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Environmental isolates of *Citrobacter braakii* that agglutinate with *Escherichia coli* O157 antiserum but do not possess the genes responsible for the biosynthesis of O157 somatic antigen

A. KHAN[1], R. K. NANDI[1], S. C. DAS[2], T. RAMAMURTHY[1], J. KHANAM[3], T. SHIMIZU[4], S. YAMASAKI[5], S. K. BHATTACHARYA[1], W. CHAICUMPA[6], Y. TAKEDA[7] AND G. BALAKRISH NAIR[1,8]*

[1] *National Institute of Cholera and Enteric Diseases, Calcutta, India*
[2] *Indian Veterinary Research Institute, Belgachia, Calcutta, India*
[3] *Jadavpur University, Jadavpur, Calcutta, India*
[4] *Institute of Basic Medical Sciences, University of Tsukuba, Japan*
[5] *Department of Veterinary Sciences, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Japan*
[6] *Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*
[7] *Faculty of Human Life Sciences, Jissen Women's University, Tokyo 181-8510, Japan*
[8] *International Centre for Diarrhoeal Disease Research, Bangladesh* (*ICDDR,B*) *Dhaka, Bangladesh*

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SUMMARY

While searching for *Escherichia coli* O157 in the aquatic environment of Calcutta using an immunodetection procedure, we fortuitously detected five strains of *Citrobacter braakii*, which cross-reacted with the commercially available O157 polyvalent antiserum. The five *C. braakii* isolates gave positive results when a sensitive dot-ELISA was performed with *E. coli* O157 monoclonal antibody. Further, the O157 monoclonal antibody recognized the bands of proteinase K treated whole cells of lipopolysaccharide of all the *C. braakii* isolates. Apart from weak reactions with two or three of the DNA probes, all the *C. braakii* strains did not hybridize with the other probes spanning the minimum region required for O157 O-antigen biosynthesis. These strains did not possess any of the virulence genes that are commonly found in the Shiga toxin-producing *E. coli* (STEC) specially the serotype O157:H7. Therefore, it appears that the serological cross-reaction between *C. braakii* and *E. coli* O157 antiserum is based on structural mimicry between the O-polysaccharide of *C. braakii* and *E. coli* O157.

INTRODUCTION

Escherichia coli is a genetically and phenotypically diverse species with serotypes normally being identified by their combination of 'O' and 'H' (and sometimes 'K') antigens [1]. Some *E. coli*, especially strains belonging to the serotype O157:H7, are capable of causing severe and fatal illness in humans and as a result have gained extensive publicity due to recent outbreaks [2]. The clinical manifestations of consuming foods contaminated with *E. coli* O157:H7 range from haemorrhagic colitis (HC) to life-threatening systemic complications, including haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Surveillance data indicate that the epidemiology of enterohaemorrhagic *E. coli* (EHEC) O157 infection is evolving. The geographic range of occurrence of the organism is growing and there is evidence of changing patterns of disease transmission [3]. Vehicles of Shiga toxin-producing *E. coli* (STEC)

* Author for correspondence: Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka-1212, Bangladesh.

infection continue to be identified, and the massive water-borne outbreak, points to environmental contaminations as major risk factors [4].

Though only few strains belonging to the *E. coli* O157 serotype have been isolated in India from sporadic cases of diarrhoea [5], these strains have not been characterized in detail and the origin of these isolates remain uncertain. In this context, we initiated a comprehensive study to understand the prevalence of *E. coli* O157:H7 and other STEC in Calcutta, India. Our study showed STEC strains were present in bovine sources and in beef samples in Calcutta but were infrequently isolated from human diarrhoea cases (1·4 and 0·7% from watery and bloody diarrhoea cases, respectively) and therefore were not construed as an important enteric pathogen among humans in this part of the world [6]. Further, in this survey, we could not isolate *E. coli* O157:H7 strains but isolated a variety of STEC isolates belonging to diverse serogroups. In this study, we extend our previous investigation to search for O157 strains of *E. coli* in the aquatic environs of Calcutta using an antibody-based method in an effort to understand if there are any reservoirs of the O157 strains of *E. coli* in the environment.

MATERIALS AND METHODS

The present study was conducted in the eastern part of the city of Calcutta (longitude 88° 20′ E, latitude 22° 33′ N). Water samples were examined from the following sites: (i) two fresh-water ponds, located in the eastern part of the city in Tangra, used by the population residing in its vicinity for different purposes including washing clothes and bathing, (ii) four fresh-water ponds located in Topsia and (iii) a fresh-water lake used by humans but which does not receive any industrial effluents.

The study was carried out over a period of 2 months from January to February 2001. Samples were collected twice every month from all the sites. After collecting the samples, 100 ml of water samples was prefiltered using a filter paper (Grade 3; size 90 mm; thickness 0·39; Whatman, USA) and then passed through a 0·22 μm membrane (Millipore Corporation, Bedford, MA, USA). The membrane was aseptically transferred to 10 ml Luria Broth (LB) (Difco, USA) and incubated at 37 °C overnight with agitation. After incubation, the enriched broths were serially diluted in 10 ml PBS (pH 7). A 100 μl volume of each dilution was spread on Luria Agar (Difco) plates in duplicate followed by overnight incubation at 37 °C. On the next day, the colonies from the plates (which contained around 300 colonies) were transferred on to a Hybond nylon membrane (Amersham, USA). The membranes were subjected to screening for *E. coli* O157 by an antigen based screening method using O157 polyclonal antibody (Difco). The colonies on the Hybond membrane were denatured by adding 1% SDS and then kept at 70 °C in a water bath. The unbound area of the membrane was blocked using 3% (w/v) bovine serum albumin phosphate buffered saline (BSA-PBS) solution containing 0·002% sodium azide. The membrane was then washed with PBS solution and incubated with appropriately diluted (1:100) O157 antiserum (Difco). This was followed by washing the membrane with PBS and adding of anti-rabbit peroxidase conjugate (Sigma, USA). The O157 colonies appeared as dark spots when the colour-developing reagent, 3,3′-diaminobe iodine (Sigma) was added. A human O157:H7 strain harbouring *stx1* and *stx2* genes VTEC3 and a K12 *E. coli* strain of DH5α were used as positive and negative controls, respectively.

Motility of the positive colonies in the antigen-based screening method was tested using motility test medium (BBL, MD, USA). For *E. coli* O157 antigen determination, slide agglutination test was performed using commercially available O157 (polyvalent) and H (H2, H4–7, H9, H10–12, H16, H18–21, H27, H28, H34, H40–42, H45 and H51) antisera (Denka Seiken Co., Japan). For O157 antigen detection, a highly sensitive and specific dot-ELISA assay (SDM, Thailand) based on monoclonal antibody (Mab) to lipopolysaccharide (LPS) of *E. coli* O157 was applied according to the manufacturer's instruction. In brief, environmental isolates were inoculated in 3 ml LB broth (Difco) and incubated for 6 h at 37 °C temperature under shaking condition. After incubation, 500 μl of each sample were taken and boiled for 20 min. Three microlitres of the boiled sample were taken and spotted on the nitrocellulose membrane provided by the manufacturer and air-dried for 5 min. After that the membrane was covered with a blocking solution for 10 min, the membrane was washed three times with Buffer A solution and incubated with the Mab for 30 min. After incubation, the membrane was washed three times with Buffer A solution. This was followed by addition of rabbit antimouse immunoglobin-biotin conjugate (Dako, Japan) (1:2000 dilution), incubated for 20 min and membrane washed twice with Buffer A and once with Buffer B solutions. Subsequently, the membrane was incubated with a substrate solution (1:2 dilution) and left in the dark for

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5 min. O157 LPS antigen positive samples appeared as purplish-blue spots while negative samples appeared as spots of brown or other non-specific colour or as clear areas.

The biochemical characterization and identification of the environmental isolates, which reacted with the O157 antiserum, were performed using a commercially available API-20E (BioMérieux, France) identification system, which uses 23 miniaturized biochemical tests. Interpretation of the results was done by referring to the identification table and matching with the analytical profile index.

For 16S ribosomal DNA (rDNA) sequencing, chromosomal DNA was extracted using Prep Man, Sample Preparation Reagent and prepared according to the manufacturer's protocol (PE Applied Biosystems, Foster City, CA, USA). Extracted DNA was used as the template for PCR amplification using MicroSeq 500, 16S rDNA PCR Module (PE Applied Biosystems). A 500-bp 16S-rDNA fragment was amplified from the 5′ end of the gene in a reaction volume of 50 μl (25 μl of MicroSeq PCR master mix, 24 μl of sterile water and 1 μl of chromosomal DNA). The amplified PCR product of 500 bp was purified with Microcon 100 microconcentrators (Amicon Inc., Beverly, MA, USA) as recommended by the manufacturer and then subjected to 16S rDNA sequencing on an automated DNA sequencer (API Prism 310). Forward and reverse sequencing reactions were performed for each amplified product. The sequence reactions consisted of 13 μl of MicroSeq sequencing mix, 4 μl of sterile molecular grade water and 3 μl of purified amplified product. Nucleotide sequences generated were aligned and analysed for identification of bacterial species using MicroSeq Analysis Software V.1.40 (PE Applied Biosystems). The database comparison, using the Full Alignment Tool of the MicroSeq software, generated a list of the closest matches with a distance score. This distance score indicated the percent difference between the unknown sequence and the database sequence. For the purpose of comparing an isolate's original identification to its MicroSeq identification, the MicroSeq identity was considered to be the closest match in the MicroSeq database no matter what the distance score was.

LPS profiles of these environmental isolates were analysed by SDS–PAGE of proteinase K-treated [7] whole cell lysates followed by silver staining [8] of the electrophoresed material. An *E. coli* O157:H7 strain (VTEC3) and a clinical strain of *Citrobacter* spp. isolated from a patient with acute diarrhoea were taken as positive and negative control strains, respectively. Immunochemical detection of O157 antigen in the LPS preparation of the environmental strains was carried out by immunoblotting experiments. LPS separated by SDS–PAGE were blotted on to Trans-Blot, Transfer Medium (Bio-Rad) by use of Semi dry Transfer Cell (Bio-Rad). Following the transfer, the blotted membrane was sequentially treated with monoclonal anti-O157 serum, followed by appropriately diluted alkaline phosphatase-labelled conjugate and developed with colour-developing solution.

The DNA probes for O-antigen biosynthesis genes were prepared by PCR on the basis of the recently published nucleotide sequence [9]. The PCR was done in a 50 μl reaction volume containing 10 μl heat-treated overnight culture of enterohaemorrhagic *E. coli* O157:H$^-$ (strain 184), 20 pmol of each primers (Table 1), 5 μl of 2·5 mM dNTP, 5 μl of 10× PCR buffer, and 2 U of r*Taq* DNA polymerase (Takara, Tokyo, Japan). The probes whose sizes are more than 1·5 kb were amplified under the following conditions: 94 °C for 30 s, 52 °C for 30 s and 72 °C for 150 s and this cycle was repeated 30 times after 94 °C for 5 min. The probes whose sizes were smaller than 1·5 kb were amplified in exactly the same way as described above except for the extension time such as 72 °C for 60 s instead of 150 s. The amplicons were analysed by 0·7 or 2% agarose gels and purified by a PCR products purification kit according to the instruction manual provided by the manufacturer (Qiagen). The DNA was labelled by the random priming method using the Multiprime DNA labelling system (Roche) and [α-^{32}P]dCTP (NEB). A colony hybridization test was done following the protocol described previously. The spot hybridized with the radiolabelled probe was analysed by BAS-2000 (Fuji Co. Ltd, Japan).

PCR for detecting both chromosomal (*stx1*, *stx2*, *eae*) and plasmid (*katP*, *espP*, *EtpD*, *hlyA*) encoded virulence genes of O157 *E. coli* strain were performed as described previously [6, 10]. The primer sequences and PCR conditions are given in Table 2. A highly sensitive bead ELISA for production of Stx1 and Stx2 and a Vero cell assay for cytotoxic effect were performed as described previously [6, 11].

To perform pulsed field gel electrophoresis (PFGE), the genomic DNA of the environmental isolates, which reacted with the O157 antiserum, was prepared in agarose plugs as described previously [12]. Agarose blocks containing genomic DNA were equilibrated in restriction enzyme buffer for 1 h at room temperature. For complete digestion of the DNA, 50 U of *Not*I

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Table 1. *PCR primer sequences used for preparing DNA probes used for determining presence of the O157 somatic antigen region*

Primer no.	Nucleotide sequence of primers*	Target
P763/P808	5′-TCTCTCCTTGAACAGCG-3′ 5′-GTCTGGATGACGGAGTA-3′	*galF*
P875/P762	5′-GGCCACGGGGAAATATA-3′ 5′-AAATATATTGCATTTTCCGC-3′	*wbdN*, *wzy*, *wbdO*
P761/P737	5′-GTGCTTTAGGTAGAGAAC-3′ 5′-CATCGAAACAAGGCCAG-3′	*wzx*, *per*
P783/P699	5′-GCGATTGTGATGTTATTCT-3′ 5′-ACAACTTCAGCTGGTCG-3′	*wbdP*, *gmd*
P710/P774	5′-GGGAAACTTGAGCCAAC-3′ 5′-GGAAAACTTCACGATGAC-3′	*fcl*, *wbdQ*, *manC*
P849/P883	5′-CGTCGATGTGCTGGATA-3′ 5′-CGTCTTCCTTGCGCATA-3′	*manB*
P742/P856	5′-ACTGTTTCTAAAAGCCCC-3′ 5′-ACATTTAGCCCCAGGAG-3′	*wbdR*
P854/P733	5′-CTGGTTCCTTACTATACG-3′ 5′-CGGCAATTTGCTTGAAGT-3′	*gnd*

* Primer sequences were based on those published by Shimizu et al. [9].

(Takara, Shuzo Co. Ltd, Japan) was used. The plugs were incubated at 37 °C overnight with restriction enzyme in fresh restriction enzyme buffer. PFGE of the *Not*I digested inserts was performed by the contour-clamped homogeneous electric field method on a CHEF mapper system (Bio-Rad, CA, USA) with 1% PFGE grade agarose in 0·5 × TBE (44·5 mM Tris HCl, 44·5 mM boric acid, 1·0 mM EDTA, pH 8·0) for 40 h 24 min with the autoalgorithm mode.

RESULTS

Of the 28 water samples examined from different water bodies in the Eastern part of the city of Calcutta, 6 colonies from 5 different water samples examined on different dates gave a brownish hue on the membrane using the O157 antibody-based immunodetection method. These colonies were picked and subcultured on Luria agar plates and checked for agglutination with a polyvalent O157 antiserum. All of the isolates gave the typical spontaneous agglutination reaction with O157 antiserum. A control strain of *E. coli* O157 (VTEC-3) was also tested at the same time to compare agglutination reactions. Although the six strains were motile, they did not react with any of the *E. coli* H antisera tested, including H7. All the six strains appeared as purplish-blue spots on the nitrocellulose membrane when the sensitive dot-ELISA (SDM, Thailand) was performed using *E. coli* O157 monoclonal antibody. The positive control strain (VTEC3) developed a purplish-blue spot while the negative control strain (DH5α) showed a clear area.

Since other non-*E. coli* organisms, which express the O157 antigen, have been identified, further biochemical or toxigenicity testing has been recommended to confirm the positive results of the screening test [13]. Therefore, the API-20E system was used to identify the six environmental strains, which agglutinated with the O157 polyvalent antiserum. The API-20E test results were unable to specifically identify these environmental strains. Of the 6 strains, 5 strains (EAK-2, EAK-3, EAK-4, EAK-5 and EAK-6) gave the same type of response in the biochemical tests while EAK-1 responded differently to some of the biochemical tests (Table 3). Of the 6 strains, 4 strains (EAK-1, EAK-2, EAK-4 and EAK-6) were chosen for precise identification using the 16S rDNA sequence and data analysis system. Of the 4 strains, 3 strains (EAK-2, EAK-4 and EAK-6) grouped within the cluster of *Citrobacter braakii* (Fig. 1) and therefore the strains were identified as *C. braakii*. The remaining strain EAK-1 was identified as *E. coli* based on the match of the 16S rDNA sequence when compared with the database (data not shown).

The LPS profile of the proteinase K-treated whole cells of the *C. braakii* revealed that the profile of LPS of EAK-2, EAK-3, EAK-4, EAK-5 and EAK-6 were

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Table 2. *PCR primers and conditions used in this study*

Primer no.	Nucleotide sequence of primers	PCR conditions*				Amplicon (bp)	Ref.
		Target	Denaturing	Annealing	Extension		
EVT1/EVT2	5′-CAACACTGGATGATCTCAG-3′ 5′-CCCCCTCAACTGCTAATA-3′	*stx1* family	94 °C, 60 s	55 °C, 60 s†	72 °C, 60 s	349	[20]
EVS1/EVC2	5′-ATCAGTCGTCACTCACTGGT-3′ 5′-CTGCTGTCACAGTGACAAA-3′	*stx2* family	94 °C, 60 s	55 °C, 60 s†	72 °C, 60 s	110	[20]
hlyA1/hlyA4	5′-GGTGCAGCAGAAAAAGTTGTAG-3′ 5′-TCTCGCCTGATAGTGTTTGGTA-3′	EHEC *hlyA*	94 °C, 30 s	57 °C, 60 s	72 °C, 90 s	1551	[21]
wkat-B/wkat-F	5′-CTTCCTGTTCTGATTCTTCTGG-3′ 5′-AACTTATTTCTCGCATCATCC-3′	*katP*	94 °C, 30 s	56 °C, 60 s	72 °C, 150 s	2125	[22]
D1/D13R	5′-CGTCAGGAGGATGTTCAG-3′ 5′-CGACTGCACCTGTTCCTGATTA-3′	*etpD*	94 °C, 30 s	52 °C, 60 s	72 °C, 70 s	1062	[23]
EAE1/EAE2	5′-AAACAGGTGAAACTGTTGCC-3′ 5′-CTCTGCAGATTAACCTCTGC-3′	*eae*	94 °C, 60 s	55 °C, 90 s	72 °C, 90 s	350	[24]

* Unless stated, PCR was done for 30 cycles.
† After 35 cycles, final extension step of 10 min at 72 °C was performed.

identical while the LPS profile of *E. coli* EAK-1 was different from the others. LPS profiles of all the six environmental strains were different from that of VTEC-3 and also from the clinical *Citrobacter* spp. strain used for comparison. In the immunoblot assay, the O157 monoclonal antibody strongly recognized the bands of LPS of proteinase K-treated whole cells of all the environmental strains.

The *wbdN* to *wbdR* genes are the minimal essential region for O157 O-antigen biosynthesis while the *galF*, *manB* and *gnd* genes react with most O serogroups in *E. coli* [9]. To see whether the *C. braakii* strains harboured O157 biosynthesis genes, a colony hybridization test using eight probes specific for various regions of the O157 biosynthetic genes were performed. In the hybridization assay, *C. braakii* strain EAK-2 reacted with probes 1, 6 and 8 while *C. braakii* strains EAK-3, EAK-4, EAK-5 and EAK-6 reacted with probes 6 and 8 (Table 4), indicating that these strains did not possess the unique O157 O-antigen biosynthesis genes although these strains agglutinated with O157 antiserum.

However, EAK-1, which was identified as *E. coli* in 16S-rDNA analysis, also included in the colony hybridization test using eight probes. As expected, EAK-1 reacted with all probes (Table 4) indicating that this strain harbours the entire region encoding the O157 O-antigen biosynthesis region.

The *C. braakii* strains and *E. coli* strain (EAK-1), which agglutinated with the O157 antiserum, did not possess any of the virulence genes usually associated with *E. coli* O157 : H7. All the five *C. braakii* strains and *E. coli* EAK-1 examined were negative for the chromosomally encoded *stx1*, *stx2* and *eae* genes or for the plasmid encoded virulence genes, *katP*, *etpD* and *hlyA*. All the *C. braakii* strains and *E. coli* O157 strain did not elaborate any toxins when a highly sensitive bead-ELISA assay was applied to detect the presence of Stx1 and Stx2 in the cell-free culture filtrates and cell lysates. None of the *C. braakii* strains or the strain EAK-1 exhibited a cytotoxic response when the strains were tested for their ability to produce Vero cell cytotoxin in the Vero cell assay. Thus, the *C. braakii* strains as well as the *E. coli* O157 strain did not express any Shiga-like toxins or any verocytotoxic toxin.

A PFGE of *Not*I-digested genomic DNA of the environmental strains was performed to determine the extent of genetic relationships among the *C. braakii* strains and between *C. braakii* and *E. coli* O157. The PFGE profiles showed that all the strains differed from each other (Fig. 2).

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Table 3. *Biochemical test reactions of the environmental strains that cross-reacted with* E. coli *O157 antiserum*

	Strain number					
Biochemical test	EAK-1	EAK-2	EAK-3	EAK-4	EAK-5	EAK-6
Ornithine decarboxylase	+	−	−	−	−	−
Citrate utilization	−	+	+	+	+	+
H_2S production	−	+	+	+	+	+
Sorbitol fermentation	−	+	+	+	+	+
Amygdaline fermentation	−	+	+	+	+	+

All the strains were positive for β-galactosidase, acetoin production and glucose, manitol, rhamnose, sucrose, melibiose and arabinose fermentation. All the strains were negative for arginine dihydrolase, lysine decarboxylase, urease, tryptophane desaminase, indole production, gelatinase, cytochrome oxidase and inositol, amygdaline fermentation.

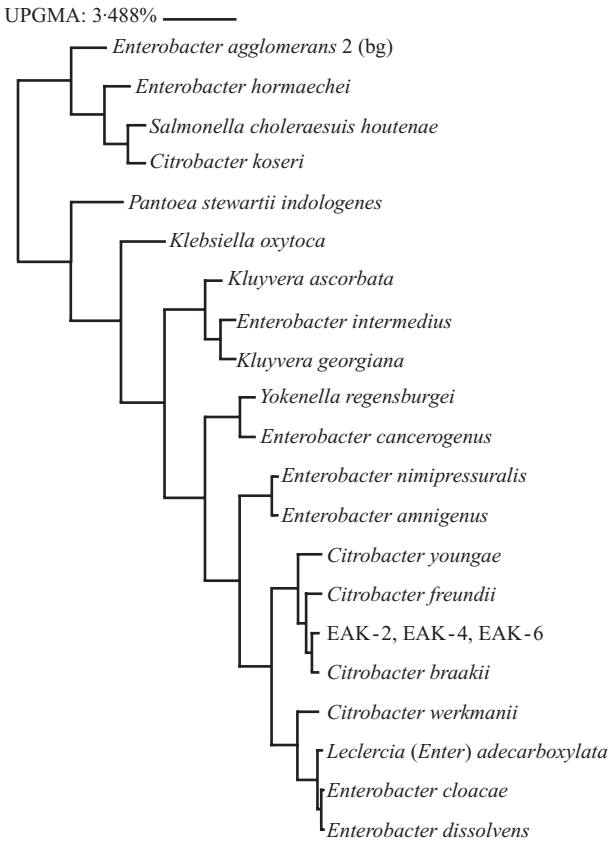

Fig. 1. Species identification by 16S sequencing (500 bp) of the environmental strains, which cross-reacted with O157 antiserum and could not be identified by phenotypic methods. Three (EAK-2, EAK-4, EAK-6) of the four strains, which were analysed, clustered with *C. braakii* strain when compared with 16S sequences in the MicroSeq database.

DISCUSSION

Cross-reactivity to O157 polyclonal antisera has been reported for *Citrobacter freundii* [14], *Yersinia enterocolitica* [15], *Pseudomonas maltophilia*, *Brucella abortus* [16], *Brucella melitensis*, *Escherichia hermannii*, *Hafnia alvei*, *Morganella morganii* and *Salmonella* group N [17]. In this study, while searching for *E. coli* O157 in the aquatic environs of Calcutta, we fortuitously detected strains of *C. braakii*, which cross-reacted with the commercially available O157 polyvalent antiserum using an immunodetection procedure. We rationalized that there could be two reasons for the cross-reaction of the *C. braakii* isolates with O157 polyvalent antiserum. One could be that the *C. braakii* isolates had an identical gene cluster as O157 *E. coli* for the synthesis of O157 antigen. The other reason could be that the LPS O-polysaccharide antigen of *C. braakii* structurally mimics that of O157. The former was a tangible possibility given that the minimum region required for O157 O-antigen expression has a GC content significantly lower than the average GC content of the *E. coli* chromosome excluding the *manC* region [9] indicating that the O157 O-antigen gene cluster was acquired by transfer from another species. The chemical composition of each O antigen is a reflection of the unique DNA sequences that are collectively responsible for the synthesis of this polysaccharide [18]. A thorough analysis of genes necessary for O-antigen synthesis has identified gene sequences unique to *E. coli* O157:H7 [1, 9, 18]. We used a set of 8 PCR-generated probes spanning the O157 O-antigen synthesis gene cluster, which is comprised of 12 genes [9], to examine the *C. braakii* strains. Our results showed that apart from weak reactions with one or two of the DNA probes, all the *C. braakii* strains did not hybridize with the probes spanning the minimum region required for O157 O-antigen biosynthesis. Therefore, it appears that the serological cross-reaction between *C. braakii* and *E. coli* O157 antiserum is based on structural mimicry and these strains do not possess all the genes necessary for the biosynthesis of the O157 antigen [9].

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Table 4. *Characterization of O157* rfb *region of the* C. braakii *isolates*

	DNA probes representing O157 *rfb* region (probe no.)							
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Strains	*galF*	*wbdN*, *wzy*, *wbdO*	*wzx*, *per*	*wbdP*, *gmd*	*fcl*, *wbdQ*, *manC*	*manB*	*wbdR*	*gnd*
E. coli								
EAK-1	+	+	+	+	+	+	+	+
C. braakii								
EAK-2	W+*	−	−	−	−	+	−	W+
EAK-3	−	−	−	−	−	W+	−	W+
EAK-4	−	−	−	−	−	W+	−	W+
EAK-5	−	−	−	−	−	+	−	W+
EAK-6	−	−	−	−	−	+	−	W+

* W+, weak reaction.

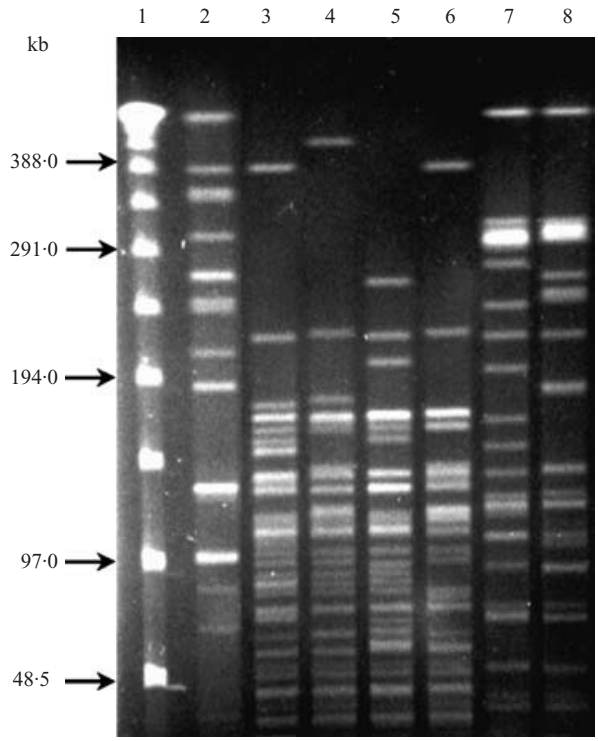

Fig. 2. PFGE profile of *Not*I-digested chromosomal DNA of *C. braakii* and O157 *E. coli* strain. NEB 48·4 kb marker (lane 1), EAK-1 (lane 2), EAK-3 (lane 3), EAK-4 (lane 4), EAK-5 (lane 5), EAK-6 (lane 6), O157:H7 serotype human origin strain VTEC1, harbouring *stx1* gene (lane 7), VTEC3 (lane 8).

While examining serological cross-reaction between the LPS O-polysaccharide antigens of *E. coli* O157:H7 and strains of *C. freundii* and *C. sedlakii*, Vinogradov et al. [19] demonstrated that the 2-substituted α-D-Rhap residue in *C. freundii* O-polysaccharide (O-PS) structurally mimics the 2-substituted 4-acetamido 4,6-dideoxy-α-D-mannopyranosyl residue (α-D-Rhap 4NAc residue) in the *E. coli* O157 O-PS. There appears to be a similar structural mimicry between the O-PS of *C. braakii* and *E. coli* O157, which results in the serological cross-reaction. According to Perry and Bundle [16], the serological and biochemical basis of cross-reactivity between O157 antiserum and other bacteria is the presence of 4-amino 4,6-dideoxy-α-D-mannopyranosyl as a constituent sugar of the LPS. Further, structural studies are required to determine the constituent sugars of the LPS of *C. braakii*, which brings about this structural mimicry. The dissimilarity in the LPS profile of *E. coli* O157 and *C. braakii* and the absence of the usual virulence genes associated with *E. coli* O157 provided additional evidence that the *C. braakii* strains did not in any way resemble *E. coli* O157 apart from structural mimicry. However, it was interesting to note that the LPS moieties of *C. braakii* reacted with the monoclonal O157 antibody. This study also shows that an immunodetection method should be approached with caution for the detection of *E. coli* O157 in the environment or in foods because of the presence of cross-reacting bacterial flora, specially the *Citrobacter* spp.

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REFERENCES

1. Wang L, Reeves PR. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. Infect Immun 1998; **66**: 3545–51.
2. Tarr PI. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin Infect Dis 1995; **20**: 1–8.
3. Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin producing *Escherichia coli* infections. Clin Micro Rev 1998; **11**: 450–79.
4. Swerdlow DL, Woodruff BA, Brady RC, et al. A water borne outbreak in Missouri of *Escherichia coli* O157:H7, associated with bloody diarrhea and death. Ann Intern Med 1992; **17**: 812–9.
5. Gupta S, Soni NK, Kaur P, et al. Verocytopathic activity of *Escherichia coli* O157 and other 'O' serogroups isolated from patients of diarrhea. Indian J Med Res 1992; **95**: 71–6.
6. Khan A, Yamasaki S, Sato T, et al. Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin producing *Escherichia coli* isolated from cattle, beef and human cases in Calcutta, India. Emerging Infect Dis 2002; **8**: 54–62.
7. Hitcheock PJ, Brown TM. Morphological heterogenicity among *Salmonella* lipopolysaccharide chemotypes in silver stained polyacrylamide gels. J Bacteriol 1983; **154**: 269–77.
8. Tsai CM, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 1982; **119**: 115–9.
9. Shimizu T, Yamasaki S, Tsukamoto T, et al. Analysis of the genes responsible for the O-antigen synthesis in enterohaemorrhagic *Escherichia coli* O157. Microb Pathog 1999; **26**: 235–47.
10. Schmidt H, Geitz C, Phillips IT, et al. Non-O157 pathogenic Shiga toxin producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. J Infect Dis 1999; **179**: 115–23.
11. Yamasaki S, Lin Z, Shirai H, et al. Typing of verotoxins by DNA colony hybridization with poly- and oligonucleotide probes, a bead-enzyme-linked immunosorbent assay, and polymerase chain reaction. Microbiol Immunol 1996; **40**: 345–52.
12. Zhao S, White DG, Ge B. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl Environ Microbiol 2001; **67**: 1558–64.
13. Park CH, Martin EA, White EL. Isolation of a non-pathogenic strain of *Citrobacter sedlakii* which expresses *Escherichia coli* O157 antigen. J Clin Microbiol 1998; **36**: 1408–9.
14. Bettelheim KA, Evangelidis H, Pearce JL, et al. Isolation of a *Citrobacter freundii* strain, which carries the *Escherichia coli* O157 antigen. J Clin Microbiol 1993; **31**: 760–1.
15. Chart H, Okubadejo OA, Rowe B. The serological relationship between *Escherichia coli* O157 and *Yersinia enterocolitica* O9 using sera from patients with brucellosis. Epidemiol Infect 1991; **108**: 77–85.
16. Perry MB, Bundle DR. Antigenic relationships of the lipopolysaccharides of *Escherichia hermanii* strains with those of *Escherichia coli* O157:H7, *Brucella melitensis* and *Brucella abortus*. Infect Immun 1990; **58**: 1391–5.
17. Rice EW, Sowers EG, Johnson CH, et al. Serological cross-reactions between *Escherichia coli* O157 and other species of the genus *Escherichia*. J Clin Microbiol 1992; **30**: 1315–6.
18. Maurer JJ, Schmidt D, Petrosko P, et al. Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. Appl Environ Microbiol 1999; **65**: 2954–60.
19. Vinogradov E, Conlan JW, Perry MB. Serological cross-reaction between the lipopolysaccharide O-polysaccharide antigens of *Escherichia coli* O157:H7 and strains of *Citrobacter freundii*, and *Citrobacter sedlakii*. FEMS Microbiol Lett 2000; **190**: 157–61.
20. Pal A, Ghosh S, Ramamurthy T, et al. Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi-urban community in Calcutta, India. Indian J Med Res 1999; **110**: 83–5.
21. Schmidt H, Beutin L, Karch H. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect Immun 1995; **63**: 1055–61.
22. Brunder W, Schmidt H, Karch H. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mol Microbiol 1997; **24**: 767–78.
23. Schmidt H, Henkel B, Karch H. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. FEMS Microbiol Lett 1997; **148**: 265–72.
24. Yu J, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157:H7. Mol Microbiol 1992; **6**: 411–7.