Chaudhary et al | Article 10662
J Pure Appl Microbiol. 2026. doi: 10.22207/JPAM.20.3.05
Received: 05 June 2025 | Accepted: 18 June 2026

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Detection and Analysis of Shiga Toxin-producing *E. coli* and Serotypes in Cattle with Diarrhea from Rajasthan

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Abstract

Colibacillosis is an infectious disease in animals, which is caused by *Escherichia coli,* leading to economic losses. The current investigation was conducted to evaluate the molecular typing of colibacillosis-associated *Escherichia coli* for determination of O157:H7 serotype and virulence factors. Overall, 100 faecal samples were screened between November 2023 to February 2024 from in and around Jaipur district of Rajasthan. Sample screening was done for isolating *E. coli* in which 77 isolates were recovered from 100 diarrhoeic cattle and its serological identification indicated presence of the most frequent serotype, encompassing O76 (5), O3 (4), O7 (4), O16 (4), O50 (4), O111 (3), O113 (2), O125 (2), O135 (2), O2 (1), O4 (1), O9 (1), O19 (1), O27 (1), O66 (1), O85 (1), O109 (1), O114 (1) and O156 (1). Finally, Molecular characterization of the recovered isolates was conducted using PCR for detecting presence of *fliCH7*, *rfbO157*, *stx1*, *stx2*, *EhlyA* along with *eaeA* genes associated with *E. coli*. Among 77 *E. coli* isolates, none were recognized as O157:H7 serotypes. Among total *E. coli* isolates, 40 comprised Shiga toxin-producing *E. coli* (STEC) genes. The *stx2* gene was the most prevalent (32.5%), followed by *stx1* (17.5%), while combinations involving *stx1*, *stx2* and *EhlyA* were detected in 10%-12.5% of isolates. The *eaeA* gene was rare, occurring in only 2.5% of the virulence-positive isolates. Results suggest that conventional culture methods, along with serological and molecular assays, are key techniques for isolating and characterizing STEC from fecal samples of diarrheic cattle.

Keyword: Colibacillosis, *Escherichia coli,* STEC, Serotype, Virulotype

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Citation: Chaudhary W, Jeph NK, Sharma SK, et al. Detection and Analysis of Shiga Toxin-producing *E. coli* and Serotypes in Cattle with Diarrhea from Rajasthan. *J Pure Appl Microbiol.* Published online 16 July 2026. doi: 10.22207/JPAM.20.3.05

INTRODUCTION

Colibacillosis is a significant neonatal disease in livestock that leads to substantial economic losses and is caused by *Escherichia coli.*[1] In calves affected by colibacillosis, morbidity rates can range between 30% and 75%, while mortality rates may vary from 10%-50%, depending on the clinical care.[2] Diarrheagenic *E. coli* (DEC) is classified into 6 primary groups, but only STEC is most significant in terms of zoonotic transmission.[3] STEC is frequently found at high rates in cattle feces, particularly in newborn calves.[4,5]

Based on the modified Kauffman scheme, *E. coli* serotyping is performed by identifying their surface antigens: O (somatic), H (flagellar) and K (capsular).[6,7] Over 400 O:H serotypes of STEC have been identified in cattle as well as humans, with more than 100 serotypes associated with human illnesses. "World Health Organization (WHO)" recognizes *E. coli* O157:H7 along with six other serogroups O45, O121, O26, O145, O111 and O103 as most prevalent STEC strains linked to human infections globally.[8] *E. coli* O157 is transmitted via fecal-oral pathway, mainly through interactions between animals and farm visitors.[9] Rise of STEC exhibits significant challenge to food safety along with global cattle industry.[10]

Production of *stx1*, *stx2*, intimin, as well as plasmid-encoded enterohemolysin is the primary cause of virulence of STEC. Shiga toxins usually inhibit synthesis of proteins, leading to host cell death along with conditions like "hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC), while intimin causes formation of attaching and effacing (A/E) lesions on intestinal" lining.[11] Enterohemolysin is regarded as an important epidemiological marker for identifying STEC strains.[12] STEC along with other *E. coli* strains can obtain virulence genes through horizontal gene transfer (HGT), leading to development of new *E. coli* pathotypes that represent a significant public health risk.[13]

MATERIALS AND METHODS

Sample collection

For the present study, 100 rectal swab samples from diarrheic cattle (irrespective of age, sex and breed) in different places were collected from in and around locations of Jaipur district of Rajasthan**.** The samples included 25 from Bassi (C-1 to C-25), 15 from Sanganer (C-26 to C-40), 10 from Chaksu (C-41 to C-50), 15 from Chomu (C-51 to C-65), 20 from Jamwa Ramgarh (C-66 to C-85), and 15 from Jhotwara (C-86 to C-100). Samples were promptly collected, properly labelled and transported to the laboratory in an ice box for bacteriological analysis.

Isolation and characterization of *Escherichia coli*

Fecal samples were "enriched in nutrient broth for facilitating identification of *E. coli*. Enriched cultures were then streaked on MacConkey agar and then incubated for 24 hrs. Colonies showing a bright pink to red coloration were chosen and then subcultured on Eosin Methylene Blue (EMB) agar". Following overnight incubation, colonies displaying typical metallic green sheen, suggestive of *E. coli* were further analysed using biochemical tests provided in a commercially available identification "kit (KB010 Hi *E. coli* Identification Kit, HiMedia)".

DNA extraction

Genomic DNA was isolated by employing HiPurA® Multi-Sample DNA Purification Kit (MB554), following protocol provided by the manufacturer. Positive control used in this study were procured from HiMedia (MBT102) and the CDSRZ Laboratory.

Screening for virulence-associated genes

Polymerase chain reaction (PCR) was carried out by employing previously published oligonucleotide primers targeting virulence-associated genes, including *stx1, stx2*, *eaeA, EhlyA*, *fliCH7* and *rfbO157* as mentioned in Table 1.[14-16] PCR mixture (10 µl total volume) comprised 2.0 µl of genomic DNA template, 1.0 µl of dNTP mix, 1.0 µl of 10× PCR buffer, 1.0 µl of each primer, 0.4 µl of Taq DNA polymerase, along with 4.6 µl of nuclease-free water. To confirm PCR amplification, electrophoresis on a 1.5% agarose gel at 100 V for 1 hr was performed. Amplified DNA fragments were visualized under UV illumination using the Fusion Solo S gel documentation system (Vilber, France), appearing as distinct bands of the expected size.

Table 1. Oligonucleotide Sequences and Thermal Cycling condition

Name of primer	Specific genomic region	Primer sequence	Amplification conditions	Size of amplicon
stx1	Shiga toxin-1	F: TTCGCTCTGCAATAGGTA R: TTCCCCAGTTCAATGTAAGAT	95 °C-5 min, 95 °C-30 sec, 54 °C-1 min, 72 °C-1.30 min, 72 °C-10 min, repeat of 35 cycles	555 bp
stx2	Shiga toxin-2	F: GTGCCTGTTACTGGGTTTTTCTTC R: AGGGGTCGATATCTCTGTCC	95 °C-5 min, 95 °C-30 sec, 51°C-1 min, 72 °C-1.00 min, 72 °C-10 min, repeat of 40 cycles	118 bp
eaeA	Intimin	F: ATATCCGTTTTAATGGCTATCT R: AATCTTCTGCGTACTGTGTTCA	95 °C-5 min, 95 °C-30 sec, 52 °C-1 min, 72 °C-1.30 min, 72 °C-10 min, repeat of 35 cycles	425 bp
EhlyA	Entero-hemolysin	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	95 °C-5 min, 95 °C-45 sec, 59 °C-45 sec, 72 °C-45 sec, 72 °C-7 min, repeat of 35 cycles	534 bp
rfbO157	*E. coli* O157	F: CGTGATGATGTTGAGTTG R: AGATTGGTTGGCATTACTG	94 °C-1 min, 94 °C-1 min, 51 °C-1 min, 72 °C-1 min, 72 °C-7 min, repeat of 35 cycles	420 bp
fliCH7	*E. coli* H 7	F: GCGCTGTCGAGTTCTATCGAGC R: CCACGGTGACTTTATCGCCATTCC	94 °C-1 min, 94 °C-1 min, 59 °C-1:30 min, 72 °C-1 min, 72 °C-10 min, repeat of 35 cycles	625 bp

Serological Identification of *E. coli* Isolates

E. coli isolates obtained during the study were sent to National Salmonella and Escherichia Centre, Central Research Institute (CRI), Kasauli (Himachal Pradesh), for serotyping analysis**.**

Hemolysin activity

Phenotypic detection of enterohemolysin (EhlyA) was carried out on 5% sheep blood agar plates.[17]

RESULTS

Isolation and Identification of *E. coli*

Among 100 diarrhoeic fecal samples analyzed, 77 *E. coli* isolates were tested positive which indicated overall incidence rate of 77%. The isolates were cultured on selective media and subjected to Gram staining, which revealed Gram-negative, medium-sized rod-shaped bacteria. Biochemical characterization of the isolates demonstrated positive results for the Indole, β-Glucuronidase, Nitrate Reduction, Methyl Red, Lysine Decarboxylase, ONPG, and sugar fermentation tests (Lactose, Glucose, and Sucrose). The isolates tested negative for Citrate utilization, Voges-Proskauer, as well as Sorbitol fermentation tests. Additionally, all isolates were oxidase-negative as well as catalase-positive, consistent with typical biochemical profile of *E. coli.*

Detection of virulence genes in *E. coli* isolates

All 77 *E. coli* isolates were assessed for prevalence of virulence-associated genes through PCR. Genes targeted included those encoding intimin (*eaeA*), Shiga toxins (*stx1, stx2*), as well as enterohemolysin (*EhlyA*). Out of the total, 40 isolates exhibited positive results for one or more virulence genes. The Distribution of virulence genes was as follows: *stx1* was detected in 7 isolates (17.5%) as shown in Figure 1, *stx2* in 13 isolates (32.5%) (Figure 2), and both *stx1* and *stx2* in 5 isolates (12.5%). As shown in Figure 3, the eaeA gene was detected in only one isolate (2.5%) and was found in combination with *stx2*. Additionally, combinations involving the *EhlyA* gene were observed as illustrated in Figure 4, with 5 isolates (12.5%) positive for *stx1* + *EhlyA*, 5 isolates (12.5%) for *stx2* + *EhlyA*, and 4 isolates (10%) for *stx1* + *stx2* + *EhlyA*.

Among 77 *E. coli* isolates tested, *stx1* gene was identified in 21 isolates (27.27%), while *stx2* gene was present in 28 isolates (36.36%). The *eaeA*

gene was identified in a single isolate (1.28%), and the *EhlyA* gene was found in 14 isolates (18.18%). None of the isolates showed amplification for the *rfbO157* or *fliCH7* genes, indicating absence of *E. coli* O157:H7 serotype within sampled population.

Virulence Gene Profiling of *E. coli* isolates

Virulotyping was conducted on *E. coli* isolates on basis of virulence genes *(stx1*, *stx2*, *EhlyA* and *eaeA)* presence. Among 77 isolates, 40 (designated as STEC) carried one or more *stx* genes, either individually or in combination. These were classified into seven distinct virulotypes (V1 to V7) as shown in Table 2. Of these, virulotype V2, characterized by the presence of *stx2* alone, was the most prevalent with 13 positive isolates, while virulotype V7 (*stx2* and *eaeA* combined) was the least common, represented by only one isolate.

Determination of serotypes in *E. coli* isolates

In the study, out of 77 isolates, there were 19 different serotypes were observed in 70

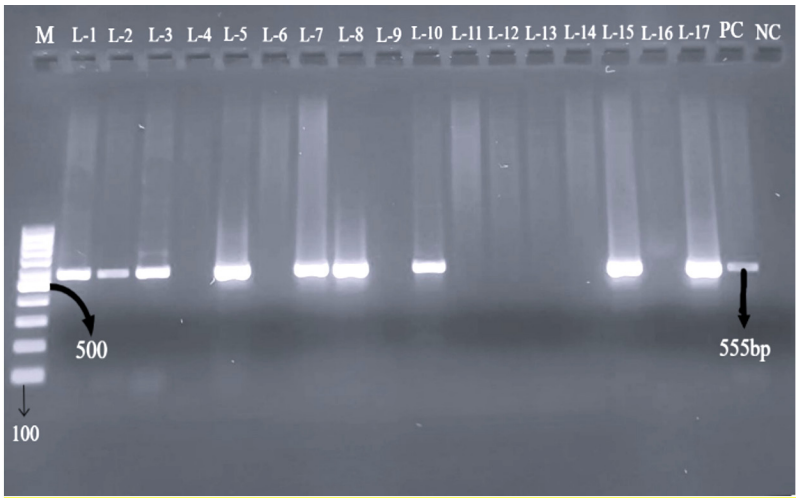

Figure 1. Agarose gel electrophoresis showing PCR products of 555 bp corresponding to the *stx1* gene in tested isolates. Lane M: 100 bp DNA ladder; Lanes L1 (C-2), L2 (C-7), L3 (C-8), L5 (C-19), L7 (C-21), L8 (C-29), L10 (C-47), L15 (C-48) and L17 (C-88) represent positive samples; PC: positive control; NC: negative control

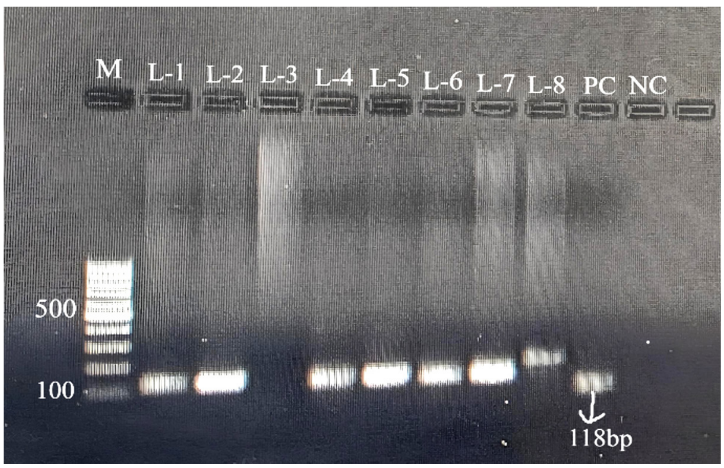

Figure 2. Agarose gel electrophoresis showing PCR amplification of the *stx2* gene with a product size of 118 bp in isolates. Lane M: 100 bp DNA ladder; lanes L1 (C-2), L2 (C-3), L4 (C-8), L5 (C-27), L6 (C-48) and L7 (C-98) show positive samples; PC: positive control; NC: negative control

randomly selected studied isolates. Out of which 40 were serotyped for "O" antigen whereas 2 were rough and the remaining 28 were untypable isolates. The most frequent serotype was reported O76 (5) followed by O3 (4), O7 (4), O16 (4), O50 (4), O111 (3), O113 (2), O125 (2), O135 (2), O2 (1), O4 (1), O9 (1), O19 (1), O27 (1), O66 (1), O85 (1), O109 (1), O114 (1) and O156 (1) as mentioned in Table 3.

Among the 40 STEC isolates, serogrouping revealed association of virulence profiles with several O-serogroups. The predominant virulotype V2 (*stx2* alone) was distributed among serogroups O7, O9, O50, O111, O156, and several untypable isolates. Serogroup O111 exhibited considerable virulence diversity, harboring V1, V2, and V3 virulotypes. The only *eaeA*-positive isolate (V7) belonged to serogroup O113. Untypable isolates displayed the greatest heterogeneity and were represented in all major virulotypes (V1-V6), indicating substantial genetic diversity among STEC strains. Serogroups O76, O111, O113 and O125 were associated with multiple virulence gene combinations, suggesting varying pathogenic potential within these serogroups. The correlation between virulotypes and serogroups of STEC

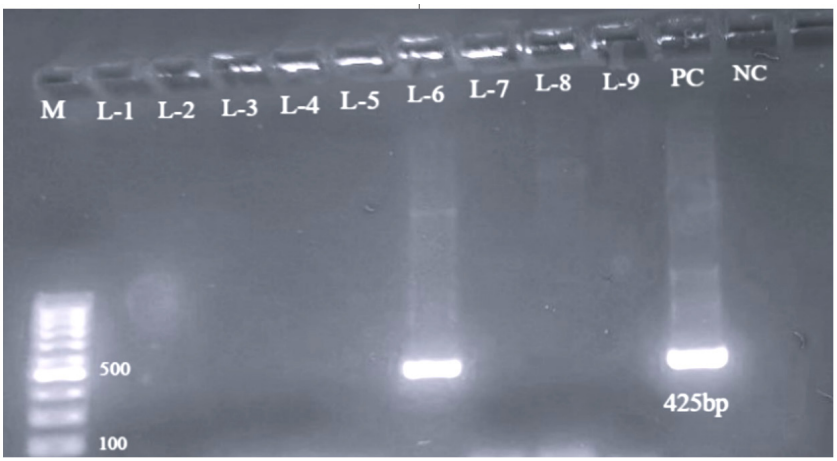

Figure 3. Agarose gel electrophoresis showing PCR amplification of the *eaeA* gene with a product size of 425 bp in the isolate C-34. Lane M: 100 bp DNA ladder; PC: positive control; NC: negative control

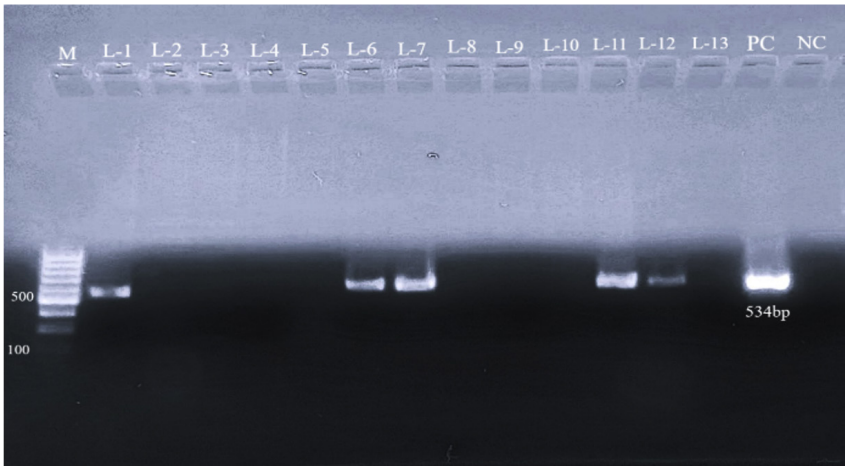

Figure 4. Agarose gel electrophoresis showing PCR products of 534 bp corresponding to the *EhlyA* gene in various isolates. Lane M: 100 bp DNA ladder; positive samples are shown in lanes L1 (C-2), L6 (C-7), L7 (C-10), L11 (C-21) and L12 (C-45); PC: positive control; NC: negative control

isolates, as well as the distribution of serogroups and their associated virulotypes, are presented sequentially in Tables 4 and 5, respectively.

Hemolysin activity

Isolates exhibiting presence of *EhlyA* gene by PCR were evaluated for hemolytic activity by employing 5% sheep blood agar. Findings

Table 2. Virulotyping of *E. coli* isolates

Virulotype	Virulence factors	Distribution in Sample (n = 40)	Sample Id
V-1	*stx1* alone	7	C-47, C-54, C-57, C-65, C-82, C-88, C-97
V-2	*stx2* alone	13	C-12, C-16, C-36, C-37, C-38, C-39, C-42, C-43, C-89, C-90, C-95, C-96, C-98
V-3	*stx1* & *stx2*	5	C-24, C-27, C-29, C-48, C-83
V-4	*stx1* & *EhylA*	5	C-7, C-10, C-21, C-45, C-58
V-5	*stx2* & *EhylA*	5	C-1, C-3, C-5, C-6 C-87
V-6	*stx1, stx2* & *EhylA*	4	C-2, C-4, C-8, C-19
V-7	*stx2* & *eaeA*	1	C-34

Table 3. Serotyping of the *E. coli* isolates

No.	Identified Serotypes	Isolate ID *E. coli*	Total no. of serogroups isolates	Prevalence of (%)
1.	O2	C-66	1	1.428%
2.	O3	C-25, C-81, C-93, C-100	4	5.71%
3.	O4	C-92	1	1.428%
4.	O7	C-36, C-38, C-54, C-72	4	5.71%
5.	O9	C-98	1	1.428%
6.	O16	C-9, C-15, C-23, C-57	4	5.71%
7.	O19	C-51	1	1.428%
8.	O27	C-19	1	1.428%
9.	O50	C-1, C-3, C-16, C-53	4	5.71%
10.	O66	C-27	1	1.428%
11.	O76	C-4, C-18, C-24, C-84, C-99	5	7.14%
12.	O85	C-6	1	1.428%
13.	O109	C-58	1	1.428%
14.	O111	C-37, C-65, C-83	3	4.28%
15.	O113	C-34, C-45	2	2.85%
16.	O114	C-71	1	1.428%
17.	O125	C-2, C-10	2	2.85%
18.	O135	C-62, C-87,	2	2.85%
19.	O156	C-12	1	1.428%
20.	Untypable (U.T.)	C-5, C-7, C-8, C-11, C-13, C-17, C-21, C-22, C-28, C-29, C-31, C-32, C-35, C-41, C-42, C-43, C-46, C-47, C-48, C-50, C-63, C-82, C-86, C-89, C-90, C-95, C-96, C-97	28	40%
21.	Rough	C-52, C-70	2	2.85%
Total			70	

*Serial numbers 20 and 21 do not represent serotypes

Table 4. Correlation between Virulotypes and Serogroups of STEC Isolates

Isolate ID	Sero-group	Virulo-type	Virulence Gene Profile
C-1	O50	V5	*stx2* + *EhlyA*
C-2	O125	V6	*stx1* + *stx2* + *EhlyA*
C-3	O50	V5	*stx2* + *EhlyA*
C-4	O76	V6	*stx1* + *stx2* + *EhlyA*
C-5	UT	V5	*stx2* + *EhlyA*
C-6	O85	V5	*stx2* + *EhlyA*
C-7	UT	V4	*stx1* + *EhlyA*
C-8	UT	V6	*stx1* + *stx2* + *EhlyA*
C-10	O125	V4	*stx1* + *EhlyA*
C-12	O156	V2	*stx2*
C-16	O50	V2	*stx2*
C-19	O27	V6	*stx1* + *stx2* + *EhlyA*
C-21	UT	V4	*stx1* + *EhlyA*
C-24	O76	V3	*stx1* + *stx2*
C-27	O66	V3	*stx1* + *stx2*
C-29	UT	V3	*stx1* + *stx2*
C-34	O113	V7	*stx2* + *eaeA*
C-36	O7	V2	*stx2*
C-37	O111	V2	*stx2*
C-38	O7	V2	*stx2*
C-39	Not serotyped/ UT	V2	*stx2*
C-42	UT	V2	*stx2*
C-43	UT	V2	*stx2*
C-45	O113	V4	*stx1* + *EhlyA*
C-47	UT	V1	*stx1*
C-48	UT	V3	*stx1* + *stx2*
C-54	O7	V1	*stx1*
C-57	O16	V1	*stx1*
C-58	O109	V4	*stx1* + *EhlyA*
C-65	O111	V1	*stx1*
C-82	UT	V1	*stx1*
C-83	O111	V3	*stx1* + *stx2*
C-87	O135	V5	*stx2* + *EhlyA*
C-88	Not serotyped	V1	*stx1*
C-89	UT	V2	*stx2*
C-90	UT	V2	*stx2*
C-95	UT	V2	*stx2*
C-96	UT	V2	*stx2*
C-97	UT	V1	*stx1*
C-98	O9	V2	*stx2*
C-90	UT	V2	*stx2*
C-95	UT	V2	*stx2*
C-96	UT	V2	*stx2*
C-97	UT	V1	*stx1*
C-98	O9	V2	*stx2*
C-90	UT	V2	*stx2*
C-95	UT	V2	*stx2*
C-96	UT	V2	*stx2*

Table 5. Distribution of Serogroups and Their Associated Virulotypes among STEC Isolates

Serogroup	Virulotype(s) Observed
O7	V1, V2
O9	V2
O16	V1
O27	V6
O50	V2, V5
O66	V3
O76	V3, V6
O85	V5
O109	V4
O111	V1, V2, V3
O113	V4, V7
O125	V4, V6
O135	V5
O156	V2
Untypable (UT)	V1, V2, V3, V4, V5, V6

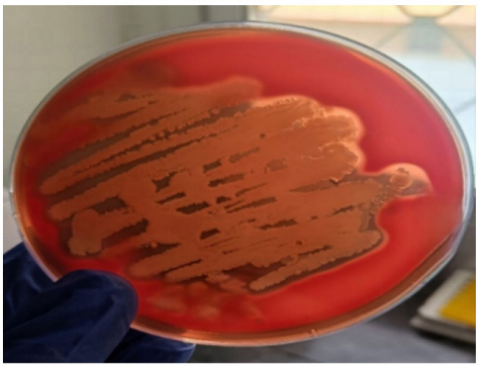

Figure 5. Enterohemolysin (*Ehly*) expression on 5% sheep blood agar plate

demonstrated that all these isolates produced clear hemolysis as shown in Figure 5 on the agar plates.

DISCUSSION

The present investigation demonstrated a considerable occurrence of Shiga toxin-producing *Escherichia coli* (STEC) among diarrhoeic cattle, with 40 of the 77 *E. coli* isolates harbouring one or more virulence-associated genes. The overall

prevalence of STEC in the 100 samples examined was 40%, which is comparable with earlier reports from India and other countries, including Shinde et al.[18] (39%), Leung et al.[19] (41.5%), Cooley et al.[20] (37.9%), and Ghoneim et al.[21] (40%). These findings indicate that cattle serve as an important reservoir of STEC and may contribute to environmental contamination and transmission of pathogenic strains.

A higher prevalence of the *stx2* gene compared to *stx1* was observed in the present study. This is of epidemiological significance, as *stx2*-positive STEC strains are more frequently associated with severe human diseases such as haemorrhagic colitis and haemolytic uremic syndrome. Similar observations have been reported by Kuhnert et al.,[22] Arya et al.,[23] and Parul et al.,[24] In contrast, some studies have reported a predominance of *stx1* (Cerqueira et al.,[25] and Buvens et al.,[26]) suggesting geographical variation in gene distribution. The *eaeA* gene was detected in only one isolate, indicating that most STEC strains may utilize alternative adherence mechanisms, as also reported in non-O157 bovine isolates.

Serotyping revealed substantial diversity with nineteen different serogroups. Although the O157 serogroup was not detected, several non-O157 serogroups of public health importance were identified. Among these, O111 and O113 are particularly noteworthy due to their established association with human infections and outbreaks.[27,28] Several STEC serotypes, including O113:H21, O26:H11, O26, as well as O111, are recognized for their "zoonotic" potential.[29] Several STEC serotypes, including O113:H21, O26:H11, O26, as well as O111, are recognized for their "zoonotic" potential.[29] Furthermore, Capps et al.[30] reported O2 and O109 as non-top STEC serogroups, with isolates carrying the *stx2* gene, particularly subtype 2a, highlighting their possible role in human infections. Similar outcomes were demonstrated by Blanco et al.,[31] who identified serotypes such as O113, O26, O91, and O8, and by Shahrani et al.[32] who documented a diverse range of non-O157 STEC serotypes, including O128, O121, and O113, among diarrheic calves in Iran.

Virulotyping identified seven distinct virulotypes, indicating marked genetic variability among isolates. The predominance of the *stx2*-only profile suggests its wide distribution in the study population. The presence of combinations of *stx1*, *stx2* and *EhlyA* genes indicates circulation of strains with enhanced virulence potential, which may be of greater public health concern.

Overall, the study highlights the epidemiological importance of non-O157 STEC in cattle. Although O157 was not detected, the presence of several zoonotically significant non-O157 serogroups underscores their potential public health risk. Continuous surveillance is essential to monitor their distribution and evolution.

The study is limited by its relatively small sample size, single geographical coverage, and analysis of a limited set of virulence genes. Further studies involving larger multi-regional sampling, Shiga toxin subtyping, antimicrobial resistance profiling, and whole-genome sequencing are recommended for a more comprehensive understanding of STEC epidemiology.

CONCLUSION

The present study demonstrated the occurrence of diverse STEC-associated *Escherichia coli* strains in diarrhoeic cattle from Jaipur district, Rajasthan. Among the 77 *E. coli* isolates recovered, a considerable proportion carried one or more virulence genes, confirming the presence of potentially pathogenic STEC in the study population. The predominance of the *stx2* gene suggests the circulation of strains with increased virulence potential, while the low detection of *eaeA* indicates that alternative pathogenic mechanisms may be involved in these isolates.

Although the O157:H7 serotype was not detected, several non-O157 serogroups, including O111, O113 and O125, were identified. These serogroups have been associated with human infections and therefore highlight the zoonotic relevance of cattle-derived STEC. The diversity of serogroups and virulence profiles observed in this study emphasizes the importance of non-O157 STEC as an emerging public health concern.

The findings confirm that diarrhoeic cattle can serve as reservoirs of potentially zoonotic STEC and reinforce the need for continuous surveillance

of non-O157 serogroups. Further studies involving larger sample populations and advanced molecular characterization are required to better understand the epidemiology, virulence attributes and public health significance of STEC circulating in cattle.

ACKNOWLEDGMENTS

The authors sincerely acknowledge the Centre for Diagnosis, Surveillance, and Response to Zoonotic Diseases (CDSRZ), Department of Veterinary Medicine, Post Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur, for providing facilities and support for this study. Authors are also grateful to the Principal Investigator, CDSRZ, and the Dean, PGIVER, Jaipur, for their valuable guidance, encouragement, and support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

NKJ and SKS conceptualized and visualized the study. WC conducted the investigation. WC, AB, SM, AC, JB and A performed data curation and developed the methodology. WC wrote the manuscript. NKJ, SKS and JB reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Animal Ethical Committee, Post Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur, India (Regd. No. 1971/GO/Re/SL/17/CPCSEA; Dated 16 June 2017).

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