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Molecular detection of verocytotoxigenic genes of *E. coli*

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Abstract
The present work was carried with the aim to detect the verocytotoxigenic genes of *E. coli* at the Navsari. During the study a total of 210 meat samples were processed, which included 60 samples of chicken, and 50 samples each, of chevon, mutton and carabeef. A total of 51 were positive for *E. coli*. included 14,11,8 and 18 samples respectively in case of chicken, chevon, mutton and carabeef. All 51 *E. coli* (VTEC) isolates were subjected to molecular analysis of verocytotoxigenic genes *stx* 1 and *stx2* and 9 (17.64%), 5 (9.8%) and 3 (5.8%), isolates contained *stx* 1, *stx* 2 and both *stx* 1as well as *stx* 2, respectively. Carabeef samples showed the highest occurrence (11.7%) followed by chicken (3.9%), chevon (3.9%) and mutton (1.9%). The *stx* 1 gene was more prevalent compared to *stx* 2 gene.

Keywords: Stx 1, stx 2, toxin genes, *E. coli*

Introduction

The *E. coli* is gram-negative, aerobic, non-spore forming, motile member of the Enterobacteriaceae family of bacteria. The intestinal pathogenic *E. coli* are categorized into 6 pathotypes viz, Verocytotoxigenic *E. coli* (VTEC), Enterotoxigenic *E. coli* (ETEC), Entero-invasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC) and Diffusely adherent *E. coli* (DAEC) (Torres *et al.,* 2010)[17].The fact that the toxins are lethal for vero cells in culture gave them the name "verotoxin," while the term "shiga-like toxin" denotes their structural and functional similarity to the shiga toxin produced by *Shigella dysenteriae*. Verotoxigenic *E. coli* (VTEC) and Shigatoxigenic *E. coli* (STEC) have also been linked to human and calf illness, respectively.

Ruminants are considered to be the chief reservoir of VTEC, which is shed through faeces. The pathogenic *E. coli* infections are spread by the fecal-oral route from food, water, animals, and the environment. It has been reported that majority of food borne illnesses were found linked to under-cooked ground beef and unpasteurized milk (Lior, 1994[11], Caprioli *et al*., 2005 [2], Erickson and Doyle, 2007 [5]; Buncic *et al*., 2014 [1]; Pires *et al*., 2019 [13]).

There are 207 VTEC serotypes in cattle and 160 in humans. Of them over 150 serotypes are frequently shared between humans and animals, indicating the possibility of the disease being spread by animals and their products (Lior, 1994[11]; WHO, 1995 [18]). Therefore, it is crucial to know its occurrence, persistence, and dependable techniques for the early identification and suppression of VTEC, to ensure product quality.

Despite the fact that, refrigeration is often thought to be effective in slowing down the development and multiplication or inhibiting the survival of bacteria, the VTEC O157:H7 is observed to survive better in an acid environment at 4 °C in fermented dry sausage (Conner and Kotrola, 1995) [3].

There are several advanced techniques which include Polymerase chain reaction (PCR) used to identify VTEC directly from the culture. The development of PCR-based methods for the detection of pathogens or virulence factors can be used for the initial screening of the presence of microorganisms in the samples (Kiranmayi and Krishnaiah, 2010 [9]).

Material and Methods

The work was carried out in the department of Veterinary Public Health and Epidemiology, College of Veterinary science & AH, Kamdhenu University, NAU campus, Navsari from December 2022 to May 2023.

Using random sampling method, a total of 210 raw meat samples weighing 100 g, which included 60 samples of chicken, 50 samples each of carabeef, chevon and mutton as mentioned

in Table 1 were collected aseptically from retail market in and around Navsari. The carabeef samples were procured from Deonar abattoir, BMC, Mumbai. These samples were collected in 3 x 4 cm sterile polyethylene bags with proper labeling.

Table 1: Details of the meat samples

Sr. No.	Type of meat	Nature of the sample					Total
		Minced	Breast/Rib muscle	Thigh muscle	Wing muscle	Giblet/Pluck	
1	Chicken	25	15	10	5	5	60
2	Chevon	20	10	10	-	10	50
3	Mutton	20	10	10	-	10	50
4	Carabeef	25	15	5	-	5	50
Total		90	50	35	5	30	210

Processing of the meat samples

Sample preparation: Approximately 10 g meat sample was cut into small pieces and then transferred into sterile mortar. The sample was triturated by addition of 90 ml NSS (1:10 dilution) to have homogenate mixture. Ten ml of the sample homogenate was mixed with 90 ml of Mac Conkey Broth followed by the incubation at 37 °C for 24 hr for enrichment. The enriched samples were streaked on Mac Conkey agar plates and incubated at 37 °C for 24 hr. The Plates with pink colour colonies were selected and inoculated on Eosin Methylene Blue (EMB) agar plate and incubated at 37 °C for 24 hr. The presumptive colonies with green metallic sheen were picked up and stored on Nutrient agar slant for further studies.

Screening biochemical tests: The spare single colony from the EMB plates were selected and streaked and stabbed on Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA) and incubated at 37 °C for 18 hr and reaction was noted. The colonies were chosen based on their standard colony morphology, Gram's staining and reaction on TSI and LIA agar. They were first subjected to catalase, oxidase and IMViC tests. The colonies showing catalase positive, oxidase negative and IMViC pattern as: +/+/-/-.

Molecular Detection by PCR: The isolates were screened for verocytotoxigenic genes by targeting the *Stx1* and *Stx 2* genes as per the standard protocol followed by Hazarika *et al.* (2007) [7]. The details of the primers used is mentioned in Table 2.

DNA extraction by *mericon* DNA Bacterial plus Kit: The isolates which were confirmed biochemically were subjected to molecular study. The isolates and standard reference cultures were grown separately in 5 ml Luria Bertani (LB) broth for 24 hours at 37 °C. The extraction of DNA was carried out using the following protocol:

1. After incubation 1ml of enriched culture was taken from the LB broth and transferred into 2ml micro centrifuge tube and centrifuged at 10000 rpm for 10 minutes.
2. The supernatant was discarded without disturbing the pellet.
3. 200 µl Fast lysis was added and vortexed vigorously.
4. Then the entire mixture was transferred into pathogen lysis tubes for horizontal vortexing in heating block of rotary shaker (800 rpm) 100 °C for 10 minutes.
5. The tubes were removed from the shaker and placed at room temperature for 10 minutes.
6. The tubes were further centrifuged at 13000 rpm for 15 minutes.
7. 100 µl supernatant was collected into 1.5 ml centrifuge and stored at -20 °C for 8 weeks till further use.

Estimation of quality, purity and concentration of DNA: The purity, quality and concentration of the isolated DNA was evaluated using agarose gel electrophoresis and Nano Drop TM 2000 Spec Spectrophotometer. The isolated genomic DNA did not shear during gel electrophoresis, demonstrating its great purity. For analysis, the DNA samples absorbance was determined at 260 and 280 nm. All samples having an A260/A280 ratio of more than 1.8 are considered to be PCR amplification-ready.

Table 2: Primers used for detection of stx 1 and stx 2

Target gene	Oligonucleotide sequence (5′ →3′)	Amplicon length (bp)	Reference
stx1	F: CAGTTAATGTGGTGGCGAAG	894	Hazarika *et al.* (2007) [7]
	R: CTGCTAATAGTTCTGCGCATC		
stx2	F: CTTCGGTATCCTATTCCCGG	478	Hazarika *et al.* (2007) [7]
	R: GGATGCATCTCTGGTCATTG		

Note: F = Forward primer, R = Reverse primer

PCR for amplification of the *stx 1* and *stx2* genes was set up in 25 µl reaction. Following initial trials with varying concentrations of components, the reaction mixture was optimized as per Table 3 and thermal cycling condition for identification as mention in Table 4 and Table 5.

Table 3: Concentration of various components of reaction mixture

Sr. No.	Components	Quantity Final	Concentration
1	DNase-RNase free water	5.5 µl	--
2	2X PCR master mix	12.50 µl	2X
3	Forward Primer (Stock cont^{n}:100 pmol/µl)	1µl	10 pmol
4	Reverse Primer (Stock cont^{n}:100 pmol/µl)	1 µl	10 pmol
5	DNA Template	5.00 µl	--
Grand Total		**25.00** µl	**--**

Table 4: Thermal cycling condition for identification of *stx1* gene

Target gene	Simplex PCR for *stx 1* gene				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
stx 1	94 °C for 5 minutes	94 °C for 30 seconds	52 °C for 30 seconds	72 °C for 1 minute	72 °C for 7 minutes
		Repeated for 35 cycles			

Table 5: Thermal cycling condition for identification of *stx2* gene

Target gene	Simplex PCR for *stx 2* gene				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
stx2	94 °C for 5 minutes	94 °C for 30 seconds	57 °C for 30 seconds	72 °C for 1 minute	72 °C for 7 minutes
		Repeated for 35 cycles			

Results and Discussion

Out of 210 meat samples 51 (24.28%) were positive for *E. coli.* The species wise spread of isolates showed that 14/60 (23%), 11/50 (22%), 8/50 (16%) and 18/50 (36%) samples were positive, respectively in case of chicken, chevon, mutton and carabeef.

From the Table 6 it is evident that 51 out of 210 were positive for *E. coli.* Among minced meat 5 (20%), 7 (35%), 4 (20%) and 8 (32%); 3 (20%), 2 (20%), 2 (20%) and 4 (26.6%) breast /rib muscle; 1 (10%), 1 (10%), 1 (10%) and 3 (60%) thigh muscle; 3 (60%) wing muscle and 2 (40%), 1 (10%), 1 (10%) and 3 (60%) giblet/pluck from of Chicken, Chevon, Mutton and Carabeef respectively were positive for *E. coli.*

Table 6: Species wise occurrence based on type of the sample

SN	Type of meat	Nature of the sample					Total
		Minced	Breast/Rib muscle	Thigh muscle	Wing muscle	Giblet/Pluck	
1	Chicken	5	3	1	3	2	14
2	Chevon	7	2	1	-	1	11
3	Mutton	4	2	1	-	1	8
4	Carabeef	8	4	3	-	3	18
	Total	24	11	6	3	7	51

The *E. coli* isolates which were confirmed by PCR were further subjected to detection of Verocytotoxigenic genes. The results of the primer-directed amplification of the *stx1* and *stx2* in the current study were 894 bp for the *stx1* and 478 bp for the *stx2*, respectively, as shown in Fig 1 and Fig 2 as predicted (Hazarika *et al.,* 2007) [7]. Hazarika *et al.* (2004) [6] reported 25.0% of *E. coli* isolates to be Verotoxin producing.

In this study, out of 51 *E. coli* isolates, 17 (33.33%) were found positive for the *stx* genes. Of which 9 (17.6%) possessed *stx 1* genes, while 5(9.8%) harbored *stx 2* genes and 3(5.8%) possessed both *stx 1* and *stx 2* genes as shown in Table 7 and Table 9. Similar result was reported by Dutta *et al.* (2011) [4] who studied a total of 42 *E. coli* isolates; of which 14 (33.33%) isolates carried at least 1 virulence gene. One carried only *stx2*, one carried *stx2 and hlyA*, four carried *stx1, stx2* and *hlyA*, two carried *stx1, eaeA* and *hlyA* genes and two carried *stx1* and *eaeA*. Takhatsinh (2009) [16] reported 54 isolates (45.38%) out of 119 were found to be positive for *stx* genes: of which 43 (36.14%) harbored *stx1* genes, while 9 (7.56%) *E. coli* isolates possesses both *stx1* and *stx2* and 2 isolates (1.68%) were positive for *stx2* gene only. Similar findings were reported by Kiranmayi *et al.* (2010) [10], who found that of the 27 samples that tested positive for *Escherichia coli* by PCR, 12 (44.4%) contained *stx1*, 7 (26%) *stx2*, and 5 (18.5%) *stx1* and *stx2* together. Compared to *stx2*, *stx1* incidence was more common.

Among the chicken samples *stx* 1 gene was detected in 2 out of 14 (14.2%), and 1 out of 14 (7.14%) was positive for *stx* 2. Whereas, 1 out of 14 (7.14%) positive for both *stx* 1 and *stx* 2. Samadpour *et al.* (1994) [14] reported that 4 out of 33 (12%) were positive for *stx* genes.

The Chevon samples demonstrated, *stx* 1 gene in 1 out of 11 (9.09%) isolates; so also 1 among 11 (9.09%) contained *stx* 2, which in agreement with the findings of Sethulekshmi *et al.* (2016) [15] where 7.92% for *stx* 1 gene, 5.61% samples positive for *stx* 2 gene. But both the genes were not present simultaneously in a single isolate.

In the mutton samples *stx 1* gene was not detected; but, 1 out of 8 (12.5%) was positive for *stx* 2. Yadav *et al.* (2007) [19] reported that 9 of 49 (18.36%) contained *stx* 1 gene, 6 of 49 (12.4%) samples for *stx* 2 gene, whereas 5 from 49 (10.20%) were positive for both the genes. However, Manna *et al.* (2006) [12] did not detect *stx* genes in the mutton samples they surveyed.

The present work found that among carabeef samples *stx 1* gene was detected in 6 of 18 (33.33%) samples; and *stx* 2 was present in 2 out of 18 (11%) samples. Additionally, 11.11% (1/18) samples were positive for both *stx* 1 and *stx* 2. Earlier works revealed that Kiranmayee and Krishnaiah (2011) [8] found 18 of 50 (36%) contained *stx* 1 gene, 7 of 15 (14%) samples revealed *stx* 2 gene, and 4 of 50 (8%) were positive for both genes. Detailed results are given Table 8.

Table 7: PCR amplification of Verocytotoxigenic genes of *E. coli* isolates

S n	Sample type	Number of samples	Number positive	Number positive for *stx* 1	Number positive for *stx* 2	Number positive for both *stx* 1 & *stx* 2
1	Chicken	60	14	2	1	1
2	Chevon	50	11	1	1	0
3	Mutton	50	8	0	1	0
4	Cara beef	50	18	6	2	2
	Total	210	51	9	5	3

Table 8: Isolate wise distribution of *stx 1* and *stx 2* genes

SN	Isolate No	*Stx* 1	*Stx* 2	SN	Isolate No	*Stx* 1	*Stx* 2
1	B-12	+		7	P-16	+	
2	B-18	+	+	8	P-50	+	+
3	B-21	+		9	G-23	+	
4	B-32	+		10	G-44		+
5	B-40	+		11	S-12		+
6	B-43	+	+				

Table 9: Occurrence of Verocytotoxigenic genes of *E. coli*

S. N.	Verocytotoxigenic gene	Samples tested	Samples positive	Occurrence
1	*Stx* 1	51	9	17.60%
2	*Stx* 2	51	5	9.80%
3	*Stx* 1 and *stx* 2	51	3	5.80%

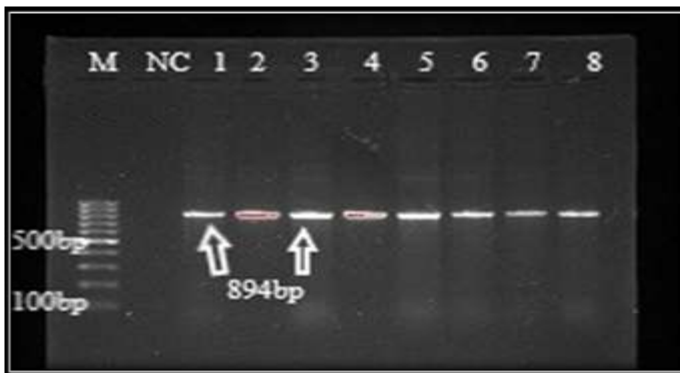

Fig 1: Representative agarose gel showing pcr amplified product of 894 bp for *stx 1* of *E. coli* isolates; Lane M: 100 bp ladder; Lane NC: Negative control (*S. aureus* ATCC 25923);Lane 1-8: Samples

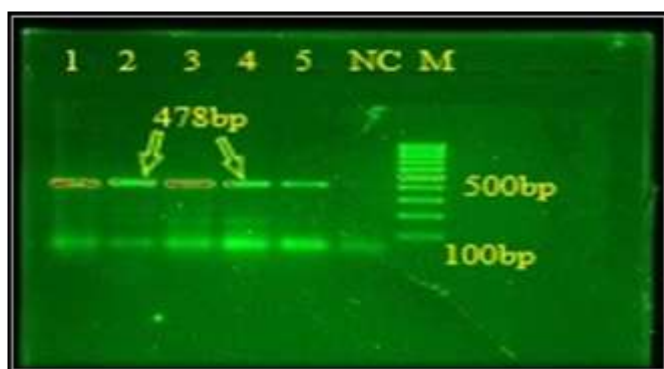

Fig 2: Representative agarose gel showing pcr amplified product of 478 bp for *stx 2* gene of *E. coli* isolates; Lane M: 100 bp ladder; Lane NC: Negative control (*S. aureus* ATCC 25923); Lane 1-5: Samples

Conclusions

Among 51 *E. coli* isolates, 9 (17.64%), 5 (9.8%) and 3 (5.8%), contained *stx* 1, *stx* 2, and both *stx* 1as well as *stx* 2 genes, respectively. It was observed that carabeef samples showed the highest occurrence (11.7%) of toxigenic genes followed by chicken (3.9%), chevon (3.9%) and mutton (1.9%). The *stx* 1 gene was more prevalent compared to *stx* 2 gene.

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