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Isolation and characterization of *Escherichia coli* O157:H7 in raw goat meat in Dhaka city using conventional and molecular based technique

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Abstract:

A total of 40 raw goat meat samples were collected from different markets in Dhaka city during September to December, 2011 and analyzed for the presence of *Escherichia coli* O157:H7, in this study. *E. coli* O157:H7 strains were isolated from 24 samples (60%) of 40 goat meat samples. From the 24 goat meat samples, total 86 presumptive *E. coli* O157:H7 colonies were isolated using different selective agar medium, however, after biochemical and immunological analysis, only 11 isolated colonies were found to be *E. coli* O157:H7, and rest were identified as different strains of *Enterobacter spp*. Further characterization using agglutination reaction with anti-O157 sensitized latex showed that only four isolates 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 polymerase chain reaction. Most clinical signs of disease arise as a consequence of the production of Shiga toxin 1 (Stx1), Stx2 or combinations of these toxins. Among the 11 immunological positive isolates, 10 isolates showed *eaeA* gene positive, which is involved in the attaching and effacing adherence phenotype. These 10 isolates were then subjected to PCR against *rfbE* and *fliC* gene**,** which encodes the ***E. coli*** O157 serotype and **E. coli** flagellum H7 serotype, respectively. All the 10 isolates showed positive result with *rfbE* gene, however, only six *E. coli* strains were fliC gene positive, indicating that these isolates were genetically H7 with flagellum antigens that were either not expressed or not detectable in serotyping tests. A multiplex PCR analysis for *eaeA, stx1* and *stx2* genes of the isolates showed similar results. Therefore, out of presumptive 86 positive *E. coli* O157:H7 isolates only 6 (7%) isolates were confirmed to be shiga-toxin producing *E. coli* O157:H7. Thus the detection/screening methods greatly influenced the estimation of *E. coli* O157:H7 and its virulence factors in goat meat samples.

Keywords: *E. coli* O157:H7, virulence factors, Conventional and molecular technique, raw goat meat, Dhaka city.

1. Introduction

Food borne illness is a major concern of the modern world. Illness associated with the consumption of fresh foods, vegetables and meat have increased in the United States during past decades [27].

The recognition of EHEC as a particular class of pathogenic *E. coli* resulted from two observations. It was reported in 1983 by Riley who investigated two outbreaks of a particular gastrointestinal illness characterized by severe crampy abdominal pain, watery diarrhea followed by bloody diarrhea, and little or no fever [25]. The second observation was made by Karmali, also in 1983, who reported the association of sporadic cases of hemolytic uremic syndrome (HUS) associated with cytotoxin producing *E. coli* in stools [18].

Escherichia coli O157:H7 is one of the hundreds serotypes of the bacterium *Escherichia coli*. *E. coli* O157:H7 has several characteristics uncommon to most other *E. coli*. The O157:H7 serotype is negative for invasiveness [24]. The ability to produce Shiga toxin, possession of attaching and effacing *(eae*) gene and 60-MDa plasmid, inability to ferment sorbitol within 24 h, inability to grow well at temperatures >44°C, inability to produce P-glucuronidase (i.e. in ability to utilize 4-methylumbelliferyl-D-glucuronide) and ability to utilize raffinose and dulcitol [13,26].

E. coli O157:H7 is the cause of many outbreaks of severe illness throughout the world. At least 30 countries in six continents have reported *E. coli* O157:H7 infection in humans [12]. The data of CDC shows that the annual disease appearance of *E. coli* O157:H7 in the United States to be more than 20,000 episodes and as many as 250 deaths [3]. In the United Kingdom, especially in England, Wales, Scotland and Northern Ireland, there has been a significant increase in the isolation rate of *E. coli* O157:H7 over the last decade. There is also report of *E. coli* O157:H7 outbreak in Turkey [31]. In most of the Asian countries, EHEC is not yet a major health problem, except in Japan, where 29 outbreaks were reported between 1991 and 1995. Few reports are available on the isolation of EHEC from Hong Kong, Thailand, Malaysia, India and Sri Lanka [19].

Cattle are thought to be the main source of *E. coli* O157:H7. This bacterium has also been isolated from other domestic and wildlife animals, such as sheep, goats, deer, dogs, horses, swine, cats, seagulls and rats [21]. In many studies, it is found that, young animals are more likely to be colonized by *E. coli* O157:H7 compared with older ones of the same herd. Prevalence of *E. coli* O157:H7 in cattle increases during warmer months of the year, which is related to the seasonal variation in human diseases [9].

A variety of foods are identified as vehicles for *E. coli* O157:H7 transmission which include ground beef, roast beef, cooked meats, mayonnaise, unpasteurized apple juice, salami, raw milk, pasteurized milk, yoghurt, cheese, cheese curds, ice cream, cake, lettuce, potatoes, radish sprouts, alfalfa sprouts and fruit/vegetable salad [21]. In one study, *E. coli* O157:H7 was isolated from 3.7% of retail beef, 1.5% of pork, 1.5% of poultry, and 2.0% of lamb samples [8]. The first documented outbreak of *E. coli* O157:H7 infection occurred in Oregon in 1982, which was associated with eating undercooked hamburgers [29]. Beef donar kebabs sold in cars are also source of *E. coli* O157:H7 in Turkey [31].

Generally direct transmission from bovines to humans has been documented in very few cases. In Canada a case of transmission of *E. coli* O157:H7 between calves and a human has reported [22] and in Cornwall and West Devon transmission of vero cytotoxin producing *E. coli* O157 has reported [28]. However, such transmission appears to be rare.

Four water-borne outbreaks of EHEC infection were reported in the United States during 1982 to 1994 [19]. Of those, two each were associated with swimming pool water and contaminated drinking water respectively. Drinking water, probably contaminated with bovine feces, has been associated to outbreaks in Scotland [6], sothern Africa [15], and subsequently with well water in Japan [2]. EHEC has been found to be in large number in seawater of Ohio [11], lake Michigan [14], Huron [1], Great lake [5].

The fecal carriage of *E. coil* O157:H7 by humans is the most common routes for person-to-person transmission of the pathogen. Fecal shedding of *E. coli* O157:H7 by patients with hemorrhagic colitis or HUS usually lasts for no more that 13 to 21 days following onset of symptoms. However, in some cases, the pathogen can excrete in feces for weeks [17]. There is a very chance of secondary transmission, which may involve direct hand-to-hand contact (e.g. among children in day care centers) or indirect contact (e.g. via contaminated water used for swimming).

The major virulence factor and a defining characteristic of *E. coli* O157:H7, is the production of Shiga toxin. This potent cytotoxin is the factor, which leads to death and many other symptoms in patients infected with *E. coli* O157:H7 [22].

In *E. coli* O157:H7 a 35-kb pathogenicity island termed locus of enterocyte effacement (LEE) is located on chromosome, which confers the ATE phenotype. EHEC may have acquired this pathogenicity island by horizontal gene transfer from other species. The AiE lesion is characterized by intimin attachment of the bacteria to intestinal cells with effacement of the underlying microvilli and accumulation of filamentous actin in the subjacent cytoplasm. *E. coli* O157:H7 produces A/E lesion in the large intestine [10].

The LEE region consists of three segments. The middle segment includes the *eae* gene, which encodes intimin and the *tir* gene, which encodes a translocated receptor for intimin. Downstream of *eae* are the *esp* genes, which encode secreted proteins responsible for inducing epithelial cell signal transduction events leading to the A/E lesion. Upstream of *eae* and *tir* are several genes (*esc* and *sep*) those encode a type III secretion system that is involved in extracellular secretion of proteins encoded *esp* genes.

Intimin is a 94-kDa to 97-kDa outer membrane protein encoded by *eae* (which stands for *E. coli* attaching and effacing) [7]. Intimin is the only potential *E. coli* O157:H7 adherence factor that has been demonstrated to play a role in intestinal colonization in vivo in an animal model. O157:H7 strains produce extensive A/E lesions in the large intestine, featuring intimate adherence of the bacteria to the epithelial cells. O157:H7 strains with mutation in the *eae* gene no longer produced A/E lesions and, did not appear to colonize any intestinal site [22]. Additional support for a role in human disease is seen with the anti-intimin immune response in HUS patients [20]. EHEC and EPEC intimins share only 49% identity [30].

In Bangladesh, no *E. coli* O157:H7 associated infection has been reported yet officially. The reasons might be the lack of proper surveillance for *E. coli* O157:H7 or this pathogen may be present but the infections due to this occur in very few numbers because of the acquired immunity in the population. 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 [16].

In this study an attempt has been taken for designing of a complete protocol for the detection and isolation of *E. coli* O157:H7 from meat sources (goat meat).

2. Materials and Methods

2.1. Sample

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

2.2. Isolation and biochemical identification

Tweenty five gram of meat sample was homogenized with 225 mL of Tryptic Soy broth (Oxoid, England) containing 20mg/L novobiocin in a stomacher (400 CIRCULATOR; Seward, England) and enriched for 6-8 h at 37 ºC. After enrichment, the broth was subjected to a series of tenfold dilution, and spread plated onto Sorbitol MacConkey Agar (SMAC; Oxoid, England) containing 0.05mg/l cefixime and 2.5mg/l tellurite (Merck KGaA, Germany). The colorless colonies were subcultured onto Eosine methylene blue (EMB; Oxoid, England) and 4-methylumbelliferyl-β-D-glucuronide [(MUG) (Difco, USA)] agar plates. The isolates showing metallic sheen on EMB agar plates and did not produce fluorescence on MUG media were subjected to other biochemical tests (Citrate, Methyl red, Voges-Proskaure, Indole, TSI and oxidase tests). The isolates, biochemically identified as *E. coli* O157:H7 were further confirmed by testing with Analytical Profile Index kits (API 20E, bioMeriuex Inc.). Eight other isolates, which were not identified as *E. coli* O157:H7 (as gave citrate positive reaction) but belong to Enterobacteriacae family (as per other biochemical reactions) were randomly picked and also tested with API kits.

2.3. Serological identification

Culturally and biochemically identified isolates were serologically analyzed 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.

2.4. Identification by amplifying specific genes

To amplify genes for virulence 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 and 2µl of

extracted template DNA was subjected to PCR amplification for the detection of virulent genes *eaeA, rfbE, fliC, stx1* and *stx2* by using specific primers and thermal condition (table-1). Isolates those gave bands of expected size were considered to carry these genes. For *eaeA, rfbE, fliC, stx1, stx2* gene specific primers 150 bp, 259 bp, 625 bp, 348 bp, 584 bp band were expected on agarose gel. Four *E. coli* non- O157:H7 were also selected to amplify the *eaeA* gene.

In order to lessen the time requirements of PCR amplification of each gene individually, we have attempted to set a multiplex PCR for amplification of three genes typical for *E. coli* O157:H7. For this, genes for attaching effacing and shiga-toxins were chosen. Primers were same and thermal conditions used were initial denaturing at 94^{o}C for 10 min, 35 cycles of denaturing at 94^{o}C for 1 min, annealing at 56^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 5 min.

Table 1: *Primer pair and thermal condition used for the detection of eaeA, rfbE, fliC, stx1 and stx2 genes*

Target gene	Primer name	Primer Sequence (5'→3')	Reference	Thermal condition
rfbE	O157F O157R	CGGACATCCATGTGATATAGG TTGCCTSTGTACAGCTAATCC	Paton and Paton, 1998	Initial denaturing at 94^{o}C for 10 min, 35 cycles of 94^{o}C for 1 min; 56^{o}C for 1 min; 72^{o}C for 1 min and final extension at 72^{o}C for 7 min
fliC	FLICH7-F FLICH7-R	GCGCTGTCGAGTTCTATCGAG CAACGGTGACTTTATCGCCATTC	Gannon *et al.* 1997	Initial denaturing at 94^{o}C for 10 min, 35 cycles of 94^{o}C for 30 sec; 65^{o}C for 30 sec ; 72^{o}C for 75 sec and final extension at 72^{o}C for 7 min
eaeA	VS8 VS9	GGCGGATTAGACTTCGGCTA CGTTTTGCCACTATTGCCC	Kawasaki *et al.* 2005	Initial denaturing at 94^{o}C for 10 min, 35 cycles of 94^{o}C for 20 min; 60^{o}C for 30 sec; 72^{o}C for 30 sec and final extension at 72^{o}C for 7 min
stx_1	LP30 LP31	CAGTTAATGTGGTGGCGAAGG CACCAGACAAATGTAACCGCTC	Vidal *et al.* 2004	Initial denaturing at 94^{o}C for 10 min, 35 cycles of 94^{o}C for 1 min; 56^{o}C for 1 min; 72^{o}C for 1 min and final extension at 72^{o}C for 5 min
stx_2	LP41 LP42	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	Vidal *et al.* 2004	Initial denaturing at 94^{o}C for 10 min, 35 cycles of 94^{o}C for 1 min; 55^{o}C for 1 min; 72^{o}C for 1 min and final extension at 72^{o}C for 5 min

3. Results and Discussion

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 investigation.

3.1. Identification of suspected *E. coli* O157:H7 isolates by cultural methods

Of 1523 sorbitol non-fermenting suspected *E. coli* O157:H7 isolates, 107 showed growths with typical green metallic sheen on EMB plates. On the other hand, the isolates grown on MUG plates 86 of them showed growth without blue fluorescence which is typical for *E. coli* O157:H7. All the MUG negative isolates were also EMB positive. The MUG negative and EMB positive isolates are presumed to be *E. coli* O157:H7 and followed up for further investigations by biochemical tests distinguishing for *E. coli*. Isolates showed pattern of biochemical reactions typical for *E. coli* O157:H7 were selected for further confirmation by a series of biochemical test, which are typical of *E. coli*. A total of 86 isolates were subjected to biochemical characterization. All of them fermented glucose and lactose with the production of gas (TSI). Eleven of 86 isolates were citrate negative which is the characteristic of *E. coli*. All the isolates were indole and methyl red positive but Voges-proskaure negative. So, it is very much likely that although most of the biochemical properties are similar to *E. coli*, but citrate positive isolates might be another member of Enterobacteriaceae.

3.2. Analytical Profile Index (API)

The isolates, which were biochemically identified as *E. coli* O157:H7 were further tested with API kits. Among them, 11 isolates were conformed as *E. coli* in API test as ′Good identification′. Randomly picked 8 isolates were identified as different species of *Enterobacter*.

3.3. Identification of *E. coli* O157:H7 by serological test

Among the biochemically positive 11 samples, 10 showed agglutination with O157 anti-sera. The 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.

3.4. Detection of *E. coli* O157:H7 by PCR amplification of *eaeA, rfbE, fliC, stx1* and *stx2* genes

Among the biochemically identified eleven isolates, ten were positive for *eaeA* gene amplification**.** All the *eaeA* gene amplification positive isolates showed positive result with *rfbE* gene amplification. In case of *fliC* gene amplification, six isolates showed positive results. Other isolates are possibly *E. coli* O157:H-. In PCR amplifications of *stx1* and *stx2* genes, 4 of 6 isolates which were positive for *eaeA*, *rfbE* and *fliC* showed positive results for both *stx1* and *stx2* genes amplification. The remaining two isolates showed positive results with only for *stx2* gene amplification. Three isolates positive for *eaeA, rfbE* but negative for *fliC* gene amplification, showed negative results with both *stx1* and *stx2* gene amplification. The other isolates which was positive for *eaeA, rfbE* but negative for *fliC* gene amplification, showed positive result only with *stx1* gene amplification.

The isolates, which were randomly picked *E. coli* non- O157:H7, none showed positive results in *eaeA* gene amplification, so no other gene amplification by PCR was done.

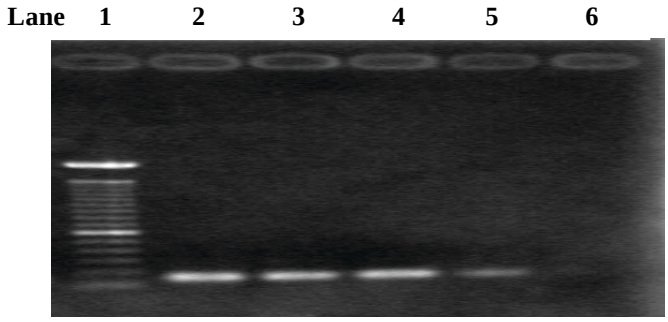

Fig. 1. *150 bp amplification products of* ***eaeA*** *gene specific primers. Lane 1: 100 bp marker, Lane 2: Positive control, Lane 3: Isolate, Lane 4: Isolate, Lane 5: Isolate, Lane 6: No template control.*

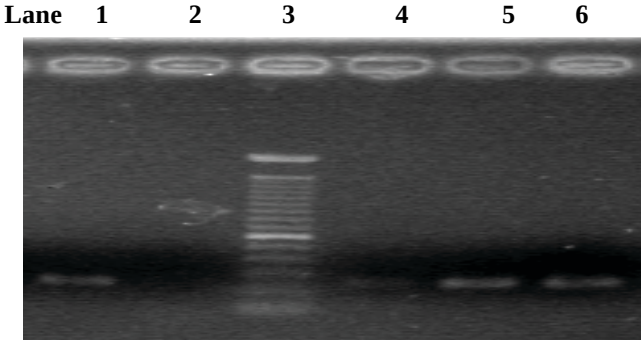

Fig. 2. *259 bp amplification products of* ***rfbE*** *gene specific primers. Lane 1: Positive control, Lane 2: No template control, Lane 3: 100 bp marker, Lane 4: Isolate, Lane 5: Isolate, Lane 6: Isolate.*

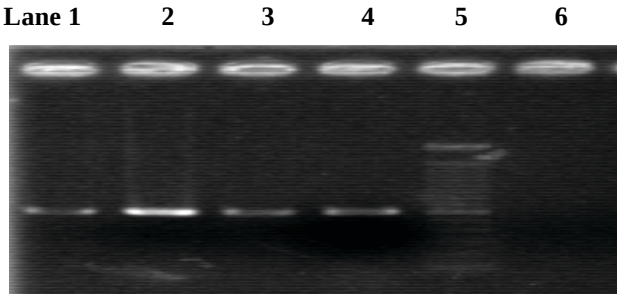

Fig. 3. *625 bp amplification products of* ***fliC*** *gene specific primers. Lane 1: Isolate, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Positive control, Lane 5: 100 bp marker, Lane 6: No template control.*

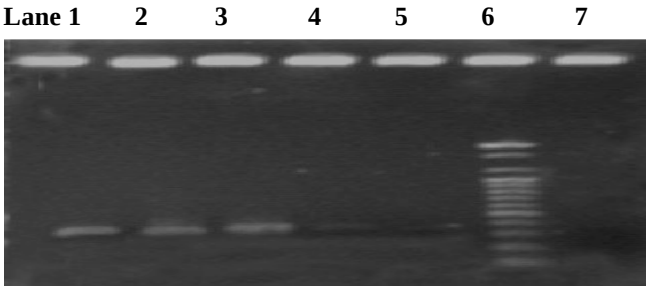

Fig. 4. *348 bp amplification products of* ***stx1*** *gene specific primers. Lane 1: Positive control, Lane 2: Isolate 3v, Lane 3: Isolate 8b, Lane 4: Isolate SN-2g, Lane 5: Isolate RM-5k, Lane 6: 100 bp marker, Lane 7: No template control.*

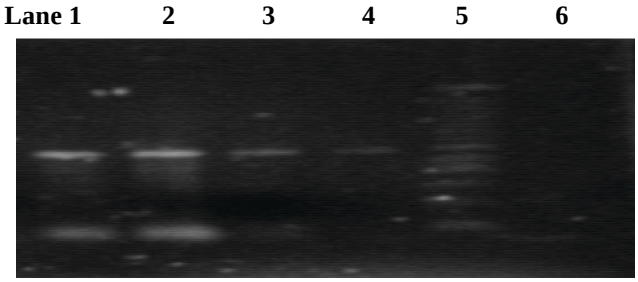

Fig. 5. *584 bp amplification products of* ***stx2*** *gene specific primers. Lane 1: Positive control, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Isolate, Lane 5: 100 bp marker, Lane 6: No template control.*

Multiplex PCR

In multiplex PCR for *eaeA, stx1* and *stx2*, the isolates showed similar result as showed in the PCRs for each gene individually.

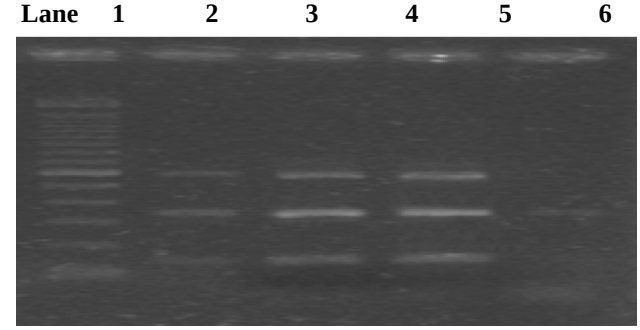

Fig. 6. *150 bp, 348 bp and 584 bp amplification products of* ***eaeA, stx1*** *and* ***stx2*** *genes specific primers in multiplex PCR. Lane 1: 100 bp marker, Lane 2: Isolate, Lane 3: Isolate, Lane 4: Positive control, Lane 5: No template control.*

4. Conclusion

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. After recovering the sorbitol negative colonies, the biochemical characteristics of the isolates are determined. For further confirmation, the isolates are checked by agglutination reactions to identify the O157 somatic and H7 flagellar antigens. Characterization based on nucleic acid is done by PCR procedures that target the shiga toxin genes (*stx-1* and *stx-2*), the attaching and effacing gene (*eaeA*), the O157 antigenic gene (*rfbE*) and the flagellar gene (*fliC*). To minimize the time, effort and the cost a multiplex PCR has also been designed. From all the isolates, the identified *E. coli* O157:H7 was low in number though the overall hygiene practices of the shoppers and the household concern is alarming. The cooking practice and the strong immunity of the people of Bangladesh may be playing a part regarding the pathogenic effect of this very organism.

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