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Competitive Research Grant

Sub-Project Completion Report

on

Biochemical and Molecular Assay for Detection of *Vibrio* spp. at Shrimp and Shrimp Farms in Bangladesh

Project Duration

May 2016 to September 2018

National Institute of Biotechnology
Ministry of Science and Technology
Ganabari, Savar, Dhaka-1349, Bangladesh

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
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Acronyms

°C	: Degree Centigrade
µl	: Micro Liter
APW	: Alkaline Peptone Water
BF	: Bagerhat Fakirhat
BS	: Bagerhat Sadar
CC	: Coxbazar Chokoria
CLSI	: Clinical and Laboratory Standards Institute
CS	: Coxbazar Sadar
DNA	: DeoxyRibo Nucleic Acid
cfu	: colony forming unit
DoF	: Department of Fisheries
KB	: Khulna Batighata
KD	: Khulna Dacope
mg	: Micro Gram
ml	: Mili Liter
mPCR	: Multiplex Polymerase Chain Reaction
MR	: Methyl Red
NA	: Nutrient Agar
NIB	: National Institute of Biotechnology
PCR	: Polymerase Chain Reaction
PS	: Pirozpur Sadar
PV	: Pirozpur Vandaria
sec	: Second
SK	: Satkhira Kaligonj
SS	: Satkhira Sadar
TBE	: Tris-borate EDTA
TCBS	: Thiosulfate Citrate Bile Salt Sucrose
VP	: Voges Proskaur

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Executive Summary

Vibrio are widely distributed in the aquatic environment and found in both marine and estuarine waters. Some *Vibrio* species can also cause infections in humans and have been isolated from a variety of intestinal and extra intestinal sites. Pathogenic mechanisms are poorly understood for the majority of *Vibrio* species except for *Vibrio cholera* O1 and O139. Bacteria belonging to the *V. cholera* O1 and O139 sero groups are almost invariably pathogenic, because of the presence of the potent cholera enterotoxin. For other *Vibrio* species, individual strains may or may not possess virulence factors or the virulence factors that are critical for disease may not be known. Such *Vibrio* species are thus considered to be potentially pathogenic from the public health perspective. This study was conducted to identify *Vibrio* spp. in shrimp and culture environment as well as to reveal the pathogenic status of the isolated strains. Considering the above fact, total 500 samples of shrimp, water and sediment were aseptically collected from 50 different shrimp farms of 10 Upazila of Khulna, Sathkhira, Bagerhat, Pirozpur and Cox'sbazar district for morphological and molecular analysis. From these samples, 607 suspected *Vibrio* strains were isolated using TCBS agar and preserved in glycerol stock for long term storage. Morphological, biochemical and molecular tests were done to characterize the selected strains. Total 231 selected isolates were analysed using two different sets of multiplex PCR to detect eight major *Vibrio* species. Among them, 13 *Vibrio cholerae*, 114 *V. parahaemolyticus*, 29 *V. alginolyticus* and 01 *V. mimicus* were identified by first set of mPCR. Six (6) *V. campbelli* and 7 *V. harvei* were identified by the second set of mPCR. Identified bacteria were further confirmed by gene specific PCR and 16S rDNA sequencing method as well. Pathogenicity study revealed that only a few of the *Vibrio* species are pathogenic and remaining majority are non-pathogenic. Twelve different commercial antibiotics were used for antibiotic sensitivity test. The results showed that isolates were highly sensitive to cotrimoxazole (92%) followed by tetracycline (85%), chloramphenicol (81%), novobiocin (73%) & ampicillin (69%) and resistant to sulphamethoxazole (92%), gentamicin (89%), trimethoprin (81%) and nalidixic acid (77%) respectively. The outcomes of the project will help to understand the present microbiological scenario of shrimp farms. In this study, to detect major *Vibrio* spp., multiplex PCR methods have been adopted. This knowledge will help others to diagnose major diseases caused by *Vibrio* species in shrimp firms. Sensitive antibiotics might be used to reduce drastic losses of shrimp due to Vibriosis. Moreover, multiplex PCR technique may be replicated in other laboratories to detect major *Vibrio* spp.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project:

Biochemical and Molecular Assay for Detection of *Vibrio* spp.at Shrimp and Shrimp Farms in Bangladesh

2. **Implementing organization:** National Institute of Biotechnology, Ganakbari, Savar, Dhaka

3. Name and full address with phone, cell and E-mail of PI/Co-PI (s):

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Co- Principal Investigator

4. Sub-project budget (Tk):

4.1 Total: **Tk. 20,00,000.00**

4.2 Revised (if any): **19,10,918.00**

5. Duration of the sub-project:

5.1 Start date (based on LoA signed): 7th May 2017

5.2 End date: 30 September 2018

6. Justification of undertaking the sub-project:

The shrimp sector plays an important role in the national economy of Bangladesh. It contributed approximately 84% to the USD 599 million frozen seafood export earnings in the financial year 2014–2015 (FRSS, 2016). The shrimp/prawn farming area and production was increased from 141,352 ha and 66,703 MT in 2002–2003 to 275,583 ha and 223,582 MT in 2014–2015, respectively (FRSS, 2016). The expansion of farming area has been unregulated and poorly coordinated (Alam SMN. *et al*., 2005; Paul BG. *et al*., 2011). The intensification of production systems leads to adverse changes in water quality and has increased the risk of diseases due to higher stocking densities and feeding rates (Nasrin, 2016). In recent years, the prevalence of known endemic diseases has increased and has been recognized as the major barrier to the development of the shrimp sector in Bangladesh (Hossain MS*. et al*., 2013; Karim *et al*., 2012). The economic loss caused by diseases was estimated at USD 832 to 3928 ha−1 per year depending on the degree of intensification of the farming systems (Jahan KM. *et al*., 2015). Several studies have reported, a wide range of diseases caused by viral and bacterial pathogens in shrimp farms in Bangladesh (Ali H. *et al*., 2016; Karim M.*et al*., 2012).

Vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all shrimp producing countries. Shrimp aquaculture is an important industry that experiences significant losses from *Vibrio* species, especially at the larval and juvenile stages. On the basis of phenotypic data, the major species causing vibriosis in shrimp are *V. alginolyticus, V. anguillarum, V. harveyi* and *V. parahaemolyticus*. (Lightner D. 1993). The main Etiological Agent of

vibriosis are *Vibrio vulnificus, V. parahemolyticus, V. alginolyticus, V. anguillarum, V. damsella, V. fluvialis* and *V. mimicus, Vibrio harveyi*.

Misidentification of bacterial pathogens in fish/shrimp farm settings may create problems in selecting appropriate prophylactic measures. There are lots of detection system for these bacteria like cultural method, PCR and Real Time PCR method, microarray method etc. In cultural method, detection of *Vibrio* spp. has consisted of enrichments steps and isolation steps on selective agar medium followed by biochemical and serological testing. Presumptive diagnosis of vibriosis has traditionally been done by isolating the causative bacteria from diseased shrimp which do not allow to identify specific pathogenic strains of Vibrio (Hedreyda*et al*., 2007). Conventional culture-based and biochemical-based methods for the detection of Vibrio are laborious and time-consuming, normally requiring more than 3 days. Molecular approach that utilizes PCR along with some other sophisticated tools overcomes many limitations.In recent years, sequencing and comparison of the 16S rRNA gene has become an important tool for the identification of bacterial species (Greenwood *et al*., 2005). DNA sequence based identification, analysis of 16s rRNA and other housekeeping gene sequence are the most popular and precise method currently used to identify closely related vibrio among other methods, ribo typing and PCR based techniques, eg. Amplified fragment length polymorphism (AFLP) fluorescence Insights Hybridization, (FISH), Random Amplified polymorphic DNA (RAPD), repetitive palindrome PCR (rep-PCR), and restriction fragment length polymorphism (RFLP) have yield the most valuable information and new insights into the identification of closely related marine bacteria.However, *V. harveyi* and *V. campbellii*share more than 97% similarity in their 16S rRNA gene sequences and show a 69% match in DNA-DNA hybridization (Gomez-Gil *et al*., 2004). The application of molecular methods such as the polymerase chain reaction (PCR), may provide an easy, less expensive, and more rapid means of identification for these *Vibriospp*. Multiplex PCR methods, which use more than 1 pair of primers to detect different microorganisms in a single reaction, have been validated as an effective simultaneous detection tools for many other food-borne pathogens (Kong *et al*., 2002;). There are several reports in the literature, which describe the use of multiplex PCR methods for the differentiation of Vibrio spp. (Tarr*et al*., 2006).

The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern, which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture (Capriol*iet al*., 2000). Antimicrobial agents have been applied to the shrimp feed and water in large quantities primarily to treat and prevent diseases in farmed shrimps. Consequently, antimicrobial agents persist in sediment and aquatic environments, leading to deteriorated environmental conditions and conferring antimicrobial resistance to the sediment bacteria. Of particular concern is the indiscriminate use of antibiotics leading to the development of multiple-antibiotic resistant pathogenic bacteria in shrimps and humans (Zanetti*et al*.,2001). In this case, shrimps could serve as delivery vehicles of antimicrobial resistance to pathogenic bacteria from aquatic environments to humans and from one country to another.

7. Sub-project goal:

The core goal is to adopt an appropriate molecular tool to diagnose of shrimp vibriosisand selecteffective antibioticagainst *Vibrio* spp. atshrimp farms in Bangladesh.

8. Sub-project objective (s):

- To identify and characterize *Vibrio* spp*.* of shrimp of respective areas of Bangladesh.
- To adopt an effective molecular diagnostic tool for characterizing*Vibrio* spp.
- To select effective antibiotic against vibriosis.

9. **Implementing location (s):** National Institute of Biotechnology, Ganakbari, Savar, Dhaka-1349

10. Methodology:

10.1 Sample collection

Total 500 samples (Shrimp, water and sediment) were aseptically collected from 50 shrimp farms from 10 Upazilla (2 upazilla in each district and 5 farms in each upazilla)of Satkhira, Bgerhat, Khulna, Pirozpur and Coxbazar District for microbiological analyses. In each farm, 4 shrimp samples (40-50 gm per sample), 3 water samples (500 ml) and 3 sediment samples (50-60 gm) from different corners of ponds were collected. Shrimps were caught using net and packed in sterile polythene bags. Water samples were collected in sterile screw-capped bottle and sediment samples in sterile polythene bags using a sterile spatula. The samples were brought to the lab in ice baskets. The samples were properly prepared for further detailed studies, as soon as possible.

Figure1: location map of sample collection

10.2 Total microbial load estimation

Total microbial load estimation was done following Bacteriological Analytical Manual standard method (Kaysner*et al*., 2004).One gram of each sample (shrimp and sediment) and one ml water sample were measured accurately using a weighing machine and measuring cylinder. Serial dilution method was used for microbial load estimation and tenfold (1:10) dilution was prepared and further diluted up to 10^{-3}. For total viable bacterial count 0.1 ml of each diluted samples were inoculated on Nutrient Agar (NA) Figureusing a sterile pipette and spreaded with the help of a spreader and incubated at 37°c for 24 hours. For *Vibrio*spp. Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar media was used. After counting of colony the cfu (colony forming unit) value was measured using the following formula-

$$\text{Viable bacterial count/ml} = \frac{\text{The total number of colonies} \times \text{Dilution factor}}{\text{The volume of sample added to the agar plate}}$$

10.3 Enrichment of shrimp, sediment and water samples

Twenty-five gram of shrimp sample was taken in a sterile stomacher plastic polythene bag and blended into small pieces with lab stomacher. Then samples were taken in a conical flask and 225ml Alkaline Peptone Water (APW) was added and mixed. Then the mixture was incubated at 35±2°c for 6 to 8 hours. One gram of sediment sample was taken and mixed with 10 ml of Alkaline Peptone Water (APW). Then APW was incubated at 35 ±2°c for 6 to 8 hours. For analysis of water sample, first water sample was enriched with APW broth. 25 ml of water sample was mixed with 225 ml of APW broth and incubated at 35 ±2°c for 6 to 8 hours (Kaysner*et al*., 2004).

10.4 Plating of enriched broth on selective media for isolation of *Vibrio* spp.

After pre enrichment, one loop full from the surface pellicle of APW culture was transferred to the surface of a dried TCBS agar Figureand was streaked in a manner that will yield isolated colonies. Then, TCBS agar plates were incubated overnight (18 to 24 hours) at 35±2°c. Greater than 2 mm size with green, blue-green and yellow colonies on TCBS agar plates were presumptively selected as *Vibrio*spp. Typically, isolated colonies were sub cultured and preserved in glycerol broth for long term storage (Kaysner*et al*., 2004).

10.5 Growth of *Vibrio* spp. on *V. Vulnificus* & *V. Parahaemolyticus* selective media

Vibrio Vulnificus Agar and *Vibrio Parahaemolyticus* Sucrose Agar media (Hi Media, India) was used for selective identification of *Vibrio vulnificus* and *Vibrio parahaemolyticus.* A single colony was isolated from TCBS agar Figurewas streaked on *Vibrio Vulnificus* Agar and *Vibrio Parahaemolyticus* Sucrose Agar media. Then the plates were incubated overnight at 35±2°c. Yellow opaque colonies (fried egg appearance) on Vulnificus Agar media was indicated the selective identification of *Vibrio vulnificus* and blue-green colony on *Vibrio Parahaemolyticus* Sucrose Agar media was indicated the selective identification of *Vibrio parahaemolyticus.*

10.6 Biochemical Characterisation

For identification of selected bacteria on TCBS agar biochemical tests were performed using Bacteriological Analytical Manual standard method (Kaysner*et al*., 2004).

10.7 Molecular Identification of the selected *Vibrio* isolates

10.7.1Genomic DNA Extraction

Genomic DNA was extracted from isolated bacteria by phenol: chloroform:isoamyl alcohol (25:24:1) method (Barker, 1998) and was preserved at -40C. The concentration of DNA was measured using nano spectrophotometer.

10.7.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones. Electrophoresis was carried out in a horizontal gel apparatus and conducted in horizontal agarose gel (2%). Agarose was added in 100 ml 1X Tris-borate EDTA (TBE) buffer (pH 8.3) and 50 ml sterile deionized water to prevent evaporation loss and dissolved by boiling in a microwave oven for two minutes. Then it was allowed to cool to 55-60°C and poured into a plastic chamber with comb containing 12 numbers of teeth. Bubbles were removed and gel was allowed to solidify for half an hour at room temperature. After removal of comb 5µl of DNA sample and 1 µl of 6X loading buffer were mixed with the help of a micropipette and loaded accordingly. 5 µl DNA markers (ladder DNA) were added to the specific well. After the gel was loaded, it was placed in the electrophoresis tank. 1X TBE-buffer was added to cover the gel to a depth of about 1 mm. Electrophoresis was carried out at 100 volts until the dye moved to the half of the chamber. The gel was viewed and photographed under a gel documentation system.

10.7.3 PCR based identification of *Vibrio* genus

The PCR reaction was performed using the following RecombinaseA gene specific primer (Kim *et al*., 2015) was used for identification of Vibrio genus. Forward primer VGC2694352 F46 (5'-GTCARATTGAAAARARTTYGGTAAAGG-3) and reverse primer VG C2694352R734 (5'-ACYTTRATRCGNGTTTCRTTRCC-3). The amplicon size was 689 bp. The thermal cycling profile was as follows: a 30 cycles of 94°C for 5 min initial denaturation, 94°C for 30 sec denaturation, 60°C for 30 sec annealing and 72°C for 45 sec, extension and final extension at 72°C for 10 min. Another set primer pair was used to confirm Vibrio genus more significantly (Tarr*et al*., 2007). Forward primer V.16S-700F (5'-CGGTGAAATGCGTAGAGAT-3') and reverse primer V.16S-1325R (5'-TTACTAGCGATTCCGAGTTC-3)'. The amplicon size was 663 bp. The thermal cycling profile was as follows: a 35 cycles of 95°C for 5 min initial denaturation, 92°C for 40 sec denaturation, 57°C for 1 min annealing and 72°C for 1.5 min, extension and final extension at 72°C for 7 min.

10.7.4 Detection of *Vibrio* spp. by Multiplex PCR

To detect vibrio spp. in shrimp, water and sediment samples, total three sets of multiplex PCR were used. Firstly, mPCR was performed using five primer pair set for identification of five pathogenic *Vibrio* spp. of shrimp, water and sediment samples. The primer sequence (Kim *et al*., 2015) and PCR product size were given in Table. Optimization was performed for each primer pair individually and then all primers in combination. Final primer concentrations were adjusted to give approximately equal signals for each gene fragment. Each reaction contained 2X master mix half of the total volume PCR mix, the various amounts of each 10M stock of the primers: VP primers, 1µl each; VC primers, 1µl each; VV primers, 1µl each; VA primers, 1µl each; VM primers, 1µl each; VG primers, 1µl each and Genomic DNA 2µl and add rest amount water. The thermal cycling profile was as follows: a 25 cycles of 94°C for 5 min for initial denaturation, 94°C for 30 sec for denaturation, 60°C for 30 sec annealing and 72°C for 45 sec, extension and final extension at 72°C for 10 min.

Primer name	Target genus or species	amplicon size (bp)	Primer sequence (5' to 3')
VP 1155272 F	*V. parahaemolyticus*	297	5'-AGCTTATTGGCGGTTTCTGTC GG-3'
VP 1155272 R			5'-CKCAAGACCAAGAAAAGC CGTC-3'
VC C634002 F	*Vibrio cholerae*	154	5'-CAA GCT CCG CAT GTC CAG AAG C-3'
VC C634002 R			5'-GGGGCGTGACGCGAATGATT-3'
VV 2055918 F79	*Vibrio vulnificus*	484	5'-CAG CCG GAC GTC GTC CAT TTT G-3'

Primer name	Target genus or species	amplicon size (bp)	Primer sequence (5’ to 3’)
VV 2055918 R			5’-ATG AGT AAG CGT CCG ACG CGT-3’
VA 1198230 F	*Vibrio alginolyticus*	199	5’-ACG GCA TTG GAA ATT GCG ACT G-3’
VA 1198230 R			5’-TAC CCG TCT CAC GAG CCC AAG-3’
VM C727581 F	*Vibrio mimicus*	249	5’-ATAAAGCGGGCTTGCGTGCA-3’
VM C727581 R			5’-GAT TTG GRA AAA TCC KTC GTG C-3’

Another multiplex PCR was performed by using five primer sets for further identification of five *Vibrio* spp. The primer sequence (Tarr*et al*., 2007) and PCR product size were given in Table.Optimization was performed for each primer pair individually and then all primers in combination similarly. Final primer concentrations were adjusted to give approximately equal signals for each gene fragment. Each reaction contained 2x master mix half of the total volume PCR mix, the various amounts of each 10M stock of the primers: Vc.sodB primers, 1µl each; Vm.sodB primers, 1µl each; Vp.flaE primers,1µl each; Vv.hsp primers,1µl each; V.16S primers,1µl each and Genomic DNA 2µl and add rest amount water. The thermal cycling profile was as follows: a 35 cycles of 95°C for 5 min initial denaturation, 92°C for 40 sec denaturation, 57°C for 1 min annealing and 72°C for 1.5 min, extension and final extension at 72°C for 7 min.

Primer name	Target genus or species	Primer sequence (5’-3’)	Amplicon size (bp)
Vc.sodB-F	*V. cholerae*	5’-AAG ACC TCA ACT GGC GGT A-3’	248
Vc.sodB-R		5’-GAA GTG TTA GTG ATC GCC AGA GT-3’	
Vm.sodB-F	*V. mimicus*	5’-CAT TCG GTT CTT TCG CTG AT-3’	121
Vm.sodB-R		5’-GAA GTG TTA GTG ATT GCT AGA GAT-3’	
Vp.flaE-79F	*V. parahaemolyticus*	5’-GCA GCT GAT CAA AAC GTT GAG T-3’	897
Vp.flaE-934R		5’-ATT ATC GAT CGT GCC ACT CAC-3’	
Vv.hsp-326F	*V. vulnificus*	5’-GTC TTA AAG CGG TTG CTG C-3’	410
Vv.hsp-697R		5’-CGC TTC AAG TGC TGG TAG AAG-3’	
V.16S-700F	*Vibrio* spp.	5’-CGG TGA AAT GCG TAG AGA T-3’	663
V.16S-1325R		5’-TTA CTA GCG ATT CCG AGT TC-3’	

Lastly, third set multiplex PCR was used to identify *V. campbelii, V. harveyi, V. parahaemolyticus and V. anguilarum*. PCR reaction was contained 2X master mix of half of the total volume PCR mix, the various amounts of each 10M stock of the primers: Vca-hly primers, 1µl each; Vh-hly primers, 1µl each; Vp-tlh primers,1µl each; Van-ami primers,1µl each and Genomic DNA 2µl and add rest amount water.

Primer name	Target genus or species	Primer sequence (5’-3’)	Amplicon size (bp)
Vca-hly5	*Vibrio campbelli*	5’-CTA TTG GTG GAA CGC AC-3’	328
Vca-hly3		5’-GTA TTC TGT CCA TAC AAA C-3’	
Vh-hly1F	*Vibrio harveyi*	5’-GAG TTC GGT TTC TTT CAA G-3’	454
Vh-hly1R		5’-TGT AGT TTT TCG CTA ATT TC-3’	
Vp-tlh1	*Vibrio parahaemolyticus*	5’-GAT TTG GCG AAC GAG AAC-3’	695
Vp-tlh2		5’-CGT CTC GAA CAA GGC G-3’	
Van-ami8	*Vibrio anguillarum*	5’-ACA TCA TCC ATT TGT TAC-3’	429
Van-ami417		5’-CCT TAT CAC TAT CCA AAT TG-3’	

Step	Vca-hly, Vh-hly, Vp-tlh	Van-ami
Initial denaturation	94°C/5min	94°C/5min

Final denaturation	94°C/30sec	94°C/30sec
Annealing	54°C/30sec	55°C/1.5min
Initial extension	72°C/30sec	72°C/45sec
Final extension	72°C/5min	72°C/10min

10.7.5 Confirmation of *Vibrio* species by 16S rDNA sequencing

PCR was performed from the genomic DNA by using 16S rDNA bacterial universal primer set of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). The PCR reaction mixture consisted of 12.5µl of 2X PCR master mix, 1µl of forward primer, 1µl of reverse primer, 9µl of nuclease free water and 1.5µl of DNA to a final volume 25µl. Amplification was carried out as follows: initial denaturation at 95°C for 5 min which was followed by 30 cycles, then the denaturation at 95°C for 30 sec, primer annealing at 56°C for 30 sec, extension at 72°C for 90 sec and final extension at 72°C for 10 min. PCR product was purified using PCR product purification kit (invitrogen) according to the manufacturer's instruction. PCR product was sequenced in both directions using an ABI 3130 automated sequencer at Molecular Biotechnology Division, NIB. 16S rDNA sequence was then aligned with known 16S rDNA sequences in Genbank database using NCBI BLAST search. A phylogenetic tree was constructed with MEGA 6.0 version using a neighbor-joining algorithm.

10.8 Study of virulence associated genes

10.8.1 Virulence associated genes of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*

Virulence associated genes of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* was done using three sets of primer (Kim *et al*., 1999).Optimization was performed for each primer pair individually and then all primers in combination. Final primer concentrations were adjusted to give approximately equal signals for each gene fragment. Each reaction contained 2X master mix half of the total volume PCR mix, the various amounts of each 10M stock of the primers: toxR primers, 1µl each; tdh primers, 1µl each; trh primers,1µl each and Genomic DNA 2µl and add rest amount water.

Primer name	Target gene	Primer sequence (5' to 3')	Amplicon size (bp)
toxR-F	toxR	5'-GTC TTC TGA CGC AAT CGT TG-3'	368
toxR-R		5'-ATA CGA GTG GTT GCT GTC ATG-3'	
M-454	tdh	5'-CGT TGA TTA TTC TTT TAC GA-3'	623
M-441		5'-TTT GTT GGA TAT ACA CAT-3'	
TRH-F	trh	5'-CTC TAC TTT GCT TTC AGT-3'	460
TRH-R		5'-AAT ATT CTG GAG TTT CAT-3'	

Step	toxR	tdh	trh
Initial denaturation	94°C/5min	94°C/10min	94°C/5min
Final denaturation	94°C/1min	94°C/1min	94°C/1min
Annealing	63°C/2min	55°C/1.5min	48°C/1min
Initial extension	72°C/1.5min	72°C/1.5min	72°C/1min
Final extension	72°C/10min	72°C/10min	72°C/5min

10.8.2 Study of virulence associated genes of *Vibrio cholerae*

Identification of virulence associated genes of *V. cholerae*was done usingseven sets of (ctxA, ompW, tcpA, rfbo1, toxR, ace and zot) primer pairs (Tarr*et al*., 2007). PCR reaction mixtures was 12.5 µl of 2X master mix, 1.0 µl of each primer set individually, genomic DNA 1.5 µl and add rest amount of nuclease free water. For ctxA gene the thermal cycling profile was a 35 cycles of 95°C for 10 min Initial denaturation, 95°C for 1 min denaturation, 61.5°C for 1 min Annealing, 72°C for 1.2 min Extension and 72°C for 10 min final extension. On the other hand, for *ompW*genes PCRcycle wasa 35

cycles of 95°C for 10 min Initial denaturation, 95°C for 1 min denaturation, 55.0°C for 1 min Annealing, 72°C for 1.2 min extension and 72°C for 10 min final extension.

Primer name	Target gene	Primer sequence (5' to 3')	Amplicon size (bp)
ompW-F	ompW	5'-CAC CAA GAA GGT GAC TTT ATT GTG-3'	588
ompW-R		5'-GAA CTT ATA ACC ACC CGC G-3'	
ctxA-F	ctxA	5'-CTC AGA CGG GAT TTG TTA GGC ACG-3'	301
ctxA-R		5'-TCT ATC TCT GTA GCC CCT ATT ACG-3'	
tcpA-F	tcpA	5'-CGT TGG CGG TCA GTC TTG-3'	805
tcpA-R		5'-CGG GCT TTC TTC TTG TTC G-3'	
rfbo1-F	rfbo1	5'-TCT ATG TGC TGC GAT TGG TG-3'	538
rfbo1-R		5'-CCC CGA AAA CCT AAT GTG-3'	
toxR-F	toxR	5'-CCT TCG ATC CCC TAA GCA ATA C-3'	779
toxR-R		5'-AGG GTT AGC AAC GAT GCG TAA G-3'	
ace-F	ace	5'-TTG ATG GCT TTA CGT GGC TT-3'	198
ace-R		5'-GCG CTT GGT CTA ACC CTA AA-3'	
zot-F	zot	5'-GGT GGC TTT TGA CAT GCA TC-3'	575
zot-R		5'-CCA AAT TGT CTA CGA GGC GA-3'	

10.9 Antimicrobial susceptibility testing

Bacterial susceptibility to antimicrobial agents was performed by the disk diffusion method (Bauer et al., 1966). A total of 12 antibiotic discs (Oxoid Ltd., UK) with Trimethoprim 5µg, Chloramphenicol 30µg, Gentamicin 10µg, Streptomycin 10µg, Tetracycline 30µg, Erythromycin 15µg, Cotrimoxazole25µg, Nalidixic acid 30µg, Ampicillin 10µg, Imipenem 10µg, Sulphamethoxazole 25µgand Novobiocin 5µg were used. Selected antibiotic discs were placed on Mueller Hinton Agar (Oxoid Ltd., UK) plates seeded with bacteria. Within 15 min of the application of the discs, the plates were inverted and incubated at 37°C. After 24h of incubation, the plates were examined and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. Then susceptible, intermediate and resistant isolates were defined according to the criteria suggested by the Clinical and Laboratory Standards Institute (CLSI), Antimicrobial Susceptibility Testing Standard, 2007 (January).

Table1: CLSI Antimicrobial Susceptibility Testing Standard

Antimicrobial Agent	Disk Content	Zone Diameter, Nearest whole mm		
		Resistance (R)	Intermediate (I)	Sensitive (S)
Imipenem	10 µg	≤ 26	27-31	32
Trimethoprim	5 µg	≤ 21	22-27	28
Chloramphenicol	30 µg	≤ 12	13-17	≥ 18
Gentamicin	10 µg	≤ 19	20-25	26
Streptomycin	10 µg	≤ 12	13-19	20
Tetracycline	30 µg	≤ 14	15-18	≥ 19
Erythromycin	15 µg	≤ 15	16-18	≥ 19
Cotrimoxazole	25 µg	≤ 10	11-15	≥ 16
Sulphamethoxazol	25 µg	≤ 10	11-15	≥ 16
Nalidixic acid	30 µg	25	-	26
Ampicillin	10 µg	≤ 13	14-16	≥ 17
Novobiocin	5 µg	≤ 12	13-15	≥ 16

11. Results and Discussion:

Disease problem in aquaculture are currently an important constraint to growth of aquaculture, which has impacted both socioeconomic development and rural livelihoods in some countries. The species associated with disease in fish and shell fish include *V. anguillarum* (isolated most commonly from marine and brackish water shellfish) *V. harveyi, V. alginolyticus, V. cholerae, V. vulnificus* and *V. parahaemolyticus* have infected in juveniles and adults. Usually most of the studies have focused on the isolation, characterization and identification of the bacterial strains present in tissues and cultural water (FAO, 2002). The present study was focused to adopt and effective molecular tools for characterizing Vibrio spp.

11.1 Sample collection

Total 500 samples (Shrimp, water and sediment) were aseptically collected from 50 shrimp farms of Satkhira, Bgerhat, Khulna, Pirozpur and Coxbazar District for microbiological analyses. Shrimps were caught using net and packed in sterile polythene bags. Water samples were collected in sterile screw-capped bottle and sediment samples in sterile polythene bags using a sterile spatula. The samples were brought to the lab in ice baskets. The samples were properly prepared for further detailed studies, as soon as possible.

Figure 2: Samples collection from different areas

11.2 Prevalence of total bacteria in shrimp and culture environment

Total viable bacterial count (Colony Forming Unit) from shrimp, water and sediment samples collected from shrimp farms of different Upazilla were summarized in Table. It was found that, the highest (8.2×10^3cfu/ml) bacterial count was found in shrimp samples collected from Coxbazar district and lowest was in Bagerhat District. But, in sediment samples it was vice versa. Similarly, total bacterial count in water samples was highest in the Khulna District and lowest was in Satkhira District.

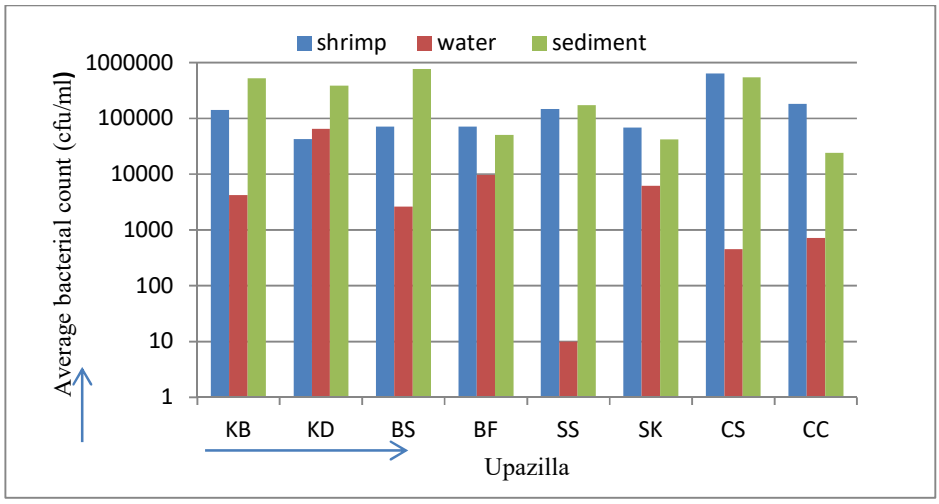

Figure 3: Total bacteria in shrimp and culture environment

11.3. Prevalence of total *Vibrio* spp. in shrimp and culture environment

Total *Vibrio spp.* in samples was also determined. From the study it was observed that the highest Vibrio in shrimp samples was found in Coxbazar and lowest was in Bagerhat. On the other hand, Vibrio was not found in water samples Satkhira and Coxbazar District but low amount was found in Khulna and Bagerhat District.

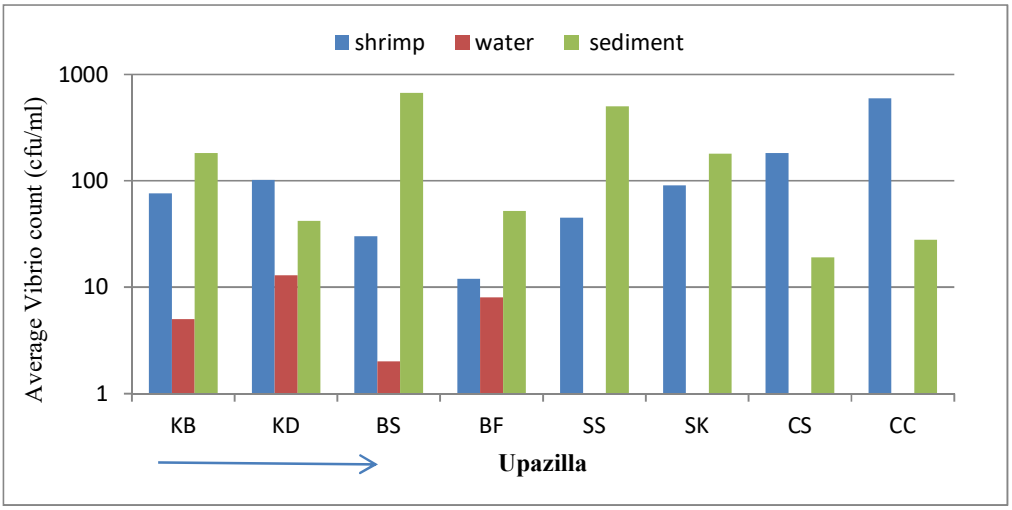

Figure 4: Total *Vibrio* spp. in shrimp and culture environment

11.4. Isolation of *Vibrio spp*. from collected samples

After pre enrichment, one loop full from the surface pellicle of APW culture was transferred to the surface of a dried TCBS agar Figure and was streaked in a manner that yield isolated colonies. Then,

TCBS agar plates were incubated overnight (18 to 24 hours) at 35±2°c. Green, blue-green and yellow colonies on TCBS agar plates were presumptively selected as *Vibrio* spp. Typical isolated colonies were sub cultured and preserved in glycerol broth for long term storage (Figure 6). Total eight hundred and forty (840) morphologically well-formed single colonies were selected from 500 samples on the basis of their morphological differences to identify and confirm the single colony of each isolates (plate).

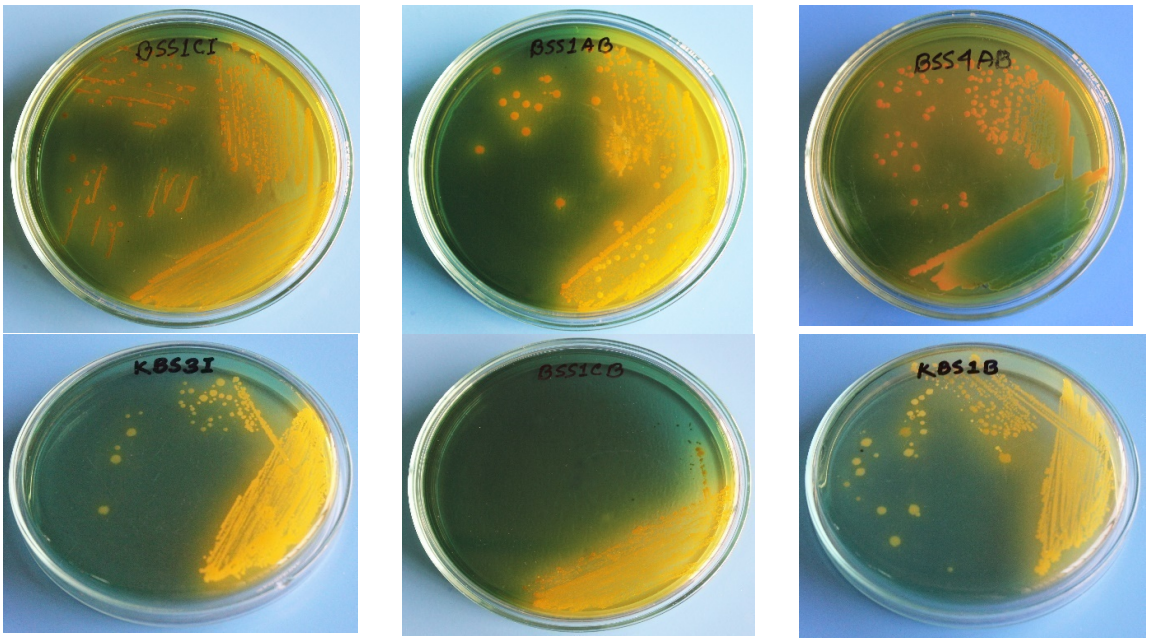

Figure 5. Pure culture of some bacterial isolates

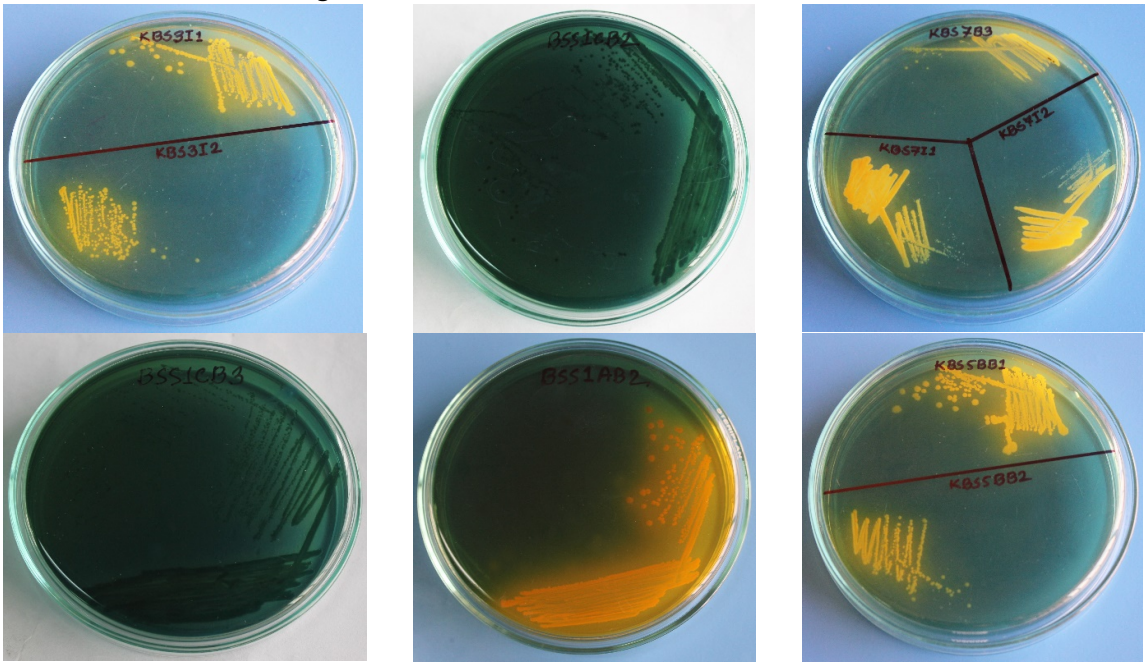

Figure 6: Sub culture of some bacterial isolates

11.5. Selection of *Vibrio* isolates for Biochemical and Molecular Identification

Total 500 samples from different regions were collected and isolated *Vibrio* spp. from each of the samples. Morphologically different isolates from each sample were selected and preserved in glycerol broth for long term storage. Then, secondary selection was conducted from the water, shrimp and sediment samples according to Upazilla and District using the following follow chart. Then selected isolates were considered for molecular identification.

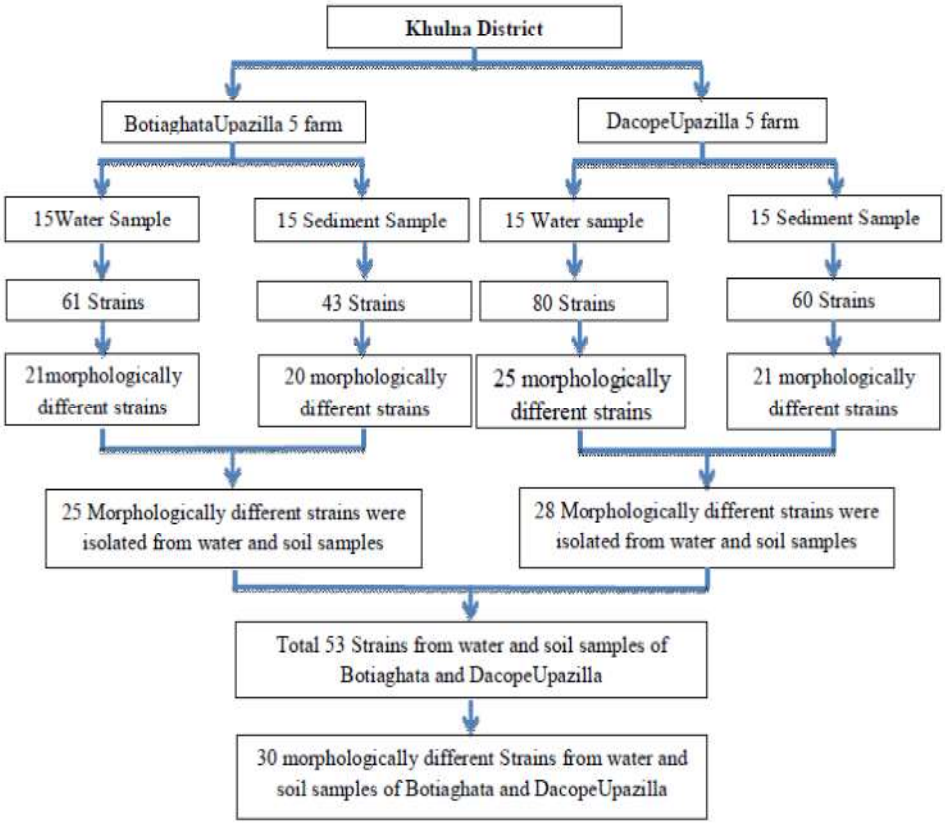

Figure 7: Schematic illustration of selection procedure of *Vibrio* isolates

11.6. Morphological and Biochemical characterisation of the selected isolates

Colony characteristics of the selected isolates were observed on TCBS agar plates and the results were presented in Table. Gram staining was also performed and observed under fluorescent microscope. 150 morphologically different isolates were selected and grown on nutrient agar plates to perform a series of conventional biochemical tests performed for identification of the selected *Vibrio* isolates to the species level. The results were summerised in Table (2).

Table2: Morphological Characterisation of the selected isolates

Strain ID	Size	Shape	Margin	Elevation	Pigment
BSS1AI1	Large	Irregular	Undulate	Raised	Yellow
BSS1AB1	Large	Circular	Entire	Convex	Green
BSS1AB2	Large	Irregular	Undulate	Raised	Yellow
BSS1CB2	Large	Circular	Entire	Convex	Green
BSS2BB2	Large	Circular	Entire	Convex	Green
BSS2CB2	Large	Circular	Entire	Convex	Greenish

BSS2CB3	Large	Circular	Entire	Convex	Yellow
KBS1I1	Large	Irregular	Undulate	Raised	Yellow
KBS4B1	Large	Irregular	Lobate	Raised	Yellow
KBS4B2	Large	Irregular	Undulate	Raised	Yellow

Table 3: Biochemical tests of the selected bacterial isolates

Strain ID	Gram Staining	Salt tolerance tests			VP Test	Oxidase Test	Indole Test	MR Test	Citrate Utilization Test	Lysine Decarboxylase Test
		0% Nacl	6% Nacl	8% Nacl						
BSS1AI1	-	+	-	-	-	+	+	+	+	+
BSS1BI1	-	-	-	-	-	+	+	+	-	-
BSS1BI2	-	+	-	-	-	+	+	+	+	+
BSS1BB2	-	+	-	-	-	+	+	+	+	-
BSS1AB2	-	+	-	-	-	+	+	+	+	-
BSS2AB	-	-	-	-	-	+	+	+	+	-
BSS2CB1	-	+	-	-	-	+	+	+	+	+
KBS1I1	-	+	+	+	-	+	+	+	-	-
KBS1I2	-	+	+	-	-	+	+	+	-	-
KBS1B1	-	+	+	+	-	+	+	+	-	-
KBS1B2	-	+	+	-	-	+	+	+	-	-

(+) indicates positive result and (-) indicates negative result

11.7. Bacterial growth on selective media for selective identification

Vibrio vulnificus Agar and *Vibrio parahaemolyticus* Sucrose Agar media were used for selective identification of *Vibrio vulnificus* and *Vibrio parahaemolyticus.* One hundred and sixty bacterial isolates were selected and these isolates were grown on these selective media. One (01) isolates were shown positive result in *Vibrio vulnificus* Agar media and ninety-nine (99) isolates were shown positive result in *Vibrio parahaemolyticus* Sucrose Agar media. These results were presented in Figure (a) and (b) respectively.

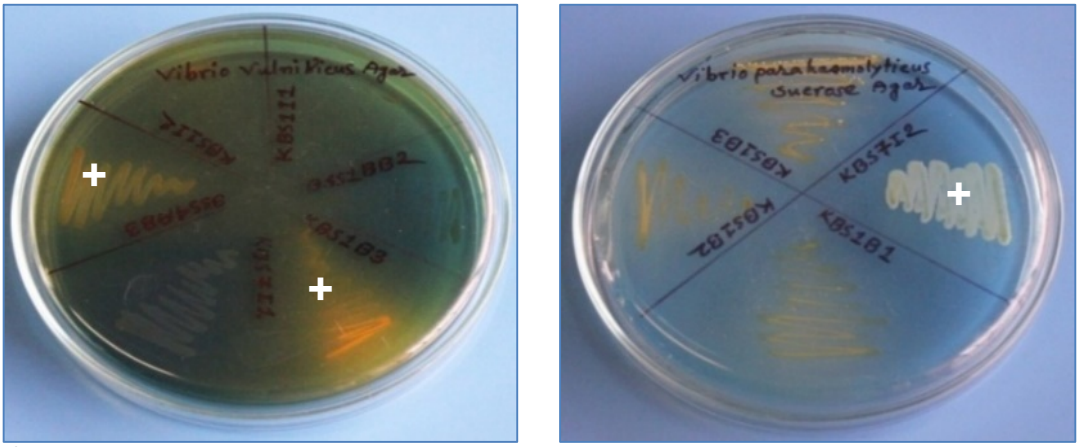

Figure 8: Growth on selective media. (a) *Vibrio vulnificus* Agar, (b) *Vibrio parahaemolyticus* Sucrose Agar
Vibrio vulnificus agar *Vibrio parahaemolyticus* agar

11.8. Molecular Identification of the selected isolates

11.8.1 Identification *Vibrio* genus using Polymerase chain reaction

Initially morphologically suspected *Vibrio* colonies were identified at genus level using gene specific primer. The PCR reaction was performed using two sets of primer. The amplicon size was 689 bp and 663 bp. Among 607 isolates, 231 isolates showed positive band and 376 isolates showed negative result.

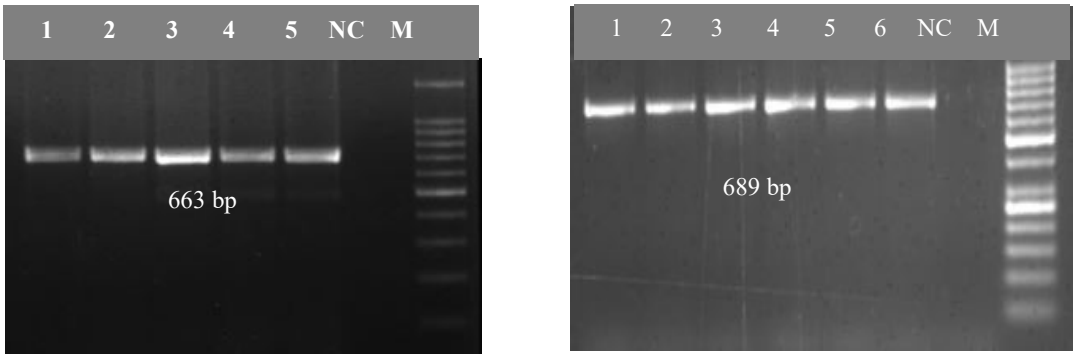

Figure 9: Identification of Vibrio genus using gene specific primer

11.8.2 Identification *Vibrio* species using Multiplex PCR and Confirmation using 16S rDNA

In the present study, two sets of multiplex PCR were used to identify total seven *Vibrio* species including *V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. mimicus, V. cholerae, V. campbelii and V. harveyi.* First set was used to identify five Vibrio species (*V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. mimicus, and V. cholerae)* at a time in a single PCR method and second set was to identify three Vibrio (*V. parahaemolyticus, V. campbelii and V. harveyi)* species in another PCR. Selected 231 isolates were initially analysed by first set of multiplex PCR according to the methods described in materials and method section. In this experiment, total 157 isolates were identified among them 13 *Vibrio cholerae*, 114 *Vibrio parahaemolyticus*, 29 *V. alginolyticus* and 01 *V. mimicus*. Rest of the 74 isolates were further analysed by the second set of mPCR. Results showed that 13 isolates were identified and among them 6 *V. Campbelli* and 7 *V. harveyi.* Sixty one (61) isolates were not identified using the two sets of multiplex PCR. Identified bacteria were then confirmed by single PCR (gene specific) and 16SRNA method also. PCR was performed with genomic DNA by using 16S rDNA bacterial universal primer set. After sequencing, sequence data was then aligned with known 16S rDNA sequences in GenBank database using BLAST search. The sequence data showed maximum similarity with *V. cholerae* sequences database that was observed by phylogenetic analysis. The sequence data and phylogenetic tree were shown in plate. Molecular identification of these isolates was conducted using multiplex PCR (Kim *et al*., 2015). Multiplex PCR is a very utilizable variant of PCR which enables the performance of more reactions in a shorter time comparing to single primer based PCR and also with less effort. The critical point in any multiplex PCR is the performance of the amplification without sacrificing the sensitivity and specificity for higher efficiency. sodB gene specific primer based PCR and 16S rDNA sequencing techniques were also used for molecular identification. These add more precision and accuracy to the phylogenetic identification and also to the true reflection of microbial diversity.

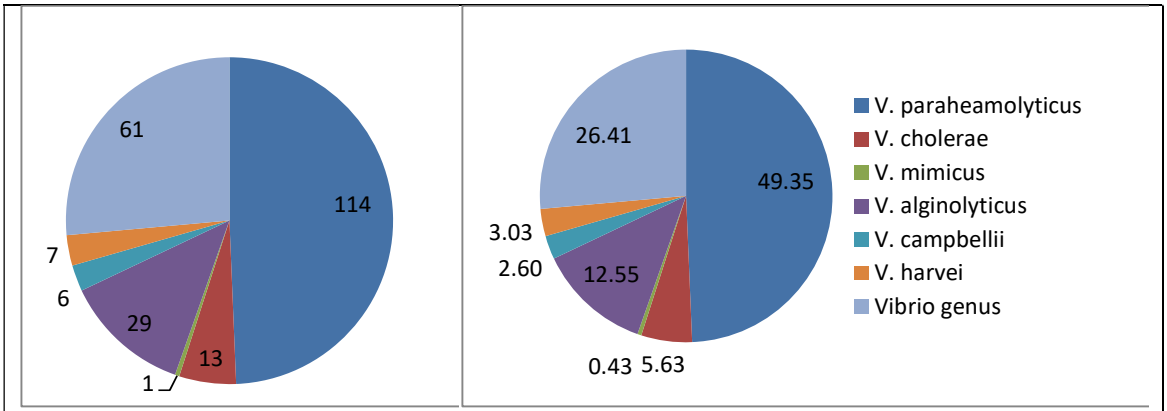

Figure 10: Distribution of *Vibrio* species (n= 231)

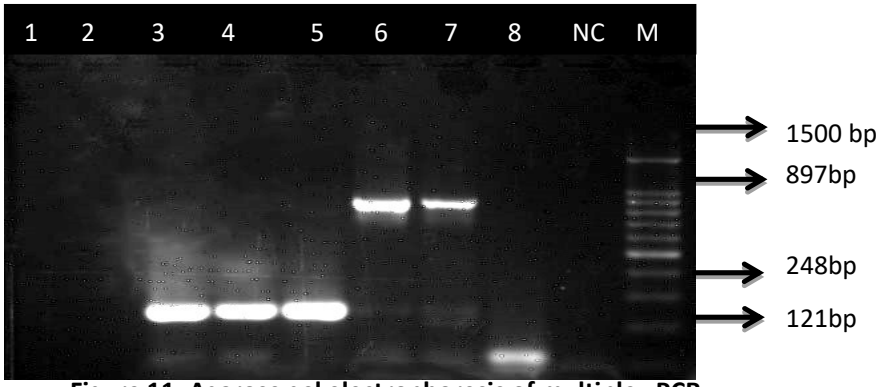

Figure 11: Agarose gel electrophoresis of multiplex PCR

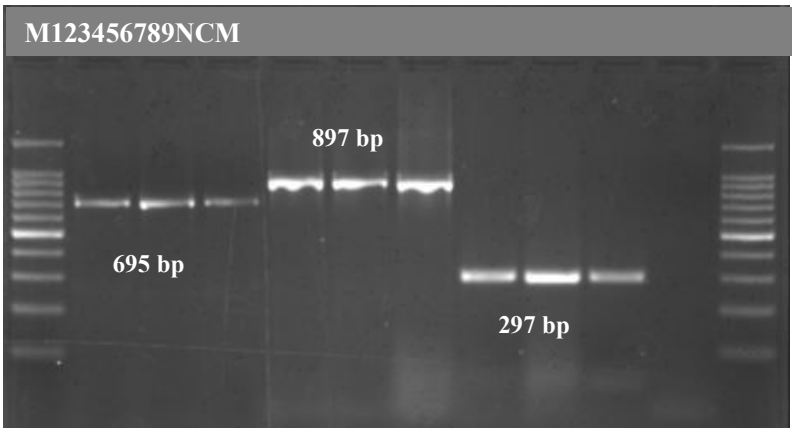

Figure 12: Identification of *V. parahaemolyticus* by gene specific primer

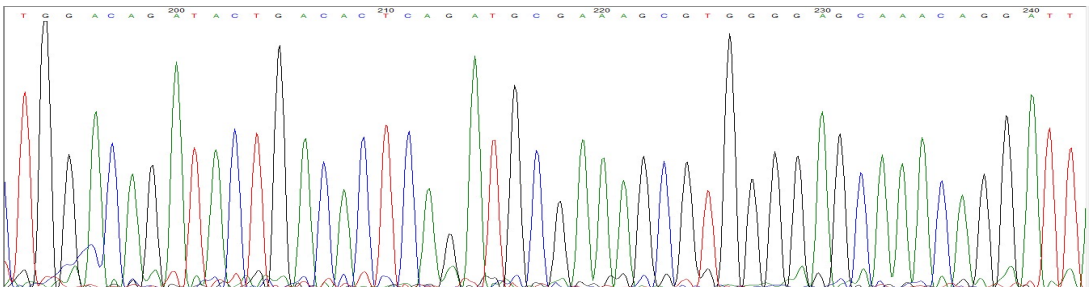

Figure 13: Sequence data of isolated *V. cholerae* strain

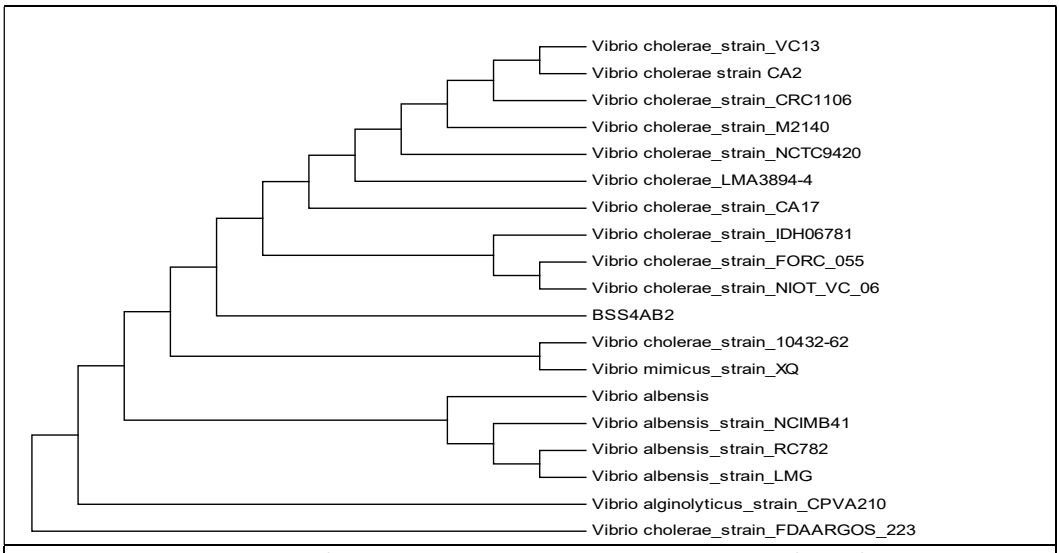

Figure 14: Phylogenetic tree of the selected isolate constructed by the use of Bioinformatics tools

11.9. Study of virulence associated genes of Vibrio parahaemolyticus, Vibrio alginolyticus and Vibrio cholerae

V. parahaemolyticus was confirmed by PCR targeting the toxR gene at 368 bp chromosomal locus specific for this species. *V. parahaemolyticus* strains possess a regulatory gene, *tox*R, which is present in all the strains (Lin *et al*., 1993) irrespective of their ability to produce *tdh*or *trh*. It is noteworthy to mention that *tdh* and *trh* like genes have been found in some strains of other *Vibrio* species such as *V. mimicus*, *V. cholera* and *V. hollisae* (Nishibuchi and Kaper, 1995). Thus their presence may explain the high level of pathogenic *V. parahaemolyticus* than actual. For this reason, in our study *tdh* and *trh* genes were also detected. Distribution of the three virulence genes (toxR, tdh and trh) were amplified from the chromosomes of all *V. parahaemolyticus* strains. All isolates (n=112) showed toxR positive. But only four strains showed positive result against tdh and nine strains against trh (table-4).

On the other hand, three sets of primer used to identify virulence genes of *Vibrio alginolyticus*. The primer sequence, target gene and PCR product sizes were mentioned in material and methods section. toxR gene was present in 58.62% isolates (n=29). Whereas, strain CCSD10B2 and CCS9BI1 were positive for all three genes (Table-5). In addition, Virulence associated genes of *Vibrio cholera* was identified using seven sets of (ctxA, ompW, tcpA, rfbo1, toxR, ace and zot) primer pairs. The primer sequence, target gene and PCR product sizes were mentioned in material and methods section. It was found that ompW was all isolates (n=13) whereas ctxA, tcpA, rfbo1, ace and zot were not found in any isolates. It was also found that toxR gene was present in six isolates (table-6).

Serajum Monir, *et al*., (2013) investigated the virulence potential, as well as phenotypic and genotypic traits of *V. parahaemolyticus* strains occurring in the fresh water environments around coastal southern parts of Bangladesh. In their study, a total of 70 suspected colonies of *V. parahaemolyticus* were isolated from water, sediment and roots of water hyacinth samples collected from four different locations of Bishkhaliriver located in Barisal district of Bangladesh. Of the 70 colonies, fourteen isolates were confirmed as *V. parahaemolyticus*. PCR detection for the presence of toxR, trh and tdh genes among these seven isolates using primers specific for toxR, trh and tdh revealed that all seven were positive for toxR but negative for trh or tdh genes. The results also indicated that the isolates under the study did not contain virulence properties that correlate to the ability of causing infection and diseases.

Table 4: Virulence genes present in *V. parahaemolyticus*

Gene	Positive number	Isolates ID
toxR	112	CCSD8B2, CCSD4I3, CCSD8B4, CCSD1I1, CCS5AI1, CCS5AI2, CCS9AI2, CCS5BI3, CCS9CI2, CCS5DI3, CCS9DI4, CCS9BI2, CCS5EI2, CCSD8B5, CCSD4B6, CCSD9B2, CCSD9I6, CCSD9I4, CCSD6I2, CCSD4I1, CCSD10I2, CCSD10I1, CCSD4I2, CCSD4B4, CCS5BI2, CCS5CB3, CCSD9I1, CCSD10B5, CCSD9B1, CCSD4B3, CCSD2B3, CCSD10I3, CCSD9I2, CCSD1I3, CCSD5I1, CCSD3B1, CCS7BB1, CCS7BI3, CCS7EI1, CCS7DI2, CCS6BI4, CCS6AI2, CCS7DI1, CCS6DI3, CCS7DB1, CCS7CI1, CCS2AB1, CCS2DB1, CSS3BI3, CSS1CI3, CSS1BI1, CSS3CB2, CSS1CI4, CSS1EI2, CSS1AI2, CSS3CI2, KDM2A1, KDW1C3, CCS7AI1, KBM12A1, CCS4BB1, CCS4DB3, CCS4DI2, CCS2DI1, CCS2DB2, CCS1CB1, CSS1EB2, CCSD4B5, CSS4CI2, CSS4EI3, CCS3DI1, CSS2BI2, CSS2AB3, CSS2DI2, CSS2CI1, CSM3B3, CSM3B2, CCS6CI1, CCS7AI2, CCS4AI1, CCS4AI2, CCS8AB2, CCS8BI2, CCS8BI1, CCS8BI3, CCS8DI2, CCS4AB2, CCS4BI1, CCS8EI2, CCS4EI2, CCS4BB3, CCS8CI1, CCS8DI1, CCS4CI1, CCS4CI2, CCS4DI1, CCS4DB1, CCS8AI1, CCS6CI2, CCS6DI4, CCS7CB2, CCS7EB2, CCS3AI1, CSS1CI2, CSS3DI2, CSS3CI1, CSS3AI4, CSS1BI3, CSS3AI2, CSS5CB2, CCS9AB1, CCS9DI2
tdh	4	CCS5AI1, CCS5AI2, CCS9BI2, CCS5EI2
trh	9	CCS5AI1, CCS5AI2 , CCS5DI3, CCS9DI4, CCS9BI2, CCS5EI2, CCS2DI1, CCS2DB2, CSS5CB2
toxR, tdh, trh	5	CCS5AI1, CCS5AI2, CCS9BI2, CCS5EI2, CSS5CB2
toxR, tdh	2	CCS9AB1, CCS9DI2
toxR, trh	4	CCS5DI3, CCS9DI4, CCS2DI1, CCS2DB2

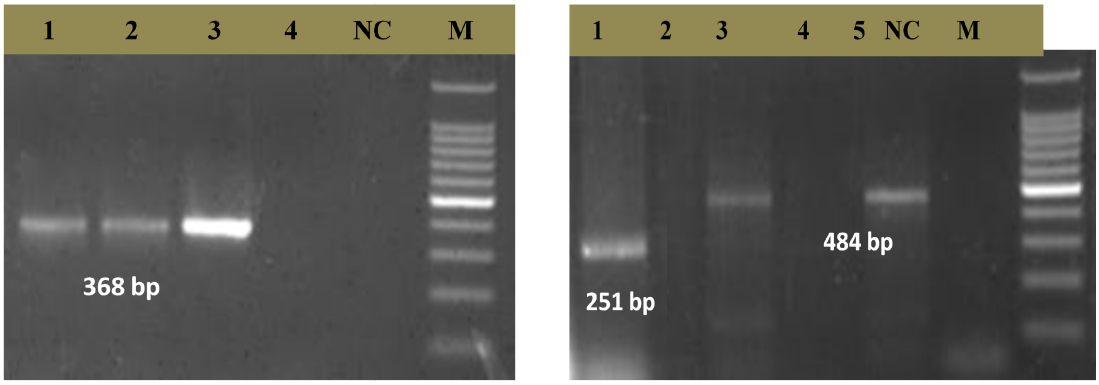

Figure 15: Identification of virulence genes of *Vibrio parahaemolyticus*

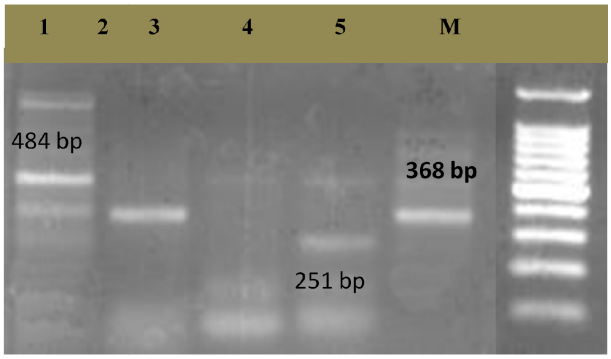

Figure 16 : Identification of virulence genes of *Vibrio alginolyticus*

Table 5: Distribution of virulence genes of *Vibrio alginolyticus*

Strain ID	toxR	tdh	trh	Strain ID	toxR	tdh	trh
CCSD1B1	+	-	-	CCS4EB1	-	-	-
CCSD10B2	+	+	+	CCS8AB1	-	-	-
CCSD10B4	+	-	-	CCS4BI2	-	-	-
CCSD9B3	+	-	-	CCS4EI1	-	-	-
CCSD2B2	-	-	-	CCS4DB2	-	-	-
CCSD2I1	+	-	-	CCS8EI1	-	-	-
CCSD2B4	+	-	-	CSS3EB1	+	-	-
CCSD2B1	-	-	-	CSS1EI3	-	-	-
CCS9DB1	+	-	-	CSS3CB1	-	-	-
CCS9EI1	-	-	-	CSS3BB2	+	-	-
CCS9AI3	-	-	-	CSS3AI1	+	-	-
CCSD6B1	+	-	-	CSS3BI1	+	-	-
CCS9BI1	+	+	+	CSS3EI1	+	-	-
CCS9EBE	+	-	-	CSS3BI1	+	-	-
CCS6BI2	+	-	-				

Table 6: Distribution of virulence genes of *Vibrio cholerae*

Strain ID	ompW	ctxA	tcpA	rfbo1	toxR	ace	zot
BSS1AI1	+	-	-	-	-	-	-
BSS1BI1	+	-	-	-	-	-	-
BSS1BI2	+	-	-	-	-	-	-
BSS1BB2	+	-	-	-	-	-	-
BSS1AB2	+	-	-	-	-	-	-
BSS2CB1	+	-	-	-	-	-	-
BSS3AB	+	-	-	-	+	-	-
BSS4AB1	+	-	-	-	-	-	-
BSS4AB2	+	-	-	-	+	-	-
BSS4AB3	+	-	-	-	+	-	-
KDW1A4	+	-	-	-	+	-	-
KDW12B3	+	-	-	-	+	-	-
KBW12B4	+	-	-	-	+	-	-

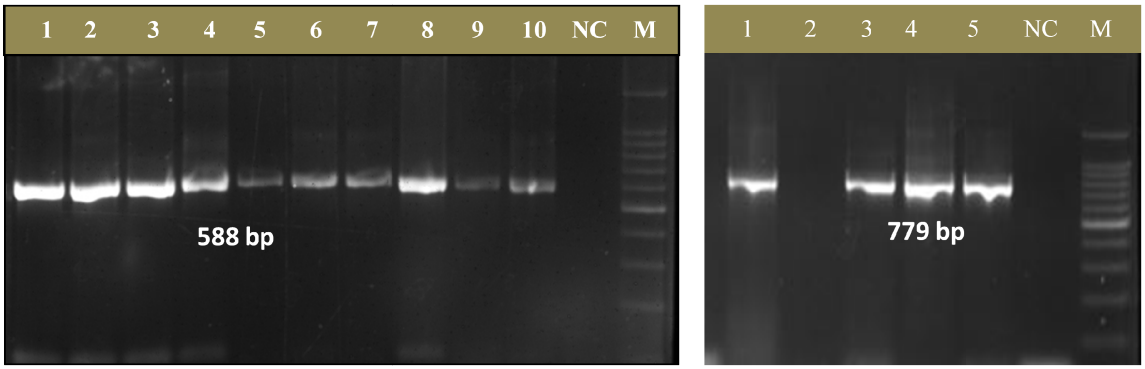

Figure 17: Identification of virulence genes of *Vibrio cholerae*

11.10. Antibiogram of the selected *Vibrio isolates*

Susceptibility of the *Vibrio* isolates to antibiotics was determined by disc diffusion tests based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). Twelve different commercial antibiotics were used against twenty-six isolates of *Vibrio* spp. Antibiotic resistance pattern showed the highest resistance to sulphamethoxazole (92%), followed by gentamicin (89%), trimethoprin (81%) andnalidixic acid (77%). All the isolates were highly sensitive to cotrimoxazole (92%), subsequently tetracycline (85%), chloramphenicol (81%), novobiocin (73%) and ampicillin (69%). Hossain MS *et al*., (2012) found that in the antimicrobial susceptibility test, highest 28.57% resistance among total vibrio species isolates was observed to penicillin and cephalexin. Khan *et al.*, (2002) reported that 100% isolates of enteropathogenic *Vibrio* species from shrimp in Bangladesh were found to show resistance to erythromycin, penicillin, ampicillin, and kanamycin, and 70 and 80% resistance, respectively to cephalexin and streptomycin. The main reason of antibiotic resistance of pathogenic bacteria may be the application of antibiotics in shrimp farming and release of shrimp pond effluent to estuarine ecosystems or postharvest contamination of shrimps with the antibiotic resistant bacteria through environment and human handling.

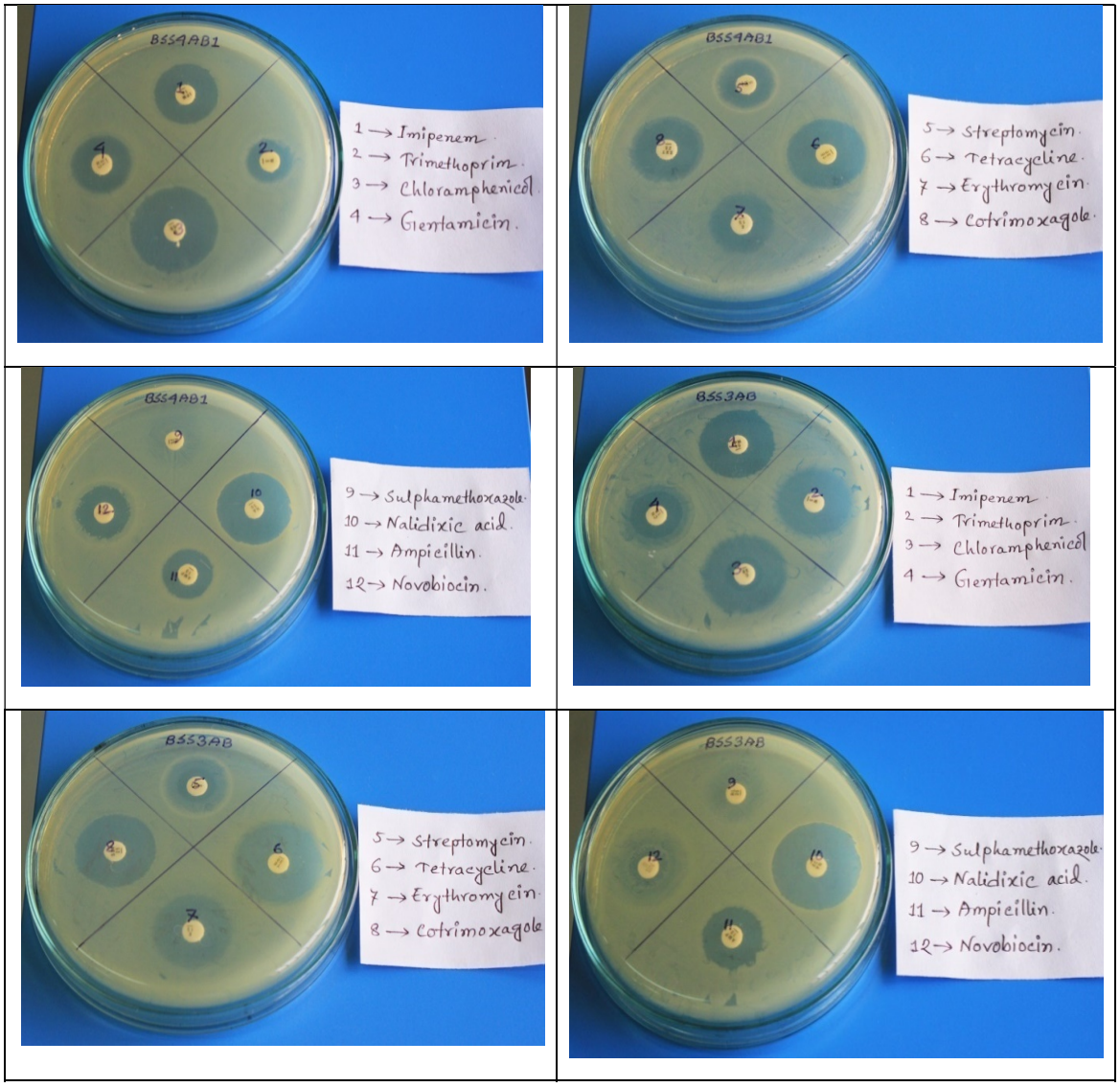

Figure 18: Antibiogram of the selected *Vibrio* spp.

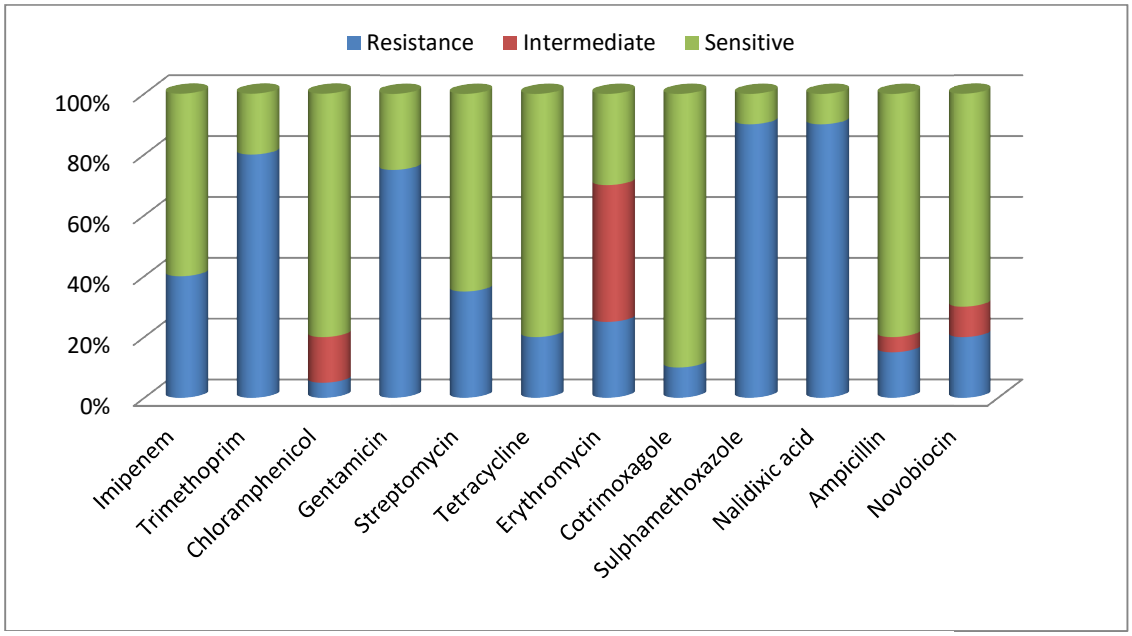

Figure 19: Antibiotic resistance and susceptibility pattern

In conclusion, the multiplex PCR assay adopted in this study can be used as a sensitive and specific assay for the simultaneous detection of *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. cholerae, V. mimicus, V. campbelli*and *V. harveyi*. This method has potential for large-scale screening of harmful *Vibrio* spp. in food and environmental samples. The method provides a fast and reliable way of identifying the main *Vibrio* spp. involved in food-borne disease. The results from the study also suggest that pathogenic *Vibrio* species pose a low risk to consumers, however a larger, more comprehensive survey is recommended.

12. Research highlight/findings:

- Five hundred (500) samples (shrimp, water & sediment) from fifty (50) shrimp firms of five (5) districts were screened for *Vibrio* spp.
- Prevalence of *Vibrio* spp. was highest in the Coxbazar district followed by Satkhira, Khulna and Bagerhat.
- Three sets of multiplex PCR were adopted to detect eight major *Vibrio* species. Total 170 isolates of seven different species were identified using mPCR. Among them, 13 *Vibrio cholerae*, 114 *Vibrio parahaemolyticus*, 29 *V. alginolyticus*, 01 *V. mimicus*, 6 *V. campbelli* and 7 *V. harveyi.*
- Pathogenicity study revealed that most of the *Vibrio* species were non-pathogenic.
- *Vibrio* spp. were highly sensitive to cotrimoxazole, followed by tetracycline, chloramphenicol, novobiocin & ampicillin.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment					
(b) Lab &field equipment	09(Stomacher-01, Refrigerator glass door-01, normal refrigerator-01, Laptop-01, Desktop computer-01, Printer-01, Digital camera-01, Scanner-01, UPS-01)	473500.00	09 (Stomacher-01, Refrigerator glass door-01, normal refrigerator-01, Laptop-01, Desktop computer-01, Printer-01, Digital camera-01, Scanner-01, UPS-01)	473500.00	
(c) Other capital items	03 (Computer table-01, computer chair-01, file cabinet-01)	28500.00	03 (Computer table-01, computer chair-01, file cabinet-01)	28500.00	

2. Establishment/renovation facilities: N/A

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: N/A

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	220820	221608	221608	0	100%	
B. Field research/lab expenses and supplies	981810	981810	981810	0	100%	
C. Operating expenses	242970	219000	219000	0	100%	
D. Vehicle hire and fuel, oil & maintenance	-	-	-	-	-	
E. Training/workshop/ seminar etc.	-	-	-	-	-	

F. Publications and printing	60000	0	0	0	-	
G. Miscellaneous	20900	15000	15000	0	100%	
H. Capital expenses	473500	473500	473500	0	100%	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output(i.e. product obtained, visible, measurable)	Outcome (short term effect of the research)
To identify and characterize *Vibrio* spp. of shrimp of respective areas of Bangladesh	Biochemical and gene specific PCR was used	*Vibrio* spp. was detected from samples	Data reveals the present status of shrimp health in different districts.
To adopt an effective molecular diagnostic tool for characterizing*Vibrio* spp.	Multiplex PCR (mPCR) method was adopted	170 different Vibrio spp. (6 categories) was identified at species level	mPCR may be used to identify major *Vibrio* spp.
To select effective antibiotic against Vibriosis	Antibiotic susceptibility test was performed	Effective antibiotic was identified	Effective Antibiotic may be prescribed in future to reduce drastic loss of shrimp due to bacterial infection

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.	N/A	N/A	
Journal publication	N/A	N/A	
Information development	N/A	N/A	
Other publications, if any	N/A	N/A	

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Multiplex PCR methods have been adopted to detect major *Vibrio* spp. in shrimp farms

ii. **Generation of new knowledge that help in developing more technology in future**

From this study, prevalence of *Vibrio* spp. in shrimp and culture environment was investigated. This might help to understand the present microbiological scenario in our shrimp culture practices. To detect major *Vibrio* spp, multiplex PCR methods have been adopted. This knowledge will help to diagnosis major threatened *Vibrio* species from shrimp firm in future. Effective antibiotics may be used in future to reduce drastic loss in this sector.

iii. Technology transferred that help increased agricultural productivity and farmers' income

Multiplex PCR method may be transferred in other laboratories to detect major *Vibrio* spp.

iv. Policy Support

Information gathered from this study, might be helpful in developing national shrimp/fish health management strategies.

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring[description & output of consultation meeting, monitoring workshops/seminars etc.): Participated in progress monitoring workshops organized by BARC at Dhaka dated 10 April 2018; 15-16 May 2018 and 19-20 September, 2019.

ii) Field Monitoring (time & No. of visit, Team visit and output): N/A

H. Lesson Learned/Challenges (if any)

i) Maximum farmers are not aware of basic shrimp farming management practices.

ii) Inadequate knowledge of farmers in shrimp disease diagnosis and preventive/control measures.

iii) Modern techniques are not available at the field labs/levels to detect Vibriosis rapidly.

I. Challenges (if any)

Sample preservation and transportation with freezing condition from remote areas to National Institute of Biotechnology, Ganakbari, Savar, Dhaka.

Signature of the Principal Investigator
Date ……
Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

J. References

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