ISOLATION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING *E. COLI* O157

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ABSTRACT

Escherichia coli (*E. coli*) is normal inhabitant of all domestic animals and human beings. Its Sorbitol-non fermenting (SNF) biotype was detected in dung of buffalo (92 %), cattle (84 %), sheep (52 %) and goat (56 %). However, *E. coli* (SNF) was not detected in droppings of rural chickens, feces of donkeys and fresh aseptically collected milk and beef samples, whereas, the *E. coli* was detected in 96 percent market raw milk and 82 percent samples from beef shops. Eighty percent milk and 53 percent beef samples contained SNF isolates of *E. coli*. All isolates of SNF *E. coli* showing green metallic sheen on eosin methylene blue (EMB) agar were further characterized using polymerase chain reaction (PCR). The isolated DNA of 100 samples when amplified using universal, *Stx1*, *Stx2* and O157 specific primers confirmed that 82 percent samples were positive for universal primers, 50 percent for O157, 60 percent for *Stx1* and 51 percent for *Stx2*. It is concluded that *E. coli* O157 is normal inhabitant of intestinal tract of domestic ruminants and presumably animal feces are acting as main source of contamination of raw milk and beef.

Key words: Sorbitol, Eosin Methylene Blue (EMB) agar, *E. coli* O157, PCR, Buffalo, Sorbitol Non Fermenters

INTRODUCTION

Escherichia coli is an important member of enterobacteriacae which contains gram negative bacilli. It is a large group of germs that are normal inhabitant of animal and human intestinal tract. On the basis of somatic (O), capsular (K) and flagellar (H) antigen, more than 700 serotypes of *E. coli* have already been identified. On the basis of sorbitol fermentation, its species are also bio-typed as sorbitol non- fermenting *E. coli* that are cause of human food poising. This biotype results a wide range of clinical manifestations, including mild illness, vomiting, sudden onset of diarrhea, hemolyticuremic syndrome and death (Su and Brandt, 1995). The incidence of food poisoning outbreaks with *E. coli* O157:H7 has increased in recent years. The disease is an increasing public health concern. The infective dose of *E. coli* O157:H7 in humans is very low (Jones, 1999).

Cattle are believed to be the primary reservoir of *E. coli* O157:H7 (Garber *et al*., 1995). Contamination of swimming pools, water supplies, raw vegetables and more commonly dairy and beef products with the cattle feces are associated with an increasingly wide variety of food poisoning (Jones, 1999). Raw or improperly pasteurized milk and meat can be source of *E. coli* O157:H7 and cause of food poisoning. Fresh beef can be contaminated with *Salmonellae*, *Campylobacter species*, and enterohaemorrhagic *E. coli* especially O157:H7 serotype and also by other enteric pathogens (Keene *et al.,* 1997).

Currently, *E. coli* are classified into distinct groups on the bases of toxigenicity, viz. Entero-toxin Producing *E. coli* (ETEC), which causes diarrhea by producing heat-stable and/or heat-labile enterotoxins. Shiga toxin or verocytotoxin-producing *E. coli* (STEC or VTEC) is an important foodborne pathogen, which may results in sporadic cases to serious outbreaks in whole world. Morbidity and mortality rates due to several recent outbreaks of STEC have proved it as a significant threat to public health. Verotoxin or Shiga toxin is the most important virulent attribute for the STEC (Staats *et al.,* 2003). Shiga toxins producing *E. coli* is an etiological agent for individual cases and outbreaks in the developed nations (Armstrong *et al.,* 1996). The resistance of powerful virulence factors to acid is mainly associated with the pathogenicity of *E. coli* O157:H7. The pathogenic *E. coli* adhere efficiently to the intestinal epithelial cells thus causing lesions (Sherman *et al.,* 1988). The present study was designed with the following objectives:

(1) Isolation and cultural/biochemical characteristics of sorbitol non fermenting *E. coli* from fecal samples of healthy domestic animals
(2) Monitoring of raw milk and beef samples from four outlets/roads of Lahore city for sorbitol non fermenting *E. coli*
(3) Molecular characterization of *E. coli* O157 using universal, specific, *Stx1* and *Stx2* primers.

MATERIALS AND METHODS

Beef and milk samples (sixty each) were collected in plastic bags aseptically from 4 major roads/outlets of city Lahore. Each sample was comprised of 250 grams of beef or 250 ml of raw unpacked milk. Fecal samples (5 grams each) from each of the 25 cattle, buffalo, sheep, goat, donkey, rabbits, guinea pigs and chicken were collected. Each of the samples was transported to the Laboratory for further analysis.

One ml of each of the raw milk and beef rinse samples were processed for total bacterial count on sterile Sorbitol MacConkey's Agar (SMAC) using sterile glass spreaders (Meng *et al.,* 1995). The white colored (sorbitol non fermenting-SNF) colony forming units (CFU) were enumerated using a Bright Field Quebec Colony Counter. The counts were expressed as standard plate count per gram of the beef sample or per ml of the milk sample. In case of the fecal samples only white colored bacterial colonies were noted. Five white colored colonies from each of the samples were separately pooled in normal saline. Each of the isolate was characterized and confirmed as *E. coli* (SNF) as per Bergey's Manual of Determinative Bacteriology.

For molecular characterization, DNA from each of the isolate was extracted by boiling method (Son *et al.,* 2000). Effect of medium, incubation time for growth, and storage temperature of extracted DNA on recovery of DNA of *E. coli* or amplification of the DNA was evaluated. For PCR, thermocycler was programmed using standard conditions (Chen and Griffiths, 1998). After polymerization, the gel was loaded with samples, positive, negative control and with 100 bp ladder and was run at 100 volts for 35 minutes. The gel was stained with Ethidium Bromide solution for 20 minutes and was scanned with Kodak Gel Logic 100 system.

RESULTS AND DISCUSSION

Fecal samples collected from mammalian and avian species when diluted and inoculated on sorbitol MacConkey's agar (SMA) showed visible white and pink colored growth. The same was confirmed by Son *et al.,* (2000) and Adam *et al.* (2008). Ninety two, 84, 52, 56, 33, 20, 0 and 0 percent fecal samples from buffalo, cattle, sheep, goat, rabbits, guinea pigs, donkey and chicken showed white colored growth on SMA medium, respectively. The differences in percent distribution of animals having sorbitol non fermenting (SNF) bacteria could be due to environmental pollution, season and fodder used for feeding the animals. The SNF *E. coli* is present in 12.9% fecal samples of cattle (Chapman *et al.* 2001), 1.4% of sheep and in 25% fecal samples of lambs (Novotna *et al.* 2005).

Bacterial DNA was extracted easily by boiling method when the isolates were grown on sorbitol MacConkey's agar or EMB agar at 37°C for 12-24 hrs. The DNA was recovered when the culture was boiled for 5-10 minutes. The DNA of each sample remained active when stored at 4°C for 48 hours or at -20°C for 7 days

All the milk and beef samples (100%) collected from different outlets were contaminated with SNF bacteria except the beef samples from Sheikhupura road where contamination was 53 percent (Table 1). It could be due to the reason that beef on the road was displayed in cabins covered with curtains. *E. coli* O157: H7 is present in 38 (76 %) raw milk samples and in 8% and 3.7% retail beef samples (Agaoglu *et al.*, 2000; Sabry and Laila, 2008; Adam *et al.* 2008). More percentage of milk samples were contaminated than beef samples. Highest contamination was recorded (7.2×10^{6} cfu/ml) from the milk samples which were collected from Multan road. The least contamination (1.65×10^{2} cfu/gm) was found in beef samples which were collected from Sheikhupura road. Minced beef contains 4×10^{5} to 7×10^{7}cfu/g and 13 x 10^{4} cfu/g while fresh beef contains 1.5×10^{6}cfu/g of SNF bacteria (Agaoglu *et al.*, 2000; Rowland *et al.* (2004). The differences in CFU among these studies could be due to variations in the environmental contamination and season.

The SNF E. coli on MacConkey's agar showed lactose fermenting/pink colonies and on Eosin Methylene Blue (EMB) agar showed green metallic sheen. Each of the isolates were gram negative, rods with rounded ends and non spore forming. Pure growth of the SNF bacteria were indole and methyle red positive while citrate and Voges Proskaur (VP) negative. The SNF *E. coli* was further characterized using Polymerase Chain Reaction (PCR). Incubation time for growth, boiling time of the culture and type of media for growth of SNF *E. coli* affected the DNA recovery. Extraction of DNA was negative at 6 h of incubation because it might be due to less growth to extract DNA. Post incubation period of 12, 18 and 24 hours was strongly positive for DNA extraction because in this incubation period fresh growth and sufficient amount of nutrients were present. But if incubation period is up to 30 hours or thereafter, the DNA extraction was negative. The growth of *E. coli* 24 hours post incubation probably enters into stationary or death phase that may be plausible reason of poor recovery of the DNA from the bacterial culture. Incubation period for growth of the culture for 12-18 hours is suitable for the extraction of DNA from *E. coli* O157:H7 (Mariela and Harrington, 2005). Growth incubation of 12-24 hours is suitable for DNA extraction (Ji-Yeon Kim *et al.,* 2005 and Shaw *et al.*, 2004). More over boiling time of pure culture of *E. coli* for 5 to 10 minutes made it possible to extract its DNA but boiling of the culture for more than 10 minutes failed to yield the DNA. Boiling of the culture for 5-10 minutes is the

effective time for recovery of the DNA (Novotna *et al.*, 2005).

The *E. coli* suspension harvested from MacConky's or EMB agar yielded its DNA on boiling but culture suspension harvested from nutrient agar, brain heart infusion broth and SMA did not yield its DNA. The DNA is effectively recovered by growing *E. coli* O157 on EMB, MacConkey's and Triple-Sugar Iron Agar (Agaoglu *et al.* 2000, Shaw *et al.* 2004 and Rowland *et al.* 2004). Exact reason for lack of recovery of the DNA from *E. coli* culture grown on such media is not known. However, sugar of the culture medium used by growing the bacteria probably supports the release of DNA on boiling.

The isolated DNA when stored at -20ºC for 7 days, 4ºC for 72 hours and 25ºC for 24 hours remained stable. Storage temperature is inversely proportional to stability of the DNA. Increase of storage temperature presumably increases activity of the nucleases that might be cause of deterioration of the isolated DNA (O'Brien, 2002).

The DNA extracted from each of the above mentioned milk and beef isolates was processed to confirm as *E. coli* O157 for Polymerase Chain Reaction (PCR). The amplican sizes of 884, 180, 256 and 259 base pairs with universal, *Stx1, Stx2* and O157 primers were obtained. The conditions used for amplification of each of the isolated DNA were adopted as described by Ji-Yeon Kim *et al.* (2005). Chen and Griffiths (1998) recorded similar results by using the similar sets of primers. Out of 100 isolates of SNF *E. coli,* 82 were positive for universal primers. From these 82 isolates, 50 (61%), 60 (73%) and 51 (62%) were positive for O157, *Stx1* and *Stx2,* respectively. Overall 31 (38%) isolates contained both *Stx1* and *Stx2* genes. Adler *et al.* (2000) detected *Stx1*, *Stx2* and both *Stx1+Stx2* genes in 1.4, 41.2 and 57.4% beef and milk isolates, respectively. Majority of the bovine isolates of SNF *E. coli* contains both the shiga toxin producing genes (Barkocy-Gallagher *et al.,* 2001). However, *Stx1* gene was detected in 3%, *Stx2* gene in 20% and both the genes in 76 % isolates of human clinical cases (Stephen *et al.,* 1989).

It is concluded that SNF *E. coli* O157 is normal inhabitant in intestine of the domestic ruminants and is presumably main source of SNF *E. coli* contamination of dairy animal products.

Table-1. Sorbitol Non-Fermenting Bacterial Count in Milk and Beef Samples

Source of Samples	Sorbitol non Fermenting Bacterial Count (Log Values)	
	Milk	**Beef**
Multan Road	7.2±0.8[a]	2.9±0.6[a]
Sandha Road	5.9±1.9[c]	2.2±1.4[c]
Wagha Road	6.3±1.8[b]	2.5±1.4[b]
Sheikhupura Road	6.1±0.4[c]	1.7±1.7[c]

Note: Each of the different dilutions was spread on Sorbitol MacConkey's Agar and Sorbitol non Fermenting bacterial colonies (white colored colonies) were counted. Datum of each sample thus obtained was transferred into antilog value and was analyzed using ANOVA (Mini Tab-7.0 Programme).

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