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Original Article

Isolation, Extraction, and Characterization of Verotoxin-producing Escherichia coli O157:H7 from Diarrheal Stool Samples

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Abstract

Background: *Escherichia coli* O157:H7 infection causes hemorrhagic colitis and is diagnosed based on symptoms such as cramps, stomach pain, and watery diarrhea. Shiga-like toxins (Verotoxin) produced by *Escherichia coli* O157:H7 damages endothelial cells of both kidney and brain, causing renal dysfunction and neurological problems.

Methods: The present study focuses on identifying the prevalence of Verotoxin-producing *Escherichia coli* O157:H7 among diarrheal inpatients at Erode Government Hospital, India, and its antibiogram. Further, the Verotoxins were characterized by using SDS-PAGE analysis. A total of 123 diarrheal stool samples were collected, and 37 of them (30.08% of the total samples) were found to have the presence of *E. coli*. The organisms were identified based on their colony morphology on various media, cell morphology, and biochemical tests. The Shiga-like toxin production was identified by non-fermentation of sorbitol on SMAC agar plates. Confirmation of Shiga-like toxin was performed using agglutination assay.

Results: In total, 12 isolates showed agglutination and these isolates were confirmed to be *E. coli* O157:H7. The molecular weight of the Verotoxin was found to be between 20 and 29 kD. The antibiogram profile of the four isolated strains against 10 standard antibiotics was determined.

Conclusion: The results of this study show the occurrence of drug resistance on hemorrhagic colitis causing *E. coli* O157:H7.

Keywords: *E. coli*, Verotoxin, diarrhea, hemorrhagic colitis

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1. Introduction

Escherichia coli is a predominant pathogenic microorganism that causes various conditions such as urinary tract infections, diarrhea, pneumonia, meningitis, and bacteremia. *Escherichia coli*-synthesizing toxins with a cytolytic effect on Vero cells were primarily termed as Verotoxin-producing *E. coli* (VTEC) [1]. Verotoxin and Shiga toxin were observed to be slightly similar in structures and the Shiga toxin-producing *E. coli* were termed as Shiga toxin-producing *E. coli* (SETC). Among the strains of SETC, the clinically isolated hemorrhagic colitis (HC)-causing serotypes were designated as enterohemorrhagic *E. coli* (EHEC) [2].

The virulent nature of EHEC is due to the bacteriophage-induced Shiga toxin. This toxin regulates diarrhea and HC to hemolytic uremic syndrome (HUS) as well. Stx 1 and Stx 2 are the two types of immunologically varied Shiga toxins synthesized in EHEC. Stx 1 was similar to the Shiga toxin synthesized by *Shigella dysenteriae*. Another virulent factor includes an external membrane protein known as intimin, which was found to be vital for colonizing the intestine. Enterohemolysin is encoded by a 60Mda plasmid called p O157. Enterohemolysin functions by lysis of RBC and providing an iron source to the pathogen [3].

The toxin produced by *S. dysenteriae* type 1 that is active on Vero cells is named Shiga toxin; two related toxins produced by *E. coli* O157:H7 are named Shiga-like toxins 1 and 2 (SLT-1 and SLT-2) or Verotoxins 1 and 2 (VT-1 and VT-2). Shiga toxin and Shiga-like toxin (SLT–1) are immunologically, biologically, and structurally closely related or identical. SLT–2 is also biologically (activity on Vero cells) and structurally (A and B subunit structures) related to but differs immunologically from Shiga toxin and SLT-1. The assay on Vero cells seems to be a useful method for their detection [4, 5].

The fecal–oral route was the common method of transmission. Oftentimes, humans are infected through carriers such as animals and fomites. Birds are identified to be evident vectors for transmission. Pathogen outbreaks are mostly related to the consumption of poorly cooked or packaged animal products; in particular ground beef, however, unpasteurized milk and dried meats, alfalfa sprouts, and other infected vegetable products have often triggered epidemics [6].

Escherichia coli O157:H7 infection causes HC; this infection is generally diagnosed based on symptoms such as cramp, stomachache, and watery diarrhea accompanied by bloody diarrhea which may be absent in the primary stages and dehydration may be probable. The infection is observed to be self-limiting and lasts about a week in healthy

adults [7]. Severe complications may occur in a small percentage of cases. Shiga-like toxins (Verotoxin) produced by *E. coli* O157:H7 damages endothelial cells of both kidney and brain, causing renal dysfunction and neurological problems. HUS can occur in 5–10% of infected patients – generally, the week after the onset of diarrhea and is evaluated by renal dysfunction, which can lead to enduring injury and hemolytic anemia. Severe complications like seizures colonic perforation, hypertension, strokes, pancreatitis, and coma may also be observed. Few patients exhibit lifelong Insulin-dependent diabetes mellitus. Although HUS can impact all age groups, children <10 years old are highly prone to this disease [8].

Therefore, the main objectives of the present study were to find out the occurrence of verotoxin-producing strains of E. coli O157:H7 among diarrheal patients, to perform the antibiogram analysis of the isolated strains, and to characterize them using SDS-PAGE analysis.

2. Materials and Methods

2.1. Sample collection and processing

A total of 123 human diarrheal stool samples were collected in a sterile wide-mouth container and processed for microbial isolation. Stool samples were collected from inpatients at the Erode Government Hospital, India. The fecal samples were homogenized (10 gm of feces in 100 ml of Trypticase soy broth) immediately after collection from both the infected person and animal [9]. It was incubated for 14 hr in Shaker at 37°C and then a loop full of culture was plated on MacConkey agar incubated at 37°C for 24 hr.

2.2. Identification of E. coli

Presumptive *E. coli* colonies were biochemically confirmed. The biochemical tests performed were oxidase test, indole production test, methyl red test, Voges–Proskauer test, citrate utilization test, Triple Sugar Iron Agar test, Gram's staining and motility analysis. The lactose-fermenting colonies were examined for hemolysis in blood agar, fermentation of lactose in MacConkey agar, and in Eosin methylene blue agar.

2.3. Antibiotic susceptibility test

The culture was tested against 13 antibiotics. The antibiotics were Ampicillin, Amoxicillin, Ciprofloxacin, Chloramphenicol, Gentamicin, Kanamycin, Neomycin, Nalidixic acid, Penicillin-G, Polymyxin-B, Rifampicin, Streptomycin, and Tetracycline

2.4. Preliminary confirmation of Shiga-like toxin-producing E. coli

The confirmed *E. coli* colonies were plated on sorbitol MacConkey agar (SMAC) by using the streak plate technique to confirm the Shiga-like toxin-producing *E. coli*. After plating, the plates were incubated at 37ºC for 24 hr [10].

2.5. Serotyping for conformation of Shiga-like toxin-producing E. coli

Escherichia coli O and H antigens were investigated based on standard protocols. Agglutination assay was performed on glass slides by adding a drop (20 μl) of antiserum in PBS with a loop full of bacteria. Agglutination reactions were examined within a few seconds. Bacterial culture in 1 drop of PBS was used as control. Agglutination reactions were evaluated by live bacterial cultures, and confirmation tests were repeatedly performed by heating the bacterial cultures for 1 hr at 100ºC.

2.6. Production of Verotoxin/Shiga-like toxin by E. coli

Cultures were oxygenated to the maximum by aeration and vigorous agitation at 300 rpm on a rotatory shaker for higher toxin production. After this, 1 ml of culture was transported to modified Syncase Broth (MSB) [11] for the detection and purification of the toxin. To 50 ml of the stock solution, 5 ml of 2% (W/V) glucose and 0.04% of tryptophan were added.

2.7. Extraction of Shiga-like toxins

The supernatant from overnight cultures was taken. Seventy percent ammonium sulfate solution was added and incubated overnight for precipitation. The pellet was suspended in 250 ml of 10 mm Tris HCl (pH − 7.4) and centrifuged at 11,950 rpm at 4ºC. The final cell pellet was resuspended in 10 ml of 10 mM Tris HCl (pH − 7.4) and dialyzed
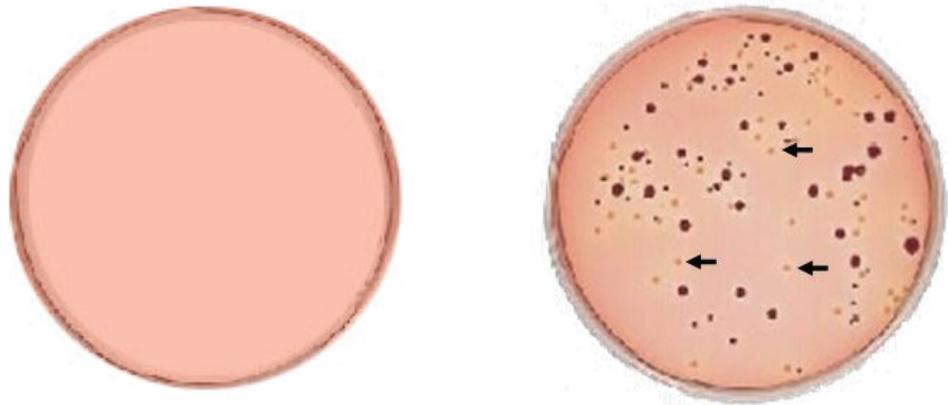
Figure 1: Identification of Verotoxin-producing *E. coli* O157:H7 in Sorbiltol MacConkey agar (SMAC) plates.

extensively against 10 mM Tris (pH − 7.4) for at least 24 hr. The dialyzed ammonium sulfate precipitate was characterized using SDS − PAGE to determine the molecular weight of the extracted toxin.

3. Results

3.1. Isolation and identification of E. coli

A total of 123 samples were collected both from the diarrheal stool. Bacteria that produce Verotoxin were isolated by using enrichment culture method followed by streak plate method by using trypticase soy agar medium. In the present study, over 37 serotypes (30.08%) of *E. coli* were isolated and the colonies on agar medium were analyzed based on their colony morphology, cell morphology, and biochemical tests.

In biochemical tests, the isolates were observed to be gram-negative rods, motile. Tryptophan was breaking down by the production of indole. Methyl red was produced. Citrate was not utilized and the result of Voges–Proskauer test was negative. Oxidase was produced. And almost all the sugars were fermented by this organism such as glucose, lactose, mannitol, and mannose, and it produced negative results in the urease test. The results are presented in Table 1. On comparing the results with Bergey's Manual of Determinative Bacteriology, the isolate was identified as *E. coli*. Most strains ferment lactose and thus grow as smooth, glossy, pink colonies on MacConkey agar and produce a clear zone of hemolysis on the blood agar medium. They produce greenish metallic sheen on Eosin Methylene Blue agar.

TABLE 1: Biochemical characterization.

Biochemical test	Results
Gram staining	G (–)ve
Motility test	+
Indole Production test	+
Methyl red test	+
Voges–Proskauer test	–
Citrate utilization test	–
Oxidase test	–
TSI test	A/A
H_2S production test	–
Glucose fermentation test	–
Lactose fermentation test	+
Mannitol fermentation test	+
Maltose fermentation test	+
Urease Test	–
Sorbitol fermentation test	S+ = V–; S– = V+

+: positive; –: negative; V–: Verotoxin negative; V+: Verotoxin positive.

TABLE 2: Antibiotic susceptibility.

Bacterial isolates	Antibiotics$^\$$/Zone of inhibition												
	A	Ac	Cf	C	G	K	N	Na	P	Pb	R	S	T
A1	17.00 (S)	18.00 (S)	21.00 (S)	11.00 (R)	18.00 (S)	18.00 (S)	13.00 (I)	0.00 (R)	0.00 (R)	0.00 (R)	10.00 (S)	13.00 (I)	17.00 (I)
A2	15.00 (I)	10.00 (R)	16.00 (I)	15.00 (I)	17.00 (S)	13.00 (R)	17.00 (S)	9.00 (R)	0.00 (R)	0.00 (R)	9.00 (S)	13.00 (I)	10.00 (R)
A3	17.00 (S)	19.00 (R)	19.00 (I)	21.00 (S)	17.00 (S)	14.00 (I)	15.00 (I)	0.00 (R)	0.00 (R)	0.00 (R)	11.00 (S)	17.00 (S)	10.00 (R)
A4	17.00 (S)	19.00 (S)	20.00 (S)	12.00 (R)	19.00 (S)	17.00(S)	14.00 (I)	0.00 (R)	0.00 (R)	0.00 (R)	10.00 (S)	18.00 (S)	12.00 (R)

$^\$$A: Ampicillin; Ac: Amoxicillin; Cf: Ciprofloxacin; C: Chloramphenicol; G: Gentamicin; K: Kanamycin; N: Neomycin; Na: Nalidixic acid; P: Penicillin-G; Pb: Polymyxin-B; R: Rifampicin; S: Streptomycin; T: Tetracycline.
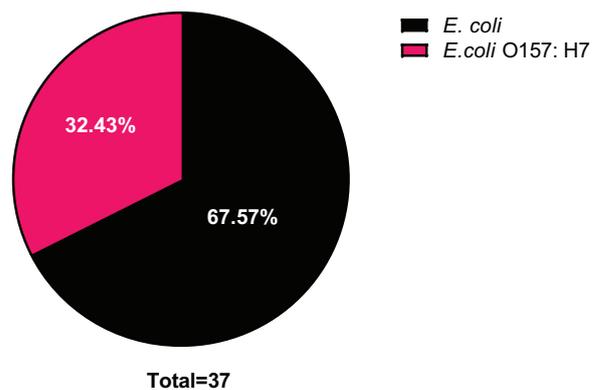

Figure 2: Occurrence of *E. coli* O157:H7 in human diarrheal samples.
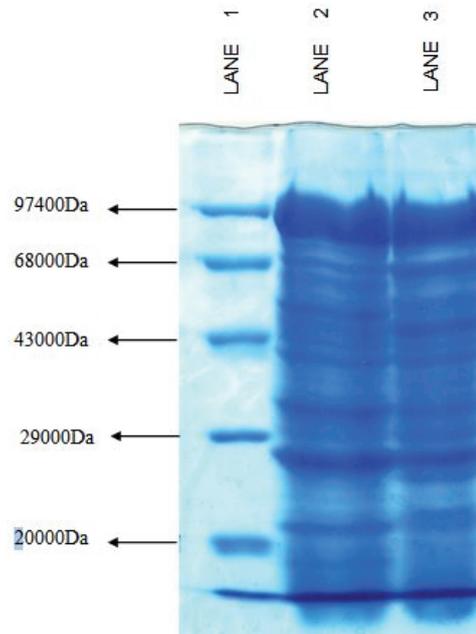
Lane 1 : Protein molecular size marker
Lane 2: Verotoxin in LB broth
Lane 3: Verotoxin in MSB

Figure 3: SDS-PAGE analysis of Verotoxin.

3.2. Preliminary confirmation of Shiga-like toxin-producing E. coli

After incubation for 24 hr, the small, pale yellow colored (Negative), round colonies appeared on the sorbitol MacConkey agar medium. While the non-Verotoxin-producing *E. coli* ferment sorbitol and produce pink-colored colonies, Verotoxin-producing *E. coli* fail to ferment sorbitol and produce white-colored colonies.

In this study, all 37 isolates were Verotoxin-positive and failed to ferment sorbitol. The colonies of this isolated strain on SMAC medium were colorless which permits presumptive identification of this organism (Figure **1**). All *E. coli* O157:H7 strains identified to date have been observed to produce Verotoxin and do not ferment sorbitol [10].

3.3. Confirmation of Shiga-like toxin-producing E. coli

Thirty-seven isolates were further tested according to the standard procedures of serotyping; of them, twelve isolates were confirmed as O157 and H7 (Figure **2**). Agglutination results show that antibody typing reagent supports the required sensitivity and specificity for identifying the *E. coli* serotype O 157:H7.

3.4. Characterization of Shiga-like toxin

The Shiga-like toxin was produced with the use of MSB and it was extracted by ammonium sulfate precipitation method. The protein was extracted from *E. coli* which are isolated from animal and human fecal samples. The molecular weight of the protein was between 20,000 and 29,000 Da (Figure **3**). On comparing to LB broth, the band which was observed in MSB gave a better result [12].

3.5. Antibiotic susceptibility test

The four isolated strains were subjected to antibiogram analysis against 10 antibiotics. After incubation, the results were noted. The size of the zone was compared with the Zone size-interpretative chart (Table 2).

4. Discussion

A cross-sectional study observed that the major pathogenic organism causing the disease was *E. coli* O157:H7, a species that was not previously reported as a human pathogen. Since then, *E. coli* O157:H7 has caused large food-borne outbreak infections worldwide. The center for disease control and prevention (CDC) has recently identified that *E. coli* O157:H7 is the fourth most predominant diarrhea-causing bacteria next to *Campylobacter* sp., *Salmonella* sp., and *Shigella* sp. This pathogen is a gram-negative motile rods belonging to the family of Enterobacteriaceae and was the primary reason for the HC in humans. *Escherichia coli* O157:H7 infection occurs globally and *E. coli* O157:H7 remains to be viable for more than three months in feces and soil, and infections can occur from weeks to months in widely used acidic foods such as mayonnaise, sausage, apple cider, and cheddar even at refrigeration temperature [13]. Thus, the present study concentrates on the identification of *E. coli* O157:H7 from diarrheal stool samples. Of the 123 samples, strains from 37 samples were found to have *E. coli*. *Escherichia coli* was confirmed by colony morphology on various media and produced green metallic sheen on EMB agar plates. Further, it was confirmed by several biochemical tests. The Shiga-like toxin production was identified by non-fermentation of sorbitol on SMAC agar plates. Confirmation of Shiga-like toxin was performed using agglutination assay. Twelve isolates showed agglutination and these isolates were confirmed to be *E. coli* O157:H7.

Escherichia coli O157:H7 has been reported in domestic animals like cattle, sheep, goats, pigs, dogs, and poultry. Cattle are the major reservoir for the pathogen and young animals shed *E. coli* in the fecal. Pathogens in feces can remain for weeks to months and can also be intermittent. Many types of STEC other than O157:H7 have been observed more commonly in cattle and most predominantly in fecal samples [14]. Similar results were observed for sheep and goats which carry other strains of STEC [15]. These observations denote that few STEC strains are mutated for colonizing the intestinal layer of their animal hosts and remain permanently for several years [16].

The effect of Shiga toxin and Shiga-like toxin 2 has been observed to differ from cell type due to the Gb_3 receptors, which is present in certain tissues like sensory neuron and renal cell. In such cells, the concentration of receptor and interaction was regulated by factors like tumor necrosis factor (TNF), interleukins, and fatty acid content of the membranes. Shiga-like toxin and Shiga-like toxin 2 differ in their effects on different tissues. Moreover, both two types have been shown to induce cell death. Shiga-like toxins were characterized by SDS-PAGE analysis [17]. The strains were cultivated in MSB and supernatants were subjected to ammonium sulfate precipitation and dialysis. The purified proteins were characterized using SDS-PAGE analysis. The molecular weight of the toxins was found to be between 20 and 29 kDa.

Such toxins have several interesting effects at the cellular level. Once the Shiga-like toxins have been enclosed, it is invariably transported through the Golgi apparatus to the rough endoplasmic reticulum where ribosomes are their major targets. The Shiga-like toxin also inhibits protein synthesis and regulates cytokines such as Il-1, Il-6, and Il-8. The Shiga-like toxin is also observed to express TNF-7, inducing F-acting de-polymerization and activating α kinase. Recent studies have shown that Shiga-like toxin is cytotoxic for primary cultures of human colonic cells. Shiga toxin can induce vascular cellular damage, as capillary lesions observed in hemolytic-uremic syndrome resemble those found in cerebral vessels of animals administered with these toxins [17, 18]. Further from the antibiogram analysis, the strains were found to be resistant to most of the commonly used antibiotics. This indicates the occurrence of drug resistance in Verotoxin-producing *E. coli* O157:H7.

5. Conclusion

A total of 123 samples were collected both from diarrheal stools and strains from 37 samples (43.02%) were found to be *E. coli*. The organisms were identified based on

their colony morphology on various media, cell morphology, and biochemical tests. The Shiga-like toxin production was identified by non-fermentation of sorbitol on SMAC agar plates. Confirmation of Shiga-like toxin was performed using agglutination assay. Twelve isolates showed agglutination and these isolates were confirmed to be *E. coli* O157:H7. The molecular weight of the Verotoxin was found to be between 20 and 29 kDa. The antibiogram profile of the four isolated strains against 10 standard antibiotics was determined.

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Ethical Considerations

Competing Interests

The authors declare no conflict of interest.

Availability of Data and Material

All relevant data of this study are available to any interested researchers upon reasonable request to the corresponding author.

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