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# Isolation and Molecular Characterization of Shigatoxigenic O157 and Non-O157 *Escherichia coli* in Raw Milk Marketed in Chittagong, Bangladesh

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## ABSTRACT

**Background:** Quality and microbial safety of milk is demanding day by day as it is considered as a host for pathogenic and spoilage microorganisms. In this study, isolation and molecular characterization of shigatoxigenic O157 and non-O157 *Escherichia coli* in raw milk marketed in Chittagong, Bangladesh were done on 186 raw milk samples in Bangladesh.

**Methods:** MacConkey agar was initially used to screen for the presence of *E. coli* and the suspected growth as evidenced by large pink colonies on MacConkey agar. Finally the organism was verified by plating through Eosin Methylene Blue (EMB) agar (a selective medium for *E. coli* where it produces metallic sheen) and applying standard biochemical tests for *E. coli*. The presence of virulent genes, Shiga-like toxin (*stx1 and stx2*), Intimin (*eaeA*), O157 antigen *rfbE* and *Enterohemorrhagic Escherichia coli (EHEC) Hemolysin* (*EHEC*) *hlyA* in the contaminating *E. coli* population was determined by polymerase chain reaction (PCR) run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore).

**Result:** Among the raw milk samples, 33 samples were identified as *E.coli* positive and among the isolates, 6 (18.18%) were identified as possible EHEC O157 and rest of the isolates (81.82%) were considered as probable non EHEC O157. About, 3.23% (186 samples) EHEC O157 was isolated from raw milk samples. Then all the 33 isolates were taken under PCR assay for the identification of five virulent genes *Stx1, Stx2, eaeA, rfbE* and *hlyA*. No virulent genes were found in non- EHEC O157 isolates, but 4 *stx2* (66.67%) and 1 *hlyA* (16.67%) gene were observed in another 4 EHEC O157 isolates out of 6, but one isolates contained the both genes and hence the prevalence of STEC was 2.15% in raw milk. Result indicated poor hygienic standard of raw milk from uncontrolled environments and the increased public health risk of those consuming raw milk from such uncontrolled sources.

**Key words:** Bacterial spores, Dairy products, Milk, Public health.

## INTRODUCTION

*E. coli* is a normal inhabitant of the intestines of animals and humans. The most reported human infections are with entero-hemorrhagic *E. coli* (EHEC O157), asymptomatic reservoirs and excretors of which are ruminants, particularly cattle. Recovery of EHEC O157 from food should be of public health concern because it can lead to severe intestinal and extra intestinal diseases when consumed. Cattle, especially the young ones, have been conceived as a principal reservoir of *E. coli* O157:H7 (Zhao *et al*., 1995). Therefore, insufficient heat-treatment of raw milk possess a potential risk for infection (Betts, 2000) while processing conditions are very crucial for the survival of the bacterium in milk.

*E. coli* has acquired specific virulence attributes associated with diarrhoeal disease and extra-intestinal infections (Kaper *et al*., 2004; Nataro and Kaper, 1998; Russo and Johnson, 2000). Among the intestinal *E. coli* there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). The group of extra-intestinal pathogenic *E. coli* comprises uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC) (Johnson and Russo, 2002, 2005; Russo and Johnson, 2000; Smith *et al*., 2007). Enterohaemorrhagic *E. coli* (EHEC) groups are verotoxin-producing *E. coli* (VTEC) that able to induce haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS)

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(Levine, 1987; Griffin and Tauxe, 1991). Report indicate that consumption of raw milk and various milk products related with occurrence of 1 to 5% of food infections and among that 53% of cases produced by enteropathogenic *E. coli* (EPEC) (Schrade and Yager, 2001).

The Stx (verocytotoxin-producing) family contains two subgroups: *Stx1* and *Stx2*(Paton and Paton, 1998). The production of Stx alone may not be sufficient for VTEC to cause disease (Beutin *et al*., 1995). Other virulence factors may play a role in VTEC pathogenicity, including intimin (encodedby the *eaeA* gene), which is required for intimate adherence of these pathogens to tissue culture cells and formation of the attaching and effacing (A/E) lesion (Mckee and O'Brien, 1996). The formation of A/E lesions is mediated by multiple genes called the Locus of Enterocyte Effacement (LEE) (McDaniel *et al*., 1995). Another virulence factor that contributes to VTEC pathogenicity is the 60-MDa plasmid borne enterohaemolysin A gene (encoded by the E-*hlyA* gene) (Paton and Paton, 1998). A gene, known as *rfbE* more specific for the O157 serotype has been identified (Desmarchelier *et al*., 1998). The various pathotypes of *E. coli* tend to be clonal groups that are characterized by shared O (lipopolysaccharide, LPS) and H (flagellar) antigens that define Serogroups (O antigen only) or Serotypes (O and H antigens) (Nataro and Kaper, 1998 and Whittam,1996). Serotypes include in EHEC groups are *E. coli* O157:H7, the non-motile organism *E. coli* O157:H- and members of other serogroups, particularly O26, O103, O111 and O145 but also O91, O104, O113, O117, O118, O121, O128 and others. The predominant serotype associated with human infection and death is O157:H7 (Levine, 1987; Griffin and Tauxe, 1991). MacConkey agar containing 1% sorbitol (SMAC), often with cefixime and potassium tellurite (CT-SMAC) (Zadik *et al*., 1993), is frequently used to identify EHEC based on lack of β-glucuronidase activity and the inability of rapid sorbitol fermentation. Plasmid profiling may aid in the genetic characterization and molecular screening of different serotypes of EHEC, *e.g*; *E Coli* O157 (Radu *et al*., 2001) and Non-O157 (Nazmul *et al*., 2012).

There are several milk-based commercial companies in Bangladesh and the demand for safe-fresh milk is increasing day by day. Taking into account this consideration, there are groups of milk vendors seen to collect raw liquid milk directly from the farmers and to sell it at the markets - may be local or city-based, or directly to the households. The producers themselves are also seen to do so. If the proper hygienic approaches are not followed through and/ or the collected milk is adulterated with different means, or unconventional materials are used to enhance its shelf life, there is every possibility that milk can be contaminated with zoonotic pathogens, such as *E. coli* with its serious pathotypes: O157 serotype or non-O157 and shiga toxin producing ones and with many more organisms like as *Campylobacter jejuni, Salmonella enterica, Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica etc*. In view of the these particulars, the present study was carried out to isolate and unravel the cultural/biochemical characteristics of *E. coli* from collected raw milk samples and to determine the molecular characterization of *E. coli* O157 and *E. coli* non-O157 using universal, specific, *Stx1, Stx2, eaeA, rfbE* and *hlyA*primers.

## MATERIALS AND METHODS

### Selection of sample collection points

186 fluid raw milk samples (250 ml) were collected where 169 Samples were from fluid-milk marketing points of Chittagong city: Shikalbaha, Sholoshahar Railway Station, Jalalabad Market, Chittagong City Gate, Halishahar, Chittagong Port and Chittagong Batali Road and other 17 samples were from a dairy farm located in Shikalbaha September 2013 to March 2014.

### Procedure of sampling

The samples were directly collected from the bulk sources of incoming fluid raw milk through proper mixing with the help of a plunger and dipper aseptically in a clean sterile bottle. Soon after collection the sample was kept into a cool box for ceasing the growth and activity of acid producing organisms and for shipment to the Department of Microbiology and Veterinary Public Health laboratory of Chittagong Veterinary and Animal Sciences University (CVASU), where the samples were kept at 0°C until investigation.

### Bacteriological investigation

Serial dilution was carried out for the initial screening of E. coli of the collected samples. 100µl of each milk sample was transferred to 900 µl sterile peptone water (0.1%) and thoroughly mixed to give 1:10 dilution, the 'first dilution'; serial dilutions were prepared by transferring one ml from first dilution (10-1) to 9 ml peptone water, (10-2) and so on (10-3, 10-4, …) as described by Harrigan and McCance (1976). Then each diluted milk sample (100 µl) was inoculated onto MacConkey agar medium (Oxoid, Basingstoke, Hampshire, UK), where *E. coli* produces large pink colour colony after incubation of 24 hrs at 37°C.

MacConkey agar medium was prepared according to the manufacturer instructions. Five large pink coloured cross-sectional colonies from MacConkey agar medium were homogenized and inoculated onto an Eosin Methylene Blue (EMB) (Oxoid, Basingstoke, Hampshire, UK) agar plate, incubated at 37°C for 24 h to verify whether such population produced colonies with metallic sheen, a diagnostic criterion for *E. coli*. (Dyes Eosin Y and Methylene Blue react with products released by *E. coli* from lactose or sucrose as carbon and energy source, forming metallic green sheen.) The isolates from MacConkey produced metallic sheen on EMB were considered as probable *E.coli*. A portion of a colony displaying characteristic metallic sheen on EMB was inoculated into TSB (Trypticase Soya Broth) broth, incubated at 37°C for 20 h and finally tested for standard biochemical tests for *E. coli*, *e.g* Catalase test, Indole, Methyl red, Voges-

Proskauer test, Nitrate reduction, Urease production, Simmon's citrate agar and various sugar fermentation tests. When a broth culture in TSB gave typical reaction was finally deduced as *E. coli* and then preserved at -80°C with 15% glycerin.

CT-SMAC (Oxoid, Basingstoke, Hampshire, UK) agar added with cefixime tellurite, which is a selected medium for EHEC O157, was initially used in this study to screen any probable *E. coli* O157 which is incapable of fermenting sorbitol and thus produces colorless colonies on medium. CT-SMAC was prepared according to the manufacturer's instructions. Briefly, 25.75gm sorbitol MacConkey agar was weighed, mixed with 500 ml of distilled water and autoclaved. The medium in the flask was then placed in a hot water bath until become cooled to 50°C when 2 ml of cefixime tellurite (potassium tellurite 1.25mg and cefixime 0.05mg) was added to it. Cefixime-tellurite (CT) added medium was poured into petridishes at the amount of about 20 ml medium per petridish and the medium in petridishes are preserved at +4°C before the use, as recommended.

Growth of a probable EHEC O157 colony on a CT-SMAC agar plate was presumptively diagnosed if it was slightly transparent, colorless with a weak pale brownish appearance and with a diameter of 1mm. Five such cross-sectional colonies were picked up and transferred to a 10 ml test tube containing 5 ml of tryptic soy broth (TSB), incubated at 37°C for 6 h and preserved at -80°C with 15% glycerin until investigation, for observing more diversity at molecular level.

**Screening the *E. coli* isolates of milk origin for the virulence genes - hlyA, stx1, stx2, eaeA and rfbE**

The diversity of all the probable EHEC isolates as found producing colorless colonies on CT-SMAC were investigated based on the presence of five virulent genes - hlyA, stx1, stx2, eaeA and rfb by Polymerase Chain Reactions (PCR). The sequences of five oligonucleotide sets of primers, respectively, used for hlyA, stx1, stx2, eaeA and rfbE genes are shown in Table 1. The reagents used for their PCR amplifications are listed in Table 2.

After thawing in room temperature, the preserved isolates were inoculated onto 5% citrated bovine blood agar and then incubated at 37°C for 24 hours. After that, 200 µl deionized water was taken in 1.5 ml Eppendorf tube for each isolate. With the help of an inoculating loop, a loop-full of fresh colonies were picked up and transferred to the Eppendorf tube. A homogeneous cell suspension was made. It was vortexed, boiled at 99°C for 15 minutes and then immediately placed upon ice. Bacterial cell wall breaks down during boiling and DNA is released. A ventilation hole was made in the lid of the Eppendorf tube using a needle. The boiled suspension was then centrifuged at 15000 rpm for 2 minutes and 100 µl of supernatant was taken in another Eppendorf tube. This collected supernatant was used as DNA template. According to the manufacturer's instructions, a stock solution containing 100 Pmol of each primer was prepared by adding molecular grade water. Working solution

**Table 1:** Oligonucleotide primers used to detect *hlyA, stx1*, *stx2,eaeA* and *rfbE*genes.

| Primer | Primer Sequence (5′ - 3′) | Target gene | Anne-aling temp. (°C) | Size of product (bp) | References |
|---|---|---|---|---|---|
| *hly F* | ACG ATG TGG TTT ATT CTG GA | *hly* | 58 | ~165 | desRosier *et al*., 2001 |
| *hly R* | CTT CAC GTG ACC ATA CAT AT | | | | |
| *stx1 F* | ACA CTG GAT GAT CTC AGT GG | *stx1* | 58 | ~614 | desRosier *et al*., 2001 |
| *stx1 R* | CTG AAT CCC CCT CCA TTA TG | | | | |
| *stx2 F* | CCA TGA CAA CGG ACA GCA GTT | *stx2* | 58 | ~779 | Manna *et al*., 2006 |
| *stx2 R* | CCT GTC AAC TGA GCA GCA CTT T | | | | |
| *eae F* | CCCGAATTCGGCACAAGCATAAGC | *eae* | 59 | ~881 | Oswald *et al*.,2000 |
| *eae R* | CCCGGATCCGTCTCGCCAGTATTCG | | | | |
| *rfb F* | CGG ACA TCC ATG TGATAT GG | *rfb* | 58 | ~259 | Desmarchelier *et al*., |
| *rfb R* | TTG CCT ATG TAC AGC TAA TCC | *O157* | | | 1998 |

**Table 2:** Reagents used for PCR amplifications of the five genes - *hlyA, stx1, stx2, eaeA* and *rfbE* in the probable EHEC isolates as produced colorless colonies on CT-SMAC.

| Name | Manufacturer |
|---|---|
| Molecular marker | Thermo scientific O'GeneRuler 1 Kb plus |
| Ethidium bromide solution (1%) | Fermantas |
| 6x Loading dye | Fermantas |
| Electrophoresis buffer 50x TAE | Fermentas |
| Agarose | Seakem® Le agarose-Lonza |
| 10x dream Taq buffer | Fermentas |
| dNTP set (100µM) | Fermentas |
| Dream Taq DNA polymerase (500 IU) | Fermentas |
| Nuclease free water pure | Thermo scientific |

having 20 Pmol concentrations of the primers were used in PCR.

The master mix for PCR of a gene was prepared according to the number of samples to be tested at a time. The proportions of different reagents used to prepare master mix for five different genes are shown in Table 3. Each PCR reaction was run with a final volume of 50 µl where 49 µl of master mix was added to 1 µl of DNA template. The readymade master mix containing Dream Taq DNA polymerase, dNTPs set, Dream Taq buffer (containing 20 mM Mgcl2) was also used to prepare PCR reaction mixture for PCR assay.

### PCR assays

PCR was run on a thermocycler (Applied Biosystem, 2720 thermal cycler, Singapore). The reactions conditions for the hlyA, stx1 and stx2 genes are listed in Table 4. The same reaction conditions were also used for the eaeAand rfbE genes.

After the PCR amplification gel electrophoresis was performed. For gel electrophoresis a gel tray was prepared and proper set up was made by placing comb in position. Then 1% agarose solution (Seakem® Le agarose-Lonza) was prepared in 1x TE buffer by boiling on a microwave oven for 2 minutes. To make 1% agarose solution 50 µg of agarose powder was added to 50 ml of 1x TE buffer. The agarose was cooled to 40-50°C in a water bath and 1 drop of ethidium bromide with a concentration of 5 µg/ml ethidium bromide was added. Finally, the agarose was poured into the gel tray and allowed 20 minutes to solidify the gel.

An electrophoresis tank which was filled up with 1x TE buffer and then the gel was placed into it with the gel tray. Then the comb was removed carefully so that it could produce smooth holes in gel. After that 5 µl of each PCR product for a gene was mixed with approximately 1 µl of a loading dye (Thermo Scientific, fermentas international Inc) and loaded into a gel-hole. First hole was loaded with 1 kb DNA marker (O'Gene Rular 1 kb plus) to compare the amplicon size of a gene product. PCR product was loaded from the second hole.

Electrophoresis was done at 110 volts, 80 Amp for 20 minutes; gel was placed in a water bath for rinsing and examined on a UV trans illuminator (BDA digital, biometra GmbH, Germany). DNA product sizes of 165, 614, 779, 881 and 259 bp were considered for the presence *of hlyA, stx1, stx2, eaeA*and *rfbE*gene, respectively.

## RESULTS AND DISCUSSION

### Proportion of milk samples positive with *E. coli*

Of the 169 raw market milk samples, 33 (18.3%) were found positive with E. coli and out of the 17 samples from a dairy farm 2 (11.8%) yielded *E. coli* characteristic growth of bacterial colonies produced on MacConkey and EMB agar plates based on which *E. coli* was delineated are shown in Fig 1 and 2, respectively. The results of the biochemical tests done with each of the isolate are shown in Table 5. These result are in conformity with other South Asian studies such as Surve *et al*. (2011), Dewangan *et al*. (2017) and Gupta *et al*. (2020).

*E. coli* is an indicator organism to show if any sample/ objects is contaminated with materials of fecal origins. *E. coli*, particularly those belonging to the shiga toxin producing O157 serotype or non-O157 but Shiga toxin producing ones themselves are pathogenic. Life threatening disease may be resulted because of consuming milk contaminated with Shiga toxin producing *E. coli* belonging to O157:H7 or O157: H- (Tarr *et al*., 2000 and Grant *et al*., 2011). This is not the only danger; the presence of *E. coli* in milk does indicate that it might also be contaminated with any other enteric

**Table 3:** Preparation of PCR master mixused for the identification of virulent genes.

| Reagent | Amount for number of samples | | |
|---|---|---|---|
| | 1 | 5 | 10 |
| MQ water (Nuclease free) | 40.8 µl | 204 µl | 408 µl |
| 10 X Dream Taq buffer (containing 20 mM $Mgcl_2$) | 5 µl | 25 µl | 50 µl |
| dNTPs set (40 µM) | 1 µl | 5 µl | 10 µl |
| Forward primer (20 Pmol/ µl) | 1 µl | 5 µl | 10 µl |
| Reverse Primer (20 Pmol/ µl) | 1 µl | 5 µl | 10 µl |
| Dream Taq DNA polymerase (0.4 u/µl) | 0.2 µl | 1 µl | 2 µl |
| Total volume | 49 µl | 245 µl | 490 µl |

**Table 4:** Cycling conditions used for the detection of three genes *hly, stx1* and *stx2* with the recommended primer sets (Nadine *et al*., 2003).

| Step | *hlyA* | *stx1* and *stx2* |
|---|---|---|
| Initial denaturation | 95°C for 3 minutes | 95°C for 3 minutes |
| Final denaturation | 95°C for 20 sec | 95°C for 30 sec |
| Annealing | 58°C for 40 sec | 58°C for 40 sec |
| Initial extension | 72°C for 1 minutes | 72°C for 1 minutes |
| Final extension | 72°C for 8 minutes | 72°C for 8 minutes |
| Final holding | 4°C | 4°C |

**Table 5:** Results of biochemical tests with each *E. coli* isolate from the study.

| Name of biochemical test | Test-result |
|---|---|
| Lactose fermentation | + |
| Catalase | + |
| Citrate utilization | - |
| Indole production | + |
| Nitrate reduction | + |
| Methyl red | + |
| Voges- Proskauer | - |
| Urease | - |
| **Acid from sugar** | |
| Glucose | + |
| Mannitole | + |
| Lactose | + |
| Salicin | + |
| Sucrose | + |

pathogens, such as any members of shigella, vibrio and others, indicating if such contaminated milk is taken, it can lead to the development of any enteric disease.

While examining the fluid milk destined to be marketed at the chittagong metropolitan area it can be concluded that a substantial proportion, *i.e* one in every five milk samples is seemingly contaminated with *E. coli* here. Milk is usually taken, having boiled in Bangladesh. However, if it is taken raw without any heat-treatment it might pose a serious health-risk to the public health because of its contamination with any enteric pathogens of animal or human origins.

### Presence of probable EHEC O157

*E. coli* from only six (18.2%) of the 33 positive samples yielded colorless colonies across the CT-SMAC, suggesting the probable presence of populations belonging to the serotype O157. Growth of probable *E. coli* O157, as evidenced by the colorless colonies on CT-SMAC compared to coloured colonies from other bacteria is shown in Fig 3. The yielding of colorless colonies on CT-SMAC from a milk sample in this study was considered as the probable presence of *E. coli* O157. This cannot be its confirmation until agglutination test is done using specific antiserum. Because of resource limitation this was not done in the study. Very virulent combination in Shiga toxin producing *E. coli* is the presence of both stx1 and stx2 genes plus the *eaeA* gene (Eelco *et al*; 2007). The findings of this study indicate that the presence of Shiga toxin producing *E. coli* in the fluid milk being marketed at Chittagong is very low and only four isolates from the study carried the *stx2* gene. None of the 33 isolates resulted from the study neither had the stx1 nor the *eaeA* gene.

### Shiga toxin producing *E. coli*

Of the probable *E. coli* O157 isolates only 4 (66.7%) had Stx2. The 779 bp sized amplicons indicating the presence of *Stx2* are shown in Fig 4. None of the other virulent genes,

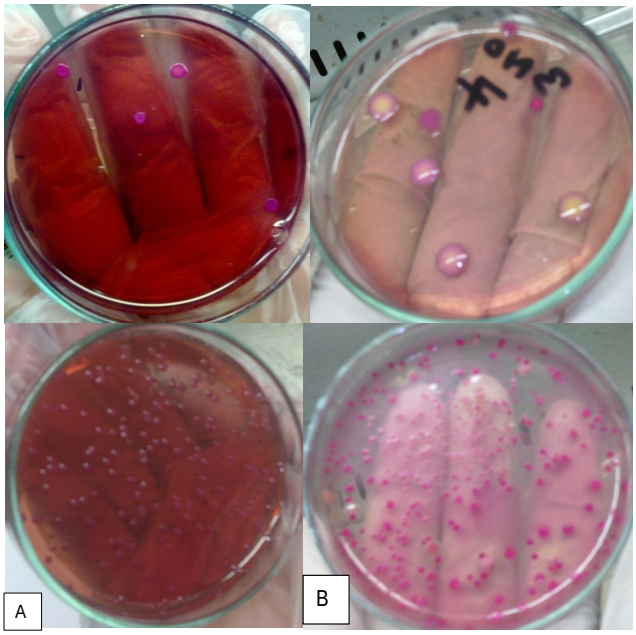



**Fig 1:** Typical large pink colonies indicating the growth of *E. coli* onto Maconkey agar plates.

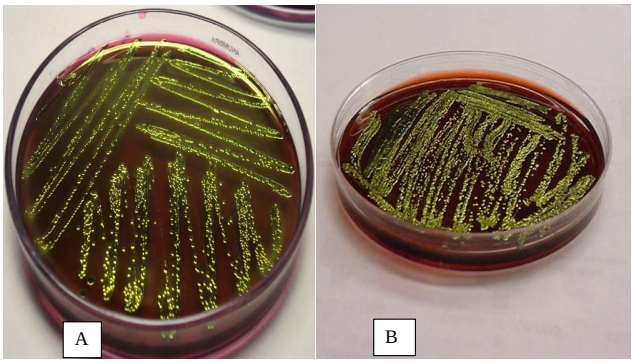



**Fig 2:** Colonies with typical metallic sheen onto EMB agar plates showing a homogenous growth of *E. coli.*

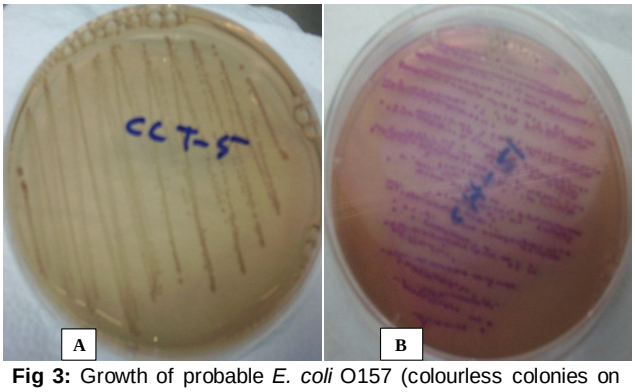



**Fig 3:** Growth of probable *E. coli* O157 (colourless colonies on CT-SMAC agar at the left plate (A) and non- O157 *E. coli* (pink coloured colonies at the right plate (B) of CT-SMAC agar.

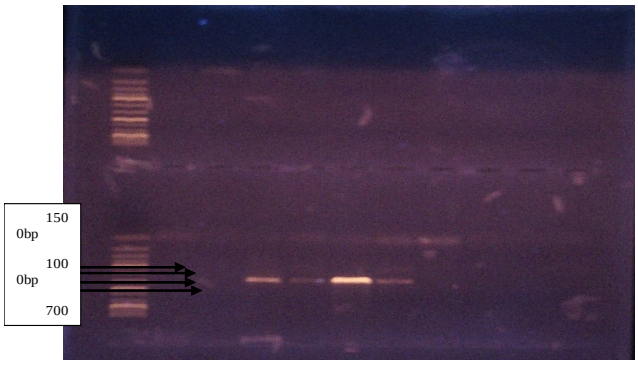



**Fig 4:** Gel picture showing the results on screening *stx2* (amplicon size: 779bp) in the *E coli* isolates.

such as Stx1, eaeA, hlyA and rfbE was found in any of the isolates investigated.

Therefore, although a substantial portion of milk samples carried *E. coli* the danger of having infections with Shiga toxin producing *E.coli*, by consuming fluid milk at Chittagong is low. While saying this low risk, however, the risk for infection with any other enteric pathogen(s) cannot be overlooked or underestimated because presence of *E.coli* in milk indicates the presence of any enteric pathogens.

## CONCLUSION

About 18% of fluid milk marketed in Chittagong contains *E. coli*. Most virulent combination of genes: *stx1, stx2* and *eaeA*in the contaminating *E. coli* seems to be absent; however, Shiga toxin 2 (stx2) producing *E. coli* could be found at a very low proportion, thus indicating some public health risk directly from the presence of *E. coli* in fluid milk. Its presence also indicates the presence of any other enteric pathogens. PCR based molecular epidemiological studies are required for detection of all types of pathogenic as well as zoonotic potential strains of *E. coli* isolates for future research.

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