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RESEARCH ARTICLE

Occurrence and Molecular Characterization of Shiga Toxin-Producing *Escherichia coli* Isolates Recovered from Cattle and Goat Meat Obtained from Retail Meat Shops in Rawalpindi and Islamabad, Pakistan

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ABSTRACT

Shiga toxin-producing *E. coli* (STEC) also known as Verocytotoxin producing *E. coli* (VTEC) have emerged as important food-borne pathogens of zoonotic importance causing outbreaks and sporadic cases of diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome. The objective of the study was to understand the distribution of various STEC in raw meat of cattle and goats collected from different meat shops of Rawalpindi and Islamabad. Raw meat samples (n=101) were analysed for presence of four virulence genes (*stx1*, *stx2*, *eae* and *ehxA*). The samples positive for one or more virulence genes were subjected to isolation using sorbitol MacConkey agar (SMAC). Of 101 samples 44 (43.5%) were found positive for one or more virulence genes. The most common combination of virulence genes in samples was *stx1*, *stx2*, *ehxA* (9/44; 20.4%). Nine *E. coli* isolates positive for one or more virulence genes were recovered from 44 positive samples. The analysis of *E. coli* isolates for serogroups O157, O26, O103, O111 and O145 using PCR indicated that three *E. coli* isolates were O111 and one was O26. The serogroup of five *E. coli* isolates could not be identified. Antibiotic susceptibility profiles of the *E. coli* isolates using the disc diffusion method indicated antimicrobial resistance ranging from 33.3 to 100% against eight antibiotics of seven different classes. Isolation of antimicrobial resistant STEC of zoonotic significance from meat samples indicated that meat sold in the shops of Islamabad and Rawalpindi may be the source of transmission of AMR STEC to humans.

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INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) are considered important food and water borne pathogens of zoonotic significance associated with various clinical manifestations ranging from diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and death (Valilis *et al.*, 2018). The first human case of HC due to STEC O157:H7 was reported in 1982 (Lupindu, 2016). Since then number of outbreaks due to STEC have been reported from different parts of the world. More than 100 serotypes of STEC have been reported to be associated in human disease causation. STEC other than O157 are commonly called as non-O157 STEC. Non-O157 STEC such as O26, O45, O103, O111, O121 and O145 have emerged as important food-borne pathogens in various parts of the world (Valilis *et al.*, 2018).

STEC possesses number of virulence factors including Shiga toxin (*stx*), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehxA*) genes (Melton-Celsa, 2014). The most important virulence marker of STEC is *stx* and most of the human cases of HC and HUS are associated with strains of *E. coli* which produce Shiga toxin (Stx). Stx has two types i.e., Stx1 and Stx2 (Melton-Celsa, 2014). Stx inhibits the protein synthesis within the cells especially renal endothelial cells (Louise and Obrig, 1995). The *E. coli* attaching and effacing (*eae*) gene is located on locus of enterocyte effacement (LEE) and encodes the outer membrane protein intimin which helps in attachment and effacement of *E. coli* to epithelial lining of intestine (Xu *et al.,* 2016). The plasmid encoded enterohaemolysin, encoded by *ehxA*, is also an important virulence marker of STEC. The presence of *ehxA* in STEC isolates from HC and HUS cases may indicate the likely role of *ehxA* in pathogenesis (Schmidt *et al.,* 1995).

Ruminants especially cattle, buffaloes, sheep and goats are considered important reservoirs of STEC (Valilis *et al.,* 2018; Kwan *et al.,* 2019). More than 400 serotypes of STEC have been isolated from healthy cattle (Bettelheim, 2003; Karmali *et al.,* 2010). Cattle and sheep play an important role in the transmission of STEC to humans as most of the human cases are associated with direct animal contact or indirectly through ingestion of contaminated food and water (Valilis *et al.,* 2018). Carriage of STEC by slaughtered animals may be responsible for contamination of carcasses during removal of hides and gastro-intestinal tracts (McEvoy *et al.,* 2003). The prevalence of STEC has been reported to range from 0.1-54.2% in ground beef and 1.1-49.6% in unspecified meat cuts (Hussein and Bollinger, 2005; Hussein, 2007). In Pakistan a study carried out in Quetta reported 10% prevalence of *E. coli* O157:H7 in meat samples collected from various retail meat shops (Samad *et al.,* 2018). However, occurrence of non-O157 STEC in retail meat from various meat shops in Pakistan is un-known. Therefore, a study was carried to determine the occurrence and distribution of various STEC in raw meat samples of cattle and goats collected from different meat shops of Rawalpindi and Islamabad.

MATERIALS AND METHODS

Raw meat samples (n=101) of cattle (n=67) and goat meat (n=34) were collected from 50 retail meat shops of 11 different localities of Rawalpindi (n=61) and Islamabad (n=40) between March 2018 to July 2018. The meat samples were collected from clinically healthy carcasses with normal physical appearance from those retail meat shops which were selling meat of the animals slaughtered in government slaughterhouses of Rawalpindi and Islamabad. Each sample of 10 grams was collected using sterile knife and tissue forceps in sterile zip lock bag. The samples were transported to the laboratory in cold conditions.

Each raw meat sample (10 gm) was homogenized with 4 ml of phosphate buffered saline (PBS) using mortar and pestle. The homogenized sample was centrifuged at 12,000g for 15 minutes. After centrifugation pellet was discarded and the supernatant was (1 ml) enriched in 9 ml of buffered peptone water (BPW) at 37°C for 24 hours. After enrichment DNA was extracted from a 1 ml aliquot of enriched broth using simple boiling method (Irshad *et al.,* 2012). The DNA extracted was analysed using multiplex PCR for presence of *stx1*, *stx2*, *eae* and *ehxA* genes (Sharma and Dean-Nystrom, 2003). The conditions for multiplex PCR including volume of each reaction are described in Table 1. PCR products were detected by electrophoresis through 2% agarose (Research Organics, Cleveland, USA) gel and observed using ethidium bromide under ultra-violet illumination.

The samples positive for one or more virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) were inoculated onto sorbitol MacConkey agar (SMAC) for isolation of STEC. SMAC plates were incubated at 37°C for 18-24 hours. Colonies of two different colours (Pink and grey) appear on SMAC. Five grey colonies indicative of sorbitol non-fermenting activity and five pink colonies indicative of sorbitol fermenting activity were selected for further analysis. DNA was extracted from each colony using simple boiling method (Irshad *et al.,* 2012). The DNA was analysed by multiplex PCR for presence of *stx1*, *stx2*, *eae* and *ehxA* (Sharma and Dean-Nystrom, 2003). Isolates positive for targeted virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) were confirmed as *E. coli* using RapIDOne kit (Remel, UK) and analysed using PCR for the detection of *E. coli* serogroups O26 (Perelle *et al.,* 2004), O103 (Fratamico *et al.,* 2005), O111 (Perelle *et al.,* 2004), O145 (Fratamico *et al.,* 2009) and O157 (Perelle *et al.,* 2004). The conditions for PCR including volume of each reaction are described in Table 1. PCR products were electrophoresed through 1% agarose (Research Organics, Cleveland, USA) gel and visualized using ethidium bromide under UV-light. The isolates were sub-cultured onto the SMAC to ensure purity and stored at -80°C in nutrient broth containing 15% (v/v) glycerol.

Antibiotic susceptibility profiles of the *E. coli* isolates were determined using the disc diffusion method on Mueller-Hinton agar (Kassim *et al.,* 2016). Briefly, an inoculum of each *E. coli* isolate was prepared using normal saline. The turbidity of inoculum was matched according to 0.5 McFarland standards. The inoculum was evenly spread on the Mueller-Hinton agar plates. Antibiotic discs were placed on the agar surface with the help of sterilized forceps. The plates were incubated at 37°C for 24 hours in aerobic conditions. The plates were observed for zones of inhibition after incubation. The diameter of zone of inhibition (clear zone) was measured around each antibiotic disc. The susceptibility and resistance of *E. coli* isolates against each antibiotic was categorized into three groups i.e., sensitive, intermediate and resistant depending upon the size of zone of inhibition around each antibiotic disc. The results were interpreted according to the criteria given by Clinical Laboratory Standard Institute 2013. The antibiotics from seven different classes (lincosamides, aminoglycosides, cephalosporin, tetracycline, fluoroquinolones, carbapenems and monobactams) tested were: lincomycine, streptomycine, neomycine, cephradine, doxycycline, enrofloxacine, imipenem and aztreonam.

Table 1: PCR condition for detection of virulence genes (*stx1, stx2, eae, ehxA*) and *E. coli* serogroups (O26, O103, O111, O145 and O157) in *E. coli* isolates recovered from meat samples of cattle and goats collected from meat shops of Islamabad and Rawalpindi. All the PCR reactions were carried out in PCR system Veriti (Applied Biosystems, Waltham, USA)

Gene	PCR conditions	PCR reaction volume
stx1, stx2, eae, ehxA	1 cycle:	2.5 µl of 10X PCR buffer
	96°C-10 min	0.15 mM $MgCl_2$
	35 cycles:	0.1 mM of each dNTP (Thermoscientific, Waltham, USA)
	95°C-45 sec	0.2 µM of each primer
	60°C-45 sec	1 unit of *Taq* DNA Polymerase (Thermoscientific, Waltham, USA)
	72°C-45 sec	2 µl of DNA
	1 cycle:	Final volume of 25 µl with sterile water
	72°C-8 min	
O26 (*wzx gene*), O103 (*wzx* gene), O111 (*wbdl* gene)	1 cycle:	2 µl of 10X PCR buffer
	96°C-10 min	0.15 mM $MgCl_2$
	40 cycles:	0.1 mM of each dNTP (Thermoscientific, Waltham, USA)
	95°C-10 sec	500 nM primer concentration for O26 and O111 and 100 nM for O103 primers
	60°C-30 sec	1 unit of *Taq* DNA Polymerase (Thermoscientific, Waltham, USA)
	72°C-10 sec	2 µl of DNA
	1 cycle:	Final volume of 20 µl with sterile water
	72°C-5 min	
O145 (*wzx1* gene)	1 cycle:	2 µl of 10X PCR buffer
	94°C-2 min	0.15 mM $MgCl_2$
	35 cycles:	0.1 mM of each dNTP (Thermoscientific, Waltham, USA)
	94°C-20 sec	500 nM of O145 primer
	60°C-60 sec	1 unit of *Taq* DNA Polymerase (Thermoscientific, Waltham, USA)
	72°C-60 sec	2 µl of DNA
	1 cycle:	Final volume of 20 µl with sterile water
	72°C-5 min	
O157 (*rfbE* gene)	1 cycle:	2 µl of 10X PCR buffer
	96°C-5 min	0.15 mM $MgCl_2$
	40 cycles:	0.1 mM of each dNTP (Thermoscientific, Waltham, USA)
	96°C-15 sec	200 nM of O157 primer
	62°C-10 sec	1 unit of *Taq* DNA Polymerase (Thermoscientific, Waltham, USA)
	72°C-10 sec	2 µl of DNA
	1 cycle:	Final volume of 20 µl with sterile water
	72°C-5 min	

RESULTS

Of 101 samples 44 (43.5%) were positive for one or more virulence genes (*stx1*, *stx2*, *eae*, *ehxA*). The number of samples positive for virulence genes was significantly higher (χ^2= 22.577; df=1; P<0.05) in meat samples collected from Islamabad (29/40; 76.4%) compared to Rawalpindi (15/61; 26.8%). Similarly, significantly higher number (χ^2= 22.573; df=1; P<0.05) of positive samples was observed in goat meat samples (26/34; 76.4%) compared to cattle meat samples (18/67; 26.8%) (Table 2). The most common combination of virulence genes in goat meat positive samples was *stx1*, *stx2*, *ehxA* (9 samples) whereas the most common combination of virulence genes in cattle meat samples was *stx1*, *eae* and *eae* (4 samples each) (Table 2). In total nine *E. coli* isolates positive for targeted virulence genes were recovered from 44 positive samples. Two isolates were recovered from cattle and seven from goat meat samples. The most common virulence profile in these isolates was *stx1* (3 isolates) followed by *stx1*, *ehxA* (2 isolates) and *ehxA* only (2 isolates each). The remaining two isolates were positive for *stx1, stx2, ehxA* (one isolate) and *stx1, stx2* (one isolate). The most commonly observed virulence gene in these isolates was *stx1* (n=7) followed by *ehxA* (n=5) and *stx2* (n=2) (Table 3). The sero-grouping of these isolates using PCR indicated that three isolates were O111 and one isolate was O26. The remaining five isolates could not be serogrouped.

The antibiotic sensitivity profile was determined for eight antibiotics of seven different classes. The antimicrobial resistance ranging from 33.3% (Doxycycline; 3/9 isolates) to 100% (Lincomycine; 9/9 isolates) was observed in *E. coli* isolates against various antibiotics including doxycycline, streptomycine, neomycine, cephradine, and lincomycine. However, all the isolates were susceptible to aztreonam and imipenem (Table 4). The results also indicated that some *E. coli* isolates were resistant to more than three classes of antibiotics providing evidence of multi drug resistance.

Table 2: Distribution of targeted virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) in cattle (n=67) and goat (n=34) raw meat samples collected from meat shops of 11 different localities of Rawalpindi (n=61) and Islamabad (n=40) between March 2018 to July 2018. The samples were analysed using multiplex PCR for *stx1*, *stx2*, *eae* and *ehxA*

Type of meat			
Cattle meat		Goat meat	
Virulence genes	No. of positive samples	Virulence genes	No. of positive samples
stx1, eae	4	*stx1, stx2, ehxA*	9
eae	4	*stx1, ehxA*	8
stx1, ehxA	3	*ehxA*	4
ehxA	3	*stx1, eae, ehxA*	1
stx1, eae, ehxA	1	*stx2, eae, ehxA*	1
stx1, stx2, ehxA	1	*stx1, eae*	1
stx1, stx2	1	*stx1, stx2*	1
stx2	1	*stx1*	1
Total	18/67 (26.8%)		26/34 (76.4%)

Table 3: Distribution of the targeted virulence genes (*stx1, stx2, eae, ehxA*) in *E. coli* isolates (n=09) recovered from cattle and goat meat samples (n=101) collected from various retail meat shops in Rawalpindi and Islamabad

Virulence genes	*E. coli* isolates from		Total
	Cattle meat	Goat meat	
stx1	1	2	3
stx1, ehxA	0	2	2
ehxA	1	1	2
stx1, stx2, ehxA	0	1	1
stx1, stx2	0	1	1
Total	2	7	9

Table 4: Antibiotic susceptibility profile of *E. coli* isolates positive for one or more virulence genes (*stx1, stx2, ehxA*). The isolates were recovered from cattle and goat meat samples collected from various retail shops in Rawalpindi/Islamabad

Isolate ID	Serogroup	Virulence profile	Antibiotic susceptibility profile against							
			Aztreonam	Imipenem	Enrofloxacine	Doxycycline	Cephradine	Neomycine	Streptomycine	Lincomycine
M1	O111	*stx1*	Sensitive	Sensitive	Intermediate	Intermediate	Resistant	Resistant	Intermediate	Resistant
M35	O111	*stx1*	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Resistant	Intermediate	Resistant
M51	O111	*ehxA*	Sensitive	Sensitive	Intermediate	Sensitive	Resistant	Resistant	Resistant	Resistant
M57	O26	*ehxA*	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
M59	NS	*Stx1*, *stx2*, *ehxA*	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Resistant	Resistant	Resistant
M61	NS	*stx1, stx2*	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
M66	NS	*stx1*, *ehxA*	Sensitive	Sensitive	Intermediate	Sensitive	Sensitive	Resistant	Resistant	Resistant
M95	NS	*stx1*, *ehxA*	Sensitive	Sensitive	Sensitive	Intermediate	Resistant	Intermediate	Intermediate	Resistant
M100	NS	*stx1*	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Intermediate	Resistant

NS = could not be serogrouped.

DISCUSSION

Although STEC O157 has been considered the most commonly associated STEC serogroup with disease in humans recent studies have reported increase in the number of human cases due to non-O157 STECs (O26, O103, O111, O121 and O145). The USA has a zero tolerance policy for STEC O157 in the meat and recently six non-O157 STECs have also been added to the list (Food Safety and Inspection Service, 2011). The most recent outbreak of STEC in Europe due to STEC O104 also highlighted the importance of STECs in disease causations other than STEC O157 (Frank *et al.,* 2011). Of 101 samples 44 (43.5%) meat samples from cattle and goats were found positive for one or more virulence genes of clinical significance using multiplex PCR. Only 9 (20.4%) isolates were recovered from these positive samples. PCR is considered more sensitive method compared to isolation which could be one possible reason for low recovery of STEC isolates from positive samples. It could also be due to the presence of dead *E. coli* isolates with virulence genes which can be detected by PCR but cannot be isolated.

All four targeted virulence genes of clinical importance (*stx1*, *stx2*, *eae* and *ehxA*) were detected in meat samples from cattle and goats. Some samples were positive for only one targeted gene while others were positive for various combinations of targeted virulence genes. Although PCR cannot differentiate whether these genes belong to one isolate or two or more isolates the presence of clinically relevant virulence genes in the samples may be indicative of the presence of STEC of zoonotic importance in the meat samples. Therefore, consumption of contaminated meat could be the source of infection for humans.

Results indicated that the rate of isolation of *E. coli* with virulence genes was higher in goat meat samples (7/26; 26.9%) compared to cattle meat samples (2/18; 11.1%). Previous studies also reported high incidence of *E. coli* in mutton compared to beef (Franco *et al.,* 2009; Momtaz *et al.,* 2013). Mutton is softer than beef due to presence of high free water contents. High water quantity in mutton may be responsible for various chemical reactions and better survival of micro-organisms like *E. coli* in goat meat (Momtaz *et al*., 2013).

STEC O26 and O111 were the most commonly observed serogroups in this study. Previous studies have also reported isolation of O26 and O111 from meat of various ruminants (Momtaz *et al.,* 2013; Valilis *et al.,* 2018). STEC O26 and O111 are included in the list of so called "gang of seven" (O157, O26, O45, O103, O111, O121, and O145) which have emerged as food borne pathogens of zoonotic significance (Food Safety and Inspection Service, 2011). Many STEC outbreaks due to O26 and O111 have been reported from different parts of the world (Valilis *et al.,* 2018). In Pakistan there is no routine surveillance and monitoring system for various STEC especially of zoonotic importance. Therefore, there is no information available about involvement of various STEC in causation of diseases in humans. In order to have better understanding about distribution of STEC in human cases there is a need to develop diagnostic facilities regarding STEC of zoonotic importance in all the major laboratories of the country. However, the isolation of STEC O26 and O111 with clinically relevant virulence genes indicates that cattle and goat meat may be responsible for transmission of STEC O26 and O111 to humans.

E. coli isolates obtained in this study were tested for serotypes O157, O26, O103, O111 and O145 using PCR. The serogroups of four isolates could not be identified indicating that *E. coli* isolates other than O157, O26, O103, O111 and O145 are also prevalent in the cattle and goat population of Pakistan. The disease causing potential of these serogroups should not be underestimated as the recent outbreak in Europe with STEC O104:H4 affecting 3222 humans including 810 HUS cases have indicated the ability of conversion of less virulent STEC to highly virulent by gaining other virulence factors (Frank *et al.,* 2011).

In this study 22.2% (2/9) isolates carried *stx2* gene. STEC virulence and *stx* genotype association has previously been reported. Previous studies have reported a high rate of isolation of STEC isolates with the *stx2* gene from HC and HUS cases (Boerlin *et al.,* 1999; Pradel *et al.,* 2008). A study carried out to investigate the association between STEC virulence genes and disease in humans reported a higher rate of isolation STEC with the *stx2* gene (39/60; 60%) from HC and HUS cases compared to STEC isolates with the *stx1* gene (29/75; 38.6%) (Boerlin *et al.,* 1999). Another study also reported a higher rate of isolation of STEC with the *stx2* gene (13/15; 86.6%) from HC and HUS cases compared to STEC isolates with the *stx1* gene (2/15; 13.3%) (Pradel *et al.,* 2008). Therefore, STEC isolates with the *stx2* gene are considered more virulent compared to STEC isolates with the *stx1* gene.

The exact role of *ehxA* in disease mechanisms is not clearly known however, its association with human disease cases highlights the importance of *ehxA* as a

virulence marker for STEC. A study indicated that most of the STEC recovered from human disease cases contained *eae* and *ehxA* genes (Gyles *et al.,* 1998). In this study most of the STEC isolates (6/7; 85.7%) were negative for *eae* and *ehxA* genes. However, recovery of STEC without *eae* and *ehxA* from HUS and diarrhoea patients has also been reported (Beutin and Martin, 2012). In addition, the isolation of STEC O104 without *eae* and *ehxA* genes from the recent European outbreak further signifies the virulence of STEC isolates without *eae* and *ehxA* genes (Frank *et al.,* 2011). Therefore, the zoonotic potential of STEC without *eae* and *ehxA* genes should not be underestimated.

AMR in *E. coli* isolates was observed against lincomycine, cephradine, neomycine, streptomycine and doxycycline. These antibiotics are widely used for treatment, prevention and as growth promoters in food producing animals in Pakistan (Rahman and Mohsin, 2019). The extensive use of these antibiotics could be the reason for development of AMR against these antibiotics as several studies have indicated association between use of antibiotics in livestock and AMR (Burow *et al.,* 2014; Chantziaras *et al.,* 2014). All *E. coli* isolates were sensitive to aztreonam and imipenem. These antibiotics are expensive and considered as last resort antibiotics therefore their use in food producing animals is minimal in Pakistan. Isolation of multidrug resistant *E. coli* indicates that ruminants may serve as reservoir for multidrug resistance *E. coli* which may be transmitted to humans through meat.

The study indicated the isolation of STEC of zoonotic significance from meat samples of cattle and goats obtained from shops of Rawalpindi and Islamabad. Therefore, it may be assumed that meat sold on the shops of Rawalpindi and Islamabad may be responsible for transmission of STEC to humans. However, these isolates should be compared with STEC isolates recovered from human patients to get better understanding of their zoonotic potential. Furthermore, it was a small scale study limited to a small geographical area, therefore a large scale study with emphasis on STEC serogroups (O157, O26, O103, O111, O121 and O145) of zoonotic importance may be carried out to clearly understand the risk of transmission of STEC to humans through meat.

Authors contribution: HI: Planning of study, collection of meat samples and data, analysis of data and writing of manuscript. IB: Assisted in collection of meat samples and data and analysis of meat samples in Lab. AA: Assisted in analysis of meat samples in Lab and compilation of results. AR: Assisted in planning of study and analysis of data. MAS: Assisted in collection of meat samples and data and analysis of samples in Lab. MQ: Assisted in planning of study and write up of manuscript. NK: Assisted in planning of study, analysis of data and write up of manuscript. WS: Assisted in planning of study and write up of manuscript. TP: Assisted in analysis of data and write up of manuscript. MH: Assisted in collection of meat samples and data. MA: Assisted in planning of study and write up of manuscript. AY: Assisted in planning of study, analysis of data and write up of manuscript.

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