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Isolation and Characterization of STEC with Particular Reference to O157 from Human and Animal Sources

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***Escherichia coli* O157 is an emerging food borne pathogen having zoonotic significance. In the present study, a total of 575 fecal sample originating from non-clinical human (n=235) and apparently healthy cattle calf (n=175), goat (n=35), pigs (n=30) and poultry (n=100) in the Pantnagar region of Uttarakhand, India, were screened for isolation of *E. coli O157* by conventional cultural method. A single strain having all characteristics *E. coli* O157 was isolated from a calf, which was confirmed by tube agglutination test with O157 antiserum and PCR based amplification of *rfb*O157 gene, but not by serotyping. This strain was motile yet, it did not either agglutinate with H7 antisera or contained *fliCH7* gene specific for H7 flagellar antigen. No *E. coli O157* isolate could be obtained from the fecal samples human, goat, pigs and poultry screened in the present study. The finding of the study suggests the need for application of molecular tools like PCR in making confirmatory identification of the *E. coli* O157 isolates.**

Key words: *E. coli* O157, *E. coli* O17, Farm animals, *fliCH7, rfbO157.*

There are more than 400 different serotypes included under the shigatoxigenic *Escherichia coli* (STEC) group but not all results in serious life threatening complications (Karmali *et al*., 2010). All these STEC serotypes carry one or two shiga toxin gene, *stx* 1 and/or *stx* 2, also referred as verotoxins 1 and 2, respectively, which plays a major role in its pathogenicity. The important seropathotypes of STEC includes O157 and other non-O157 serotypes like O26, O103, O111, O118, O121, O145 (Tzschoppe, *et al*., 2012). Among them, *E. coli* O157 is considered as important emerging food-borne pathogen, having zoonotic significance. It results in illness ranging from mild watery diarrhea to life threatening conditions like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in human (Gavin and Thomson, 2004; Waswa *et al*., 2007; Oporto *et al*., 2008). Children below 5 years of age, elderly and immune-compromised individuals are at higher risk, and females compared to males are at greater risk of developing hemolytic uremic syndrome (Park *et al*., 2002 and Rivas *et al*., 2006, Kiranmayi *et al*., 2010). Ruminants are considered as the major reservoir for these pathogens (Naylor *et al*., 2005; Oporto *et al*., 2008), but other animals including wild animals and birds could act as natural reservoirs (Khan *et al*., 2002; Karch *et al*., 2005; Tzschoppe *et al*., 2012). Transmission occurs mainly by ingestion of food and water contaminated with feces of infected animals and humans. Outbreaks are often associated with ingestion of foods of animal origin like undercooked ground beef and milk (Oporto *et al*., 2008, Ateba and Bezuidenhout, 2008; Kiranmayi and Krishnaiah, 2010). The organism can survive in the environment for more than 10 months, increasing the risk of acquiring infection from the environment long after contamination (Karch *et*

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al., 2005). Contact with ruminant feces either directly or indirectly plays key role in human *E. coli* O157 infection (Alhelfi *et al*., 2013).

In conventional methods of isolation, the samples are subjected to an enrichment step for 6-18h and then plated onto selective medium, followed by biochemical and serological confirmation (Waswa *et al*., 2007; Karmali *et al*., 2010). Unlike most *E. coli* strains, serotype O157 does not ferment sorbitol present in sorbitol MacConkey agar (SMAC) (Shelton *et al*., 2008). But, organisms such as *Proteus* and *Morganella* also could grow as NSF on SMAC medium and therefore to improve recognition of *E. coli* O157 on SMAC supplements like Cefixime-Tellurite are added to the medium. Cefixime inhibits *Proteus* (Chapman *et al.*, 1991) while Tellurite, improves *E. coli* O157 isolation by inhibiting or suppressing, other non-sorbitol fermenting organisms (Zadik *et al.*, 1993). In addition, approximately about 96% of *E. coli* isolates produce the enzyme β-D-glucuronidase which cleaves the MUG substrate, providing a fluorescent end product, methylumbelliferone, detectable under a long-wave UV light source. But *E. coli* O157 lacks this enzyme therefore, MUG when added to the media it is not cleaved no fluorescent product is produced thereby facilitating its identification (Khandaghi *et al.*, 2010). Numerous reports of isolation of *E. coli* O157:H7 from the gastrointestinal tract of animals are available worldwide; cattle (Breum and Boel, 2010; Jeong *et al* 2013; Sallam *et al*., 2013), sheep (Dipineto *et al*., 2013), goat (Dontorou *et al*., 2004), pigs (Wong *et al*., 2009), wild boars (Sanchez *et al*., 2010). But in India, only a meager amount of literature is available, *E. coli* O157 were detected from milk (Kumar *et al*., 2013); Shrimp (Surendraraj *et al*., 2010), sheep (Wani *et al*., 2003), calves (Dutta *et al*., 2000; Manna *et al*., 2006) and carabeef (Hazarika *et al*., 2004). Sehgal *et al*. (2008) reported that over a period of 10 years, only 0.5% of the isolates received at the National *Salmonella* and *Escherichia* Centre, India, were found to be of the *E. coli* O157. Khan *et al*. (2002) and Chattopadhyay *et al*. (2003) were unable to isolate *E. coli* O157 from the fecal sample of human, animals and food origin and Sharma *et al*. (2003) also was unable to detect *E. coli* O157 from water samples. The aim of the present study was to understand the fecal carriage status of human, farm animals and poultry for *E. coli* O157.

MATERIALS AND METHODS

Sample collection

A total of 575 fecal samples originating from non-clinical human (n=235) and apparently healthy cattle calf (n=175), goat (n=35), pigs (n=30) and poultry (n=100) were collected in sterile sample collection vials from different locations of within Pantnagar region of Uttarakhand, India. The human stool samples were obtained from the residents within Pantnagar. The samples collected were brought to the laboratory with minimum delay maintaining the cold chain and processed.

Isolation and identification

The samples were subjected to selective enrichment in modified-EC enrichment broth containing 20 mg/l novobiocin and incubated at 37°C for 24 h. After incubation, the samples were plated onto MacConkey Lactose agar (Himedia, India) and incubated at 37°C for 24 h. Lactose fermenting pink color colonies were picked up and streaked on EMB agar and incubated for further 24 h at 37°C. The culture with a typical metallic sheen appearance on EMB agar, were then streaked onto nutrient agar plates and incubated further at 37°C for 24 h and a single colonies were picked up on nutrient slants as a pure culture. The pure culture isolated was subjected to various biochemical tests, viz., indole test, methyl red test, Voges Proskauer test, citrate utilization test, triple sugar iron test, urease test, and sugar fermentation tests (glucose, lactose, dulcitol, mannitol, sucrose) as described by CDC (1994) and Agarwal *et al*. (2003).

Identification of *E. coli* O157:H7 using SMAC

For identification of *E. coli* O157:H7, the biochemically confirmed isolates were plated onto Sorbitol MacConkey agar (Himedia, India) supplemented with cefixime (0.05 mg/l), potassium tellurite (2.5 mg/l) and MUG (4-methylumbelliferyl-β-D-glucuronide) substrate (CT-MUG-SMAC) and incubated at 37°C for 24 h. The plates were examined for the appearance of characteristic non–sorbitol fermenting (NSF) colorless colonies on CT-MUG-SMAC plates which were then examined under long wavelength UV light and only non fluorescent colonies, suspected to be *E. coli* O157:H7 was picked up onto the nutrient agar slant for further confirmation.

Testing against O157 and H7antisera

Suspected isolates were tested with O157

and H7 antisera (Difco) by the tube agglutination method as described by the manufacturer. Tubes showing 50% agglutination of cells; supernatant fluid moderately cloudy (2+) or greater agglutination are considered as positive. Before proceeding with testing with H7 antisera, flagellar motility test was performed as recommended by the manufacturer. The suspected isolates were inoculated by stabbing in mannitol motility agar and incubated for 18-24 h and this was repeated 3 times for 3 consecutive days to ensure full growth of flagella. The positive reaction was indicated by growth along the line of stabbing and a change in the color of medium from red to yellow. Mannitol motility test positive isolates were then checked with H7 antisera by the tube agglutination method as per manufacturer's recommendation.

Serotyping

To cross check our results the isolates were also got serotyped from National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

Molecular characterization

The putative *E. coli* O157 isolates obtained were subjected to molecular characterization using polymerase chain reaction targeting *E. coli* O157 specific *rfb*O157 gene, H7 specific *fliC*H7 gene, shiga toxin specific *stx* gene. The details of the oligonuleotide primers used are given in table 1. All the amplification reactions were carried out with GeneAmp 9700 thermal cycler (Applied Biosystems) in a 25 µl reaction volume consisting of 2.5 µl 10X PCR assay buffer, 2 µl of DNA template, 200 mM of dNTPs, 10 pmol of each primer and 1 U of Taq DNA polymerase (Genei, India). The amplicons were analyzed by 1.5% agarose gel electrophoresis and documented.

Genomic DNA extraction

The genomic DNA was extracted using Hi-pura DNA purification kit (Himedia, India) as per manufacturer's recommendation and stored at -20^{0}C until used**.**

PCR for amplification of *rfb*O157 and *fliC*H7 genes

PCR for *rfb*O157 gene and *fliC*H7 gene was performed according to Jamshidi *et al.* (2008). The cycling condition employed is as follows: initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 30 s) and extension (72°C for 1 min) and a final extension step (72°C for 5 min).

PCR for *stx* gene

PCR based detection of *stx* (Shiga toxin) gene was performed using the oligonuleotide primers described by Lei *et al.,* (2008) with the following cycling conditions: initial denaturation (95°C for 5 min), followed by 35 cycles of denaturation (95°C for 1 min), annealing (55°C for 90 s) and extension (72°C for 90 s) and a final extension step (72°C for 5 min).

RESULTS AND DISCUSSION

Shiga-toxigenic *E. coli* (STEC), especially *E. coli* O157 are a potential threat to public health due the morbidity and mortality associated with its infection. Epidemiological studies are required to understand the prevalence of *E. coli* O157 and to facilitate designing of risk based control strategies (Alhelfi *et al*., 2013). Based on literature survey, the prevalence of *E. coli* O157 was found to be very low and in many studies no *E. coli* O157 isolate could be isolated (Alhelfi *et al*., 2013). In this study we attempted to isolate and identify *E. coli* O157 from stool/fecal samples collected from human, cattle, goat, pigs and poultry. Of the 575 samples analyzed, a total of 212 biochemically confirmed *E. coli* isolates were obtained. These 212 isolates were processed further for identification of *E. coli* O157:H7; 19 isolates consisting of 5 from human, 9 from calf, 1 from pig and 4 from poultry were found to produce characteristic non–sorbitol fermenting (NSF) colorless colonies. These 19 NSF *E. coli* isolates when examined under long wavelength UV light, only 7 were found to be non fluorescent which was considered as putative *E. coli O157:H7* isolates (Table 2).

Of the 7 isolates, only one isolate originating from calf was found to produce a characteristic agglutination reaction with O157 antisera in tube agglutination test. Further, among the seven isolates only 3 were motile by mannitol motility test; two from calf, including the isolate positive by O157 antisera and one from poultry but, none exhibited positive reaction with H7 antisera. To confirm our results, these three isolates were sent for serotyping, wherein the isolate obtained from poultry was identified as O17 serotype while the two calves isolates were found

Table 1. Details of primers targeting specific gene

Gene	Sequence of primer (5'-3')	AmpliconSize (bp)	Reference
*rfb*O157	F: CGG ACA TCC ATG TGA TAT GG R: TTG CCT ATG TAC AGC TAA TCC	259	Jamshidi *et al*., 2008
*fliC*H7	F: GCG CTG TCG AGT TCT ATC GAG R: CAA CGG TGA CTT TAT CGC CAT TCC	625	Jamshidi *et al*., 2008
Stx	F: TGG GTT TTT CTT CGG TAT CC *R:* CCA GTT CAG AGT GAG GTA CA	632	Lei *et al*., 2008

Table 2. Cultural and serological characterization of *E. coli*

Species	Total samples	*E. coli* isolates	Non sorbitol fermenting isolates	Non-fluorescent colonies under UV	O157 antisera	Motility	H7 antisera
Human	235	98	5	1	0	0	0
Calf	175	63	9	5	1	2	0
Goat	35	3	0	0	0	0	0
Pig	30	10	1	0	0	0	0
Poultry	100	38	4	1	0	1	0
Total	575	212	19	7	1	3	0

to be rough. In PCR, all three isolates revealed the presence of *stx* gene specific amplicon, confirming that these isolates belonged to the shigatoxigenic group of *E. coli* (STEC) (Fig.1.). The presence of *rfb* O157 specific amplicon was detected in one of the calf isolate (Fig. 2.) which was previously identified as O157 using antisera and none revealed the presence of the *fliC*H7 specific amplicon characteristic of H7 flagellar antigen.

In this present study, we could obtain one *E. coli* O157 isolates from cattle calf giving a prevalence rate of 0.57% and no other sample derived from goat, pigs, poultry or human yielded positive isolate. It was also interesting to note that the isolate obtained from cattle was identified as O157 using by tube agglutination test with O157 antisera and PCR, but serotyping could not demonstrate the presence of O157 antigen in the

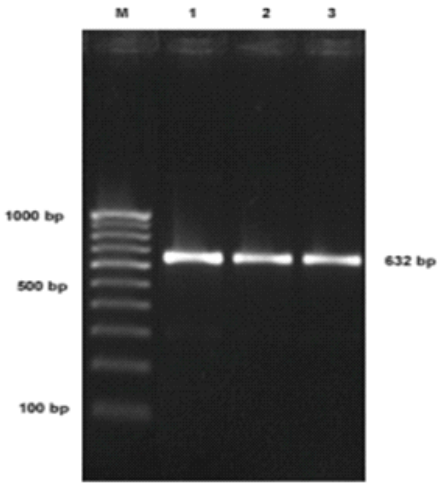

Fig. 1. Agarose gel showing *stx* gene specifc amplicon

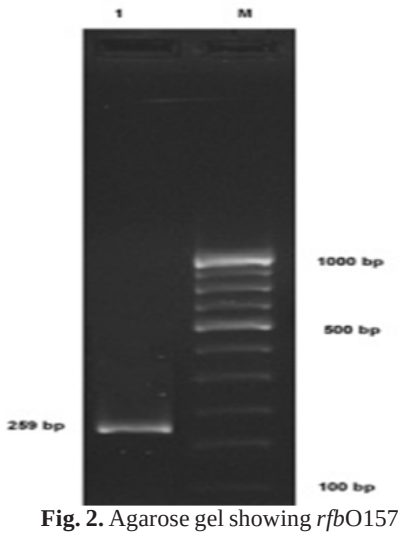

Fig. 2. Agarose gel showing *rfb*O157 gene specific amplicon

same isolate. This finding suggests that identification of O157 by serotyping cannot be considered as confirmatory for all isolates, hence PCR based detection of specific *rfb*O157 locus should always be carried out in making confirmatory identification. Additionally, in our study the *fliC*H7 gene specific amplicon was not observed indicating the absence of H7 flagellar antigen but at the same time the isolates were found to be motile by motility test suggesting that the isolate is an O157: H+ possessing flagellar antigen other than H7.

The overall prevalence of *E. coli* O157 obtained in this study was very low (0.17%) which could be because we screened samples by culture based method followed by confirmation using PCR, rather than direct PCR based screening. In addition, the human samples screened were of non clinical in origin. And we selected only non fluorescent, non sorbitol fermenting colonies as presumptive *E. coli* O157 isolates but, Hamner *et al.* (2007) reported identification of *E. coli* O157 isolates from sorbitol fermenting colonies. Sallam *et al.* (2013) also reported that out of 15 *E. coli* O157 isolates obtained, 11 were found to be sorbitol fermenting and only 4 were non sorbitol fermenting type. In some reports MUG positive *E. coli* O157 isolates were also obtained (Dontorou *et al*., 2004).

Besides, in our study we also obtained an *E. coli* O17 strain from poultry, based on literature survey this is the first report of its isolation from poultry in our country. Earlier, Wani *et al*. (2003) reported its isolation from cattle samples. The *E. coli* O17 serotype was reported as a uropathogenic strain for human (Ramchandani, *et al*., 2005, Cortes *et al*., 2010). Thus its isolation from poultry in this study suggest that further studies are required to understand the zoonotic nature of this strain and the potential role of poultry as a reservoir for it.

In conclusion, the very low prevalence obtained in this study indicates that the incidence or fecal carriage of *E. coli* O157 is very low in this region. But the detection of one *E. coli* O157:H+ isolate from cattle could have significant public health impact. It also suggests the need for further studies involving a number of sample screenings by conventional culture based or other methods, along the application of molecular tools like PCR to make confirmatory identification of the isolate.

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