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Research Article

DETECTION AND PATHOTYPE ANALYSIS OF SHIGA TOXIN-PRODUCING *E. COLI* (STEC) SEROGROUPS: O26, O111 AND O157

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ABSTRACT

Escherichia coli is a ubiquitous inhabitant of the gut microbiome in humans, animals and birds. The majority of *E. coli* strains maintain a harmless presence, however, certain high-risk serogroups such as O26, O111 and O157 acquire specialized virulence factors that enable them to cause a spectrum of clinical syndromes. In this study, we employed a PCR-based serogrouping approach to detect and characterize three major key O-serogroups (O26, O111 and O157) among *E. coli* isolates recovered from 341 diverse samples collected from cow and buffalo milk, rectal swabs of cows, buffaloes and goats as well as poultry cloacal swabs from Sambhal district of Uttar Pradesh, India. Initial biochemical screening was followed by molecular confirmation using *uid*A and *cyd*A gene targets to identify *Escherichia coli*. The isolation rate of *E. coli* was 73.02% (256/351). Subsequent PCR analysis revealed five O26, two O111 and one O157 isolates, corresponding to prevalence rates of 2.0%, 0.8%, and 0.4%, respectively. Notably, all the serogroup-positive isolates were Shiga toxin-producing *E. coli* (STEC), with various combinations of *stx*1, *stx*2 and *hly*A gene presence. Distinct antimicrobial resistance profiles were observed among the isolates, with universal susceptibility to amikacin and chloramphenicol, higher resistance to Ampicillin, Ceftazidime and Ceftriaxone and resistance to Imipenem amongst O157 isolates, highlighting that resistance traits are not solely determined by serotype or pathotype. The data provides critical insights into the occurrence of highly virulent STEC serogroups in livestock and poultry, emphasizing their potential impact on food safety and public health.

Keywords: *Escherichia coli*, Molecular serogrouping, O26, O111, O157, Pathotypes

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Escherichia coli, a key member of the *Enterobacteriaceae* family, is a common inhabitant of the gastrointestinal tracts of humans, animals and birds, as well as in environmental reservoirs. Although most *E. coli* strains exist harmlessly as commensals, certain clones have acquired unique virulence factors that enable them to cause a wide range of diseases. Pathogenic strains of *E. coli* are implicated in diverse clinical syndromes, including gastrointestinal infections that cause diarrhea, urinary tract infections, and severe systemic illnesses such as sepsis and meningitis. Shiga toxin-producing *E. coli* (STEC) are especially concerning on a global scale, being major foodborne pathogens responsible for outbreaks ranging from watery diarrhea and hemorrhagic colitis to life-threatening hemolytic-uremic syndrome (HUS). Historically, *E. coli* O157:H7 has been identified as the most important STEC serotype, accounting for a significant proportion of infections and fatalities (Scallan *et al.*, 2011). However, non-O157 serogroups such as O26, O45, O103, O111, O121 and O145 are emerging as major contributors to illness. Ruminants are the primary reservoirs, with additional evidence pointing to wild animals as carriers. India is a country of immense geographical diversity, data on *E. coli* serogroups and their pathogenic profiles remain limited. This study aims to assess the prevalence of O26, O111, and O157 serogroups in *E. coli* isolates from diverse sources, pathotyping and antimicrobial resistance profiling to inform effective public health interventions.

MATERIALS AND METHODS

A total of 341 samples comprising 65 cow milk samples, 64 buffalo milk samples, 66 cow rectal swabs, 64 buffalo rectal swabs, 33 goat rectal swabs and 49 poultry cloacal swabs were randomly collected from Sambhal district of Uttar Pradesh, India.

Milk samples were centrifuged at 3500 rpm for 10 minutes and the pellet was inoculated into MacConkey broth at 37° C for 18-24 hours, rectal and cloacal swabs were also inoculated into MacConkey broth under similar conditions. After incubation, broth was streaked onto MacConkey Lactose Agar plates from which the selected colonies were streaked onto eosin methylene blue (EMB) agar and incubated for 24 hours. After incubation plates were examined, typical *E. coli*-like colonies were selected and screened for the IMViC tests. The cultures giving typical (+, +, −, −) reaction were confirmed as *E. coli*. Further molecular conformation was done by targeting *uid*A and *cyd*A gene. A total of 249 isolates were confirmed as *E. coli* through phenotypic and molecular methods. The confirmed isolates were screened for the presence of the O26, O111 and O157 serogroups using PCR. Following molecular confirmation of these O serogroups, all positive isolates were further screened by PCR for the presence of

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pathotypes (STEC, EPEC, ETEC and EAEC) to identify the serogroups-pathotype relationship. The primers, their sequences and the annealing temperatures are listed in Table 1.

Each PCR reaction was performed in a 200 µL Eppendorf tube with a total reaction volume of 25 µL. The reaction mixture consisted of 12.5 µL of 2X master mixture (containing dNTPs, Taq DNA polymerase, along with reaction buffer 1.5 mM $MgCl_2$) and 5 to 10 pmol of both forward and reverse primers. Additionally, 4 µL of DNA template was added and the volume was adjusted to 25 µL using nuclease-free water. The PCR cycling conditions for gene amplification were: denaturation at 94° C for 5 minutes, followed by 30 cycles of denaturation at 94° C for 30 seconds, annealing at the temperature specified for each primer (as listed in Table 1) and extension at 72° C for 45 seconds. A final extension was carried out at 72° C for 5 minutes. To prevent contamination, a non-template control (NTC) was included in each PCR run. PCR products (8 µL) were separated by electrophoresis on 1.5% agarose gels in 1X Tris-acetate-EDTA (TAE) buffer. The gels were stained with ethidium bromide (1 mg/mL) and visualized under UV light.

In addition, all verified O26, O111 and O157 strains were subjected to antimicrobial susceptibility testing via the disc diffusion method, following the protocols established by the Clinical and Laboratory Standards Institute (CLSI). For anti-microbial susceptibility pattern following antibiotics including ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), ceftriaxone (30 µg), cefpodoxime (10 µg), aztreonam (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg, amikacin (30 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), imipenem (10 µg), nalidixic acid (30 µg), sulphamethoxazole-trimethoprim (1.25-23.75 µg) and tetracycline (30 µg) were used.

RESULTS AND DISCUSSION

A total of 249 *E. coli* isolates were recovered from 341 samples by targeting *uid*A and *cyd*A gene using PCR assay, resulting in an isolation rate of 73.02%. The sample sources included 50.76% cow milk samples, 54.68% buffalo milk samples, 86.36% cow rectal swabs, 85.93% buffalo rectal swabs, 81.81% goat rectal swabs and 85.71% poultry cloacal swabs. Conventional serotyping, the standard method for detecting O-antigens in *E. coli* isolates, involves labor-intensive agglutination reactions with specific antisera. This study used a PCR-based O-typing method utilizing 3 primer pairs, referred to as *E. coli* PCR-based serogrouping, which is a more efficient approach. The selected primers represent the most commonly employed molecular techniques for the identification of pathogenic bacteria, facilitating rapid and reliable screening of O types in *E. coli* isolates. In this study, we targeted the three most virulent O-serogroups of *E. coli* using a PCR assay. The serogroups included O26, O111 and O157. These O serogroups were detected by using PCR assay by targeting various genes such as *wzx* and *rfb*E. We found five O26, two O111 and one O157 serogroups with a positivity of 2.00%, 0.80% and 0.4%, respectively, among the 249 *E. coli* isolates screened. The O157 strain was isolated from buffalo fecal sample. Supporting our findings, Kumar *et al.* (2022) also reported that O157 was less common and associated with STEC. Abike *et al.* (2015) identified nine serogroups in dairy products, with O111 being the most prevalent. Pathotyping revealed that O111, O26 and O157 consistently harbored STEC-associated genes (*stx*1/*stx*2/*hly*A). Details of positive O serogroups isolates and its relationship with pathotype are mentioned in table 2.

All positive samples from PCR-based O antigen serogrouping were screened for the presence of various pathotypes, including enteropathogenic (EPEC), Shiga toxic *E. coli* (STEC), enterotoxigenic (ETEC), and enteroaggregative (EAEC) *E. coli* by detecting specific genes such as *stx*1, *stx*2, *hly*A, *bfp*, *lt*, *st*II and *agg*R, respectively. Pathotyping results revealed that all the O26, O111 and O157 isolates carried STEC virulence genes and were identified as STC pathotype. The O157 and O111 confirmed isolates revealed the presence of all the three (*stx*1, *stx*2, *hly*A) STEC genes. Among the five O26 isolates, four were having two STEC genes (*stx*1/*stx*2/*hly*A) whereas one isolate derived from buffalo rectal sample possessed only one (*stx*1) STEC gene. Amongst these STEC genes, *sxt*1 gene was present in all the O26, O111 and O157 serogroups whereas *hly*A gene was present in the only four isolates. Similar to our findings, Renter *et al.* (2005) confirmed that all O26, O111 and O157 serogroups possessed STEC genes. Merwad *et al.* (2014) identified STEC serogroups O26 and O111 in milk and fecal samples. In contrast to our findings, Malvi *et al.* (2015) detected the O26 serogroup within enteropathogenic *E. coli* (EPEC) strains, while Cardonha *et al.* (2004) identified the O111 serogroup in enteropathogenic *E. coli* from sewage and nearby coastal areas in Brazil. Tamaki *et al.* (2005) analyzed 42 isolates of the O111 serogroup, classifying 11 as EPEC, 6 as STEC and 14 as EAEC. Similarly, among 49 O26 isolates, three were identified as EPEC, nine as STEC, one as enterotoxigenic *E. coli* ETEC and one as EAEC.

The Shiga toxin-producing *E. coli* (STEC) is a significant foodborne pathogen that can cause gastroenteritis, hemorrhagic colitis and hemolytic-uremic syndrome (HUS). The O157 serogroup is particularly associated with HUS, while other non-O157 important pathogenic serogroups include O26, O103, O111, O121 and O145. Emerging serogroups, such as O104:H4, have also been implicated in outbreaks. The most prevalent pathogenic non-O157 STEC serogroups in the United

Table 1. Details regarding the primers, including their specific annealing temperature and product size

S.No.	Type	Gene	Primer	Annealing temp. (°C)	Product size (bp)	Reference
1.	*E. coli*	*cyd*A	F-CGCCGATGCAGATATTCG R-GCTGTGACGCACAGTTCATAG	60	398	Chopra *et al.*, 2021
2.		*uid*A	F- CGCCGATGCAGATATTCG R- GCTGTGACGCACAGTTCATAG	60	603	Chopra *et al.*, 2021
3.	O26	*wzx*	F-GGGGGTGGGTACTATATTGG R-AGCGCCTATTTCAGCAAAGA	67	241	Paddock *et al.* (2012)
4.	O111	*wzx*	F-CAAGAGTGCTCTGGGCTTCT R-AACGCAAGACAAGGCAAAAC	67	451	Paddock *et al.* (2012)
5.	O157	*rfb*E	F-CAGGTGAAGGTGGAATGGTTGTC R-TTAGAATTGAGACCATCCAATAAG	64	296	Bertrand and Roig (2007)
6.	STEC	*stx*1	F-CAGTTAATGTGGTGGCGAAGG R-CACCAGACAATGTAACCGCTG	62	348	Cebula *et al.* (1995)
7.		*stx*2	F-ATCCTATTCCCGGGAGTTTACG R-GCGTCATCGTATACACAGGAGC	62	584	Cebula *et al.* (1995)
8.		*hly*A	F-AGCTGCAAGTGCGGGTCTG R-TACGGGTTATGCCTGCAAGTTCAC	62	569	Wang *et al.* (2002)
9.	ETEC	*st*II	F-AAAGGAGAGCTTCGTCACATTTT R-AATGTCCGTCTTGCGTTAGGAC	62	129	Vidal *et al.* (2004)
10.		*lt*	F-GCACACGGAGCTCCTCAGTC R-TCCTTCATCCTTTCAATGGCTTT	62	218	Vidal *et al.* (2004)
11.	EPEC	*bfp*	F- GGAAGTCAAATTCATGGGGGTAT R- GGAATCAGACGCAGACTGGTAGT	62	254	Vidal *et al.* (2004)
12.	EAEC	*agg*R	F-GTATACACAAAAGAAGGAAGC R-ACAGAATCGTCAGCATCAGC	52	254	Ratchtrachenchai *et al.* (1997)

Table 2. Details of positive O serogroups and its relationship with pathotype

Isolate No.	Sample type	O serogroups	Pathtyping gene	Pathotypes
E736	Poultry cloacal	O26	*stx*1 and *hly*A	STEC
E797	Buffalo rectal	O26	*stx*1	STEC
E1083	Cow milk	O26	*stx*1 and *stx*2	STEC
E1090	Buffalo rectal	O157	*stx*1, *stx*2 & *hly*A	STEC
E1139	Poultry cloacal	O26	*stx*1 and *stx*2	STEC
E1158	Cow rectal	O26	*stx*1 and *stx*2	STEC
E1168	Buffalo rectal	O111	*stx*1, *stx*2 & *hly*A	STEC
E1160	Cow rectal	O111	*stx*1, *stx*2 & *hly*A	STEC

States are O26, O45, O103, O111, O121 and O145, causing over 70 to 83% of the total non-O157 STEC illnesses (Bettelheim, 2007).

The antimicrobial resistance patterns of O26, O111 and O157-confirmed isolates exhibited distinct variations. All three serotypes were found to be susceptible to Amikacin and Chloramphenicol. The highest resistance rates were recorded against Ampicillin (50%) followed by Ceftazidime and Ceftriaxone (37.5%) across all positive serotypes. Resistance against cephalosporin antibiotics has been reported previously by Bairwa *et al.* (2023). However, resistance to Imipenem was observed exclusively in O157-positive isolates. A detailed antimicrobial resistance pattern is presented in Table 3. There was no observed correlation between antimicrobial resistance patterns and serogroups or pathotypes, as these characteristics exhibited significant variation. Similar to our research Mora *et al.* (2005) also found that the resistance profiles of both O157:H7 and non-O157 STEC (which include serogroups such as O111 and O26) varied significantly among isolates, with no clear correlation between the resistance patterns and the serogroup or pathotype. This indicates that, despite classification into different serogroups (e.g. O111, O157, O26), the antimicrobial resistance traits are not strictly linked to these groups but rather show high variability.

CONCLUSION

This study revealed a low prevalence of highly virulent serogroups (O26, O111 and O157) among diverse samples collected from Sambhal district, Uttar Pradesh. All these O-types possessing STEC genes. The data further revealed that while O157 and O111 isolates consistently possessed all three virulence determinants, O26 strains displayed variable gene carriage, with some isolates harboring only *stx*1. This variability in virulence profiles, even at low prevalence, highlights the complex epidemiological landscape of STEC and underscores their significant public health potential. Overall, these findings advocate for the continued implementation of targeted molecular surveillance and enhanced food safety protocols to mitigate the risks associated with emerging STEC strains in livestock, poultry, and related food products. The study revealed that while all isolates were susceptible to amikacin and chloramphenicol, high resistance was observed to Ampicillin,

Table 3. Details of Anti-microbial susceptibility pattern of conformed O-types isolates

Isolate ID(O-type)	E736 (O26)	E797 (O26)	E1083 (O26)	E1090 (O157)	E1139 (O26)	E1158 (O26)	E1168 (O111)	E1160 (O111)
Ampicillin	I	R	S	R	S	R	S	R
Amoxicillin-clavulanic acid	S	S	I	R	S	S	I	S
Amikacin	S	S	S	S	S	S	S	S
Aztreonam	S	S	S	S	R	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Ceftazidime	R	S	S	S	R	I	S	R
Cefotaxime	S	S	S	S	R	S	I	S
Ceftriaxone	S	I	S	R	R	S	S	R
Cefoxitin	S	S	S	R	R	I	S	S
Cefpodoxime	S	S	S	S	R	S	S	S
Enrofloxacin	S	R	S	S	S	S	S	S
Imipenum	S	S	S	R	S	S	S	S
Nalidixic acid	I	S	S	S	S	R	I	R
Tetracycline	S	S	S	S	S	R	S	S
Trimethoprim-Sulfamethoxazole	S	S	S	S	S	R	S	S

* S- Sensitive *I- Intermediate *R-Resistance

Ceftazidime and Ceftriaxone with Imipenem resistance found exclusively in O157 isolates. These findings underscore that antimicrobial resistance traits vary significantly among serogroups and are not strictly linked to serotype or pathotype classifications.

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