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Biochemical and PCR Assay for Detection of Pathogenic Bacteria at Shrimp and Shrimp Farms in Bangladesh

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Abstract

The study was conducted to detect faecal coliforms and pathogenic bacteria in shrimp and shrimp farms. Although coliforms are widely distributed in the environment, but only a small percentage are pathogenic to humans. The presence of pathogenic bacteria in shrimp causes several waterborne infections in humans that are worldwide concerning issues. In order to better determination of the health risks that are associated with the exposure to pathogenic bacteria, a multiplex PCR system was used for the rapid detection of pathogenic *Aeromonas sp., Clostradium sp., Listeria sp., Salmonella sp., Shigella sp., Vibrio sp.* and *Staphylococcus sp.* in the water, sediment and shrimp. The target genes were chosen for this investigation included: Aer gene for *Aeromonas sp.*, hilA gene for *Salmonella sp.*, virA gene for *Vibrio sp.*, Sec gene for *Staphylococcus aureus,* neurotoxin gene type A, B, E, F for *Clostridium botulinum,* internalin gene for *Listera sp.* andipaH gene for *Shigella sp.* Seven pairs of specific primers were used to amplify internal fragments of these genes by PCR to generate PCR products that could be analyzed and confirmed with relative ease by gel electrophoresis. The presence of *Aeromona* and *Vibrio sp.* were found in the water, sediment as well as in the shrimp that were dominant among other bacteria species. On the other hand, *Salmonella* and *Clostridium* group was found in the sediment of one sample but there is no evidence of *Listeria, Staphylococcus and Shigella* group in the farms. All of the samples contained relatively large number (1100 to ≥2400 cfu/g) of coliform bacteria. The unhygienic condition and polluted water source could be the major reasons for the occurrence of these types of pathogenic bacteria in the shrimp farms in this area. The result revealed that the PCR-based rapid detection system described in this study is a powerful method for routine monitoring and risk assessment of water quality in the shrimp farms and could be an effective tool for disease studies in our shrimp sector.

Keywords: Shrimp; Pathogenic bacteria; *Salmonella; E. coli;* PCR detection

Introduction

Fish is the primary source of animal proteins for millions of people in Bangladesh. As a riverine country, Bangladesh poses an immense potential for fisheries which provide 60% of total animal protein supply needed in the nationwide. The fishery sectors in Bangladesh currently contribute about 4.43% of the Gross Domestic Production and more than 2.73% of the export earnings. Shrimp culture is one of the most important divisions in the total fishery sector. In the 2011-2012 fiscal years, the total production of shrimp is about 239460 mt. ton in which 52.05% is from culture basis [1].

The main cultured species is the tiger shrimp, *Penaeus monodon* that is a marine shrimp and is cultivated in brackish water. With strong international demand and high prices culture of this shrimp in the brackish-water habitats in the coastal areas has become popular. Currently *P. monodon* culture is practiced not only in Khulna region but also in the district of Cox's Bazar.

South-western region of Bangladesh, especially Khulna, Satkhira, Bagerhat are most suitable for aquaculture and particularly for shrimp culture. Ponds, Ghers (Closed water field), and rivers are the main source of aquatic foods among the coastal communities of Bangladesh. The major shrimp producing districts are Bagerhat, Satkhira, Pirojpur, Khulna, Cox's Bazar and Chittagong, recently farmers especially in the Bagerhat and Pirojpur districts have begun shrimp farming in their paddy fields. Traditionally shrimp farming began by trapping tidal waters in nearby coastal enclosures known as 'Gher' where no feed, fertilizers or other inputs were applied, with an increasing demand from both national and international markets farmers started to switch over into improved extensive and semi-intensive systems.

Shrimp farming in the south and southeastern coastal belt of Bangladesh began in the early 1970s. From less than 20,000 ha of brackish water ponds in 1980, the area under cultivation expanded to approximately 140,000 ha by 1995. The last complete survey to estimate the total area under shrimp cultivation was carried out in 1993-94; it has not been updated since then. Paul [2] estimated that the total area under farming has expanded to 203,071 ha in 2003-2004.

Shrimp farming concentrated largely in tropical developing countries for export to the west, has experienced spectacular growth over recent decades. Currently, aquaculture industry in Bangladesh and other parts of the world especially southwest region has been facing serious problems due to microbial diseases. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens [3]. In culture conditions various diseases are found which are acting like as natural disaster. Bacterial and viral diseases have most serious losses occurred. Like fish, shrimp lack an antigen/antibody system and so cannot be vaccinated in the way fish can be. Prevention is the only cure.

Coliforms are bacteria that are always present in the digestive tracts

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of animals, including humans, and are found in their wastes. They are also found in plant and soil material.

Total coliforms include bacteria that are found in the soil, in water that has been influenced by surface water, and in human or animal waste.

Fecal coliforms are the group of the total coliforms that are considered to be present specifically in the gut and feces of warm-blooded animals. Because the origins of fecal coliforms are more specific than the origins of the more general total coliform group of bacteria, fecal coliforms are considered a more accurate indication of animal or human waste than the total coliforms. *E. coli* is the major species in the fecal coliform group. Of the five general groups of bacteria that comprise the total coliforms, only *E. coli* is generally not found growing and reproducing in the environment. Consequently, *E. coli* is considered to be the species of coliform bacteria that is the best indicator of fecal pollution and the possible presence of pathogens.

Pathogenic bacteria are bacteria that cause bacterial infection. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. Pathogenic bacteria contribute to other globally important diseases. The pathogenic bacteria belonging to the group *E. coli*, *Salmonella, Vibrio, Staphylococcus, Shigella, Aeromona, Listeria, Clostridium botulinum,* are common and widely distributed in the aquatic environment in various parts of the world. The more psychrotropic organisms (*Listeria*) are common in colder climates, while the more mesophilic types (*V. cholerae, V. Parahaemolyticus*) are representing part of the natural flora of fish from coastal and estuarine environments of temperate or warm tropical and sub-tropical zones [4] Faecal coliform bacteria are available in water wherever it is contaminated with faecal waste of human or animal excreta. Faecal coliforms are the indicator of the presence of the presence of bacterial pathogens such as *Salmonella sp., Shigella, Vibrio cholera and E. coli.* These organisms can be transmitted via digestive tract by contaminated water and may lead to diseases such as gastroenteritis, salmonellosis, dysentery, cholera and typhoid fever. Higher concentration of faecal coliforms in water will indicate a higher risk of contracting waterborne disease. For years, total coliforms and fecal coliform were the most widely used indicators but, more recently, the abundance of *E. coli* has been shown to be more related to the sanitary risk than that of coliforms [5].

The classical species of coliforms are *E. coli* and *Enterobacter aerogenes* which are also known as faecal coliforms have relation to other pathogenic enteric bacteria such as *Salmonella, Shigella, Klebsiella, Proteus, Serratia* etc. that may cause dangerous fever, diarrhea and dysentery [6]. Even members of coliforms are sometimes opportunistic to cause enteric diseases, urinary tract infections, wound infections and bacteraemia [7]. In contrast to non-faecal, faecal coliforms have the capability to grow at elevated temperatures (44-44.5°C) and are associated with the faecal materials of warm-blooded animals [6].

There are various types of bacteria that present in the water body and create various types of disease that economically very harmful in our country. Disease is inevitable in uncontrollable tradition and semi-intensive culture systems. In addition, the use of antibiotics to control bacteria population and maintain healthy environment for shrimp culture becomes popular. A wide range of antibiotics is now being used to treat bacterial diseases and to control bacterial population in the hatcheries and prawn farms [8]. The potential consequences of used antibiotics for treatment may arise various antibiotic resistant, antibiotic-resistant bacteria. The phenomenon resistance was transfer to pathogenic bacteria, and led to reduce efficacy of antibiotic treatment for disease caused by the resistant pathogens [9]. Moreover, all the fish have normal bactericidal activity in their blood serum. Current state of knowledge regarding fish health is still greatly needed. Few attempts have been taken in order to assess the bacterial population in aquatic environment and their involvement in causing disease. Representatives of upwards of 25 bacterial genera have been implicated at various times as pathogens of fresh water, marine shrimp and fish species [10].

Types and levels of bacterial populations associated with farmed brackish water shrimp (*P. monodon*) are the important indicators for the assessment of quality and safety of shrimp. In addition, most diseases in *P. monodon* are caused by opportunistic pathogens which are prevalent in the rearing environment.

Identification of bacteria is essential for the diagnosis of diseases in shrimp. Conventional identification of bacteria involves cultivation of bacteria followed by their biochemical identification. Biochemical identification methods are laborious, time-consuming and at times misleading due to the presence of variants among bacterial species [11]. There are also many bacterial species that are not cultivable by standard methods. PCR has been proved to be a simple and rapid way to identify bacteria.

PCR is an in vitro method for selective, repeated duplication of a specific segment of DNA. The concept of PCR was developed by Kary Muillis and Saiki and co-workers during the early 1980s. The development of nucleotide sequencing methods, the storage of this information in a computer researchable database, invention of automated oligonucleotide synthesis methods, discovery of thermo stable DNA polymerase have all contributed to the rapid implementation and widespread use that PCR enjoys today [12].

In the 40 years since the advent of molecular biology, developments in DNA technology have revolutionized biological research. The study of fish disease is no exception and great advances have been made in this field, particularly in the last 10 years. The time has now come to reap the benefits of work that has provided the means to improve the diagnosis and control of fish disease [13]. Prior to the advent of molecular methods, diagnosis of disease relied largely on culture of the causative organism in media or cells, analysis of phenotypic or serological properties of the pathogen or histological examination of the effects on host tissue. Nucleic acid technology presents the opportunity to detect the pathogen directly, targeting the genetic material and augmenting or replacing culture, serological or histological techniques [14,15].

The presence or absence of a product following PCR may be sufficient to indicate whether a sample is infected by a certain pathogen. In other situations, the specificity of diagnosis can be improved by further manipulations of PCR products and the amplification is only carried out in order to enable these other reactions. Restriction digestion, probe hybridization or even nucleotide sequencing using PCR products can often provide further detail on the identity of the original sample material [16]. When dealing with pathogens, such as *Renibacterium salmoninarum*, which require lengthy culture prior to identification by other methods, PCR may greatly increase the speed of diagnosis [17].

The specificity of assays using primers is usually conferred by further manipulations such as digestion or sequencing. Despite their ready availability for prokaryotic and eukaryotic organisms, universal primers carry an increased risk of false positive results, as they are potentially able to anneal to a wide variety of bacteria or parasites, thus detecting harmless organisms in addition to pathogens. Template

preparation is an important step of any diagnostic test. Traditional methods of nucleic acid preparation involve extractions that use harmful chemicals and may take a considerable time to perform. Many commercial extraction kits are now available that are more user-friendly, but can result in reduced yields and therefore may affect the sensitivity of PCR. The reaction conditions must be optimized. In many cases, development work is undertaken using pure cultures or cloned DNA. The practical application of PCR in diagnostic testing does require significant attention to development of suitable methodologies and validation to ensure greatest possible sensitivity and specificity are obtained. Despite the possible difficulties involved with this technique, PCR will play an integral part in molecular diagnosis of fish and shellfish diseases [18].

The PCR has seen numerous recent applications to pathogen detection and shrimp pathology research. In PCR, small, often undetectable, amounts of DNA can be amplified to produce detectable quantities of the target DNA. This is accomplished by using specific oligonucleotide primers designed for the target DNA sequence. The resultant PCR product may then be compared to a known standard using gel electrophoresis, by reaction with a specific DNA probe of PCR products blotted directly onto a membrane or to the PCR products in southern transfers [19].

Fish importers oftenly complain that pathogenic bacteria are found in the sea foods imported from Bangladesh. Multifarious gastroenteritis problem is common among the coastal communities. It is believe that the main sources of these diseases are water and aquatic foods. So, it is very important to focus a special research on the identification of all possible sources of pathogenic bacteria in aquatic food chain and aquatic environment in the coastal region of Bangladesh.

One of the inherent difficulties in the detection of pathogens in foods and environment is that they are generally present in very low numbers (<100 cfu/g) in the midst of up to a million or more other bacteria and these microbes may be lost among a background of indigenous microflora in the sample. The isolation of these bacteria in the samples is frequently complicated by the presence of nontoxigenic strains that phenotypically and getically resemble.

For pathogenic bacteria several PCR based detection methods have been reported during the last decade [4,19-25]. Compared to conventional methods these protocols provide rapid and sensitive detection of these organisms. The Multiplex PCR method targeting species specific gene may provide a more sophisticated approach, enabling a simultaneous and specific detection of pathogenic bacteria.

The objectives of the study were:

To determine the total coliforms, faecal coliforms, *salmonella sp.* and *vibrio sp.* by biochemical method.

To detect pathogenic bacteria (*Aeromonas sp., Clostridium sp., Listeria sp., Salmonella sp., Shigella sp., Vibrio cholera and Staphylococcus aureus*) by PCR in shrimp and shrimp farms.

Materials and Methods

The Experiment was conducted at Genetics Laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University from July to October 2012.

Study area

Samples (water, sediment and fresh shrimp) were collected from five different shrimp farms which are situated at Khesra and Jalalpur Union of Tala upazilla of Satkhira district.

Sample collection

The samples were collected from five different location of shrimp farm. Mainly water, sediment and shrimp samples were collected. During sampling, the some other information were also checked, such as, the size of the farm, status of sanitation system, culture system, water source, stocking density, disease problem, as well as production of each farm.

Both water and sediment sample were collected from five different point of each shrimp farm by a pasteurized tube and was kept in a sterile 10 ml plastic vial. Fresh shrimp, *Penaeus monodon* (average body weight 15.7 ± 2.5 g) also collected from each farm which was kept in individual polythene bag. The sample containing plastic vials were transported to Molecular Biology Laboratory, FMRT Discipline, Khulna University. Intestine of the collected shrimp was separated by dissecting the body and was kept each in 1.5 ml tube with 1 ml distill water. To avoid cross contamination, separate surgical blade, dissection tray were used for each individual. All the samples were properly labeled and stored in 4°C temperature (Figure 1).

Biochemical test

Biochemical test for total coliform, faecal coliform, *Vibrio cholera* and *Salmonella sp* was done in FMRT Laboratory.

Total coliform: Twenty gram polled sample (water) was weighed and aseptically taken into a sterile warming blender jar and then blended. After blending, the sample was added into conical flask containing 180 ml. of 0.1% peptone water. Ten fold serial dilutions were made with this suspension in McCarty's bottles containing 9.0 ml of 0.1% peptone water. One milliliter of each dilution was transferred into three screw-capped test tubes autoclaved LSTB (10.0 ml) with Durham's tubes.

The tubes were inverted to ensure that Durham's tubes did not contain gas bubbles. These tubes were incubated at 37°C for 24 hours. The formation of gas within 24 to 48 hours was considered as evidence for the presumptive positive test for Coli form organisms.

A loopful of suspension from all LSTB broth tube was transferred into 10. ml. volumes of Brilliant Green Lactose Bile (BGLB) broth. These tubes were incubated at 37°C for 24 hours.

Gas production in BGLB tubes considered as sufficient evidence for the confirmatory test for Total Coli form organisms. Number of positive tubes out of three was recorded for each dilution. Using the MPN charts, the MPN of Total Coliform was calculated based on the portion of conformed LSTB tubes (gas production) for three consecutive dilutions in BGLB broth at 37°C.

Faecal coliform: Those tubes of LSTB, which were positive for gas production in total Coliform estimation tests, were considered. Subcultures were made from all positive tubes of LSTB into a 10 ml volume of BGLB and incubated at 44.5 + 0.5°C up to 48 hours. Any tube showing gas production was considered positive for the presence of presumptive faecal coliforms.

All positive tubes were sub-cultured by streaking onto plates of EMB agar. Then these plates were incubated at 37°C for 18-24 hours. After incubation, the plates were examined for suspicious Faecal Coliform colonies i.e. black or dark centered with the greenish metallic sheen.

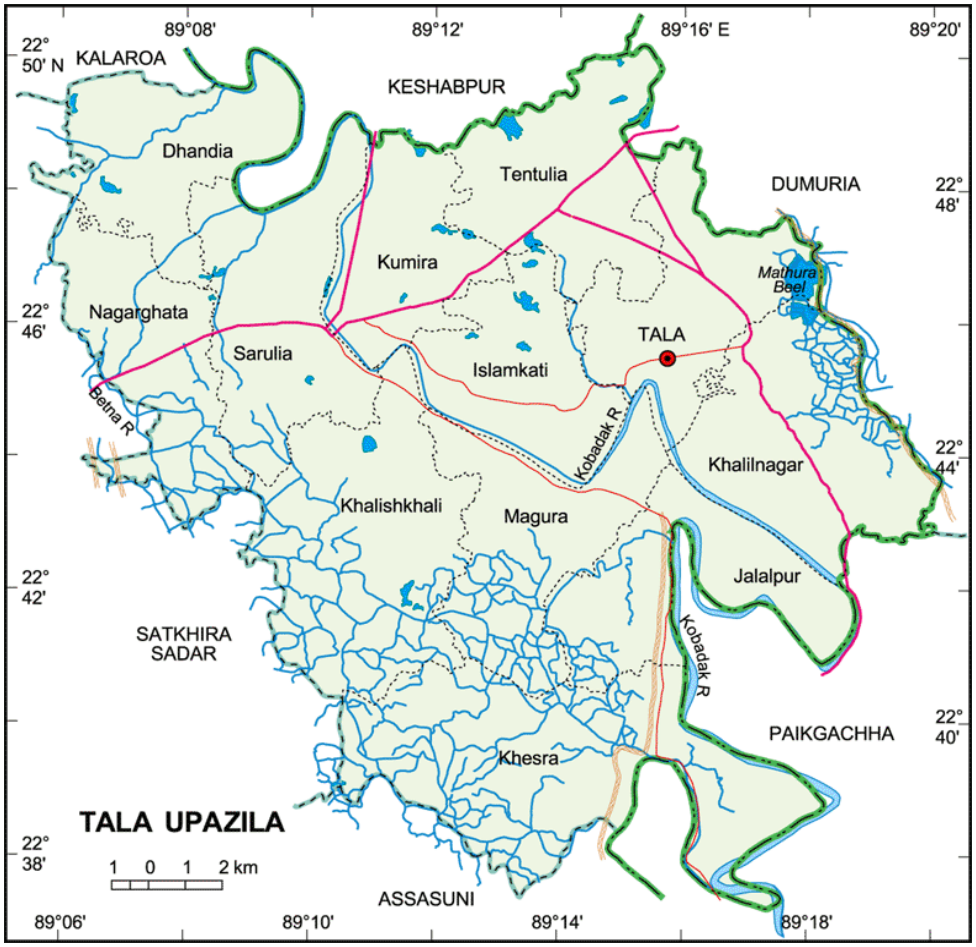

Figure 1: Sampling area at Tala upazilla; the circle indicates the approximate location of sampling farm.

***Vibrio cholerae*:** Twenty five gram's of the sample was added with approximately 225 ml of alkaline peptone water in a warring blender flask. Then the sample containing alkaline peptone water was blended for 1 minute. The sample was incubated at 37°C for 6-8 hrs.

At the end of the incubation period, a loopful of the alkaline peptone water was streaked onto TCBS agar plates. The plates were then incubated at 37°C for 24 hrs. At the end of incubation, plates were checked for the characteristic colony of *Vibrio cholerae*. On TCBS agar colonies appeared as large as (2-3 mm), smooth and yellow, slightly flattened, with opaque center and translucent peripheries.

***Salmonella sp.*:** Two gram of part A and 1.33 gm's of part B of selenite broth was suspended in 100 ml distilled water. It was warmed to mix well. It was dispensed and sterilized in a boiling water bath or in free flowing steam for 10 minutes. It was not done in autoclave because excessive heating is detrimental.

Twenty five gram of sample was weighed and taken into a warring blender flask. 225 ml. of buffered peptone water was added into the sample and then homogenized for 1 minute. Sample was incubated at 37°C for 24 hours.

The resuscitated culture was gently mixed. 1.0 ml of the mixture was added each to 10.0 ml. of Selenite broth and 10.0 ml of Tetrathionate broth. The selective enrichment brothwas then incubated at 37°C for 24 hours. After incubation loopful of each of the two selective enrichment broths were transferred to the surface of the selective agar media, i.e. XLD agar. The plates were then incubated at 37°C for 24 hours and observed for characteristics colonies for *Salmonella sp*. After 24-48 hours; *Salmonella sp*. shows pink colonies with black centre of H_2S on XLD.

PCR test

Preparation of media and culture of organism: Luria-Bertani (LB) broth (Difco Laboratories, MI, USA) was prepared by mixing 2 g LB powder in 100 ml water and then it was autoclaved (121°C, 20 min), then 1 ml LB broth was taken into 1.5 ml centrifuge tube (Eppendrof, Hamburg, Geramny) where 10 µl samples (water, sediment and shrimp intestine) were added and culture was carried out in a shaking incubator at 37°C overnight. A pair of 1.5 ml tube was taken into culture for each sample (water, sediment and shrimp intestine). Following overnight culture, the bacteria sample was used for DNA extraction.

DNA Extraction: According to the Invitrogen manual for DNA zol Reagent, bacterial DNA was extracted from the cultured bacteria. DNAzoL Reagent is a complete and ready-to-use reagent for the isolation of genomic DNA isolation solid and liquid samples. The DNAzoL Reagent procedure is based on the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. DNAzoL Reagent is an advanced DNA isolation

reagent that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzoL Reagent protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes.

During the isolation, a biological sample is lysed (or homogenized) in DNAzoL Reagent and the genomic DNA is precipitated from the lysate with ethanol. Following an ethanol wash, DNA is solubilized in 8 mM NaOH. The procedure can be completed in about 30 min with DNA recovery of 70-100%. The isolated DNA can be used without additional purification for applications polymerase chain reaction (PCR).

Qualitative and quantitave determination of extracted DNA: Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Sambrook. Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus at a constant volt of 100 with a 1% agarose gel containing 1.2 µl ethidium bromide in 120 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK). The detailed procedure was mentioned in appendix. B (Figures 2-6).

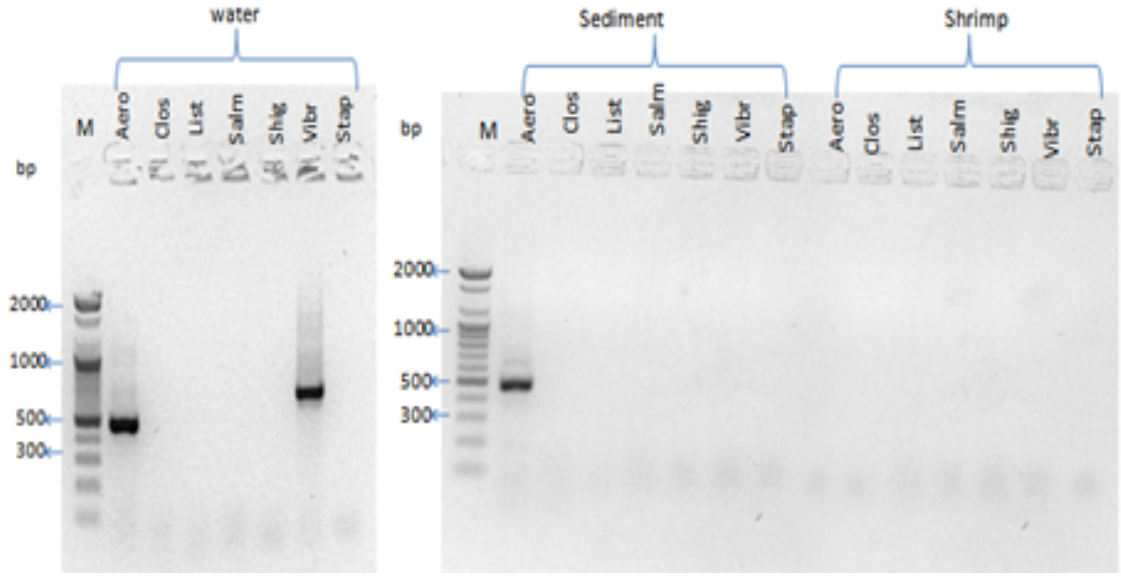

Figure 2: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

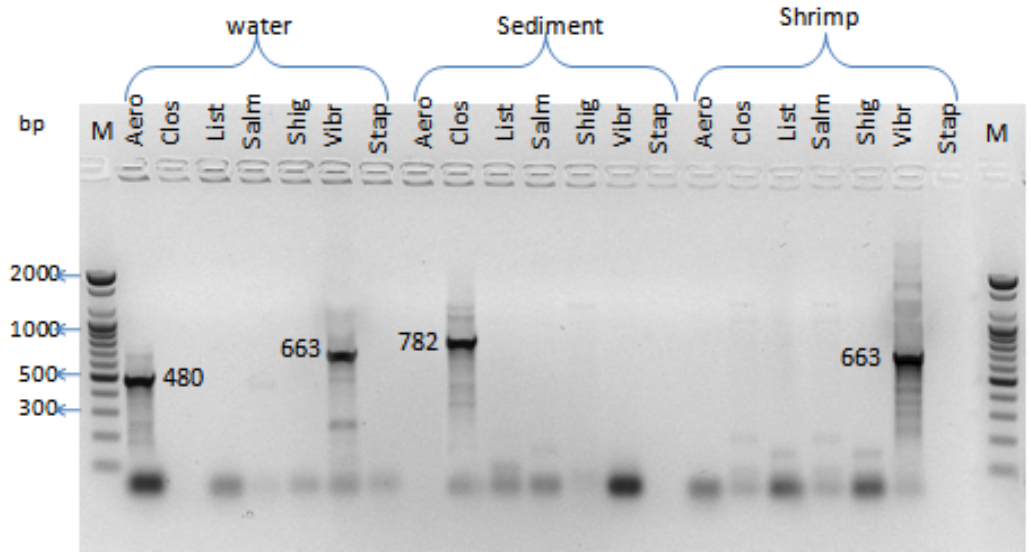

Figure 3: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

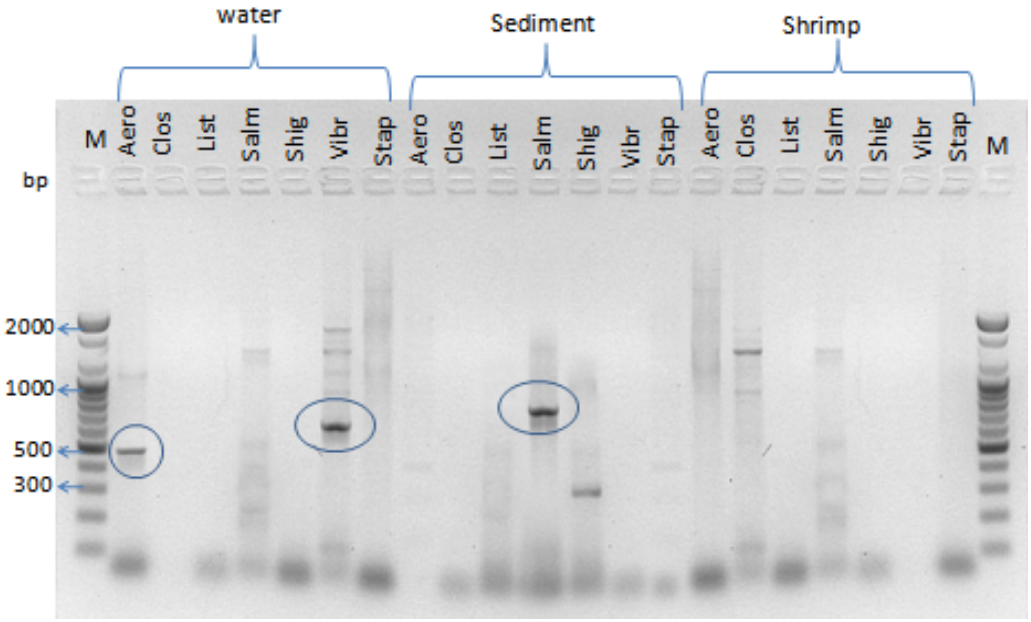

Figure 4: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

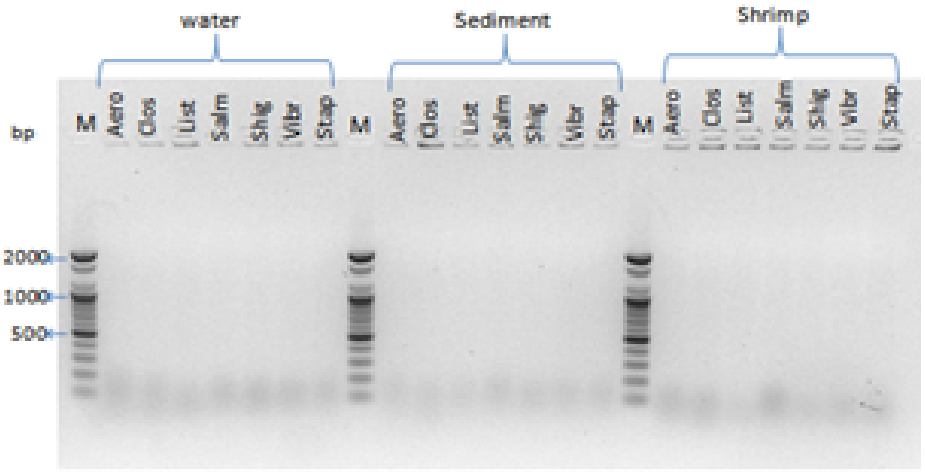

Figure 5: UV visualization of PCR product of farm 4 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 9 to 15 and 17 to 23 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

Components for DNA amplification in PCR

Description of Target genes and primers

Target genes: The target genes were chosen for this investigation included: Aer gene for *Aeromonas*, hilA gene for *Salmonella sp*, virA gene for *Vibrio*, Sec gene for *Staphylococcus aureus,* neurotoxin gene type A, B, E, F for *Clostridium botulinum,* internalin gene for *Listera* ipaH gene for *Shigella*. Seven pairs of specific primers were designed from above mentioned gene sequence (Table 1).

Oligonucleotide primers: The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands that are complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, seven pairs of primers were used. Sequences of the seven PCR primer pairs and size of expected amplification products are shown in Table 2.

The final mixture for the PCR test was 25 µl. In the mixture, to prepare 1 unit *Taq* DNA polymerase, it was 3 times diluted with de-ionized water. The components of the mixture are shown in Table 3.

Top DNA polymerase enzyme: The enzyme was isolated room recombinant E. coli. Strain containing the DNA polymerase gene from *Thermus thermophillus*. It exhibits highest activity at pH 9.0 and 72°C (Bioneer, Korea). To prepare 1 unit Top DNA Polymerase from

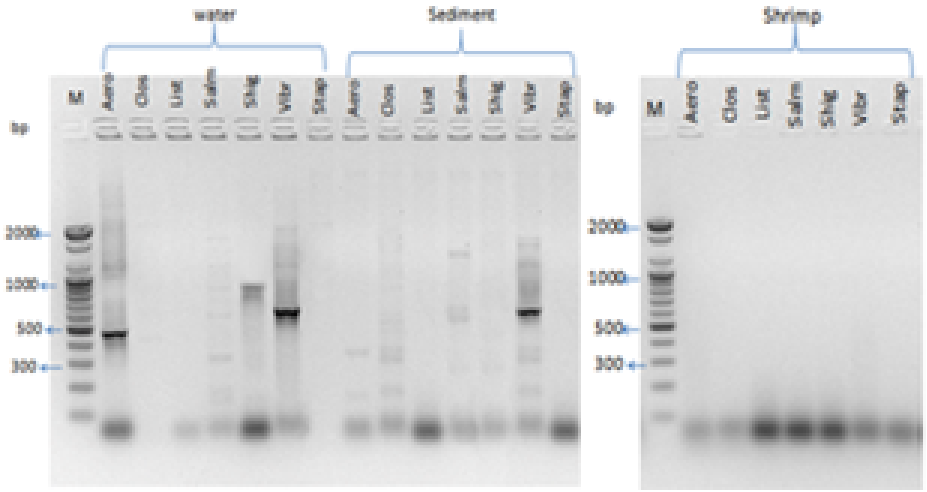

Figure 6: UV visualization of PCR product of farm 5 following electrophoresis in a 2% agarose gel. Lanes 1 to 7 and 8 to 14 represent water, sediment respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. For intestine of shrimp sample Lane 1 to 7 which represent *Aero, Clos, List, Salm, Shig, Vibr, Stap* serially. Lane M represents 100 bp DNA ladder or marker.

Specie	Specific gene	Accession No.	References
Aeromonas	aerolysin toxin (Aer) gene	M 84709	Kannan et al. [19]
Clostridium Botulinam	botulinum neurotoxin gene type A, B, E, or F		Lindstrom et al. [20]
Listeria monocytogenes	internalin gene	M67471	Ingianni et al. [3]
Salmonella	hilA gene		Pathmanathan et al. [22]
Shigella	invasion plasmid antigen H gene (ipaH)	M32063	Thiem et al. [29]
Vibrio	rpoB, toxR gene, vvhA gene, virA gene	D26468	Tarr et al. [24]
Staphylococcus aureus	Sec gene		Mehrotra et al. [29]

Table 1: Specific gene for pathogenic bacteria detection.

Specie	Primers	Sequence (5' →3')	Product size (bp)	Temp
Aeromonas	Aero-F	ATGACCCAGTCCTGGCACGG	480	61
	Aero-R	GCCGCTCAGGGCGAAGCCGC		
Clostridium botulinam	Clos-F	AGCTACGGAGGAGCTATGTT	782	52.5
	Clos-R	CGTATTTGGAAAGCTGAAAAGG		
Listeria monocytogenes	List-F	ACTATCTAGTAACACGATTAGTGA	250	51
	List-R	CAAATTTGTTAAAATCCCAAGTGG		
Salmonella	Salm-F	CGGAACGTTATTTGCGCCATGCTGAGGTAG	784	65.5
	Salm-R	GCATGGATCCCCGCCGGCGAGATTGTG		
Shigella	Shig-F	TGCTGATGCCACTGAGAGCT	939	51.5
	Shig-R	CGTCGATTGTTATATCAGTCTA		
Vibrio	Vibr-F	CGGTGAAATGCGTAGAGAT	663	49.5
	Vibr-R	TTACTAGCGATTCCGAGTTC		
Staphylococcus aureus	Stap-F	AGATGAAGTAGTTGATGTGTATGG	451	50
	Stap-R	CACACTTTTAGAATCAACCG		

Table 2: Primers and expected size of PCR amplified gene targets of pathogenic bacteria.

purchased 5 units, it was diluted with enzyme dilution buffer (50 mM Tris-HCL, 0.1 mM EDTA, 1 mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

Deoxynucleotide Triphosphates (dNTPs) mixture: In the present experiment, 10 mM dNTP mixture (each dNTP 2.5 mM) was used (Bioneer, Korea).

Buffers used for PCR: 10× Reaction buffer (Tris; pH 9.0, 15 mM $MgCl_2$) was used for PCR test.

DNA amplification by a thermal cycler: Desired DNA amplification and target gene amplification were conducted by PCR analysis. In this method, following DNA extraction from samples with forward and reverse primers and *Taq* polymerase. PCR amplification was performed in a Gene Cycler under the following conditions: In brief, heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. This is followed by incubation at 72°C for 10 min followed by cooling at 4°C (Table 3) and the PCR product was analyzed by agarose gel electrophoresis.

Before PCR, made PCR sample by the following way, in Brief, 3 µl

Component	Final volume
1. **Di- ionized water**	13 µl
2. **10X Reaction Buffer** (Tris; pH 9.0, 15 mM $MgCl_2$)	5 µl
3. **10 mM dNTP mixture** (each dNTP 2.5 mM)	2 µl
4. **Template**	2 µl
5. **Primers**	2 µl (Forward 1 µl + Reverse 1 µl)
6. **A *Taq* Polymerase** (5 units/µl; Top DNA Polymerase, Bioneer, Korea)	1 µl

Table 3: The component of reaction mixture for the PCR.

Farm	Sample	Total Coliforms/ gm	Faecal coliforms/ gm	*Presence of*	
				Salmonella	*Vibrio*
F-1	Water	4.39×10^4	3.10×10^4	-	+
	Sediment	4.47×10^4	2.40×10^3	-	-
	Shrimp	2.40×10^2	2.4×10^2	-	-
F-2	Water	6.50×10^4	4.50×10^3	-	+
	Sediment	4.47×10^4	2.40×10^3	+	-
	Shrimp	2.4×10^2	1.1×10^2	-	-
F-3	Water	1.30×10^4	1.70×10^3	-	+
	Sediment	1.50×10^4	1.40×10^2	-	-
	Shrimp	1.10×10^2	15	-	+
F-4	Water	2.40×10^4	1.30×10^4	-	-
	Sediment	2.20×10^4	1.40×10^2	-	-
	Shrimp	2.40×10^2	0	-	-
F-5	Water	4.40×10^4	2.50×10^4	-	+
	Sediment	2.47×10^4	5.40×10^3	-	+
	Shrimp	1.10×10^2	23	-	-

Table 4: The biochemical test result of the total coliform ,faecal coliforms and the presence of *vibrio cholerae* and *salmonella sp*. in 5 water sample, 5 sediment sample and 5 shrimp sample are represented below in graphically.

DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 1 µl primer (Forward 0.5 µl + Reverse 0.5 µl), 2 µl 10x Reaction buffer, 2 µl dNTPs, 1 µl 1 unit Top DNA Polymerase enzyme as well. These all components were mixed in 13 µl distill water and thus a total of 20 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendrof, Humburg, Germany) to operate in a thermal cycler.

Evaluation of PCR products: After completion of the thermal cycling, a sample of 8 µl from each PCR products was analyzed electrophoretically by running on a 2% agarose gel and the amplified product size was determined by comparison with a 100 bp DNA size marker.

Results and Discussion

To analyze the bacteriological quality of fresh shrimps, five habitats (as described in materials and methods) were chosen as representative habitat in Tala Upazilla of Satkhira district of Bangladesh. The water quality of selected five farms was in unhygienic condition. Among the five farms, farm-4 was comparatively in better quality. Its water quality was good than others. Semi-intensive culture was practiced in farm four and traditional culture was practiced in other four farms.

The MPN of total and faecal coliforms in pooled samples was enumerated using LSTB and BGLB by three tubes of three different 10-fold dilutions. Table 4 showed that the MPN of total coliforms is at least 1.30×10^4 per 100 ml of water sample in farm-3 and highest in farm-2 that represents the MPN of total coliforms 6.50×10^4 per 100 ml of water sample. For sediment samples, the MPN of total coliforms is highest in both farm-1 and farm-2, that is 4.47×10^4 per gm of sediment and, the MPN of total coliforms is lowest in farm-3, which is 1.50×10^4 per gm of sediment. The MPN of faecal coliforms was comparatively low than total coliforms in shrimps of that farms.

However, fresh shrimps of five farms had comparatively less number of total and faecal coliforms. But for sediment and water sample, coliforms were comparatively high when we compare to fresh shrimp sample.

Besides the counting of total coliforms and faecal coliforms, we have detected and isolated the *Vibrio cholera* and *Salmonella sp.* by biochemical tests. Among the 15 samples of 5 farms, only one sample represent *Salmonella sp* and 4 samples represent *Vibrio cholera.*

We have done PCR test to sure the presence of pathogenic bacteria in collected sample. Seven pairs of oligonucleotide primers were designed in this study to simultaneously amplify various virulence-associated genes of pathogenic bacteria *(Aeromonas, Clostridium botulinum, Listeria monocytogenes, Salmonella sp, Shigella sp, Vibrio cholera, Staphylococcus aureus)* in a single tube. In the PCR analysis, the PCR product was amplified with designed primers and bacterial extracted DNA of five shrimp farms. The summery of the results were shown in Table 5.

Farm-01: In the case of farm 1, in water, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp* and *Vibrio cholerae* in water sample of farm-1. For sediment sample only *Aeromonas sp* is observed but for shrimp sample no pathogenic bacteria was observed. The water quality of farm-1 was polluted. Traditional culture was practiced and there was no monitoring of water quality.

Farm-02: In the case of farm 2, in water, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp* and *Vibrio cholerae* in water sample of farm-2. For sediment sample pathogenic *Salmonella* sp is observed at 784 bp position but for shrimp sample no pathogenic bacteria was found.

Farm-03: In the water sample of farm 3, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp* and *Vibrio cholerae* in water sample of farm-3. For sediment sample pathogenic *Clostridium botulinum* is observed at 782 bp position but for shrimp sample pathogenic *Vibrio cholera* was present at 6663 bp position,

Farm-04: There was no evidence of pathogenic bacteria *(Aeromonas, lostridium botulinum, Listeria monocytogenes, Salmonella sp, Shigella sp, Vibrio cholera, Staphylococcus aureus)* in the water, sediment and shrimp sample of farm 4.

Farm-05: *Aeromonas, Vibrio cholera* in water sample and *Vibrio cholera* in sediment sample for farm5 is detected by PCR where *Aeromonas* was observed on 480 bp position and *Vibrio cholera* was observed on 663 bp position. For shrimp sample, pathogenic bacteria was not found.

In this study, all shrimp farms sample represent a moderate quantity of total coliform and faecal coliform. In the farm 3, 4 and 5 there were small portion of faecal coliform in shrimp intestine but high in water and sediment. Because most of the selected farms were located in unhygienic condition. There was hanging latrine and the water

Farm		*Aeromonas*	*Clostridium*	*Listeria*	*Salmonella*	*Shigella*	*Vibrio*	*Staphyloccus*
F-1	Water	+	-	-	-	-	+	-
	Sediment	+	-	-	-	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-2	Water	+	-	-	-	-	+	-
	Sediment	-	-	-	+	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-3	Water	+	-	-	-	-	+	-
	Sediment	-	+	-	-	-	-	-
	Shrimp	-	-	-	-	-	+	-
F-4	Water	-	-	-	-	-	-	-
	Sediment	-	-	-	-	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-5	Water	+	-	-	-	-	+	-
	Sediment	-	-	-	-	-	+	-
	Shrimp	-	-	-	-	-	-	-

Table 5: Summery of the PCR results.

monitoring system is very bad. In most of the water and sediment sample pathogenic *Vibrio cholera* and *Aeromonas sp* was found.

The entire bottom water sample contains higher bacterial load then surface water samples. It may happen due to that the most of the organic materials; debris and living organism including shrimp exist in the bottom of the pond that acts as substratum for the bacteria.

In any aquatic system, environmental parameters such as temperature, salinity, pH and dissolved oxygen play a foremost part in the temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and distribution of bacteria [26]. But, pond being a confined environment the optimum environmental parameters (temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and DO 5.1-5.9 ppm) could be maintained throughout the culture period by proper pond management involving water exchange and lime application. The bacterial loads might have changed due to the organic matter deposited at the pond bottom irrespective of environmental factors and water exchange, which was supported by Palaniappan [26] They suggested that environmental parameters did not influence the distribution of bacterial load in the pond ecosystem because there was no dramatic change in the environmental parameters.

Due to the influence of turbidity, heavy organic matter plays a major role in the distribution of bacterial population in pond water [27]. High organic stuff is possible in shrimp culture ecosystem due to organic manure, fertilizers, high stocking density, feed waste, fecal matter, algal bloom and human interference [28].

Shrimp ponds are stressful environments compared to estuaries or other enclosed water bodies [28] (Direkbusararam et al.). This is mainly due to high organic matter and dissolved oxygen fluctuations which affect the composition of natural bacterial communities. Under normal conditions, temperature increments will also bring about a greater diversity of *Vibrio* species [29,30]. When high temperatures and high salinity conditions prevail, some species such as *Vibrio parahaemolyticus* will predominate [31].

In shrimp culture ecosystem, most of the bacteriaplay a negative role as they compete with shrimps for food and oxygen, causing stress and disease [31]. Generally gram-negative bacteria were found to be the dominant forms in the shrimp culture ponds as noticed in the present investigation.

The Total Coliform, *Salmonella and Vibrio* bacteria play a vital role in shrimp culture ecosystem [32] damage water quality causing diseases and mortality to the shrimp as primary and secondary pathogens. *Vibrio* sp. is the autochthonous flora of the coastal pond ecosystem [33]. A sudden increase of bacterial load could develop bacterial infection directly and making shrimps susceptible to infection indirectly as found by Ping and Meimei [34].

Efficient sample treatment methods are needed to fully exploit the potential of the PCR technique to detect pathogens due to the presence of PCR inhibitors [35] (Rossen et al.) Consequently, several treatments to concentrate DNA for the PCR detection of pathogenic bacteriastrains (*Salmonella, Shigella, Vibrio, Listeri sp.*) in shrimp farms and to remove PCR inhibitors have been suggested e.g., lysozyme, proteinase K, detergents by centrifugation and filtration use of proteinase K and organic solvents such as phenol, chloroform and iso-amyl alcohol for deproteinizing cell digest [36].

During the baseline survey, hanging toilet, cattle field were observed near the farms or at the canals or rivers which is the main source of water in shrimp farms. So, these could be the possible source of *E. coli.* in the farms [11].

The present study was carried out to investigate the availability of pathogenic seven types of bacteria strains in the shrimp farms. The presence of various strains was observed among the four studied farms except farm 4.

However considering higher count and prevalence of total and faecal coliforms in almost all of the samples under investigation, it can be inferred that the shrimp cultures in Satkhira district of Bangladesh are exposed to faecal contamination, indicating the contamination by other pathogens also, and further suggested that these shrimps may contain bacterial toxin even after processing and may cause risk for health. The PCR test of pathogenic bacteria confirmed some pathogenic strain in the samples that show the unhygienic condition of the farms.

Depending on the results of this study, the following suggestions may be made to prevent bacterial contamination of shrimps- (i) hygienic conditions for shrimp cultivation should be maintained, (ii) routine microbial analysis of feed is necessary before use, (iii) the feed manufacturer should maintain the microbial quality, (iv) aseptic shrimp seed multiplication farm should be established, (v) water quality should be routinely tested and (vi) awareness should be developed among the personnel associated with this industry. This study will contribute to detect pathogenic bacteria in short time, so that one can understand the fact of bacteriological quality of fresh shrimps and shrimp farms, and to adopt strategies to improve the shrimp quality.

Conclusion

Bangladesh is a country with high incidence of bacterial diseases in shrimp farms due to the most of our water body is not hygienic. Every year vast amount of shrimps is affected in bacterial diseases. It is threatened of human health and disease our total shrimp production. From our present study it may be concluded that shrimp culturing ponds in southern part of Bangladesh are getting contaminated with pathogenic bacteria. Therefore, it is very important to pay attention to good rearing practice, hygiene, use of antibiotic supplemented feed and pollution in the shrimp rearing ponds. Furthermore, investigation and research are required to find out the root cause of different pathogenic bacteria such as *Salmonella sp, Vibrio cholera, Shigella sp, Aeromonas sp, E. coli* and *Staphylococcus sp.* in shrimp rearing ponds in Satkhira, Bangladesh. The application of PCR based techniques is beginning a new era of excellence in the field of fish disease analysis. The findings reported in this study describe a versatile, reliable and highly sensitive PCR system for the rapid detection of pathogenic bacterial species in shrimp farm samples. At present, the main goal of the shrimp industry is to meet the growing demand in a sustainable manner without damaging the environment. Further research is required to enable high-quality validation and trial of these methods as we move into the next century of shrimp health research and development.

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