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ORIGINAL ARTICLE

Molecular detection of Shiga toxin-producing *Escherichia coli* (STEC) O157 in sheep, goats, cows and buffaloes

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Abstract

Background Shiga toxin-producing *E. coli* (STEC) are important foodborne pathogens that causing serious public health consequences worldwide. The present study aimed to estimate the prevalence ratio and to identify the zoonotic potential of *E. coli* O157 isolates in slaughtered adult sheep, goats, cows and buffaloes.

Materials and methods A total of 400 Recto-anal samples were collected from two targeted sites Rawalpindi and Islamabad. Among them, 200 samples were collected from the slaughterhouse of Rawalpindi included sheep (n = 75) and goats (n = 125). While, 200 samples were collected from the slaughterhouse of Islamabad included cows (n = 120) and buffalos (n = 80). All samples were initially processed in buffered peptone water and then amplified by conventional PCR. Samples positive for *E. coli* O157 were then streaked onto SMAC media plates. From each positive sample, six different Sorbitol fermented pink-colored colonies were isolated and analyzed again via conventional PCR to confirm the presence of *rfbE* O157 gene. Isolates positive for *rfbE* O157 gene were then further analyzed by multiplex PCR for the presence of STEC other virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*) simultaneously.

Results Of 400 RAJ samples only 2 (0.5%) showed positive results for *E. coli* O157 gene, included sheep 1/75 (1.33%) and buffalo 1/80 (1.25%). However, goats (n = 125) and cows (n = 120) found negative for *E. coli* O157. Only 2 isolates from each positive sample of sheep (1/6) and buffalo (1/6) harbored *rfbE* O157 genes, while five isolates could not. The *rfbE* O157 isolate (01) of sheep sample did not carry any of STEC genes, while the *rfbE* O157 isolate (01) of buffalo sample carried *sxt1*, *stx2*, *eae* and *ehlyA* genes simultaneously.

Conclusion It was concluded that healthy adult sheep and buffalo are possibly essential carriers of STEC O157. However, *rfbE* O157 isolate of buffalo RAJ sample carried 4 STEC virulent genes, hence considered an important source of STEC infection to humans and environment which should need to devise proper control systems.

Keywords *Escherichia coli* (STEC) O157 · RAJ · Conventional PCR · Multiplex PCR

Introduction

Shiga toxin-producing *E. coli* (STEC) are considered important foodborne pathogens of zoonotic importance which causing mild to severe bloody diarrhea with the emergence of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) which is a life-threatening disease [1, 2]. There are nearly more than 200 serotypes of STEC are recognized and the most frequent outbreaks of STEC are documented to be related to serotype O157: H7 strain throughout the globe [3]. *E. coli* O157: H7 serotype is the most important strain in hundreds of the other *E. coli* serogroups which live inside healthy humans and animal's digestive organs it delivers an intense toxin that can cause serious public complications [4]. The toxin produced by STEC is in similarity with *Shigella*

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dysentery producing toxin is also called Shiga-like toxins, or verodoxins [5]. In 1982 the pathogen STEC O157: H7 was recognized for the first time during an outbreak in the United States (US) [6, 7]. Since then, for public health importance nowadays *E. coli* O157: H7 is widely recognized as a food-borne pathogen [8, 9]. STEC primary transmission occurs through fecal–oral route by either indirectly use of a broad preparation of unhygienic foods, through contaminated water ingestion or directly through animals contact and their condition as well as from individual to individual straightforwardly [5, 10]. Ruminant animals especially cattle, goats and sheep serve as a natural reservoir for STEC, which exist in the guts of these animals and appear to be the supportive hosts for STEC O157: H7. Thus, when animals are butchered, bacteria from animal intestines may contaminate their meat [11, 12]. In most human cases cattle have been considered the suspected domestic ruminant of the source of infection [13]. Sheep have also been suggested as a source of human infection and a major cause of contamination to the food industry [14–16]. Like cattle and sheep, goats have also been considered the sub-clinical carrier of STEC O157, as they are the asymptomatic shedder of these bacterial pathogens [17]. In 2010, there are 1.78 STEC infection cases for each 1 lac populace are reported in the United States (US) [18]. In the European Union, STEC infection rate in 2011, is 1.93 cases per 1 lac populace [19]. Similarly, in New Zealand in 2011, the documented rate of STEC is 3.5 cases for each 1 lac populace (154 cases) [20]. In Argentina, between 2002 and 2015 only 4 cases of HUS were reported which are connected with food intake and these all cases were linked with STEC O157: H7 *ehxA, eae* and stx_2 [21–23]. STEC contamination rate in 2012, reported in Australia is 0.5 cases for every 1 lac populace [24]. In Pakistan, surveillance data regarding this organism is very sparse. Although demonstrated by several studies reported by [25–30].

Therefore, the present study was designed to estimate the prevalence rate and recognize the zoonotice potential of *E. coli* O157: H7 disseminating from adult sheep, goats, cows and buffaloes slaughtered in the slaughterhouses of District Rawalpindi and islamabad, Pakistan.

Materials and methods

Study area

The present study was carried out at the Bacteriology laboratory of Animal Health Program, Animal Sciences Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan in a duration of 8-months from May 2017 to December 2017. Two local government slaughterhouses located at District Rawalpindi and Islamabad, Punjab Pakistan, included slaughterhouse of Rawalpindi which distributed the products of healthy slaughtered sheep and goats, while slaughterhouse of Islamabad which distributed the products of healthy slaughtered cows and buffalos to the other parts of the country.

Recto-anal samples collection

A total of 400 Recto-anal junctions (RAJ) samples were collected with the help of sterile labeled cotton-tipped swab sticks from two targeted sites Rawalpindi and Islamabad. Among them, 200 samples were collected from the slaughterhouse of Rawalpindi included healthy slaughtered adult sheep (n = 75) and goats (n = 125). While, 200 samples were collected from the slaughterhouse of Islamabad included healthy slaughtered adult cows (n = 120) and buffalos (n = 80). The complete data history such as age, sex, weight, and species with each sample was recorded. In the studied animals, the output variable was the status of *Escherichia coli* O157. Slaughterhouses were visited seven and six times respectively, during the hot months from May to July 2017, because STEC O157 can easily survive in warm temperatures. On each visit, Twenty-five samples were randomly collected from both regions which included adult sheep, goats, cows and buffaloes. These RAJ samples were placed into modified Stuart's transport medium (Bacti Swab NPB, Thermo Scientific, Lenexa, KS), and maintained approximately at 4 °C until processed in the Laboratory.

RAJ swab samples processing

RAJ swab samples were initially processed in 20 ml of buffered peptone water (BPW) taken in the sterilized universal bottle. Each RAJ swab sample was enriched in the enrichment broth (BPW) by cutting the swab sample inside each universal bottle using a sterilized scissor and incubated for 24 h at 37 °C.

DNA extraction from enriched broth (BPW)

After enrichment, DNA extraction was carried out from enriched broth (BPW; Difco™, Becton, USA) by boil cell lysate method [31, 32]. A 1-ml aliquot of enriched broth was taken and centrifuged at 13,000 rpm for 3 min. The supernatant was discarded after centrifugation and the pellet was re-suspended in 500 µl double distilled water (ddH_2O). Vertexing was done at high speed for 10 s. The aliquot was heated at 95 °C for 10 min. Suspension of the lysed bacterial cell was then cooled at 4 °C for 5 min and was re-centrifuged again at 13,000 rpm for 3 min. The supernatant containing the DNA was then collected and transferred to another eppendorf tube. It was then subjected towards conventional PCR to detect *rfbE* O157 gene [33].

Initial screening for *rfbE* O157 serotype by conventional PCR

The *rfbE* gene is responsible for the production of the lipopolysaccharide (LPS) O side chain of the STEC O157: H7 cell surface and is a highly preserved gene specific to the serotype *E. coli* O157: H7 [34]. Conventional PCR was performed in the Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia). Already standardized Oligonucleotide specific sequence of *rfbE* O157 primers along with the amplified product size were specifically used for the synthesis of the *rfbE* O157 gene (Table 1). Chemical components contained buffer 2.5 µl (Invitrogen, NZ), each primer 0.5 µl, dNTP 0.6 µl (Fermentas), 2.5 µl of MgCl2 (Invitrogen, NZ), 0.3 µl unit of *Taq* DNA polymerase (Invitrogen, NZ), 16.1 µl of nuclease-free water completed to a final volume of 25 µl with the addition of 2 µl of extracted DNA. Thermocycling conditions were programmed for 7 min at 95 °C, followed by 35 cycles for 24 s at 95 °C, 45 s for 60 °C, 45 s at 72 °C, with final extension for 8 min at 72 °C, followed by maintenance at 4 °C.

Table 1 Primers used for amplification of *E. coli* O157 antigen-specific gene in conventional PCR

Target gene (serogroup)	Primer sequence (5′–3′)	GC%	Amplicon size (bp)	GenBank accession number/reference
rfbE (O157)	F-5′TTTCAC ACTTAT TGGATG GTCTCA A′3	36%	88	AF163329 [35]
	R-5′CGA TGAGTT TATCTG CAAGGT GAT′3	41.7%		

E. coli O157 isolation and confirmation by conventional PCR

Agar media is one of the important sources for desire colonies isolation and for the use of further confirmation purposes [36]. STEC O157 can typically and also be effectively recognized by its capability to ferment Sorbitol in 24 h on Sorbitol MacConkey agar media as compared to other *E. coli* strains. The RAJ swab samples suspected positive for *E. coli* O157 gene by conventional PCR were then streaked onto Sorbitol MacConkey Agar media plates (SMAC) and incubated at 37 °C for 24 h. After the incubation period, only Sorbitol fermented pink-colored colonies were grown on SMAC media plates. About 6 different isolated colonies were selected from each plate for further analysis. DNA extraction was carried out from each colony using the simple boiling method [31, 32]. The extracted DNA was then analyzed via conventional PCR under similar conditions to conform the presence of *rfbE* (O157) gene.

Multiplex PCR for STEC virulent genes (*stx1, stx2, eae* and *hlyA*)

Isolates positive for *rfbE* O157 serogroup by conventional PCR were then briefly subjected towards multiplex PCR (Gene Amp PCR system 9700; Applied Biosystems, Melbourne, Australia) to detect the presence of STEC other virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*) simultaneously. Already standardized (Oligonucleotide) specific sequence of primers and its desirable base-pair sizes were used by multiplex PCR assay for the amplification of STEC virulent genes (Table 2). Chemical components contained 2.5 µl buffer (Invitrogen, NZ), 0.5 µl each of the 8 primers (4 primer pairs) *stx1, stx2, eae* and *hlyA*, 0.6 µl of each dNTP (Fermentas), 2.5 µl $MgCl_2$ (Invitrogen, NZ), 0.3 µl unit of *Taq* DNA Polymerase (Invitrogen, NZ) and 13.1 µl of Nuclease-free water completed to final volume 25 µl along with 2 µl of extracted isolate DNA. Thermocycling conditions were programmed for 7 min at 95 °C, followed by 40 cycles for 45 s at 95 °C, 45 s for 60 °C, 45 s at 72 °C, with final extension

Table 2 Primers used for amplification of STEC (*stx1*, *stx2*, *eae* and *hlyA*) specific genes in multiplex PCR

Target gene	Primer sequence (5′–3′)	GC%	Amplicon size (bp)	References
stx1	F-5′GAC TGC AAA GAC GTA TGT AGA TTC G′3	44	150	[37]
	R-5′ATC TAT CCC TCT GAC ATC AAC TGC′3	45.8		
stx2	F-5′ATT AAC CAC ACC CCA CCG′3	55.6	200	[37]
	R-5′GTC ATG GAA ACC GTT GTC AC′3	50		
eae	F-5′GTA AGT TAC ACT ATA AAA GCA CCG TCG′3	40.7	106	[37]
	R-5′TCT GTG TGG ATG GTA ATA AAT TTT TG′3	30.8		
hlyA	F-5′GCATCATCAAGCGTACGTTCC′3	52.4	534	[38]
	R-5′AATGAGCCAAGCTGGTTAAGCT′3	45.5		

for 8 min at 72 °C, followed by maintenance at 4 °C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel (Invitrogen, NZ) and visualized using ethidium bromide under Gel documentation system. The isolates were then sub-cultured onto the SMAC media plates to confirm pure growth and stored at − 80 °C in nutrient broth containing 15% (v/v) glycerol.

Results and discussion

In the present study, of 400 RAJ swab samples, only 2 (0.5%) showed positive results for *E. coli* O157 gene, included sheep 1/75 (1.33%) and buffalo 1/80 (1.25%). However, goats (n = 125) and cows (n = 120) showed negative results for *E. coli* O157 (Table 3). From each positive sample (sheep and buffalo), 6 different Sorbitol fermented pink-colored colonies were isolated onto two SMAC agar media plates (Fig. 1). DNA was extracted from each colony using simple boil cell lysate method and analyzed via conventional PCR to confirm the presence of *rfbE* O157 gene. Results indicated that only 2 isolates from each positive sample of sheep (1/6) and buffalo (1/6) harbored *rfbE* O157 genes (Figs. 2 and 3), While, rest of the five isolates showed negative results.

In comparison with a recent study, a high prevalence ratio 10/320 (6.3%) of STEC O157 was reported in cattle samples collected from the fecal rectum (n = 160) and hide brisket area (n = 160) at the abattoir in Northern Italy [39]. This is much higher than the prevalence rate of 2/400 (0.5%) observed in our study. Similarly, a total of 12/1200 (1.0%) of STEC O157 strains were isolated from bovine 8/620 (1.3%), caprine 1/130 (0.8%) and ovine 3/230 (13%) [40]. Followed by another study, in which a total of 8 (0.8%) STEC O157: H7 isolates were recovered from fecal samples of sheep 7/361 (1.9%) and goats 1/178 (0.6%) in Central Greece [41]. Whereas in our findings the prevalence rate of STEC O157 reported in RAJ sample of sheep was 1/75 (1.33%), while no STEC O157 was detected in goat samples. In contrast to our study, a higher *E. coli* O157 was reported in hides and fecal samples of cattle (49.4%), sheep (6.3%) and goats (2.5%), respectively [42]. Similarly, out of 210 samples of beef, buffalo and lamb meat the prevalence rate of *E. coli*

Table 3 Overall prevalence ratio of *E. coli* O157 estimated in 400 RAJ samples of sheep, goats, cows and buffaloes

Species of animals	Total RAJ samples	*E. coli* O157 positive sample	STEC virulent genes detected	Overall prevalence %
Sheep	75	1	Not detected	1.33
Goats	125	Negative	Not detected	0
Cows	120	Negative	Not detected	0
Buffalos	80	1	*stx1, stx2, eae,* and *hlyA*	1.25
Total	400	2		0.5

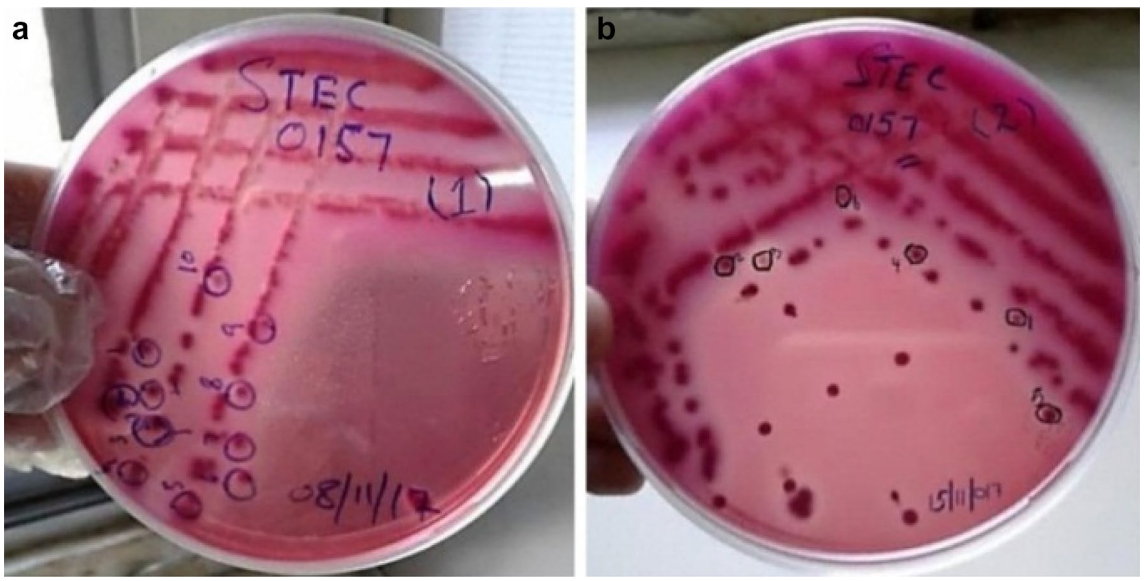

Fig1 Isolated colonies from *E. coli* O157 positive RAJ sample of sheep (**a**) and buffalo (**b**) for *rfbE* O157 gene confirmation

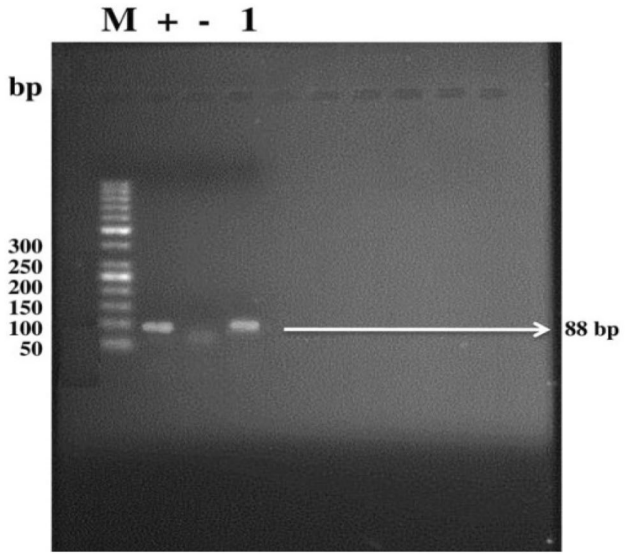

Fig 2 Single *rfbE* O157 isolate from positive RAJ sample of Sheep (well 1, 88 bp) by conventional PCR assay

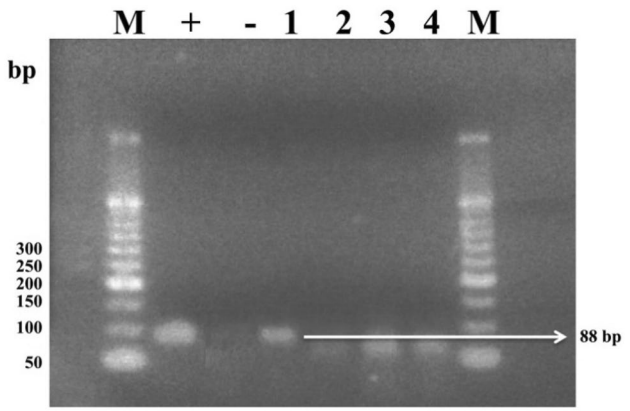

Fig. 3 Single *rfbE* O157 isolate from positive RAJ sample of Buffalo (well 1, 88 bp) by conventional PCR assay

O157: H7 was reported as (2.8%) in beef and (1.4%) in buffalo. However, lamb meat samples showed a negative result for this serogroup [43]. In addition, the prevalence rate of *E. coli* O157 was also recovered from fecal samples of camel (4.3%), goats (2%) and cattle (1.46%). However, none of the *E. coli* O157 was recovered from sheep fecal samples [44].

To concern the observed variation of our findings with these studies could be attributed to the differences in a wide range of sample collection, culture and molecular-based methods being applied for screening, detection and characterization of STEC. In a recent study, samples collected from ovine and bovine hosts via fecal palpitation and recto-anal junction swabs were reported most appropriate for the identification of STEC [45, 46]. For *E. coli* O 157 colonization the RAJ site is a good indicator to collect a sample [47]. According to these reports, we acquired the same appropriate methodology for a sample collection from the studied animals.

During the enrichment process, the recovery of high STEC and other *E. coli* O157 strains may be difficult to isolate because of the presence of other competing flora in the medium. Hence, this could be one of the obvious reasons behind the recovery of a low number of STEC while testing samples [48, 49]. Additionally, the temperature required for STEC detection during the enrichment process may be preferred to particular serotypes to show enough growth [50, 51].

Similarly, culture-based methods (selective and differential media) is almost difficult to differentiate STEC from other *E. coli* strains, as STEC can only be recognized by its capability to produce Shiga toxins, however, it cannot be used as a phenotypic marker for the identification of STEC when there is an availability of mixed culture [52]. Besides, there is no assurance for the accuracy, specificity and safe use of these cultural methodologies [53]. As in the O serogroup of STEC a huge variability has also been observed [54, 55]. As compare to immunomagnetic separation techniques, Sorbitol MacConkey agar has also been recommended for direct STEC O157: H7 isolation. However, its less sensitive factor was confirmed [56].

While some appropriate molecular techniques have been applied in recent studies for the quantification of STEC in bovine feces [36, 57, 58], in ovine feces [59] and in agricultural food matrices [60]. However, these molecular techniques are more costly to apply in less facilitative areas as compare to culture-based methods. The misidentification of a culture-positive sample, giving false-negative and false-positive results, the targeted genes need to analyze may be present in different viable cells and the detection of a gene does not indicate if it may be expressed or not are the certain limitations and apparently main reasons behind the detection of a limited number of targeted samples [52]. It is also stated that polymerase chain reaction (PCR) may sometimes incapable to differentiate between live and dead cells, as the amplified DNA from dead STEC cells and the presence of background flora in the sample sometimes makes the PCR more vulnerable to give the exact prevalence ratio of STEC [61].

The DNA extracted from each single *rfbE* O157 isolate of sheep and buffalo RAJ samples were then briefly subjected towards multiplex PCR to detect the presence of STEC other virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*) simultaneously (Fig. 4). Results revealed that the single *rfbE* O157 isolate (01) of sheep sample did not carry any of STEC genes (Fig. 5). It only harbored *rfbE* O157 gene and thus possessed rear chances of dissemination in the region of Rawalpindi. On the other hand, the single *rfbE* O157 isolate (01) of buffalo sample carried four STEC clinical virulent genes (*sxt1*,

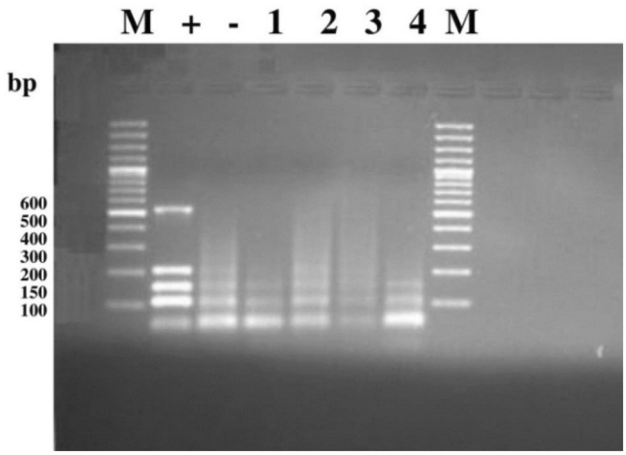

Fig. 4 STEC virulent strains (*hlyA* (534 bp), *stx2* (200 bp), *stx1* (150 bp) and *eae* (106 bp)) by multiplex PCR assay. *rfbE* O157 isolate of sheep RAJ sample (well 1, 2, 3 and 4) showed negative results for STEC targeted genes

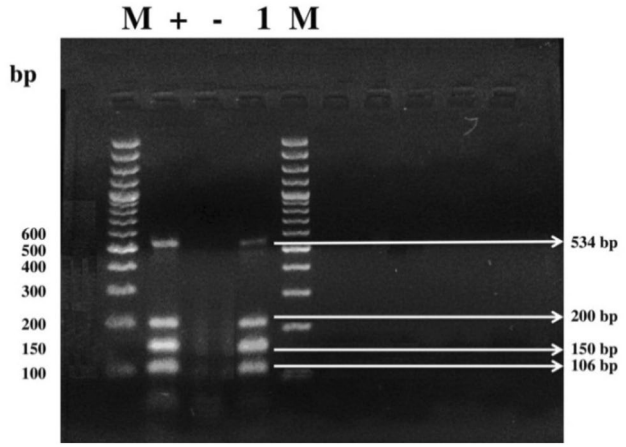

Fig. 5 STEC virulent strains (*hlyA* (534 bp), *stx2* (200 bp), *stx1* (150 bp) and *eae* (106 bp)) by multiplex PCR assay. *rfbE* O157 isolate of Buffalo RAJ sample (well 1) showed a positive result for all STEC targeted genes

stx2, *eae* and *ehlyA*) at the same time (Fig. 5), thus it possessed high zoonotic potential of transferring to human and environment in the region of Islamabad.

In consistence with our findings, a total of 6 (4%) *E. coli* O157: H7 was separated from fecal samples and all isolates were found that contained *ehxA, stx*$_2$c and *eaeA*-γ1. The non-O157 STEC observed in 2 (1.5%) fecal samples contain one isolate which carried *ehxA, stx*$_2$*c*; *stx*$_2$*a* and *stx*$_1$ and the other isolates containing *stx*$_1$*a* only [62]. STEC O157 was also reported as (81–87%) in Irish cattle and beef-derived isolates. Among them, the most predominant virulent strain was *stx2* and *eae* [63, 64]. Followed by similar results were noted among *E. coli* O157 isolates from cattle in France [65]. Likewise, a total of 55/1317 (4.18%) STEC O157 was also recovered from RAMS samples of cattle in Ireland. Amongst 50/55 *E. coli* O157 isolates harbored *stx2* genes and all were *eae* positive [66].

In the current study, none of the *E. coli* O157: H7 was detected in RAJ samples of goats and cows. The reason may be due to very fewer chances of *E. coli* O157 colonization in intestinal hosts of these animals. As goat cannot be colonized exclusively with *E. coli* O157 and they have been considered the sub-clinical carrier of STEC [67, 68]

The present study revealed a very low prevalence ratio of *rfbE* O157 recovered from healthy slaughtered adult sheep and buffalo in the region of Rawalpindi and Islamabad, Punjab Pakistan. One of the possible reasons for the low prevalence rate 2 (0.5%) reported in our study is the inclusion of healthy adult ruminant animals (sheep, goats, cows and buffaloes). The animals bring to the slaughterhouses for buttering in these regions are majority of the adult age.

Studies indicated that when sheep and cattle get older so changes in the composition of gut microbiota (gastrointestinal tract and recto-anal site) of these animals occur consequently the prevalence rate of STEC decreases [69, 70]. In the United States, STEC prevalence rate was reported higher in fecal samples of younger sheep (22.7%) at slaughter than older animals (0–1.9%) at pasture [71]. Similarly, in New Zealand, a higher prevalence rate of STEC was reported in slaughtered lamb (3.8%) than ewes (0.9%) at pasture [72]. Another study was reported on a group of older Scottish beef cattle potentially related with a lower risk of *E. coli* O157 shedding [73].

Even though several factors such as study design, sample collection and isolation methods used have a profound impact on the prevalence rate of *E. coli* O157. Despite this, the intrinsic factors (age, sex etc.) and extrinsic factors (season, diet, and climate etc.) have also a significant impact on the prevalence rate of *E. coli* O157 [74]. Keeping in view these differences thus limits the application of our study which could be one of the noticeable reasons behind the low prevalence rate 2/400 (0.5%) of *E. coli* O157 reported in our findings.

Conclusion

The present study revealed a low prevalence rate of 2/400 (0.5%) reported in sheep 1/75 (1.33%) and buffalo 1/80 (1.25%) RAJ samples at District Rawalpindi and Islamabad, Pakistan. However, it cannot be underestimated as healthy adult sheep and buffalo was possibly essential carriers of STEC O157 in these regions. But, as compared to Rawalpindi, the Islamabad region was at high risk because STEC O157 with 4 clinical relevant virulent genes (*stx1, stx2, eae* and *hlyA*) were detected in positive RAJ sample of buffalo

which may possibly act as a serious public health consequence in future.

Recommendations

Data about the study of disease transmission of STEC O157 in these specific areas Rawalpindi and Islamabad as well in other parts of Pakistan are rare. Subsequently, more data is required for the study of disease transmission of STEC O157, distribution of virulence genes and their subtypes in *E. coli* separates from small and large ruminants and transmission of STEC O157 from these living organisms is to devise proper control systems. These control procedures would ultimately help in diminishing the increasing number of human STEC cases in Pakistan and further keep away from possible losses to Pakistan's economy.

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