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ORIGINAL ARTICLE

# Occurrence, virulence characteristics and antimicrobial resistance of *Escherichia coli* O157 in slaughtered cattle and diarrhoeic calves in West Bengal, India

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## Abstract

**Aims:** (i) To study the occurrence of *Escherichia coli* serotype O157 in cattle stool in West Bengal, India, and (ii) the virulence properties and antimicrobial resistance of the *E. coli* isolates.
**Methods and Results:** Following enrichment in modified EC broth and plating onto HiCrome MS.O157 agar, a total of 14 strains of *E. coli* serotype O157 was isolated from faecal samples from two (2.04%) slaughtered cattle and six (7.59%) diarrhoeic calves. By multiplex PCR, Shiga toxin genes were detected in all the isolates. The enterohaemolysin phenotype was found in all, but one strain. Among 14 strains, ten were resistant to at least one of the antimicrobial agents tested. Multiple antibiotic resistance was frequent.
**Conclusions:** The study showed that occurrence of Shiga toxin-producing and multiple antibiotic-resistant *E. coli* O157 among cattle population in this region of India is significant.
**Significance and Impact of the Study:** Considering routine human contacts with cattle, a large human population in this region may be at risk for exposure to Shiga toxin-producing *E. coli* O157.

## Introduction

Shiga toxin-producing *Escherichia coli* (STEC) have drawn wide attention as a causative agent of foodborne diarrhoea, haemorrhagic colitis (HC) and fatal haemolytic uraemic syndrome (HUS). Among various STEC serotypes, *E. coli* O157 is one of the most predominant human pathogens in many parts of the world and is commonly associated with large outbreaks (Griffin and Tauxe 1991; Armstrong *et al.* 1996; Paton and Paton 1998). In many cases, the infection has been linked to foods of cattle origin or contact with cattle (Griffin and Tauxe 1991; Armstrong *et al.* 1996). Cattle are the principal reservoir of STEC strains, including those belonging to serotype O157 (Armstrong *et al.* 1996; Chapman *et al.* 1997). Field surveys identified that about 0.3% to over 15% of cattle in North America and some parts of European and Asian countries are carriers of *E. coli* O157 (Griffin and Tauxe 1991; Armstrong *et al.* 1996; Blanco *et al.* 1996; Chapman *et al.* 1997; Sekiya 1997).

Literature on prevalence of *E. coli* O157 in India are very sparse. Excepting in sporadic cases (Gupta *et al.* 1992), *E. coli* O157 has not been identified as an aetiological agent of human diarrhoea (Bhan *et al.* 1989; Dutta *et al.* 2000; Khan *et al.* 2002a; Chattopadhyay *et al.* 2003). However, the serotype has been isolated from foods of cattle origin, namely, raw minced beef samples (9%, $n = 22$) (Dutta *et al.* 2000), beef surface swabs (3.7%, $n = 27$) and milk samples (2.4%, $n = 81$) (Manna *et al.* 2006). The human diarrhoea isolates have not been characterized (Gupta *et al.* 1992). All the food isolates were identified to carry Shiga toxin genes (Dutta *et al.* 2000; Manna *et al.* 2006), the principal virulence determinants responsible for cytotoxicity to

 

human colonic, ileal epithelial and vascular endothelial cells that lead to diarrhoea, HC–HUS and thus were potentially pathogenic. Recently, *E. coli* O157 has been identified as one of the predominant serotypes from buffalo meat (Hazarika *et al.* 2004) and bovine and ovine diarrhoea faecal samples (Wani *et al.* 2003) indicating possible spread of the pathogen in India. Considering the presence of *E. coli* O157 in foods of cattle origin but paucity of information on prevalence of this serotype in cattle, this work was undertaken to investigate the occurrence, virulence properties and antimicrobial susceptibility of *E. coli* serotype O157 in stools from slaughtered cattle and diarrhoeic calves.

## Materials and methods

### Faecal samples

All the samples were collected during March– August 2003. Rectal faecal samples ($n = 98$) were collected from adult cattle immediately after slaughter in abattoir at Kolkata, India. Diarrhoeic stool samples from calves, within about 4 months of age, were also tested. About 15–30 g of faeces was collected per rectum by finger palpation, wearing sterile gloves from two state-owned dairy cattle farms, namely Haringhata farm ($n = 24$) and Kalyani farm ($n = 17$) in Nadia district near Kolkata, and from a private farm ($n = 10$) at Bandel in Hooghly district, West Bengal, India. Faecal samples were also collected from 28 diarrhoeic calves that were brought by rural farmers to Animal Health Centres for treatment in districts of West-Midnapore and Hooghly, West Bengal. All the samples were collected in sterile polypropylene containers, transported to the laboratory under ice cover and were processed within 4 h.

### Isolation and identification of *Escherichia coli* O157

About 10 g of faecal sample was enriched in 90 ml of EC broth (DIFCO) supplemented with sodium novobiocin (18 mg $l^{-1}$) for 12–16 h at 37°C. The enriched culture medium was streaked onto HiCrome MS.O157 agar (HiMedia Laboratories, Mumbai, India) supplemented with potassium tellurite. Inoculated plates were incubated at 37°C for 24 h. Sorbitol-negative and β-D-glucuronidase-negative colourless colonies were further streaked onto Levine EMB agar (DIFCO) for isolation in pure culture. The presumptive *E. coli* isolates were identified by BIOLOG MicroLog$^{TM}$ automatic identification system. Serotyping of somatic antigen of the *E. coli* strains was carried out at National Salmonella and Escherichia Centre, Central Research Institute (CRI), Kasauli, Himachal Pradesh, India.

### Detection of enterohaemorrhagic *Escherichia coli* haemolysin

The *E. coli* O157 strains were examined for phenotypic expression of enterohaemorrhagic *E. coli* (EHEC) haemolysin (E-hly) following method described by Beutin *et al.* (1989). Briefly, bacterial isolates were streaked onto two types of nutrient agar plates supplemented with 5% unwashed Sheep RBC (SRBC), and 5% washed SRBC and 10 mmol $CaCl_2$. Characteristic narrow zone of haemolysis on the washed SRBC-containing agar plates after 16 h but not after 3 h of incubation at 37°C, combined with no haemolysis on the unwashed SRBC-containing plates was considered to be due to EHEC E-hly.

### Detection of Shiga toxin-1 and Shiga toxin-2 genes

The test strains were grown overnight in brain heart infusion broth at 37°C. Bacteria were pelleted by centrifugation at 12 000 ***g*** for 1 min at 4°C. The pellet was washed twice with phosphate-buffered saline (PBS), pH 7·4 and finally it was dispersed in sterile distilled water, vortexed and incubated at 100°C for 10 min to release the DNA. The samples were then snap-cooled in ice, centrifuged at 12 000 ***g*** for 1 min and 1-$\mu$l supernatant of each sample was used for DNA amplification. Multiplex PCR for detection of *stx1* and *stx2* (Shiga toxin genes 1 and 2) gene sequences was performed following methods and using oligonucleotide primers described by Gannon *et al.* (1992). The amplified DNA fragments were resolved by standard submarine gel electrophoresis with 1·4% (w/v) agarose gel in TBE buffer (89 mmol Tris, 89 mmol boric acid, 2·5 mmol ethylenediaminetetraacetic acid). Gels were stained with ethidium bromide (0·5 $\mu$g $ml^{-1}$) and visualized with Bio-Rad GelDoc 2000 imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

### Antimicrobial resistance determination

Antimicrobial susceptibility test was performed on Mueller-Hinton agar plates by disc diffusion method (Bauer *et al.* 1966) using antimicrobial discs from HiMedia.

## Results

### Occurrence of *Escherichia coli* O157

Examination of a total of 98 stool samples from slaughtered cattle and 79 diarrhoeic faeces from the calves revealed the presence of sorbitol-negative and β-D-glucuronidase-negative *E. coli*. Slaughtered cattle and diarrhoeic calves were sampled because previous studies indicated

that these animals are more likely to shed STEC than healthy cattle in farm condition (Suthienkul *et al.* 1990; Zhao *et al.* 1995; Blanco *et al.* 1996; Armstrong *et al.* 1996; Chapman *et al.* 1997). Serotyping of the *E. coli* isolates identified *E. coli* serotype O157 strains from two (2.04%) slaughtered cattle and six (7.59%) calves. In Kalyani farm, only one calf and in Haringhata farm, five calves were found to be excreting the pathogen in their stool. Presence of *E. coli* O157 could not be detected among diarrhoeic calves in the private farm in Bandel and those belonging to different rural farmers. Among six positive calves shedding the pathogen, four were aged between 4 and 6 weeks and the other two between 11 and 12 weeks.

### Virulence characteristics of *Escherichia coli* O157 isolates

A total of 14 strains of *E. coli* O157 were isolated in this study. By multiplex PCR, the *stx* gene sequences were detected in all the isolates: 12 (85.71%) isolates carried s*tx2* gene only and two (14.28%) isolates had both the s*tx1* and s*tx2* genes. The E-hly phenotype wasfound in all but one *E. coli* O157 strains (Table 1).

**Table 1** Virulence characteristics and antibiotic resistance of *Escherichia coli* O157 isolates

| Strain | Virulence traits | | | Antimicrobial resistance |
|---|---|---|---|---|
| | *stx1* | *stx2* | E-hly | |
| TB8* | – | + | + | G, Nf, T, Ts |
| TW23* | – | + | + | Nf, Nx T, Ts |
| TB72* | + | + | + | – |
| HC4† | – | + | + | Nf |
| HC5† | – | + | + | A, G, Nf, Ts |
| HC10† | – | + | + | A, Ch, Nf |
| HC12† | – | + | – | – |
| HC15† | – | + | + | Cf, G, Nf, Nx, Ts |
| HC16† | – | + | + | Nf, Nx, T, Ts |
| HC18† | + | + | + | – |
| HC19† | – | + | + | Nf, T, Ts |
| HC23† | – | + | + | – |
| LKC8† | – | + | + | Nf |
| LKC13† | – | + | + | A, Nf, T |

+, Virulence trait present; –, virulence trait absent.

*, Strains isolated from slaughtered cattle.

†, Strains isolated from diarrhoeic calves.

E-hly, enterohaemorrhagic *E. coli* haemolysin; *stx1* and *stx2*, Shiga toxin genes 1 and 2.

Antimicrobial agents used: A, ampicillin 10 $\mu$g; Ak, amikacin 30 $\mu$g; C, chloramphenicol 30 $\mu$g; Cf, ciprofloxacin 5 $\mu$g; Ch, cephalothin 30 $\mu$g; G, gentamicin 10 $\mu$g; Na, nalidixic acid 30 $\mu$g; Nf, nitrofurantoin 300 $\mu$g; Nx, norfloxacin 10 $\mu$g; T, tetracycline 30 $\mu$g; Ts, trimethoprim 1.25 + sulphamethoxazole 23.75 $\mu$g.

### Antimicrobial resistance

Prevalence of antimicrobial resistance among *E. coli* O157 isolates is presented in Table 1. Among three isolates from the slaughtered cattle, two were resistant to nitrofurantoin, tetracycline and co-trimoxazole. Resistance to gentamicin and norfloxacin was also recorded. Prevalence of antimicrobial resistance was also common in the calf diarrhoea isolates. Excepting amikacin, chloramphenicol and nalidixic acid resistance was recorded against all other agents. One strain isolated from slaughtered cattle and three strains from calf stools were however, susceptible to all the antimicrobial agents tested. Overall, occurrence of resistance was most frequent against nitrofurantoin (eight strains) followed by co-trimoxazole (four strains), tetracycline (three strains), ampicillin (three strains), gentamicin (two strains), norfloxacin (two strains), cephalothin and ciprofloxacin (one strain each). Ten (71.43%) isolates were resistant to at least one of the antimicrobial agents tested: two (14.28%) isolates were resistant to one antibacterial compound, three (21.43%) isolates were resistant to three compounds, four (28.57%) isolates were resistant to four compounds and one (7.14%) isolate showed resistance to five antimicrobial agents.

## Discussion

Cattle are the major reservoir of *E. coli* serogroup O157 and frequently identified as direct or indirect sources of infection to man. In the present study, two (2.04%) of the slaughtered cattle and six (7.59%) diarrhoeic calves were found to be the carriers of this serotype. Distribution frequency of positive faecal samples showed that *E. coli* O157 strains were isolated from two organized herds located in Nadia district, but none from diarrhoeic calves belonging to different farmers and private farms in the other two districts of West Bengal. This is in contrast to previous studies that could not detect presence of this serotype in a total of 428 cattle faecal samples examined from same dairy cattle farms or semiurban community near Kolkata (Pal *et al.* 1999; Khan *et al.* 2002a; Chattopadhyay *et al.* 2003). But, this supports the observation of Dutta *et al.* (2000) that a small but significant (3.28%) population of diarrhoeic calves excrete *E. coli* O157 in these state-owned farms. As this study targeted specific groups of animals most suspected for excreting the pathogen, this prevalence rate cannot be extrapolated for healthy cattle in farm or amongst farmers. Unlike in developed countries, cattle farming in India are household practice. Considering routine contacts with cattle in day-to-day life a large human population in this region may be at risk.

Compared with the adult slaughtered cattle, occurrence of *E. coli* O157 was more in diarrhoeic calves in this study.

It was more prevalent in calves of about 4–6 weeks of age. This is in agreement with earlier observations that carriage rate and shedding of *E. coli* O157 in calves and heifers is higher and longer than in adult cattle (Suthienkul *et al.* 1990; Zhao *et al.* 1995; Cray and Moon 1995; Rahn *et al.* 1997). Moreover, faecal samples from cases of gastroenteritis had higher incidence of contamination than non-diarrhoeic faecal samples (Blanco *et al.* 1996; Richards *et al.* 1998).

The most important pathogenic features of *E. coli* O157 are the production of Shiga toxins, called Stx1 and Stx2 (Paton and Paton 1998). Multiplex PCR showed that all the *E. coli* O157 strains carried the Shiga toxin gene and majority of the isolates (12; 85.71%) had only *stx2* gene. Stx2 is strongly cytotoxic to human renal microvascular endothelial cells and STEC strains producing Stx2 only are more commonly associated with serious human disease, than those producing Stx1 or both the toxins (Paton and Paton 1998). Thus, majority of the strains isolated in the present investigation were potentially dangerous to human health.

Examination of phenotypic E-hly showed that all but one of the *E. coli* O157 strains isolated in this study expressed this cell-associated haemolysin. STEC strains producing E-hly are more commonly associated with human disease than strains that lack it (Schmidt *et al.* 1994; Gyles *et al.* 1998). The exact role of enterohaemolysin in STEC infection is unknown; haemoglobin released by its action may provide a source of iron for bacterial growth in the intestine (Paton and Paton 1998).

The present study revealed high prevalence of antimicrobial resistance among the *E. coli* O157 isolates: ten (71.4%) isolates were resistant to one or more antibiotics tested. Even higher prevalence of antibacterial resistance has been documented among non-O157 STEC isolates from the same dairy cattle farms (Khan *et al.* 2002b; Chattopadhyay *et al.* 2003). Likely explanation for this high resistance is the frequent and nonjudicious therapeutic use of antimicrobial compounds in the cattle in diarrhoea and other ailments. Oxytetracycline, gentamicin, co-trimoxazole and ampicillin are the most frequently used antibacterials in the region and as expected, prevalence of resistance was high against all of them. Resistance against norfloxacin and ciprofloxacin was also recorded. This is in contrast to an earlier report (Dutta *et al.* 2000) on the same dairy farms where all the calf *E. coli* O157 isolates were sensitive to gentamicin, norfloxacin and ciprofloxacin indicating that the bacteria have gained resistance against these drugs in the last 5 years or so. This is a possible consequence of frequent use of norfloxacin, enrofloxacin and other quinolone derivatives in cattle, swine and poultry husbandry, as well in human practices. High prevalence of antimicrobial resistance may confer advantage to the resistant *E. coli* O157 strains in colonizing the intestinal tract of cattle and thereby increasing the incidences of faecal shedding during such antimicrobial therapy. Moreover, resistance factors may be transferred to susceptible population conferring resistance to numerous antimicrobials.

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