Influence of Detection Methods in Characterizing *Escherichia coli* O157:H7 in Raw Goat Meat Using Conventional and Molecular Methods

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Presence of *Escherichia coli* O157:H7 on fresh goat meat samples (n= 40) of Dhaka city was analyzed using conventional and molecular methods. A total of 86 presumptive *E. coli* O157:H7 colonies were isolated from 60% of the samples using selective agar plating method. After conventional biochemical assay followed by API 20E assay, only 11 isolates were found to be *E. coli* O157:H7. Further serological test identified only four isolates that has strong agglutination reaction against anti-H7 sensitized latex. The biochemically and serologically confirmed isolates were then screened for major virulence factors include eaeA, rfbE, fliC, stx1 and stx2 genes by PCR. PCR analysis of positive isolates showed, 10 isolates were eaeA and rfbE genes positive but fliC gene was only in six, indicating that these isolates were H7 positive with flagellum antigens which might not expressed or detected in serotyping tests. Multiplex PCR against eaeA, stx1 and stx2 genes of the isolates showed similar results as when done individually. These results revealed that only 7% of the primary presumptive *E. coli* O157:H7 was found to be stx producing *E. coli* O157:H7 and thus greatly influenced the detection of the pathogen in meat samples.

**Key words**: *Escherichia coli* O157:H7 / Virulence factors / Conventional and molecular technique / Fresh goat meat / Dhaka city.

Shiga toxin-producing *Escherichia coli* (STEC) bacteria, including the O157 serogroup, are well-described human pathogens associated with bloody diarrhea, hemolytic-uremic syndrome (HUS), and death (Gould et al., 2009). Illnesses are often attributed to the consumption of contaminated food or water; however, transmission by animal contact may also be important (Hale et al., 2012, Rangel et al., 2005). Ruminants, particularly cattle, are considered the primary reservoir for *E. coli* O157:H7, where the organism typically colonizes the lower gastrointestinal tract (Low et al., 2005) and is shed in the feces. Recent outbreaks of human illness associated with small-ruminant contact at livestock exhibitions (Griffin et al., 2012) have demonstrated the importance of these animals as reservoirs for STEC, including *E. coli* O157:H7. *E. coli* O157:H7 and *Salmonella* are significant foodborne pathogens that can be found in the feces and on the hides of meat animals. When hides are removed during the harvest process, the carcass and subsequent meat products can become contaminated. Camels, cattle, sheep, and goats are harvested for meat in many countries including Bangladesh. The prevalence of *E. coli* O157:H7 and *Salmonella* are unknown in these animals, and it is assumed that if the animals carry the pathogens in their feces or on their hides, meat products are likely to become contaminated. Hoque et al., (2008) reported that unhygienic and poor sanitary conditions are prevailing in slaughter yards and meat stalls and higher fecal coliform was also found in fresh goat meat. As there are no official surveillance exists for meat and meat products in Bangladesh, therefore, the pathogen...
contamination is obvious and the cooking practices help inactivating pathogen and therefore no outbreak event reported. Only non-O157:H7 Shiga toxin-producing E. coli associated diarrhea in Bangladesh has been investigated among hospitalized patients with diarrhea including children and the urban slum community of Dhaka city. Shiga toxin genes were detected by multiplex PCR in 2.2% of hospitalized patients and 6.9% of community patients (Islam et al., 2007). In this study low cost detection method has been sought for the detection and isolation of E. coli O157:H7 from fresh goat meat.

A total of 40 sliced, fresh goat meat samples (approximately 100g) were collected randomly from different markets around Dhaka city. Samples were collected weekly between September to December, 2011 and were transported to the laboratory in a cooling box and processed as early as possible.

Twenty five gram of meat sample was homogenized with 225 ml of Tryptic Soy broth (Oxoid, England) containing 20 mg/L novobiocin in a stomacher (400 CIRCULATOR; Seward, England) and enriched for 6-8 h at 37°C. After enrichment, serial dilution was made and spread plated onto Sorbitol MacConkey Agar (SMAC; Oxoid, England) containing 0.05 mg/l tellurite and 2.5 mg/l tellurite (Merck KGaA, Germany). The colorless colonies were subcultured onto Eosine methylene blue (EMB; Oxoid, England) and 4-methylumbelliferone-l-β-D-glucuronide [(MUG) (Difco, USA)] agar plates. The isolates showing metallic sheen on EMB agar plates and non-fluorescence produce on MUG media were subjected to other biochemical tests (Citrate, Methyl red, Voges-Proskauer, Indole, TSI and oxidase tests). Biochemically identified as E. coli O157:H7 were further confirmed by API kits (API 20E, bioMeriuex Inc.).

Culturally and biochemically identified isolates were serologically analyzed for ‘O157’ and ‘H7’ antigen using commercial ‘Wellcolex E. coli O157:H7 rapid latex agglutination test kit’ (Ramel, USA). To determine the specificity of serological test performed, 4 culturally and biochemically identified non-E.coli isolates were also randomly picked (from the isolates which were tested with API kit) and tested by the same kit.

The genes of E. coli O157:H7 were amplified either singly or in combination. Template DNA was prepared from the isolates which are culturally, biochemically and serologically identified as E. coli O157:H7. DNA was prepared by boiling method as described by Kawasaki et al., 2005 and 2µl of extracted template DNA was subjected to PCR amplification for the detection of genes eaeA, rfbE, fliC, stx1 and stx2 by using specific primers and thermal condition (Table 1). Isolates that gave bands of expected size in electrophoresis were considered to carry these genes. For eaeA, rfbE, fliC, stx1, stx2 gene specific primers 150, 259, 625, 348, 584 bp bands were expected on agarose gel. Four E. coli non O157:H7 were also selected to amplify the eaeA gene. A multiplex PCR for amplification of three genes typical for E. coli O157:H7 was also done to reduce the experiment time and work load. For this, genes for attaching effacing and shiga-toxins were chosen. Primers were same and thermal conditions used were initial denaturing at 94°C for 10 min, 35 cycles of denaturing at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min.

A total of 40 goat meat samples were included in this study. Of 40 samples, 1523 Sorbitol non-fermenting colonies were selected and proceed for further investi-

| Target gene | Primer name | Primer Sequence (5'-3') | Reference | Thermal condition |
|-------------|-------------|-------------------------|-----------|-------------------|
| rfbE        | O157F       | CGGACATCCATGTGATATAGG   | Paton and Paton, 1998 | Initial denaturing at 94°C for 10 min, 35 cycles of 94°C for 1 min; 56°C for 1 min; 72°C for 1 min and final extension at 72°C for 7 min |
|             | O157R       | TTGCTSTGTACAGCTAACATCC  |           |                   |
| fliC        | FLICH7-F    | GCGGTGCGAGTTATCGAG      | Gannon et al. 1997 | Initial denaturing at 94°C for 10 min, 35 cycles of 94°C for 30 sec; 65°C for 30 sec ; 72°C for 75 sec and final extension at 72°C for 7 min |
|             | FLICH7-R    | CAA CGG TCG ATTTAGCCATCC |           |                   |
| eaeA        | VS8         | GGC GG AGTAG CACTCGGATA | Kawasaki et al. 2005 | Initial denaturing at 94°C for 10 min, 35 cycles of 94°C for 20 min; 60°C for 30 sec; 72°C for 30 sec and final extension at 72°C for 7 min |
|             | VS9         | CG TTG GCC ACTATTGCCC   |           |                   |
| stx1        | LP30        | CAGT TAATGGTGGCGGAAG    | Vidal et al. 2004 | Initial denaturing at 94°C for 10 min, 35 cycles of 94°C for 1 min; 56°C for 1 min; 72°C for 1 min and final extension at 72°C for 5 min |
|             | LP31        | CACCAGACA AAT GTAACCGCTC |           |                   |
| stx2        | LP41        | ATGCC TATCCC GGGAG GTT TACG | Vidal et al. 2004 | Initial denaturing at 94°C for 10 min, 35 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 1 min and final extension at 72°C for 5 min |
|             | LP42        | GCGT CAT CGT TAC ACA GAGC |           |                   |
INFLUENCE OF DETECTION METHODS IN CHARACTERIZING *Escherichia coli* O157:H7

Only 7% of the primary presumptive *E. coli* O157:H7 was found to be stx producing *E. coli* O157:H7 and thus greatly influenced the detection of pathogenic *E. coli* O157:H7 in meat samples. Most identified strains of *E. coli* are able to ferment sorbitol but *E. coli* O157:H7 cannot. So isolation of *E. coli* O157:H7 is based on the inability of this organism to ferment sorbitol. For further confirmation, the isolates were checked by agglutination reactions to identify the O157 somatic and H7 flagellar antigens. Presence of O157 somatic antigen confirms the isolates to be *E. coli* O157 and the presence of H7 flagellar antigen confirms the presence of flagella.

Of 1523 sorbitol non-fermenting suspected *E. coli* O157:H7 isolates, on MUG plates 86 showed growth without blue fluorescence which is typical for *E. coli* O157:H7. The MUG negative isolates are presumed to be *E. coli* O157:H7 and followed up for further investigations by biochemical tests distinguishing for *E. coli*. A total of 86 isolates were subjected to biochemical characterization. For further confirmation the isolates which were biochemically identified as *E. coli* O157:H7 were tested with API kits. Among them, 11 isolates were confirmed as *E. coli* with API test kits. Then biochemically positive 11 samples were screened for the presence of O157 antigen and H7 antigen, which are very common characteristic of this pathogenic strain. Of the 11 strains, 10 showed agglutination with O157 anti-sera and remaining one sample did not show any agglutination. On the other hand, only 4 of 11 isolates showed agglutination against H7 anti-sera. None of the four randomly picked isolates gave agglutination reaction against O157 or H7 anti-sera. The biochemically and serologically confirmed isolates were then screened for major virulence factors include *eaeA*, *rfbE*, *flIC*, *stx1* and *stx2* genes by PCR. Among the 11 immunological genes only six showed positive isolates, indicating that these isolates were H7 with flagellum antigens which might not expressed or detected in serotyping tests (Table 2). A PCR analysis revealed that 10 isolates to be positive, which were *eaeA* and *rfbE* gene positive (Figure 1A). Further screening against *eaeA*, *stx1* and *stx2* genes by multiplex PCR of the isolates showed similar results (Table 2). These results revealed that only 7% of the primary presumptive *E. coli* O157:H7 was found to be stx producing *E. coli* O157:H7 and thus greatly influenced the detection of pathogenic *E. coli* O157:H7 in meat samples. Most identified strains of *E. coli* are able to ferment sorbitol but *E. coli* O157:H7 cannot. So isolation of *E. coli* O157:H7 is based on the inability of this organism to ferment sorbitol. For further confirmation, the isolates were checked by agglutination reactions to identify the O157 somatic and H7 flagellar antigens. Presence of O157 somatic antigen confirms the isolates to be *E. coli* O157 and the presence of H7 flagellar antigen confirms the presence of flagella.

**TABLE 2.** Serological agglutination test and specific virulence genes detection by PCR

| Isolated strain number | Serological Test | PCR Detection |
|-----------------------|-----------------|---------------|
|                        | O157 anti-sera  | H7 anti-sera  | *eaeA* | *rfbE* | *flIC* | *stx1* | *stx2* |
| 8b                    | +               | +             | +       | +      | +      | +      | +      |
| 7l                    | +               | -             | +       | +      | -      | -      | -      |
| 3v                    | +               | +             | +       | +      | +      | +      | +      |
| 1l                    | +               | -             | +       | +      | +      | -      | -      |
| SN-2g                  | +               | +             | +       | +      | -      | -      | -      |
| SN-4b                  | -               | -             | ND      | ND     | ND     | ND     | ND     |
| ML-3k                  | +               | -             | +       | +      | -      | +      | +      |
| ML-3j                  | +               | -             | +       | +      | -      | +      | -      |
| ML-4a                  | +               | +             | +       | +      | +      | -      | +      |
| RM-6n                  | +               | +             | +       | -      | -      | -      | -      |
| RM-5k                  | +               | -             | +       | +      | -      | -      | +      |

**FIG. 1.** Agarose Gel Electrophoresis of the amplified genes by PCR method.

(A) Agarose gel electrophoresis showing 150, 348 and 584 bp amplification products of *eaeA*, *stx1* and *stx2* genes specific primers in multiplex PCR. Lane 1: 100 bp marker, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Positive control, Lane 5: No template control. (B) Agarose gel electrophoresis showing 259 bp amplification products of *rfbE* gene specific primers. Lane 1: Positive control, Lane 2: No template control, Lane 3: 100 bp marker, Lane 4: Isolate, Lane 5: Isolate, Lane 6: Isolate. (C) Agarose gel electrophoresis showing 625 bp amplification products of *flIC* gene specific primers. Lane 1: Isolate, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Positive control, Lane 5: 100 bp marker, Lane 6: No template control.
However, not all the biochemically confirmed isolates showed this pattern. Characterization based on nucleic acid sequence is done by PCR procedures that target the shiga toxin genes (stx1 and stx2), the attaching and effacing gene (eaeA), the O157 antigenic gene (rtbE) and the flagellar gene (flIC). Though it was expected that all the biochemically confirmed isolates should possess the genes but not all the confirmed isolates showed these genes. On the other hand, serological test was also observed not enough because two isolates containing the flagellar gene (flIC) showed negative results in serology. In this study, only 7% of goat sample was found contaminated with E. coli O157:H7 even though unhygienic practices of the slaughter house and stalls were evident while purchasing the meat sample.

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