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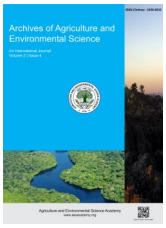

ORIGINAL RESEARCH ARTICLE

Identification of pathogenic *Escherichia coli* strain from river and sewage water in Bangladesh

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ABSTRACT

The study was conducted to isolate and identify the presence of Shiga toxin producing *Escherichia coli* (STEC) strains in water samples of the Old Brahmaputra River and Sewage water at Mymensingh Municipality. A total of 20 water samples were collected for this experiment. Samples were cultured on EMB agar, stained and PCR was done to detect the pathogenic *E. coli*. The Old Brahmaputra River is used as a sink of all types of municipal sewage, agricultural wastes, domestic wastes and religious ritual wastes through unplanned sewerage to the river water body. Low quality sanitation system and open defecation are also considerable problems to deteriorate river water quality. Due to accumulation of municipal untreated wastes to river body, it is possible to contain various pathogens. After investigation and identification, fourteen isolates of *E. coli* was found to contain *stx-1* gene with none of *stx-2* gene among twenty isolates which indicate pathogenic STEC. There may present major health risk to human and animal due to STEC. Several human diseases like mild diarrhea, bloody diarrhea or even severe hemolytic-uremic syndrome (HUS) may occur for drinking of untreated river water. Domestic ruminants can act as a reservoir for STEC and play a significant role in the epidemiology of human infections.

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INTRODUCTION

Bangladesh is a low lying flat country with big inland water bodies, including some of the biggest rivers in the world and is extremely vulnerable because of its geographical characteristics (Matin and Kamal, 2010). Old Brahmaputra River is one of the most important water sources in Bangladesh. It passes through the area near Bangladesh Agricultural University in Mymensingh Sadar Upazila. It has a great impact directly and indirectly to the economy of Mymensingh. Authority of Mymensingh Municipality provides drinking water through supply pipes. Untreated sewage discharges in the Old Brahmaputra River body due to lack of proper treatment plant and sewerage system of Municipality authority. Water is essential to humans and all other life forms. In both developed and developing countries, water quality is a major health concern. In assimilating or carrying off industrial and municipal waste water and runoff from agricultural fields, streets and roads, rivers play a great role that causes river water pollution (Stroomberg *et al*., 1995; Ward and Elliot, 1995). The rivers that flow though Bangladesh comprises of the third largest sources of water to discharge to the oceans (Ali, 2002). Old Brahmaputra River is now on a threat to be polluted at a

large scale due to municipal sewage and other pollutant sources. Safe water and improved sanitation are powerful determinants of health. The WHO program in Bangladesh aims at promoting preventive approaches to water quality management using Water Safety Plans (WSP) to enable utilities, small communities and the households to ensure safety of drinking water. Since the quality of drinking water is closely associated with human health, providing safe drinking water is one of the most important public health priorities. An estimated 80 percent of all diseases and one third of deaths in developing countries are caused by the consumption of contaminated water (Kormoker *et al*., 2017). Generally river is considered as sink of storage of all kind of wastage. Municipal area residents usually depends on supply water and river water is supplied in municipal area without any treatment procedure. River water and sewage water contains various pathogens harmful for livestock and human beings. Among all, Shiga Toxin producing *E. coli* (STEC) 0157:H7 is responsible for mild to life-threatening diarrheal diseases, depending on the type of *E. coli* involved. *E. coli* O157:H7 is an important bacterial pathogen linked with large gastrointestinal illness outbreaks (Manning *et al*., 2008). According to the WHO (1993) guidelines for microbial quality of drinking water, *E. coli* or coliform bacteria must not be detectable in any 100 ml of drinking water sample (Yassi, 2001). *E. coli* O157:H7 is fecal pathogen frequently isolated from waters (Bavaro, 2009) and had found to be the major causative agent of gastrointestinal disease outbreaks (Bopp *et al*., 2003). *E. coli* O157:H7 outbreaks had been found associated with contaminated water sources (Shelton *et al*., 2004) like crop irrigation water, and wastewater on the basis of environmental, epidemiological, and microbiological studies. *E. coli* O157:H7 causes approximately illnesses of 73,000 people, 2000 hospitalizations, and 50–60 deaths each year in the United States (Spickler, 2010). Wastewater is treated to remove pathogens and prevent waterborne disease, a large number of studies indicate that conventional wastewater treatment does not guarantee their complete elimination (Espigares *et al*., 2006). The microbiological quality of wastewater can also pose several potential risks in public health and environment when considering the possible reuse of wastewater effluents (Levantesi *et al*., 2010). Different pathogenic bacteria have been found in effluents including Shiga toxin producing *E. coli* (STEC). The detection of pathogenic microorganisms in wastewater samples is time-consuming and difficult. Indicator microorganisms (e.g. *E. coli*, faecal enterococci) are generally used to assess the microbiological quality of wastewater. Polymerase chain reaction (PCR) has resolved some problems in pathogen detection research. PCR has high specificity, speed and sensitivity (Shannon *et al*., 2007). These traits are required to assess public health risks accurately, quickly identify contaminated wastewater and minimize human exposure (Bertrand and Roig, 2007). The goal of this study was to assess the occurrence and presence of pathogenic *E. coli* O157:H7. To evaluate the occurrence and presence of *E. coli* O157:H7, *E. coli* virulence genes in sewage water and river water samples, PCR was tested.

MATERIALS AND METHODS

Study area and water sampling

The study was carried out through experimental method at Old Brahmaputra River and sewage water at Mymensingh Municipality. Sewerage water samples from sewerage channels and river water samples from Old Brahmaputra River at Mymensingh Municipality were collected and analyzed. It takes three months (September to November, 2017) to carry out the research work, under the department of Environmental Science of Bangladesh Agricultural University. There were five sampling stations such as: (i) Uttara Police Line area (ii) Circuit House area (iii)Mymensingh Zero Point area (iv) Kewatkhali Railway colony area and (v) Boishakhi Chottor area of Banladesh Agricultural University campus (Figure 1). The water samples were collected for bacteriological analysis from five stations of the surrounding aquatic environment vicinity to sewage channels. Total twenty samples (10 from river water body and 10 from sewerage channels) were collected for bacteriological analysis with 100ml Falcon tube. Samples were collected between 9:30 and 11:30 am. The samples were taken from the mid-stream and few centimeters above the surface of drains or channels. These samples were placed in a light-proof box to protect from direct sunlight and then taken to the laboratory for analysis. Necessary information for each sample, date of collection, location etc were recorded in a note book. Samples were brought to the Bacteriological Laboratory of Department of Microbiology and Hygiene at Bangladesh Agricultural University. In the laboratory, samples were kept in dark and dry place.

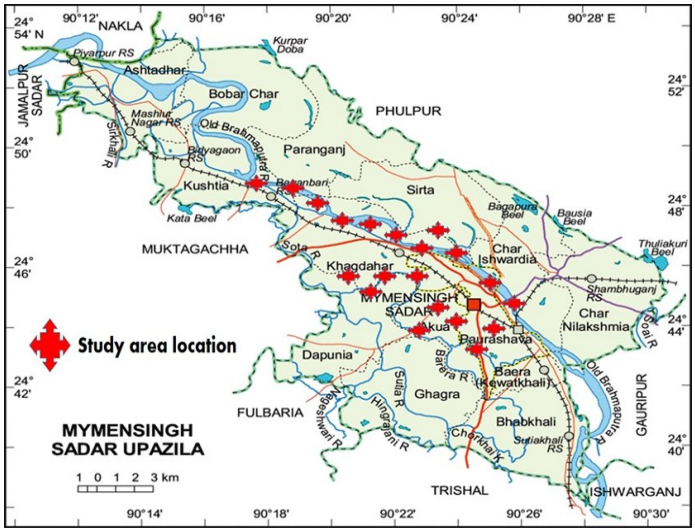

Figure 1. *Map showing study area for collection of water samples.*

Media and reagents for bacteriological analysis

The solid culture media was Eosin-Methylene-Blue (EMB). The liquid media used for this study were Nutrient broth (NB). For Gram's staining Crystal violet, Gram's iodine, Safranine were used and Acetone alcohol, 70% ethyl alcohol was used for hand wash prior to collection of sample and content to work in the laboratory. The different types of glass wares and appliances used during the experiment were as follows: Test tubes, petridishes, falcon tube, immersion oil, centrifuge machine, water bath, inoculating loop,

Table 1. The sequence of primers used to detect *E. coli*.

Primer Name	Gene Targeted	Primer Sequence (5´-3´)	Amplicon size (bp)	Reference
EC16S rRNA F	16Sr RNA	5´GACCTCGGTTTAGTTCACAGA3´	585	Schippa *et al.*, 2010
EC16S rRNA R		5´CACACGCTGACGCTGACCA3´		
EC *Stx*-1 F	*Stx*-1	5´CACAATCAGGCGTCGCCAGCGCACTTGCT3´	606	Heuvelink *et al.*,1995
EC *Stx*-1R		5´TGTTGCAGGGATCAGTCGTACGGGGATGC3´		
EC *Stx*-2 F	*Stx*-2	5´CCACATCGGTGTCTGTTATTAACCACACC3´	372	
EC *Stx*-2R		5´GCAGAACTGCTCTGGATGCATCTCTGGTC3´		

conical flask (100 ml, 500 ml, and 1000 ml), cotton, slides and cover slips, eppendrof tube, test tube stand, pipette, micropipette, incubator, refrigerator, sterilizing instruments, hot air oven, autoclave machine, electronic machine, glass bit, compound microscope, whirly mixture machine.

Materials required for PCR

Primers used for PCR

PCR was performed to amplify the DNA of *E. coli* by using primer. The details about primers are presented in Table 1.

Reagents and applianes used for PCR mixture

Genomic DNA template, PCR master mix 2X (Promega, USA), Forward and reverse primers, Nuclease free water, Eppendorf tube, PCR tube, PCR tips, Micropipette, Thermocycler.

Materials and reagents used for agarose gel electrophoresis

Noble agar, Gel electrophoresis apparatus, Micropipette, Tips for micropipette, UV Solo TS imaging system (Biometra, Germany), Microwave oven, Electronic balance (Precision 0.01g), Loading Buffer 6X (Promega, USA), 100bp DNA ladder (Promega, USA), Ethidium Bromide Solution (0.5 nicrogram/ml), Tris Acetate EDTA (1X TAE) buffer.

Methods of sample and media preparation

Preparation of samples for inoculation

All collected water was centrifuged at 10000 rpm for 3 minutes. Supernatants were discarded and sediments were resuspended with sterile distilled water used for culture.

Preparation of culture media

Nutrient Broth

Thirteen gram of dehydrated nutrient broth (NB) base (Himedia, India) was dissolved in 1000 ml of distilled water, heated gently by an electric heater to dissolve properly and then sterilized by autoclaving at 121°C under 15 lbs pressure per square inch (1kg/cm^2) for 15 minutes (1 kg/cm^2). Five ml broth was transferred in sterile tubes and then stored at 4°C in the refrigerator until use.

Eosin Methylene Blue (EMB) agar media

Thirty six grams powder of EMB agar base (Hi-media, India) was suspended in 1000 ml of distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely in water. The medium was autoclaved for 30 minutes under 15 lbs pressure per square inch (1 kg/ cm^2) to make it sterile. After autoclaving the medium was put into water bath maintaining 45°C and 10-20 ml of medium was poured into small and medium size sterile petridish to make EMB agar plates. After solidifying the medium, the plates were kept in the incubator at 37°C for overnight to check their sterility.

Preparation of 70% Glycerin Solution

300 ml of distilled water after autoclave was added to 700 ml of glycerin (100% available in the market) and dispensed in screw capped tubes or suitable containers. Sterilization was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Isolation of *E. coli* by culturing of sample into bacteriological media

Primary culture was obtained when resuspended samples were inoculated in NB and inoculated at 37°C for overnight. Enriched culture from nutrient broth was streaked on to selective agar media and incubated at 37°C for 24 hours. Single colony appeared on the selective media was further streaked onto selective media to obtain pure cultures. Stock culture was mixed with a medium prepared by adding 200 µl of 70% sterilized glycerol in 300 µl of pure culture in nutrient broth and this was stored at -20°C for further use.

Molecular identification of *E. coli* by PCR

Extraction of genomic DNA from *E. coli*

A pure bacterial colony of each isolates was inoculated in NB and incubated at 37°C overnight. Then one ml of cultured broth centrifuged at 12000 rpm for 3 min and supernatant was discarded and resuspended with 200 µl distilled water. The tube then transferred to boiling water and boiled for 20 minutes then immediately to ice for cold shock about 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. Supernatant were collected and used as DNA template during PCR.

Preparation of PCR mixture for *E. coli*

In a PCR tube PCR mixture (25 µl) for *E. coli* was prepared with following reagents by keeping them on ice. Such as: Master mixture (Promega, USA) = 12.5 µl, Forward primer (20 pmol/µl) = 1 µl, Reverse primer (20 pmol/µl) = 1 µl, DNA template = 2 µl, Nuclease free water = 8.5 µl.

Thermal profile

Thermal profile for the amplification of 16SrRNA, *Stx-1*and *Stx-2* gene of *E. coli* by PCR (Table 2)

Agarose gel electrophoresis of PCR product

The amplified PCR products were resolved in 1.5% agarose gel at 100v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator. The procedure of gel electrophoresis was as follows:

I. Gel casting tray was assembled with gel comb of appropriate teeth size and number then 1.5% agarose solution was prepared in TAE buffer by melting in a microwave oven.

II. Molten agarose was poured onto the casting tray and allowed to solidify on the bench and the hardened gel in its tray was transferred to the electrophoresis tank (Biometra Power Pack P 25, Germany) containing sufficient amount TAE.

III. 5 µl of each PCR product was mixed with 1µl loading dye and the negative and positive sample was loaded to the appropriate well of the gel and 5 µl DNA size marker was loaded in one well.

IV. The leads of the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 100 V for 30 mins. When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading bufter, the power supply was switched off.

V. The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place then the gel was destained in distilled water for 10 minutes. After electrophoresis and staining the PCR products were documented under UV trans-illuminator (Biometra, Germany).

Table 2. Thermal profile for the amplification of 16SrRNA, *Stx-1* and *Stx*-2 gene of *E. coli* by PCR.

Conditions	Temperature (°C)		Time	Cycle(s)	References
	16s rRNA	*Stx-1* and *Stx-2*			
Initial denaturation	95	95	5 min	30	Schippa *et al*., 2010
Denaturation	94	94	0.5 min		
Annealing	58	56	1 min		
Extension	72	72	1 min		
Final extension	72	72	10 min		
Holding	4	4	Until use		

RESULTS AND DISCUSSION

PCR results and pathogenic *E. coli* detection

Cultural characteristics of the isolated *E. coli*

All the samples were found positive for *E. coli* when cultured on EMB agar media. Smooth, circular, black or green color colonies with metallic sheen on EMB agar media (Figure 2) was produced by each isolate.

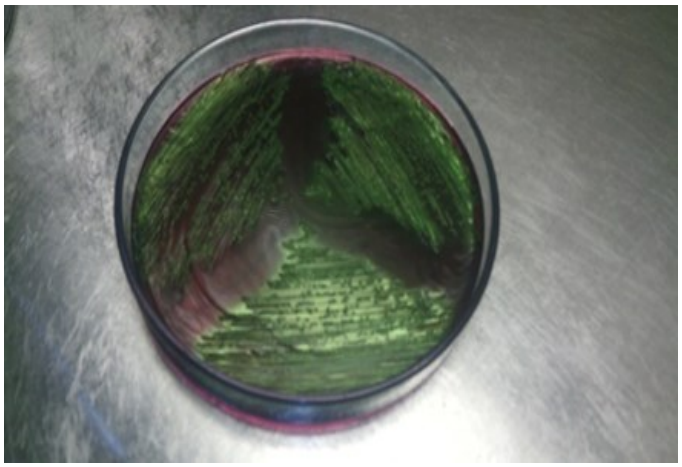

Figure 2. *Greenish black metallic sheen of E. coli on EMB agar.*

Results of *E. coli* detection by PCR

A total of 20 isolates were confirmed as *E.coli* (585 bp) by amplifying genus specific 16S rRNA primers (Figure 3). Among the positive isolates, 14 isolates were found to contain *stx-1* gene (Figure 4) and none were found to contain *stx-2* gene (Table 3).

The cultural properties of *E. coli* like production of metallic sheen on EMB agar was similar with the findings of others Hossain *et al.* (2008); Nazir *et al.* (2005) and Sharada *et al.* (1999).Two specific primers ECO-1 and ECO-2 for *E. coli* were used targeting 16S rRNA gene where PCR product size was 585 bp which was observed in agarose gel electrophoresis that was similar to the findings of other authors (Bashir *et al*., 2015; Schippa *et al*., 2010). Shiga toxin producing *stx-1* gene specific primers produced band size 606 bp.

Prospective human health effect of *Stx-1* positive gene containing *E. coli*

Diseases acquired from contact with contaminated water can cause gastrointestinal illness, skin, ear, respiratory, eye, neurologic, and wound infections. The most commonly reported symptoms are stomach cramps, diarrhea, nausea, vomiting, and low-grade fever resulting from *Stx-1* positive gene containing *E. coli.* People who contract gastroenteritis from drinking water contaminated with *E coli* are at an increased risk of developing high blood pressure, kidney problems and heart disease in later life (Clark *et al*., 2010). *E. coli* O157:H7 infections cause up to 120,000 gastro-enteric illnesses annually in the US alone, resulting in over 2,000 hospitalizations and 60 deaths. However, the long term health effects of *E. coli* infection in adults are largely unknown (Gaffield *et al*., 2003; Clark *et al*., 2010). *Stx-1* positive gene containing *E. coli* is one of hundreds of strains of the bacterium *E. coli*. Although most strains are harmless and live in the intestines of healthy humans and animals, this strain

produces a powerful toxin and can cause severe illness. Infection often causes severe bloody diarrhea and abdominal cramps; sometimes the infection causes non-bloody diarrhea. Frequently, no fever is present. It should be noted that these symptoms are common to a variety of diseases, and may be caused by contaminated drinking water. Children under 5 years of age and the elderly, the infection can also cause a complication called hemolytic uremic syndrome, in which the red blood cells are destroyed and the kidneys fail. About 2%-7% of infections lead to this complication. In the United States, hemolytic uremic syndrome is the principal cause of acute kidney failure in children, and most cases of hemolytic uremic syndrome are caused by *E. coli* O157:H7. Hemolytic uremic syndrome is a life-threatening condition usually treated in an intensive care unit. Blood transfusions and kidney dialysis are often required. With intensive care, the death rate for hemolytic uremic syndrome is 3%-5%.

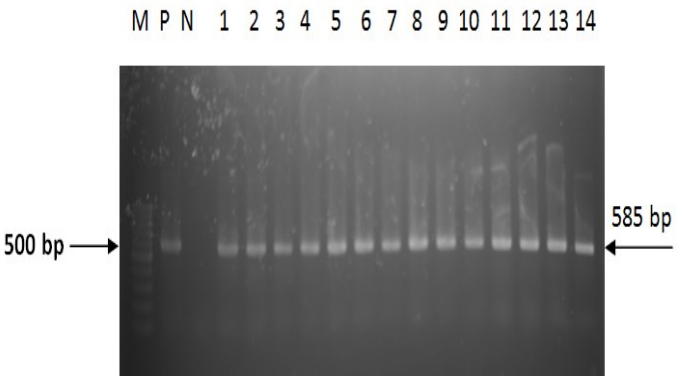

Figure 3. *Agarose gel electrophoresis (1.5%) of PCR products after amplification of 16S rRNA for E. coli. Lane M: 100 bp DNA ladder, Lane P: positive control Lane N: Negative control Lane 1-14: Extracted DNA suspected culture.*

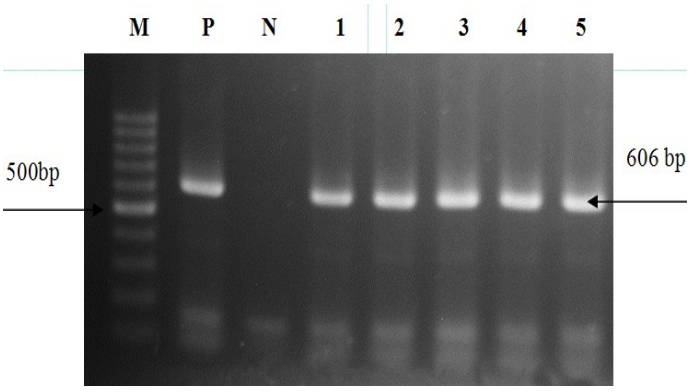

Figure 4. *Amplification of Stx-1 gene; Lane M: 100 bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1-5: Extracted DNA of 16S rRNA positive E. coli isolates.*

Table 3. Summary of prevalence of *E. coli* from collected samples.

Source of samples	No. of total samples	No. of *E. coli* positive samples on the basis of cultural properties	No. of 16S rRNA positive samples	*Stx-1* positive	*Stx-2* positive
Drain and River water	20	20	20	14	00

Conclusion

In Bangladesh life and river are closely related. Fishery, agriculture and even sanitation are directly or indirectly depend on river system in Bangladesh. Due to discharges of municipal sewage and other wastes through sewerage channels to river body, water quality of Old Brahmaputra River is on a threat to be degraded with low water quality parameters and presence of various pathogens. The effects of water pollution are not only harmful to people, but also to animals, fish, and birds. Polluted water is unsuitable for drinking, recreation, agriculture, and industry. The overall test results reveal that the water of Old Brahmaputra River is suitable for irrigation but presence of pathogenic *E. coli* can cause harm to human and animal health when they are in contact with the *E. coli* contaminated water. From the study, it is proved that *E. coli* is present in all water samples with 70% of them are pathogenic. *E. coli* is present mostly in sewage water (found 8 samples are pathogenic) and river water is contaminated with Shiga Toxin producing *E. coli* due to continuous discharge of sewage water in river (found 6 samples are pathogenic from river). However, it is recommended to suggest a way forward in achieving proper management though effective planning for the conservation of this river in near future. Routine research work with wide public awareness, government participation and government regulations can save the water of Old Brahmaputra River and thus a safe and sound water environment can be made for future generations.

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Conflict of interest

No any conflict of interest is declared by the authors.

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