured shrimp can act as vehicles of antibiotic resistant vibrios, especially to β-lactam and tetracycline (Costa et al., 2015). Due to the international trade of live shrimp and its products, shrimp aquaculture can play a major role in the dissemination of antimicrobial resistance globally (Thornber et al., 2020). So, it is necessary to detect the *Vibrio* spp. with antibiotic resistance in shrimp farming areas.

Identification of the pathogenic bacteria up to the species level is crucial in disease epidemiology to trace the exact source of the new outbreak and to formulate strategies to minimise the losses due to disease. But the classical method of detecting bacteria based on phenotypes and biochemical reactions is not as accurate as identification through molecular approach. Moreover, traditional identification techniques are time consuming and ambiguous. Ransangan and Mustafa (2009) suggested that only the biochemical characteristics cannot be used to accurately identify disease causing bacteria in aquaculture. Instead, 16S rRNA gene sequence can be used to identify non-popular, phenotypically variant strains, and can lead to the identification of novel pathogenic strains and non-cultured bacteria (Clarridge, 2004). Furthermore, the 16S rRNA gene sequence (about 1500 bp) is large enough for bioinformatics purposes (Patel, 2001). Therefore, in this study, 16S rRNA gene sequencing was employed for the identification of *Vibrio* isolates.

In Bangladesh, the shrimp aquaculture industry has emerged as a sector for foreign exchange earnings. Due to the occurrences of several diseases in shrimp, especially vibriosis, the sector has been facing serious production losses annually. Most of the vibrios are autochthonous inhabitants of marine water and this water is used for shrimp aquaculture. *Vibrio* spp. are opportunistic pathogens that cause serious disease outbreaks when it's found suitable host such as shrimp.

The objective of the work was to investigate the diversity of *Vibrio* species in the cultured shrimp *P. monodon* of Bangladesh. In addition, the antibiotic resistance pattern of representative bacterial isolates was also investigated.

Materials and Methods

Sample collection

In the present study, shrimp samples were collected using cast net from 16 farms in Bagerhat and Satkhira districts of Khulna division (Fig. 1). The freshly caught shrimp samples were kept in an icebox maintaining at –4 °C and transferred to the laboratory. All samples were collected following the method of American Public Health Association (APHA, 1998).

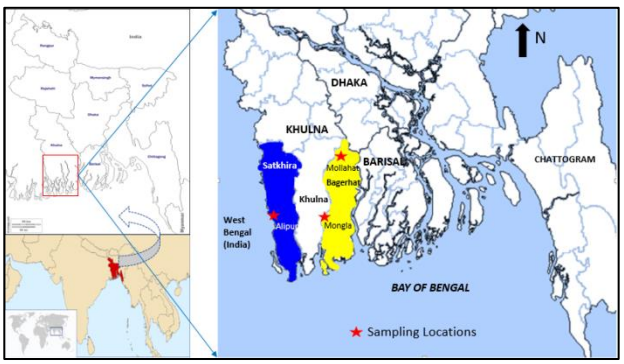

Fig. 1. Location of farms investigated for *Vibrio* diversity in Satkhira and Bagerhat districts of Khulna Division, Bangladesh.

Isolation of Vibrio *spp. from shrimp samples*

Physiological saline (0.85 % NaCl) was added to shrimp samples (hepatopancreas and whole shrimp) and the samples were ground in a mortar aseptically. Alkaline peptone water (APW) was used for the enrichment of the samples in order to provide a suitable environment for *Vibrio* species' to grow and reach a detectable level for the presumptive identification. Then 1 mL of blended solution from each sample was taken in 9 mL APW in a test tube. These tubes were kept in an incubator at 37 °C for 6 to 24 h.

After the incubation period, 2–3 loop full of bacteria were transferred from APW tube to TCBS (Oxoid, UK)/ HiChrome (HiMedia, India)/ CHROMagar (France) vibrio media aseptically and then streaked. The streaked TCBS/HiChrome/CHROMagar plates were incubated at 37 °C for 24 h. The single colonies from agar plates were further screened on their morphological appearance and only isolates that showed variations were stored in Luria-Bertani (LB) broth supplemented with 30 % glycerol at -80 °C for further use.

Molecular identification and phylogenetic analysis using 16S rRNA gene sequences

Heat extracted chromosomal DNA of the selected isolates were used for the amplification of partial 16S rRNA gene by Polymerase Chain Reaction (PCR) using the following primer pairs: 27F 5′-AGAGTTTGATCCTGGCTCAG -3′ and 1492R 5′-CGGTTACCTTGTTACGACTT - 3′ (Weisburg et al., 1991). Amplification was performed in a reaction volume of 25 µL that comprises 12.5 µL of Hot Start Colorless Master Mix, 1 µL of DNA template, 9.5 µL of Nuclease-free water, 1 µL of forward and reverse primers. PCR amplification was performed in an oil-free thermal cycler (Applied Biosystems 2720 Thermal Cycler) with the following program: 95 °C for 5 min for denaturation, then 32 cycles at 95 °C for 30 sec, 48 °C for 30 sec and 72 °C for 1 min 30 sec, followed by a final extension step at 72 °C for 5 min. Successful amplification of the desired segment was visualised by running the PCR products in 1 % agarose gel (w/v) stained with 2 µL of ethidium bromide (H5041, Promega, USA). Alphalmager mini gel documentation system (ProteinSimple, USA) was used to observe and photograph DNA bands (Fig. 2). Before sending for sequencing, amplified DNA fragments were further purified with the Wizard PCR SV Gel and PCR clean-up system kit (Promega, USA) according to the manufacturer's instruction.

The PCR products were sent to First Base Laboratories Sdn. Bhd., Malaysia, for sequencing where BigDye Terminator v 3.1 Cycle Sequencing Kit was used according to manufacturer's instruction. The sequences were obtained by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystem, USA). Both forward and reverse sequences were done for all the 16 representative bacterial isolates.

The 16S rRNA sequences of the bacterial isolates were compared with other sequences using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov) program in NCBI (National Center for Biotechnology Information) to determine the closest matched sequences in GenBank. From the scoring of relatedness shown in the search result, the best matched strains were considered as the identified isolates. MEGA X was used for the comparative analysis of DNA sequence data which was used for reconstructing the phylogenetic tree of *Vibrio* species (Kumar et al., 2018).

Antibiotic susceptibility test

Pure culture of *Vibrio* isolates was grown in Muller Hinton broth, MHB (Oxoid, UK) for antibiotic sensitivity testing. Mueller Hinton agar (MHA) was used as solid media and the disc diffusion method was applied to check antibiotic susceptibility (Hudzicki, 2009). The antibiotics *viz*. amoxycillin (10 µg), ampicillin (10 µg), azithromycin (15 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg), nitrofurantoin (300 µg), polymyxin B (300 unit), sulphamethoxazole (25 µg) and tetracycline (30 µg) were used in the present study. After enrichment in MHB for 18-24 h at 37 °C, the cultures were streaked on MHA plates using a cotton swab. The antibiotic discs (Oxoid, UK) were placed on

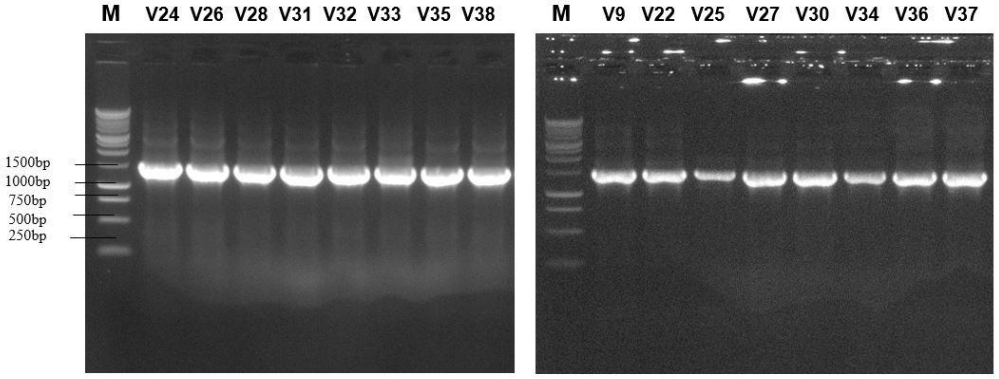

Fig. 2. Gel electrophoresis of 16S rRNA gene PCR product using 27F & 1492R universal primers generated from 16 different bacterial isolates sampled from *Penaeus monodon* farms; M denotes 1kb DNA ladder (Marker).

the agar surface with enough space to avoid overlapping of the inhibition zones. After 30 min of pre-diffusion time, the plates were incubated at 37 °C for 18–24 h. After the incubation period, the diameter of the inhibition zones was measured and compared with the interpretive chart of Performance Standards for Antimicrobial Disk Susceptibility Tests and classified as resistant, intermediate and sensitive (CLSI, 2009).

Multiple antibiotic resistance (MAR) index of the studied strains

The studied 16 isolates were observed for multiple antibiotic resistance (MAR) against 12 antibiotics. According to Schwarz et al. (2010), the bacterial strains resistant against three or more antibiotics can be considered as multidrug resistant strains. Following Krumperman (1983), the MAR index values of the bacterial isolates were calculated as: a/b; where 'a' represents the number of antibiotics the strain was resistant to and 'b' denotes the total number of antibiotics the strain was tested.

The reported sequences in this study have been deposited to the GenBank database under accession numbers MH244235 to MH244247.

Results

Isolation of Vibrio *spp. from shrimp samples*

A total of 38 isolates were isolated from three *Vibrio* selective media. Among them, 16 isolates were selected based on colony morphology and purified for detailed study towards molecular identification. Colonies of the selected isolates were found to be different in their form, size and colour (Table 1). Most of the selected colonies were entire, round and smooth in nature.

Identification of bacterial isolates based on 16S rRNA gene sequence

The identification of the 16 representative isolates (V9, V22, V24, V25, V26, V27, V28, V30, V31, V32, V33, V34, V35, V36, V37 and V38) based on 16S rRNA gene sequences through nucleotide BLAST of NCBI is summarised in Table 2. The sequences of isolates (except V30 and V36 due to noise in sequence) were matched with the strains presented in their respective following column. Table 2 also provides information about scoring *viz.* maximum score, percentage of query coverage, E value and percentage of identity of the sequences with NCBI GenBank accession number of the matched sequences.

The amplified PCR products (Fig. 2) were sequenced and aligned with the 16S rRNA gene sequences available in GenBank. Most of the sequences showed >99 % homology and some of them showed 100 % homology. Two strains V32 and V38 showed 99.93 % identity with *V. azureus* strain CHB-23. Both V9 and V33 strains showed 100 % query coverage and identity with *V. fluvialis* strain LCB1. V28 and V31 showed 100 % similarity with *V. vulnificus* strain CMCP6 and WN141012, respectively. On the other hand, V24, V26, V35 and V38 strains exhibited 99 % query coverage and identity with *V. parahaemolyticus*, whereas V22 as *Photobacterium damselae* subsp. *damselae* CSMA-T3 and V25 as *Aeromonas hydrophila* strain I-T-1-1. V27 isolate showed 100 % query coverage and 99.93 % identity with *V. cholerae* strain 2011V-1043.

Phylogenetic analysis

Phylogenetic analysis performed by constructing the phylogenetic tree based on the partial 16S rRNA gene sequences of the representative 14 isolates using the neighbour-joining method confirmed the taxonomic position of the isolates (Fig. 3). The phylogenetic tree

Table 1. Sampling information and colony morphology of the 16 isolates suspected as *Vibrio* spp. isolated from shrimp *Penaeus monodon* samples.

Serial No.	Strain ID	Sample ID	Sampling region	*Gher* volume (Acres)	Culture medium	Culture condition	Colony morphology
1	V9	M2H6	Satkhira	8	TCBS	Direct plate	Yellow, large
2	V22	ShW1	Satkhira	6	TCBS	Direct plate	Green, small
3	V24	ShW2	Satkhira	8	TCBS	Direct plate	Greenish, medium
4	V25	AnW2	Satkhira	4	TCBS	Direct plate	Greenish, medium
5	V26	AbW1	Satkhira	0.6	TCBS	6 h enrichment	Yellow, small
6	V27	AbW2	Satkhira	0.6	TCBS	Direct plate	Yellow, Large
7	V28	BaW1	Satkhira	8	TCBS	Direct plate	Yellow, small
8	V30	BaWM	Mongla	1	TCBS	6 h enrichment	Yellow ,small
9	V31	MiWB	Bagerhat	2	TCBS	6 h enrichment	Yellow, small
10	V32	MiHB	Bagerhat	2	TCBS	6 h enrichment	Yellow, small
11	V33	JoHB	Bagerhat	4	ChromAgar	Direct plate	Yellow, large
12	V34	AfWB	Bagerhat	3	ChromAgar	Direct plate	Yellow ,small
13	V35	SeWB	Bagerhat	3	ChromAgar	Direct plate	Yellow, small
14	V36	MaHB	Bagerhat	9	TCBS	Direct plate	Green small
15	V37	IsHM	Mongla	4	HiChrome	6 h enrichment	Turquoise, small
16	V38	JoWB	Bagerhat	4	HiChrome	6 h enrichment	Turquoise, small

Table 2. Molecular identification of representative 16 bacterial isolates based on the 16S rRNA gene sequences.

Serial No.	Isolates ID with strain designation	Description of closest relative (obtained from GenBank BLAST search)	Base pairs (bp)	Max score	Query coverage (%)	E value	Identity (%)	Accession number of corresponding sequence	Accession number of strains under present study
1	V9 (msr1)	*Vibrio fluvialis* strain LCB1	1426	2634	100	0.0	100	KC210808.1	MH244235
2	V22 (msr2)	*Photobacterium damselae* subsp. *damselae* CSMA-T3	1422	2627	100	0.0	100	MK482019.1	MH244236
3	V24 (msr3)	*Vibrio parahaemolyticus* strain CZN-34	1424	2630	100	0.0	100	KR347270.1	MH244237
4	V25 (msr4)	*Aeromonas hydrophila* strain I-T-1-1	1417	2591	99	0.0	99.79	KU570301.1	MH244238
5	V26 (msr5)	*Vibrio parahaemolyticus* strain 20140829008-1	1425	2627	100	0.0	99.93	CP034294.1	MH244239
6	V27 (msr6)	*Vibrio cholerae* strain 2011V-1043	1419	2615	100	0.0	99.93	CP046837.1	MH244240
7	V28 (msr7)	*Vibrio vulnificus* strain CMCP6	1413	2610	100	0.0	100	CP037931.1	MH244241
8	V30	Bad chromatogram	Excluded from identification						-
9	V31 (msr8)	*Vibrio vulnificus* strain WN141012	1415	2614	100	0.0	100	KU245729.1	MH244242
10	V32 (msr9)	*Vibrio azureus* strain HHN-3	1425	2623	99	0.0	99.93	KR270154.1	MH244243
11	V33 (msr10)	*Vibrio fluvialis* strain LCB1	1423	2628	100	0.0	100	KC210808.1	MH244244
12	V34 (msr11)	*Vibrio parahaemolyticus* strain 461	1427	2630	100	0.0	99.93	JN188418.1	MH244245
13	V35 (msr12)	*Vibrio parahaemolyticus* strain CZN-34	1424	2625	100	0.0	99.93	KR347270.1	MH244246
14	V36	Bad chromatogram	Excluded from identification						-
15	V37	*Vibrio xuii* strain 0104 (Not in full length)	1008	1722	99	0.0	97.81	KP236239.1	-
16	V38 (msr13)	*Vibrio azureus* strain CHB-23	1423	2623	100	0.0	99.93	KR347286.1	MH244247

indicates that V32, V38, V24, V26, V35 and V36 were closely related to allocated strains *V. azureus* CHB-23 and *V. parahaemolyticus* strains CZN-34. The phylogenetic tree further confirmed the taxonomic position of V25 supporting their similarity with the allocated strain *A. hydrophila* strain I-T-1-1, V27 as *V. cholerae* strain 2011V-1043 and, V28 and V31 as *V. vulnificus* WN141012. From the tree, it is found that V9 and V33 were closely related to *V. fluvialis* strain LCB1, while V37 strain is similar with *V. xuii* strain 0104.

The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.64045822 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed from the number of base substitutions per site using the maximum composite likelihood method (Tamura et al., 2004). The analysis involved 23 nucleotide sequences with 1508 positions in the final dataset. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Green circular shape indicates the position of the studied strains.

Antibiotic susceptibility test of bacterial strains of Vibrio *spp.*

In the present study, 16 bacterial isolates were tested against 12 common antibiotics. Results of antibiotic susceptibility test are shown in Table 3 and Table 4. Except for four isolates (V25, V27, V30, V31), rest of the twelve strains were resistant to amoxycillin (10 µg), whereas all 16 strains showed sensitivity to nitrofurantoin, sulphamethoxazole, chloramphenicol, ciprofloxacin and tetracycline. Among all strains, 11 strains of *Vibrio* showed sensitivity to kanamycin but five strains (V22, V28, V31, V32, and V36) were found intermediate to it. Moreover, 15 strains were found sensitive against azithromycin while only V33 exhibited an intermediate pattern. Among all the 16 strains, nine strains were found resistant to ampicillin.

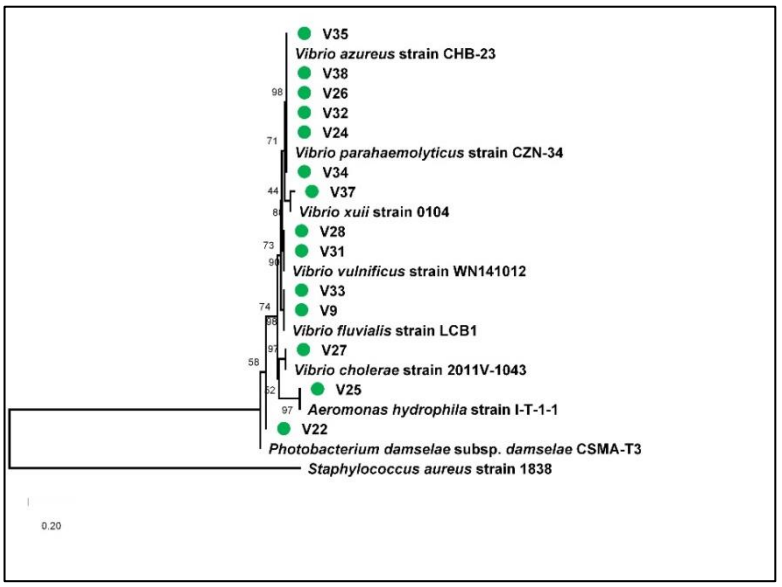

Fig. 3. Molecular phylogenetic analysis by neighbour-joining method using MEGA X of representative 14 strains collected from *Penaeus monodon* farms.

Table 3. Results of antibiotic susceptibility test against 12 antibiotics for 16 bacterial isolates collected from *Penaeus monodon* farms.

Isolate ID	Identified species	Antibiotic susceptibility		
		Sensitive	Intermediate	Resistant
V9	*Vibrio fluvialis*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP
V22	*Photobacterium damselae*	AMP, AZM, C, CIP, F, PB, SXT, TE	E, CN, K	AMC
V24	*V. parahaemolyticus*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP
V25	*Aeromonas hydrophila*	AMC, AMP, AZM, C, CIP, CN, K, F, PB, SXT, TE	E	-
V26	*V. parahaemolyticus*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP
V27	*V. cholerae*	AMC, AMP, AZM, C, CIP, E, CN, K, F, SXT, TE	-	PB
V28	*V. vulnificus*	AZM, C, CIP, E, CN, F, SXT, TE	K	AMC, AMP, PB
V30	Not identified	AMC, AMP, AZM, C, CIP, E, CN, K, F, PB, SXT, TE	-	-
V31	*V. vulnificus*	AMC, AMP, AZM, C, CIP, F, SXT, TE	E, CN, K	PB
V32	*V. azureus*	AZM, C, CIP, F, PB, SXT, TE	CN, K	AMC, AMP, E
V33	*V. fluvialis*	C, CIP, CN, K, F, SXT, TE	AZM, E	AMC, PB
V34	*V. parahaemolyticus*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP
V35	*V. parahaemolyticus*	AZM, C, CIP, E, CN, K, F, SXT, TE	-	AMC, AMP, PB
V36	Not identified	AMP, AZM, C, CIP, F, PB, SXT, TE	E, CN, K	AMC
V37	*V. xuii*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP
V38	*V. azureus*	AZM, C, CIP, CN, K, F, PB, SXT, TE	E	AMC, AMP

Amoxycillin (AMC), Ampicillin (AMP), Azithromycin (AZM), Chloramphenicol (C), Ciprofloxacin (CIP), Erythromycin (E), Gentamycin (CN), Kanamycin (K), Nitrofurantoin (F), Polymyxin B (PB), Sulphamethoxazole (SXT), Tetracycline (TE).

Four isolates (V22, V32, V31, and V36) showed an intermediate sensitivity/resistance towards gentamycin, whereas the other 75 % strains represented sensitivity against it (Table 4). Five strains exhibited resistance against polymyxin B. Only one strain (V32) was resistant to erythromycin but 11 strains showed intermediate sensitivity/resistance and the remaining four strains were found to be sensitive. The descending order of resistance patterns of studied strains was V28, V32, V35 (3 resistances) > V9, V24, V26, V33, V34, V37, V38 (2 resistances) > V22, V27, V31, V36 (1 resistance). Therefore, among 16 bacterial strains, 14 strains (87.5 %) were resistant to at least one antibiotic.

Table 4. Percentage of antibiotic sensitivity, intermediate and resistance for 16 strains of *Vibrio* against 12 antibiotics.

Name of antibiotics	Isolates of *Vibrio* spp. (n = 16)		
	R %	I %	S %
Amoxycillin	12 (75)	0 (0)	4 (25)
Ampicillin	9 (56.25)	0 (0)	7 (43.75)
Azithromycin	0 (0)	1 (6.25)	15 (93.75)
Chloramphenicol	0 (0)	0 (0)	16 (100)
Ciprofloxacin	0 (0)	0 (0)	16 (100)
Erythromycin	1 (6.25)	11 (68.75)	4 (25)
Gentamicin	0 (0)	4 (25)	12 (75)
Kanamycin	0 (0)	5 (31.25)	11 (68.75)
Nitrofurantoin	0 (0)	0 (0)	16 (100)
Polymyxin B	5 (31.25)	0 (0)	11 (68.75)
Sulphamethoxazole	0 (0)	0 (0)	16 (100)
Tetracycline	0 (0)	0 (0)	16 (100)

S = Susceptible, I = Intermediate, R = Resistant, n = Number of isolates.

The resistance rates of studied isolates to amoxicillin, ampicillin, polymyxin B and erythromycin were found to be 75 %, 56.25 %, 31.25 % and 6.25 %, respectively. A 100 % sensitive response by the isolates was observed against nitrofurantoin, tetracycline, chloramphenicol, sulphamethoxazole and ciprofloxacin.

Multiple antibiotic resistance (MAR) index of the studied strains

Multiple antibiotic resistance (MAR) was observed for three studied strains *viz* V28, V32 and V35 which were identified as *V. vulnificus*, *V. azureus* and *V. parahaemolyticus*, respectively. MAR index value of these three isolates was 0.25 (Fig. 4).

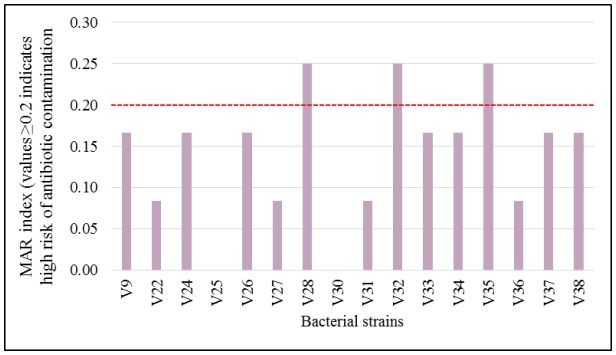

Fig. 4. Multiple antibiotic resistance (MAR) index values of 16 diversified bacterial isolates from *Penaeus monodon* farms against 12 antimicrobial agents.

Discussion

Classical approach of identification and characterisation of bacterial isolates is based on phenotypic traits from biochemical reactions that make the study of diverse *Vibrio* spp. difficult (Alsina and Blanch, 1994a, b); and has the disadvantage of the requirement of strains to grow and produce a detectable reaction. The use of 16S rRNA gene sequences has been considered as the solution to this problem. By phenotypic characterisation, Ransangan and Mustafa (2009) identified four bacterial isolates as *V. harveyi*, 16 as *V. parahaemolyticus* and one as *V. alginolyticus*, whereas 16S ribosomal DNA sequencing showed all 21 isolates as *V. harveyi*. In this work, 16S rDNA was applied to estimate the diversity of pathogenic *Vibrio* associated with tiger shrimp cultured in brackish waters of Satkhira and Bagerhat districts of Bangladesh. Among 16 isolates of the present study, 16S rRNA sequences of 14 isolates were identified as seven different bacterial species viz. *V. fluvialis* (V9,V33), *P. damselae* (V22), *V. parahaemolyticus* (V24, V26, V34, V35), *V. cholerae* (V27), *V. vulnificus* (V28, V31), *V. azureus* (V32, V38), *V. xuii* (V37) under Vibrionaceae family and one *Aeromonas hydrophila* (V25) under Aeromonadaceae family. Heenatigala and Fernando (2016) in one of their studies used 16S rDNA gene and identified only four *Vibrio* species viz., *V. alginolyticus*, *V. parahaemolyticus*, *V. damselae* and *V. anguillarum*, though more than 30 pathogenic *Vibrio* spp. have been identified in shrimp culture systems (Farmer III, 1992). Manilal et al. (2010) worked on the virulence of vibrios isolated from diseased black tiger shrimp and the isolated 29 distinct colonies were under six species including luminescent *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. fischeri*, *V. parahaemolyticus*, and *P. damselae*.

Felix et al. (2011) used 16S rDNA to identify *Vibrio* species and found that of seven strains of *Vibrio* sp., five were 97 % homologues to *V. alginolyticus*, *V.*

parahaemolyticus, V. harveyi, V. shilonii and V. vulnificus. Another two strains isolated in their study were not found to be homologous to any of the GenBank listed strain and therefore were considered as indigenous *Vibrio* spp. from Indonesia. Hagstrom et al. (2000) stated that isolates those shared greater than 97 % similarities with the 16S rDNA sequence may represent the same species. While the sequence similarities between 93- 97 % can represent identity at the genus level but different at the species level. In our study, we have found 97.81 to 100 % similarity of studied isolates with that of closest relatives obtained from NCBI GenBank.

Though popularly known to cause gastrointestinal illness in humans, *V. parahaemolyticus* is also one of the several causative agents of shrimp disease causing Acute Hepatopancreatic Necrosis Disease (AHPND) (Tran et al., 2013). This disease has gained much importance due to high mortality rates in the early stage of post larvae (PL) stocking resulting in severe production losses (Kongrueng et al., 2014). This bacterium also caused disease outbreak and mass mortalities in cultured Pacific white shrimp (*Penaeus vannamei* Boone, 1931) in the grow-out ponds of India under unfavourable environmental conditions (Kumar et al., 2014). Moreover, this bacterium was isolated from shrimp farms in India but was found AHPND negative (Paria et al., 2019). *Vibrio parahaemolyticus* was also found as dominant bacteria in infected shrimp facing red disease and tail necrosis (Jayasree et al., 2006). *Vibrio cholerae* causes cholera. *Vibrio cholerae* is being reported to be isolated from fish during cholera outbreaks and also isolated when there were no outbreaks as this bacteria are indigenous to marine waters (Feachem et al., 1981; cited in ICMSF, 2005). Species of the genus *Vibrio* are the most common bacterial agents in contaminated shellfish causing food poisoning (Levine et al., 1993; cited in ICMSF, 2005). *Vibrio vulnificus* is a human pathogen found in coastal environments. After *V. parahaemolyticus*, it is the second most common bacterial agent that causes food-borne infection and due to the high mortality, it poses significant health risk (Kushawaha et al., 2010). *Vibrio vulnificus* has also been found to be associated with loose shell syndrome (LSS) of shrimp with other *Vibrio* species (Jayasree et al., 2006). LSS was observed in all ages of shrimp with mortality rate reached 100 % within 10 days of onset of infection. *Vibrio fluvialis* is another halophilic emerging food-borne human pathogen that is commonly found in coastal water (Liang et al., 2013). *Vibrio fluvialis* is also known to cause white faeces syndrome (WFS) in *P. monodon* (Kumara and Hettiarachchi, 2017). *Vibrio azureus* is a luminous marine bacterium, and still not recognised as shrimp pathogen though reported recently in India associated with running mortality syndrome (RMS) of *P. vannamei* (Alavandi et al., 2019). Considered as an environmental species, *V. xuii* has been isolated from shrimp culture environments (Thompson et al., 2003) and demonstrated either non- or low virulence in the aquatic animal models (Austin et al., 2005). *Photobacterium damselae* subsp. *damselae* is a marine bacterium under the family Vibrionaceae that is identified as an emerging pathogen of diverse aquaculture species, including fish, molluscs, and crustaceans. It was found in the internal organs of *P. monodon* and it showed pathogenicity to shrimp and also reported in the black gill diseased *P. monodon* in India (Vaseeharan et al., 2007). *Aeromonas hydrophila* infections are another major threat in commercial aquaculture and a large number of fish and shellfish species are very much prone to this infection. Bacterial diseases associated with *Aeromonas* in prawn are "black-spot" bacterial necrosis and gill obstruction (Lombardi and Labao, 1991a, b). Moreover, identified *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were categorised as high risk vibrios whereas *V. fluvialis* and *P. damselae* as lower risk organisms in terms of zoonotic potential (Austin, 2010).

In the present study, chloramphenicol was found to be 100 % sensitive to *Vibrio* isolates, but the scenario was opposite in Indian shrimp farms where all six *Vibrio* species showed 100% resistance, as chloramphenicol is highly used in India (Manilal et al., 2010). Heenatigala and Fernando (2016) used antibiotics to control the vibriosis diseases of shrimp and found that all *Vibrio* isolates were resistant to ampicillin, chloramphenicol, gentamycin, kanamycin, oxytetracycline and trimethoprim which are commonly used antibiotics in aquaculture. *Vibrio parahaemolyticus* isolated from *P. vannamei* shrimp cultured in low saline waters in India were found to be resistant to β-lactam antibiotics and cephalosporin (Sanathkumar et al., 2014). In the present study, several *Vibrio* species were found to be resistant to amoxicillin, ampicillin, erythromycin and polymyxin B.

MAR index values ≥0.2 means that the bacterial strains were exposed to several antibiotics or isolated from contaminated sources, whereas, the strains with values <0.2 are less likely to be exposed to antibiotics (Noorlis et al., 2011). Lee et al. (2018) isolated 165 strains of *V. parahaemolyticus* from 240 fish samples (both freshwater and marine) in Malaysia. They discovered the antibiotic resistance patterns of those isolates and found that 70 % were resistant to 3 to 8 types of antibiotics having MAR index values >0.2 (Lee et al., 2018). In a study on AMR and virulence genes of *Vibrio* strains isolated from ready-to-eat shrimps in Nigeria, Beshiru et al. (2020) found that 10.9 % of *V. parahaemolyticus* were resistant to 18 antibiotics with a MAR index of 0.75 and 21.4 % of *V. vulnificus* were resistant to 10 antibiotics with a MAR index of 0.42. The present study supports the results of these previous research findings. Therefore, antibiotic resistance of *Vibrio* spp. is a potential barrier of controlling infectious diseases of aquatic animals as well as it is a global public health issue.

Hossain et al. (2012) found the main reason for antibiotic resistance of *Vibrio* species was due to the indiscriminate use of antibiotics in shrimp farming of Bangladesh. Kumara and Hettiarachchi (2016) found varying degrees of antibiotic resistance among *Vibrio* species and recommend strict bio-security measures and best management practices to control the pathogenic vibrios. Moreover, the antibiotic resistance phenomenon is going to be a 'slow-moving pandemic' that at least 700,000 people die each year globally (https://amr-review.org/), and the yearly death toll is predicted to rise to 10 million by 2050 which is higher than death toll caused by cancer today, as reported by Foreign Policy news (https://foreignpolicy.com/2020/05/21/china-farms-antibiotic-resistance-antimicrobial-amr/).

Conclusion

This study was conducted to check the presence of *Vibrio* species diversity in farmed shrimp in south-western farming regions of Bangladesh. The study found several species of *Vibrio* that are potentially pathogenic to shrimp, as well as to human. Varying degrees of antimicrobial resistance were also observed among the isolated vibrios. Further investigations are needed to identify the risk factors that could trigger disease outbreaks in shrimp farms with *Vibrio* strains. Shrimp health surveillance programme should be implemented effectively to prevent disease outbreaks and will help to improve shrimp production in Bangladesh.

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