Molecular Detection Of Shiga Toxin-Producing *Escherichia Coli* (Stec) O157 In Sheep, Goats, Cows And Buffaloes

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Research Article

Keywords: Escherichia coli (STEC) O157, RAMS, Conventional PCR, Multiplex PCR, Bacterial Toxicon

Posted Date: April 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-430892/v1

Version of Record: A version of this preprint was published at Molecular Biology Reports on August 10th, 2021. See the published version at https://doi.org/10.1007/s11033-021-06631-3.

Abstract

A total of 400 Recto-anal mucosal swabs samples (200 from each region) were collected and processed initially by conventional PCR. Among them, only two (n=2) RAMS samples were detected and positive for *E. coli* O157 gene, included one in RAMS samples of sheep from slaughterhouse of Rawalpindi, while another in RAMS samples of buffalos from slaughterhouse of Islamabad. The rest of the samples from goats and cows showed negative results for *E. coli* O157 in both regions. *E. coli* O157 isolates were obtained from each positive sample onto two different Sorbitol MacConkey Agar media plates and analyzed again through conventional PCR, resulted only single 1/6 isolated colony confirmed from both samples which possessed *rfbE* genes. The overall prevalence of *rfbE* (O157) genes came up to (0.5%). Furthermore, for the presence of STEC other virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*), these both positive *rfbE* isolated colonies were then briefly subjected towards multiplex PCR. Results revealed that none of the STEC virulent genes were detected in single (01) positive *rfbE* O157 isolated colony of sheep sample. While fortunately 4 STEC virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*) were detected in single (01) positive *rfbE* O157 isolated colony of buffalo sample. It was concluded that healthy adult sheep and buffalo are possibly essential carriers of STEC O157. However, buffalo sample possessed 4 STEC virulent genes, hence considered an important source of STEC infection to humans either through direct or indirect transmission which should devise proper control systems.

1. Introduction

Shiga toxin-producing *E. coli* are considered important foodborne pathogens of zoonotic importance which causing mild to severe bloody diarrhea with the emergence of the hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) which is a life-threatening disease (Karmali et al., 2010; Chileshe and Ateba, 2013).

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 (Abrar *et al*., 2020). *E. coli* O157: H7 serotype is the most important strains in hundreds of the other *E. coli* serogroups which live inside healthy humans and animals digestive organs it delivers an intense toxin that can cause dangerous disease (Gyles, 2007).

The toxin produced by STEC is in similarity with *Shigella dysentery* producing toxin is also called Shiga-like toxins, or verotoxins (Hunt, 2010). In 1982 the pathogen STEC O157: H7 was recognized for the first time during an outbreak in the United States (US) (Acha and Szyfres, 2003; Fernandez, 2008). Since then, for public health importance nowadays *E. coli* O157: H7 is widely recognized as a foodborne pathogen (Xia et al., 2010).

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 (Alelis et al., 2009; Hunt, 2010).

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 (Fatima et al., 2020).

In 2010, there are 1.78 STEC infection cases for each 1 lac populace are reported in the United States (US), (Anonymous, 2012). In the European Union, STEC infection rate in 2011, is 1.93 cases per 1 lac populace (ECDC, 2013). Similarly, in New Zealand in 2011, the documented rate of STEC is 3.5 cases for each 1 lac populace (154 cases), (Lim et al., 2012). 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 (Carbonari et al., 2016). STEC contaminations rate in 2012, reported in Australia is 0.5 cases for every 1 lac populace (Group, 2012).

In Pakistan, surveillance data regarding this organism is very sparse. Although demonstrated by a number of studies reported by (Mohsin et al., 2007; Ali et al., 2010; Mohsin et al., 2010; Shahzad et al., 2013 and Razzaq et al., 2016; Irshad et al., 2020).

Sheep have been suggested as a source of human infection many times (Allison et al., 1997). Goat cannot be colonized exclusively with *E. coli* O157, however, its natural behavior implies that it has also a major role in causing zoonotic disease either by direct or indirect transmission (Keen et al., 2006; Fox et al., 2007). Very little information on the prevalence of STEC O157 in goats are available as compared to sheep (Cortés et al., 2005; Espie et al., 2006; Rey et al., 2006; La Ragione et al., 2009).

In most human cases cattle have also been considered the suspected domestic ruminant of the source of infection (Qayyum *et al.*, 2020). There are several studies have been reported which indicates the presence of STEC O157 and non-O157 genes in cattle and small ruminants (Vold et al., 2001; Johnsen et al., 2001; Geue et al., 2002; Kuhnert et al., 2005b; Kiranmayi et al., 2010; Rahimi et al., 2012). The present study aimed to estimate the prevalence ratio and to identify the zoonotic potential of *E. coli* O157 isolates obtained from sheep, goats, cows and buffaloes slaughtered in the slaughterhouse of Rawalpindi and Islamabad, Pakistan.

2. Materials And Methods

2.1 Sample collection and transportation

The present study was carried out at Bacteriology laboratory of Animal Health Program, Animal Sciences Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan in duration of 8-months from May 2017 to December 2017. Two local government slaughterhouses located at Rawalpindi and Islamabad, Punjab Pakistan regions, 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.

A total of 400 Recto-anal mucosal swabs (RAMS) samples were collected with the help of sterile cotton-tipped swabs sticks, included 200 RAMS samples from different healthy slaughtered sheep (n = 75) and goats (n = 125) at local government slaughterhouse of Rawalpindi region. While 200 RAMS samples were collected from healthy slaughtered cows (n = 120) and buffalos (n = 80) at local government slaughterhouse of Islamabad region along with a complete data history such as age, sex, weight, and species with each sample. The output variable was the status of *Escherichia coli* O157 in the studied animals. 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 temperature. On each visit, Twenty-five samples which included species sheep, goats and cows, buffaloes both were selected systematically.

These RAMS samples were placed into modified Stuart's transport medium (Bacti Swab NPB, Thermo Scientific, Lenexa, KS), and maintained approximately at 4ºC until processed in the Laboratory.

2.2 Sample processing

All 400 RAMS samples were initially processed in 20 ml autoclaved buffered peptone water enriched media under sterilized condition. All enriched media universal bottles were then kept for 24 hours at 37 ºC in the incubator.

2.3 DNA Extraction from Buffered Peptone Water

After successful enrichment, DNA extraction was carried out by the boil cell lysate method as described previously by (Qayyum *et al.*, 2020; Jeshveen et al., 2012). A 1-mL aliquot of enriched broth was taken and centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded after centrifugation and the pellet was re-suspended in 500µl double distilled water (ddH2O). Vortexing was done at high speed for 10 seconds. The aliquot was heated at 95 ºC for 10 minutes. Suspension of the lysed bacterial cell was then cooled at 4 ºC for 5 minutes and was re-centrifuged again at 13,000 rpm for 3 minutes. The supernatant containing the DNA was then collected and transferred to another eppendorf tube. It was then subjected for amplification purpose to detect *rfbE* (O157) gene using conventional PCR (Irshad et al., 2012). The *rfbE* gene is responsible for the production of the lipopolysaccharide (LPS) O side chain of the STEC O157: H7 cell surface and are highly preserved gene specific to the serotype *E. coli* O157: H7 (Fortin et al., 2001).

2.4 Conventional PCR for *rfbE* (O157) virulence marker

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) shown in (Table 1). Chemical components contained buffer 2.5µl (Invitrogen, NZ), each primer 0.5µl, dNTP 0.6µl (Fermentas), 2.5µl of MgCl2 (Invitrogen, NZ), 0.3µl unit of *Taq* DNA polymerase (Invitrogen, NZ), 16.1µl of nuclease-free water completed to final volume of 25 µl with addition of 2µl of extracted DNA. Thermocycling conditions were programmed for 7 minutes at 95˚C, followed by 35 cycles for 24 seconds at 95˚C, 45

seconds for 60˚C, 45 seconds at 72˚C, with final extension for 8 minutes at 72˚C, followed by maintenance at 4˚C.

Table 1
Primers used for amplification of O157 antigen specific genes in Conventional PCR assays

Target gene (serogroup)	Primer sequence (5' – 3')	GC%	Amplicon size (bp)	GenBank accession number/Reference
rfbE (O157)	F-5'TTTCACACTTATTGGATGGTCTCAA'3 R-5'CGATGAGTTTATCTGCAAGGTGAT'3	36% 41.7%	88	AF163329 (Perelle et al., 2004)

2.5 STEC O157 isolation

STEC O157 can typically and also effectively recognized by its capability to ferment Sorbitol on Sorbitol MacConkey agar media plate within the duration of 24 hours as compared to other *E. coli* strains. The RAMS sample of sheep and buffalo both showed positive results for *E. coli* O157 by conventional PCR was then streaked onto two separated Sorbitol MacConkey agar (SMAC) media plates. After 24 hours incubation period, only Sorbitol fermented pink colored colonies were grown on SMAC media plates. About 6 different isolated colonies were selected from each plate and its DNA extraction was carried out by boil cell lysate method as described previously by (Radu *et al.*, 2001; Jeshveen et al., 2012). The extracted DNA was then amplified once again by using conventional PCR under similar conditions to confirm the presence of *rfbE* (O157) gene in both positive RAMS samples (sheep and buffalo). Amplification results revealed that only single 1/6 isolated colony was confirmed in each positive sample which possessed *rfbE* (O157) gene.

2.6 Multiplex PCR for STEC virulence markers (*stx1, stx2, eae* and *hlyA*).

Briefly, multiplex PCR was then performed in Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia) to identify STEC other virulent genes (*stx1, stx2, eae* and *hlyA*) in both single isolated *E. coli* O157 colonies confirmed by conventional PCR. Already standardized (Oligonucleotide) specific primers and its desirable base pair sizes were utilized by multiplex PCR assay for the synthesis of STEC virulent genes (Table 2) show that. Chemical components contained 2.5µl buffer (Invitrogen, NZ), 0.5µl each of the 8 primers (4 primer pairs) *stx1, stx2, eae* and *hlyA*, 0.6µl of each dNTP (Fermentas), 2.5µl MgCl2 (Invitrogen, NZ), 0.3µl unit of *Taq* DNA Polymerase (Invitrogen, NZ) and 13.1µl of Nuclease-free water completed to final volume 25 µl along with 2µl of extracted isolate DNA. Thermocycling conditions were programmed for 7 minutes at 95˚C, followed by 40 cycles for 45 seconds at 95˚C, 45 seconds for 60˚C, 45 seconds at 72˚C, with final extension for 8 minutes at 72˚C, followed by maintenance at 4˚C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel [illegible]nder Gel documentation system.

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

Target gene (serogroup)	Primer sequence (5' – 3')	GC%	Amplicon size (bp)	Reference
stx1	F-5'GAC TGC AAA GAC GTA TGT AGA TTC G'3 R-5'ATC TAT CCC TCT GAC ATC AAC TGC '3	44% 45.8%	150	(Sharma and Dean-Nystrom, 2003)
stx2	F-5'ATT AAC CAC ACC CCA CCG'3 R-5'GTC ATG GAA ACC GTT GTC AC'3	55.6% 50%	200	(Sharma and Dean-Nystrom, 2003)
eae	F-5'GTA AGT TAC ACT ATA AAA GCA CCG TCG'3 R-5'TCT GTG TGG ATG GTA ATA AAT TTT TG'3	40.7% 30.8%	106	(Sharma and Dean-Nystrom, 2003)
hlyA	F-5'GCATCATCAAGCGTACGTTCC'3 R-5'AATGAGCCAAGCTGGTTAAGCT'3	52.4% 45.5%	534	(Mori et al., 2014)

3. Results And Discussion

In the present study, only two $(n = 2; 0.5\%)$ out of 400 RAMS samples showed positive results for *E. coli* O157 gene by conventional PCR, included one $(n = 1; 1.33\%)$ RAMS sample was detected in $75/200$ RAMS obtained from healthy slaughtered sheep, while the remaining $125/200$ RAMS samples obtained from goats showed negative results in the region of Rawalpindi. Similarly, another positive RAMS sample $(n = 1; 1.25\%)$ was detected in $80/200$ RAMS collected from healthy slaughtered buffalos, while the remaining $120/200$RAMS samples collected from cows showed negative result for *E. coli* O157 in the region Islamabad as shown as in (Table 3).

Table 3
Shows overall prevalence ratio of *E. coli* O157 estimated in 400 RAMS samples of Sheep, Goats, Cows and Buffaloes.

Species of animals	Total RAMS Samples	*E. coli* O157 positive gene by conventional PCR	STEC genes (*stx1, stx2, eae*, and *hlyA*) by multiplex PCR	Overall prevalence ratio
Sheep	75	1	None of STEC genes were detected	1.33%
Goats	125	0		0%
Cows	120	0		0%
Buffalos	80	1	STEC 4 virulent genes (*stx1, stx2, eae*, and *hlyA*) were detected	1.25%
Total	400	2		0.5%

For *E. coli* O157 isolation, each positive sample was plated onto two different Sorbitol MacConkey agar media plates and 6 different Sorbitol fermented pink colored colonies were picked out from each plate (Figure 1 and 2) and analyzed once again through conventional PCR, resulted only 1/6 single isolated colony confirmed from each positive RAMS sample of sheep and buffalo which possessed *rfbE* O157 genes (Figure 3 and 4). The overall prevalence of *rfbE* (O157) genes came up to (0.5%). In comparison with relevant studies, a total of 0.2% VTEC 0157 occurrence was reported in Rome, central Italy (Battisti *et al*., 2006). In the UK the *E. coli* O157: H7 isolation rate from a sheep was 1.4% along with monthly variations from 0% to 4.8% reported by (Chapman *et al*., 2001; Paiba *et al*., 2002). Similarly only one sample (0.3%) *E. coli* O157: H7 was recovered from a total of 10.8% STEC strains isolated from caprine and ovine bulk tank milk samples in Spain (Rey *et al*., 2006), followed by (Blanco *et al*., 2003) who isolated (0.4%) *E. coli* O157 from five lambs in Spain. In dissimilar studies reported by (Lenahan *et al*., 2007; Dontorou *et al*., 2004; Johnsen *et al*., 2001) no *E. coli* O157: H7 serogroup was isolated in analyzed samples which could be characterized into different examinations of sampling and differences in cultural methods. As compared to these studies we used an appropriate culturing and molecular based detection for the identification of *E. coli* O157 strains *as a result, two (n=2) sample showed resemblance to E. coli O157.*

Furthermore, to detect STEC other virulent genes (*sxt1*, *stx2*, *eae* and *ehlyA*), these both (n = 2) single isolated colonies of sheep and buffalo positive for *rfbE* O157 genes were then briefly subjected towards multiplex PCR (Fig. 5). In our investigation, none of the STEC genes (*stx1, stx2, eae* and *hlyA*) from Sorbitol-fermented single (01) isolated colony of sheep were identified thus, keep rear chances of transferring because they are non-motile and do not contain flagellar section through which they can move to their targeted host only will be conceivable in exceptionally raising conditions (Fig. 6). While, fortunately, there were 4 STEC genes (*stx1, stx2, eae* and *hlyA*) identified in Sorbitol-fermented single (01) isolated colony of buffalo which possessed high zoonotic potential of *E. coli* O157: H7 (Fig. 7). In consistence with our findings, a total of 6 (4%) *E. coli* O157: H7 was separated from fecal samples and all

isolates found which 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 (Perera et al., 2015). Similarly, in another study a total of 154 isolates were observed for (*hlyA*) gene which were 75.3%, (*eaeA*) 56.5%, (*stx*$_1$/*stx*$_{2)}$ 41.6%, (*stx*$_{2)}$ 25.5 and (*stx*$_{1)}$ were found 11.0%. Amongst the O157 isolates which were found positive for *hlyA* and/or *eaeA*, while 7.6% were found negative for all four virulence genes and 24.5% were found negative for *stx* genes (Ojo et al., 2010). Moreover, previous studies have been reported by (Mora et al., 2004; Salmanzadeh-Ahrabi et al., 2005; Inat and Siriken, 2010; Iguchi et al., 2011; Atabakhsh et al., 2012; Amezquita-Lopez et al., 2012; Miri et al., 2014; Hessain et al., 2015; Fierz et al., 2017 and Brusa et al., 2017) shows a strong interaction evidences with our findings.

As opposed to sheep and buffalo there was no such kind of *E. coli* O157: H7 organism was isolated from RAMS samples of goats and cows as isolated in previous studies reported by (Hiko et al., 2008; Solomakos et al., 2009; Mersha et al., 2010). The reason might be due to very fewer chances of *E. coli* O157 colonization in these animals intestinal hosts as goat has been considered the sub-clinical carrier of STEC. current study showed that the occurrence of *rfbE* O157 positive STEC in healthy sheep and buffalo in these two regions Rawalpindi and Islamabad, Punjab Pakistan was low. The reason for low prevalence might be due to the proper hygienic conditions had been accommodated the transportation of these species to the slaughterhouses for butchering because of which the risk of contamination has been decreased. Another reason might be the age factor of the ruminant animals which have a major role in the STEC colonization due to favorable environmental conditions in their intestinal tract. As compared to healthy ruminants the neonate's ruminants have more chances of colonization from STEC, while we collected samples from healthy sheep, goats, cows and buffaloes because of which low prevalence ratio (0.5%) of *E. coli* O157 was reported.

Similarly, as compared to Rawalpindi in Islamabad region the *rfbE* O157 gene was present in buffaloes RAMS sample possessed a low prevalence ratio, however, this same positive sample for *rfbE* gene also contain 4 other STEC clinical relevant virulent genes (*stx1, stx2, eae* and *hlyA*) which consist high potential to cause serious public health consequences.

4. Conclusion

The evidence provided by this investigation that healthy adult sheep and buffalo are possibly essential carriers of STEC O157 in the District Rawalpindi and Islamabad Punjab province, Pakistan. However, its prevalence rate was significantly very low in these specific areas but, as compare to Rawalpindi, Islamabad region is at high risk because STEC with 4 clinical relevant virulent genes (*stx1, stx2, eae* and *hlyA*) were detected in positive RAMS sample of buffalo which may act as a serious public health consequence in future. In addition to public health importance, it has extraordinary economic importance

n of STEC O157 in these specific areas

Rawalpindi and Islamabad as well in other parts of Pakistan are rare, consequently, 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 sheep and buffalo and transmission of STEC O157 from these living organisms is to devise proper control systems. These control procedures would help in diminishing the increasing number of human STEC cases in Pakistan and furthermore to keep away from possible losses to Pakistan economy.

Declarations

Ethics approval and consent to participate:

Hereby, I Abrar Hussain Mian consciously assure that for the manuscript **Molecular Detection Of Shiga Toxin-Producing *Escherichia Coli* (Stec) O157 In Sheep, Goats, Cows And Buffaloes** the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Date: 17/04/2021

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Declaration of interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding: No funding

Consent to Participate/Publish: All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Authors Contributions: **Concept & Methodology**: Formal analysis and investigation:; Writing - original draft preparation -: was done by Abrar Hussain Mian,

Funding acquisition: No Funding; Resources:

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Isolation of Sorbitol-fermented pink color colonies from single positive RAMS sample of Sheep for rfbE O157 gene detection.

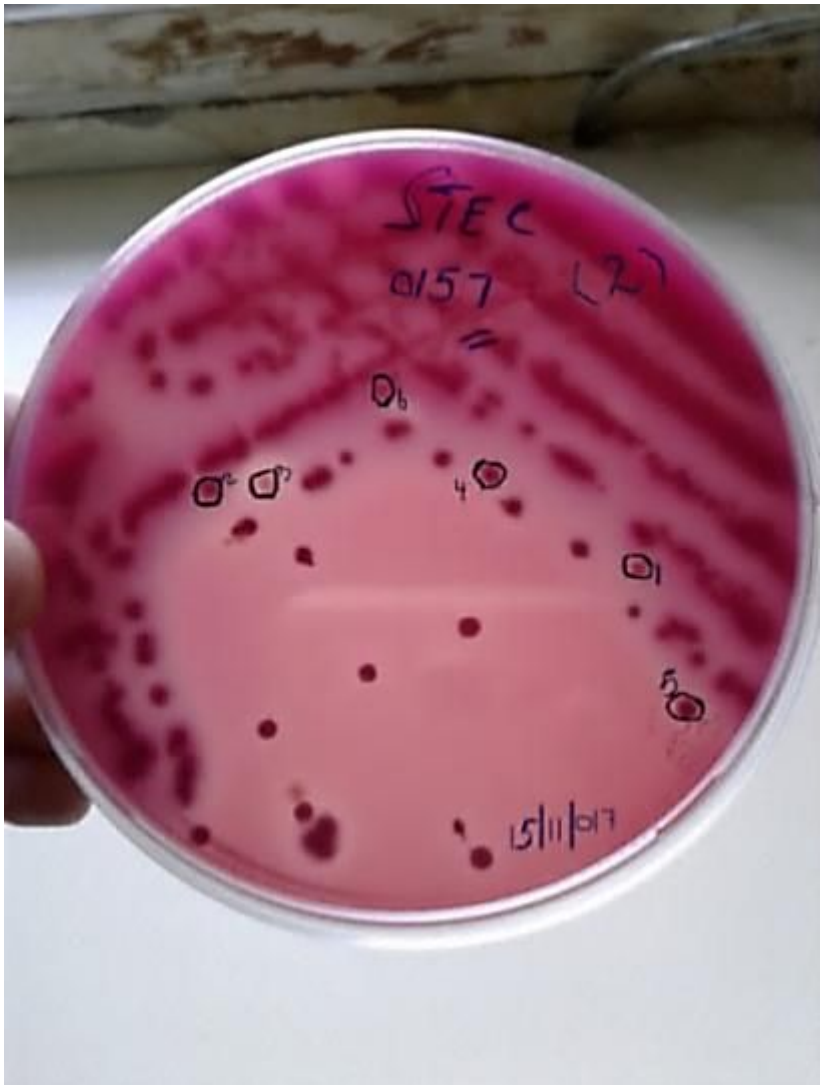

Figure 2

Isolation of Sorbitol-fermented pink color colonies from single positive RAMS sample of Buffalo for rfbE O157 gene detection.

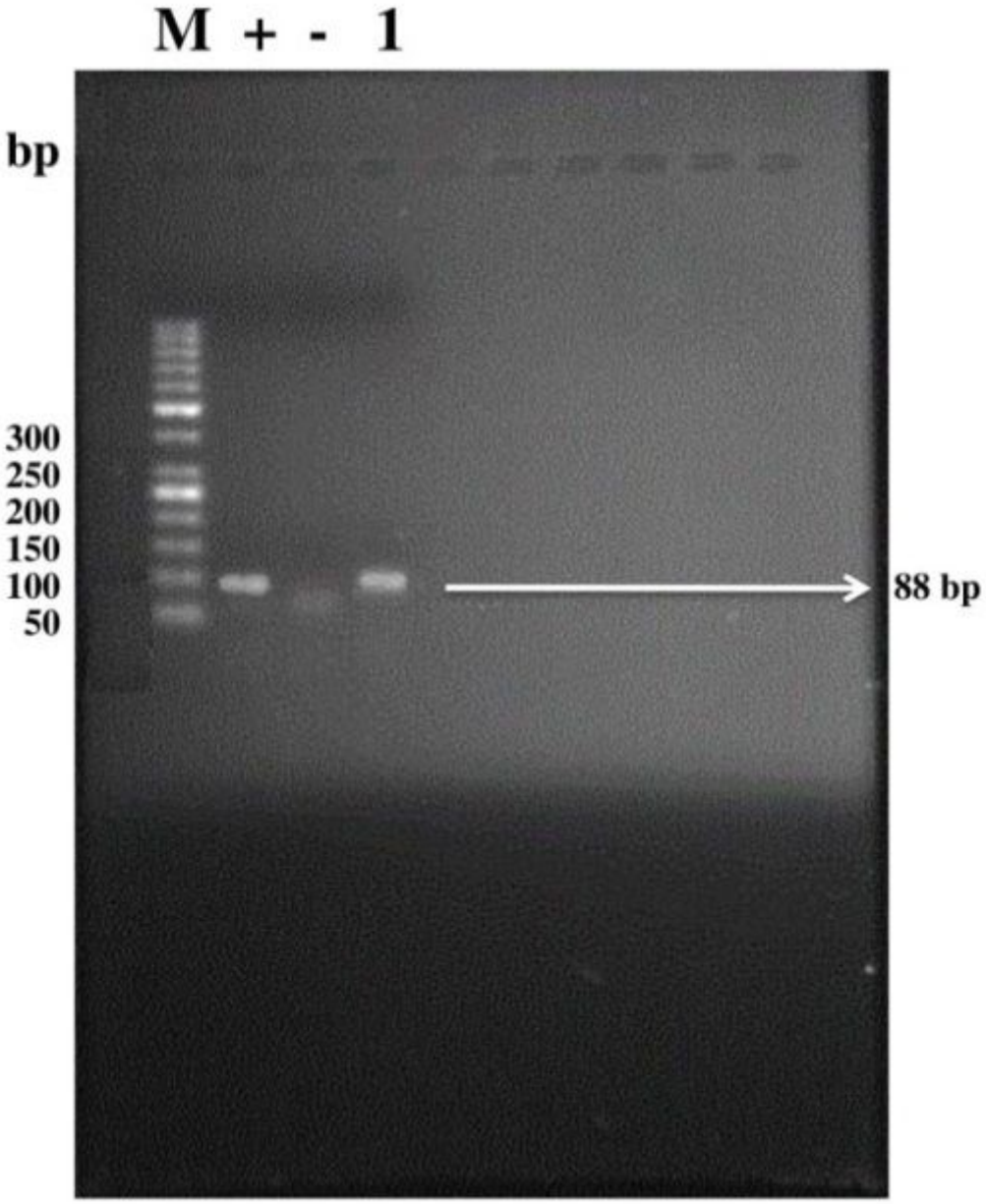

Figure 3

STEC O157 strain isolated from positive RAMS sample of Sheep (well 1, 88bp) by conventional PCR assay.

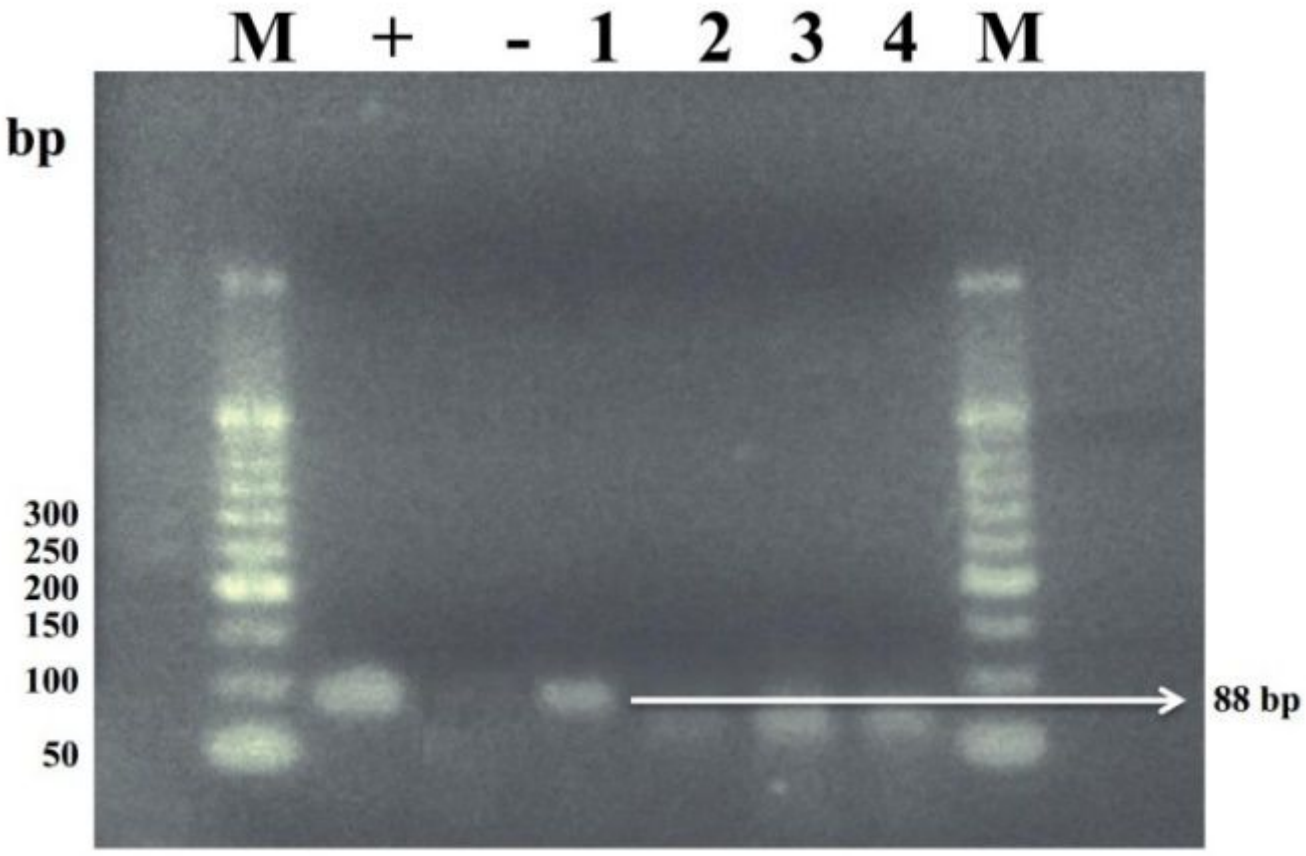

Figure 4

STEC O157 strain isolated from positive RAMS sample of Buffalo (well 1, 88bp) by conventional PCR assay.

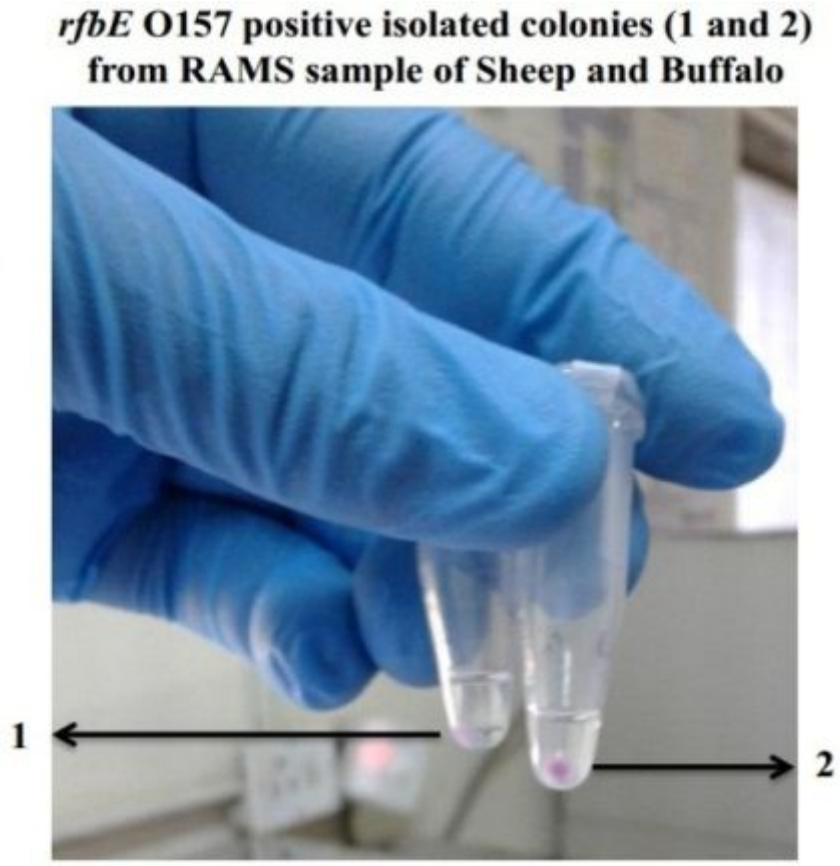

Figure 5

DNA extraction from rfbE O157 positive single (01) isolated colonies of RAMS samples of Sheep and Buffalo (1 and 2) for the presence of STEC virulent genes (sxt1, stx2, eae and ehlyA).

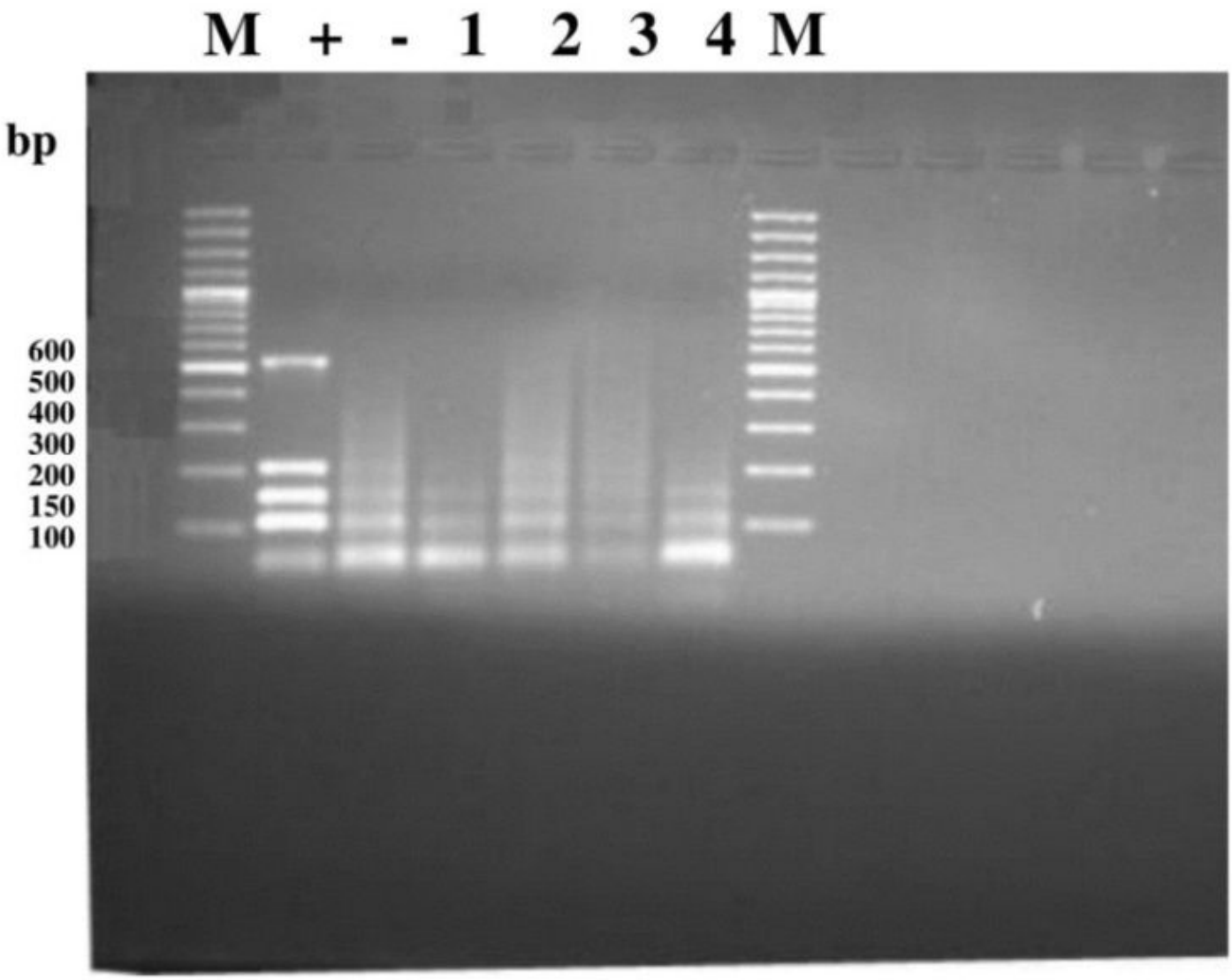

Figure 6

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

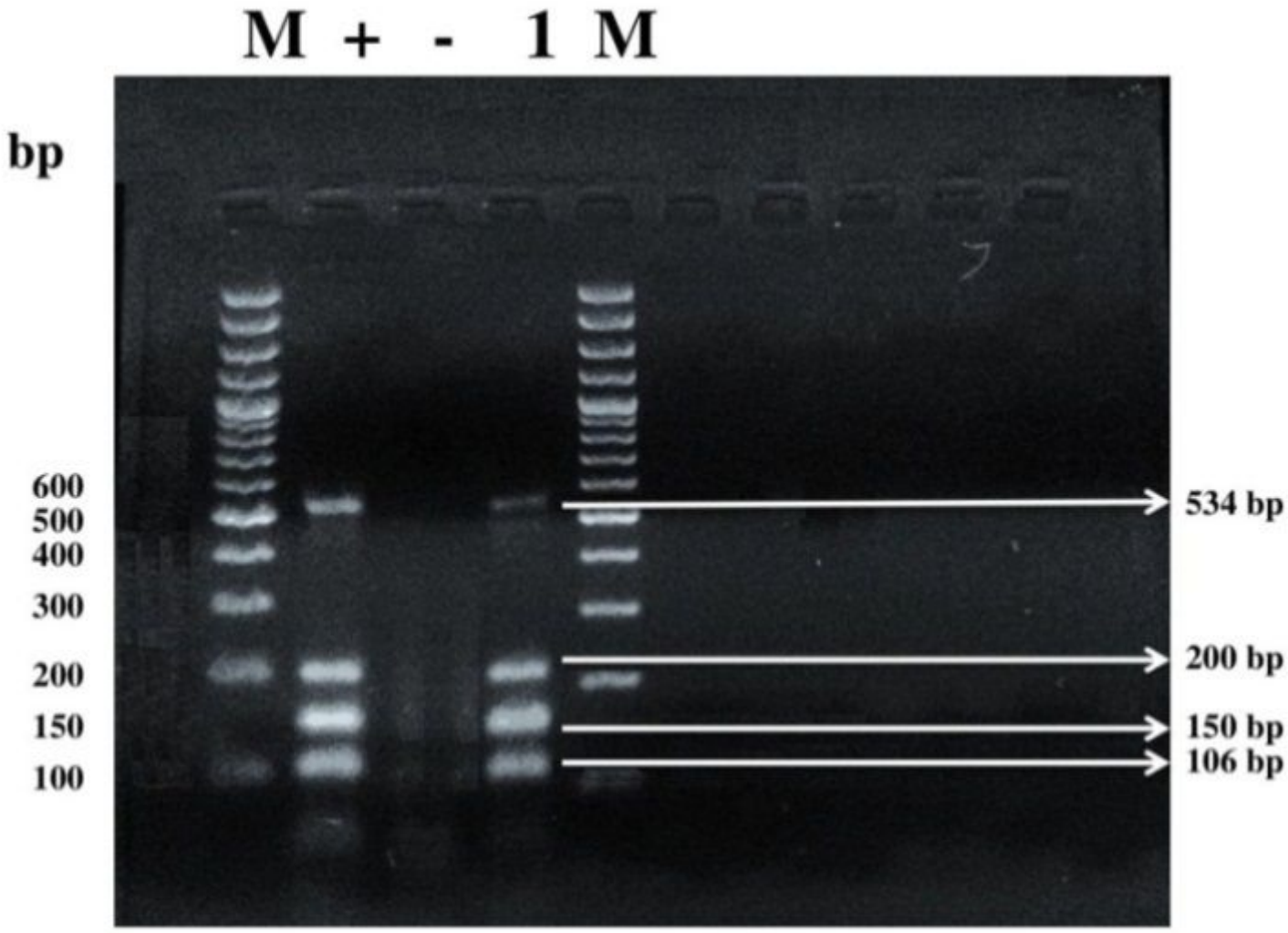

Figure 7

STEC virulent strains (hlyA (534 bp), stx2 (200 bp), stx1 (150 bp) and eae (106 bp)) by multiplex PCR assay. Positive O157 RAMS sample of Buffalo (well 1) showed positive result for all these targeted genes.