Antibiotic Resistance, Virulence Gene, and Molecular Profiles of Shiga Toxin-Producing *Escherichia coli* Isolates from Diverse Sources in Calcutta, India

Asis Khan,¹ S. C. Das,² T. Ramamurthy,¹ A. Sikdar,² J. Khanam,³ S. Yamasaki,⁴
Y. Takeda,⁵ and G. Balakrish Nair^{1,6*}

National Institute of Cholera and Enteric Diseases, Beliaghata,¹ Eastern Regional Station, Indian Veterinary Research Institute, Belgaichia,² and Jadavpur University, Jadavpur,³ Calcutta, India; Department of Veterinary Sciences, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Osaka,⁴ and Faculty of Human Life Sciences, Jissen Women's University, Tokyo,⁵ Japan; and International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh⁶

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Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin-producing *Escherichia coli* (STEC) non-O157 strains isolated from human stool samples, cow stool samples, and beef samples over a period of 2 years in Calcutta, India, were determined. Resistance to one or more antibiotics was observed in 49.2% of the STEC strains, with some of the strains exhibiting multidrug resistance. The dominant combinations of virulence genes present in the strains studied were *stx*₁ and *stx*₂ (44.5% of strains) and *stx*₁, *stx*₂, and *hlyA* (enterohemorrhagic *E. coli* hemolysin gene) (19% of strains). Only 6.4% of the STEC strains harbored *eae*. The diversity of STEC strains from various sources was assessed by random amplification of polymorphic DNA (RAPD). STEC strains that gave identical or nearly similar DNA fingerprints in RAPD-PCR and had similar virulence genotypes were further characterized by pulsed-field gel electrophoresis (PFGE). Identical RAPD and PFGE profiles were observed in four sets of strains, with each set comprising two strains. There was no match in the RAPD and PFGE profiles between strains of STEC isolated from cows and those isolated from humans. It appears that the clones present in bovine sources are not transmitted to humans in the Calcutta setting although these strains showed evolutionary relatedness. Maybe for this reason, STEC has still not become a major problem in India.

During the past decade, Shiga toxin-producing *Escherichia coli* (STEC) has evolved from a clinical novelty to a global public health concern. STEC infections have been reported from over 30 countries and can cause a spectrum of human illness ranging from symptom-free carriage to severe bloody diarrhea and even life-threatening sequelae such as hemolytic-uremic syndrome (HUS) (4, 31). STEC is a serologically diverse group of food-borne, zoonotic pathogens, of which the serotype O157:H7 has been epidemiologically significant worldwide because of its notoriety of being associated with life-threatening disease (18). However, in some geographic areas, non-O157 strains are more commonly isolated from persons with diarrhea or HUS than are O157 STEC strains (40).

The ability of STEC to cause serious disease in humans is related to the production of one or more Shiga-like toxins (Stx1, Stx2, or their variants), which inhibits protein synthesis of host cells, thus leading to cell death (26, 27). Additional virulence factors—including the presence of a pathogenicity island designated the locus of enterocyte effacement (LEE) and especially *eae*, one of the constituent genes of LEE, which is responsible for attaching and effacing lesions (17, 28)—are also shown to be necessary for STEC infection. The large plasmid of STEC O157 carries determinants characteristic for STEC that presumably harbor additional virulence factors:

hlyA (the enterohemorrhagic *E. coli* hemolysin gene), which acts as a pore-forming cytotoxin on eukaryotic cells (38); the bifunctional catalase peroxidase (KatP) (6); the *etp* gene cluster (36); and the secreted serine protease (EspP) (5, 35).

Cattle and sheep are the primary reservoirs of STEC, while other animals such as deer, horses, dogs, and birds are recognized as a major risk factor (1). However, newer vehicles of STEC infection continue to be identified, and the massive waterborne outbreak in Missouri points to environmental contamination and intensive farming as major risk factors (41). Rural drinking water and recreational water are increasingly being recognized as important vehicles (7).

Epidemiological investigation of STEC is complicated by its ubiquitous nature and lack of heterogeneity between clonal strains. The discriminatory capacity of many methods used to subtype strains of *E. coli* causing enterohemorrhagic disease is restricted by clonal strains that are responsible for the majority of cases of infection. The phenotypic and genotypic methods are particularly useful in this context, maximizing the likelihood of detecting the often minimal amount of variation between strains (2, 11, 13, 33, 35). These typing methods are of great use to identify the sources and routes of transmission of organisms and make intervention strategies. In this study, one of our main objectives was to determine the relatedness of STEC strains isolated from different sources and thereby obtain clues on whether transmission occurs between human and bovine sources in our setting. This represents the first systematic study of strains of STEC in India, where these organisms are still not a potential cause of human diarrhea and do not

* Corresponding author. Mailing address: Laboratory Sciences Division, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka-1212, Bangladesh. Phone: 880-2-9886464. Fax: 880-2-8823116. E-mail: gbnair@icddr.org.

TABLE 1. PCR primers and conditions used in this study

Primer	Nucleotide sequence	Target	PCR conditions ^a			Amplicon size (bp)	Reference
			Denaturing	Annealing	Extension		
EVT1	5'-CAAACTGGATGATCTCAG-3'	<i>stx</i> ₁ family	94°C, 60 s	55°C, 60 s ^b	72°C, 60 s	349	29
EVT2	5'-CCCCCTCAACTGCTAATA-3'						
EVS1	5'-ATCAGTCGTCACCTCACTGGT-3'	<i>stx</i> ₂ family	94°C, 60 s	55°C, 60 s ^b	72°C, 60 s	110	29
EVC2	5'-CTGCTGTACAGTGACAAA-3'						
hlyA1	5'-GGTGCAGCAGAAAAAGTTGTAG-3'	<i>hlyA</i>	94°C, 30 s	57°C, 60 s	72°C, 90 s	1,551	38
hlyA4	5'-TCTCGCCTGATAGTGTGTTGTA-3'						
wkat-B	5'-CTTCTGTCTGATTCTTCTGG-3'	<i>katP</i>	94°C, 30 s	56°C, 60 s	72°C, 150 s	2,125	5
wkat-F	5'-AACTTATTTCTGCATCATCC-3'						
D1	5'-CGTCAGGAGGATGTTTCAG-3'	<i>etpD</i>	94°C, 30 s	52°C, 60 s	72°C, 70 s	1,062	36
D13R	5'-CGACTGCACCTGTTCTGATTA-3'						
EAE1	5'-AAACAGGTGAAACTGTTGCC-3'	<i>eae</i>	94°C, 60 s	55°C, 90 s	72°C, 90 s	350	43
EAE2	5'-CTCTGCAGATTAACCTCTGC-3'						

^a Unless stated, PCR consists of 30 cycles.^b After 35 cycles, a final extension step of 10 min at 72°C was carried out.

portray the same threat as they do in other Western countries (19).

MATERIALS AND METHODS

Bacterial strains. Sixty-three STEC strains were isolated while surveillance of sporadic cases of diarrhea in humans, cow stool samples, and beef samples from a city abattoir was being conducted from January 1999 to December 2000. The virulence properties and serotypes of 30 of these strains have been reported recently (19). The remaining 33 strains were isolated using similar procedures detailed previously (19) and characterized in this study.

Serotyping. For serotyping, the slide agglutination test was performed using commercially available O157 (polyvalent) antisera (Denka Seiken Co., Tokyo, Japan) for *E. coli* O157 antigen determination.

PCR detection of virulence factors. PCR for detecting both chromosome (*stx*₁, *stx*₂, and *eae*)- and plasmid (*hlyA*, *katP*, and *etpD*)-encoded virulence genes was performed as described earlier (19) in a total volume of 20 µl containing a 2.5 mM concentration of each deoxynucleoside triphosphate, a 30 µM concentration of each primer, 2 µl of 10× PCR buffer, and 1 U of recombinant *Taq* DNA polymerase (both from Takara, Shuzo, Otsu, Japan) using a thermal cycler. The primer sequences and PCR conditions are given in Table 1.

Antibiotic resistance. The STEC strains were examined for resistance to ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), gentamicin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg), tetracycline (30 µg), cephalothin (30 µg), amikacin (30 µg), ceftazidime (10 µg), kanamycin (30 µg), and ceftriaxone (30 µg) using commercial disks (Hi Media, Bombay, India) by the disk diffusion test. *E. coli* ATCC 25922, which is sensitive to all the drugs, was used as the control strain. The characterization of strains as being susceptible, having reduced susceptibility, or being resistant was as recommended by the National Committee for Clinical Laboratory Standards (22).

RAPD of STEC strains. Genomic analysis of STEC strains was done by RAPD-PCR analysis using a single primer, 1247 (5'-AAGAGCCCGT-3') (35). Genomic DNA (10 to 20 ng) was used as a template. PCR for RAPD-PCR analysis was done using the Gene Amp PCR system 9600 (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) in a volume of 50 µl containing a 200 µM concentration of each deoxynucleoside triphosphate, 30 pmol of primer, 5 µl of 10-fold-concentrated polymerase synthesis buffer, 3 mM MgCl₂, and 2.0 U of *Taq* DNA polymerase (Takara). After a hot start at 80°C for 5 min, the DNA was subjected to 35 cycles of denaturing at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min. A final extension step was done for 10 min at 72°C. The PCR products were separated on 1.5% (wt/vol) agarose gels in 1× TAE (0.04 M Tris acetate, 0.001 M EDTA [pH 8.0]) buffer at 100 V for 5 h. After being stained with ethidium bromide, the gels were digitized for computer-aided analysis (Gel Doc system; Bio-Rad, Hercules, Calif.).

Interpretation of RAPD-PCR results. Using Gel Doc 2000 (Bio-Rad), gel images are stored in a PC that runs on the Windows system. All the images were

retrieved and aligned using Adobe software and analyzed in the Diversity Database fingerprinting software (Bio-Rad). Comparison of differences in the patterns of RAPD-PCR bands were made to ascertain the phylogenetic relationship between isolates. An unrooted phylogenetic tree was made with the neighbor-joining method that is available in the software. The minimum and maximum distance values between nodes on the sorted branches were removed to present the tree in a compressed form.

Pulsed-field gel electrophoresis (PFGE). A 1-day PFGE protocol described by Zhao et al. (44) was adapted in our study. Briefly, bacterial strains were grown overnight on Luria agar (LA) plates at 37°C. Bacterial colonies were suspended in cell suspension buffer (100 mM Tris-HCl, 100 mM EDTA [pH 8.0]) and adjusted to an optical density of 1.3 to 1.4 at 610 nm. The cell suspension (200 µl) was mixed with 10 µl of proteinase K (20 mg/ml) and an equal volume of melted 1% SeaKem agarose containing 1% sodium dodecyl sulfate. The mixture was carefully dispensed into a sample mold (Bio-Rad). After solidification, the plugs were transferred to a 2.0-ml Eppendorf tube containing 1.5 ml of cell lysis buffer (50 mM Tris-HCl, 50 mM EDTA [pH 8.0], 1% sarcosyl) and 0.5 mg of proteinase K per ml. Cells were lysed for 2 h in a water bath with continuous agitation. After lysis, the plugs were washed twice with sterile distilled water and four times with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) for 15 min per wash at 54°C with vigorous shaking. The agarose-embedded DNA was equilibrated in restriction enzyme buffer for 1 h at 37°C. For complete digestion of the DNA, 50 U of *Xba*I (5'-TCTAGA-3'; Takara) was used. The resulting fragments were resolved by the contour-clamped homogeneous electric field method on a CHEF Mapper system (Bio-Rad) 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]) at 6 V/cm for 40 h 24 min at 14°C using the autoalgorithm mode. Gels were stained with ethidium bromide and digitized for computer-aided analysis.

RESULTS

Of the 63 strains isolated during the study period, 40 (63.5%) were from cow stool samples, 19 (30.2%) were from human stool samples, and the remaining 4 (6.3%) strains were isolated from beef samples obtained from a city abattoir.

Antibiotic sensitivity profile. The STEC strains were tested for resistance against 15 antimicrobial agents. Resistance was observed most commonly to ampicillin (25.4%), tetracycline (23.8%), and streptomycin (14.3%) and less frequently to cephalothin (11.1%), co-trimoxazole (9.5%), nalidixic acid (6.4%), and neomycin (3.2%) (Fig. 1). More than one-third of the strains (35%) showed reduced susceptibilities to different antimicrobial agents but were not completely resistant to any of the antibiotics, and 14.3% were sensitive to all the antibi-

FIG. 1. Antibiotic resistance patterns of STEC strains. Abbreviations: A, ampicillin; Ch, cephalothin; G, gentamicin; Ak, amikacin; Cf, ciprofloxacin; Ca, ceftazidime; K, kanamycin; T, tetracycline; C, chloramphenicol; Ci, ceftriaxone; Co, co-trimoxazole; Na, nalidixic acid; Nx, norfloxacin; S, streptomycin; N, neomycin.

otics used in this study. Multidrug resistance was seen in 14 strains, and there was no common resistance pattern among the strains.

Virulence gene profile. In STEC strains, *stx*₁, *stx*₂, and *eae* are chromosomally encoded, while *hlyA*, *etpD*, and *katP* are plasmid-encoded virulence marker genes (2, 35). We used a PCR-based method for detection of these virulence genes. Of the 63 strains, 36.5% carried *stx*₁ and 19% carried *stx*₂, while 44.5% of strains gave positive amplicons for both *stx*₁ and *stx*₂. Nineteen percent of the STEC strains possessed *stx*₁, *stx*₂, and *hlyA* genes; 4.8% of the strains carried *stx*₂ and *hlyA* genes; and one strain (P-179-10) isolated from a cow stool sample carried the *stx*₁ and *hlyA* genes (Table 2). The *eae* gene was found in 14.3% of strains and was exclusively associated with *stx*₁-positive strains in this study. Of the 63 strains of STEC, the *etpD* gene was found in 3 strains isolated from cows, and in all 3 of these strains it was associated with *stx*₁. Two strains which carried *stx*₁ and *eae* genes were positive for *etpD* and *katP*. Two strains, AK38 and P-33-2-26, which were isolated from cow stool samples, carried all the potential virulence genes searched for in this study (*stx*₁, *eae*, *hlyA*, *etpD*, and *katP*). Only one of the STEC strains (AK47) isolated from a human stool sample possessed more than three virulence marker genes (*stx*₁, *eae*, *hlyA*, and *katP*). A strain (AK17) isolated from a beef sample gave positive amplicons for *stx*₁, *stx*₂, *hlyA*, and *katP*. Overall, the most-prevalent toxin genotype was that of STEC strains possessing both *stx*₁ and *stx*₂ (23.8%), and this was followed by that of strains carrying *stx*₁, *stx*₂, and *hlyA* (19%) (Table 2).

RAPD profile of STEC strains. All 63 STEC strains were characterized by RAPD-PCR to analyze the genetic diversity among the strains. All the strains were typeable with primer 1247, with amplified fragment sizes ranging from 0.2 to 6.0 kb. The RAPD profile of strains SD8, SD10, and SD12, isolated from different cow stool samples during different time periods, were identical (Fig. 2, panel I, lanes 4, 5, and 6). Three sets of bovine isolates (SD2 and SD5 [Fig. 2, panel I, lanes 2 and 3], SD4 and SD7 [Fig. 2, panel II, lanes 2 and 3], and AK48 and

AK54 [Fig. 2, panel IV, lanes 2 and 3]) which had the same virulence genotype had the same RAPD profile, while SD1 and SD3 gave the same profile in RAPD-PCR (Fig. 2, panel II, lanes 4 and 5), despite having a different virulence genotype (Fig. 2). Two human strains (SDH2 and SDH3) were closely identical in RAPD profile, with only a one-band difference. Interestingly, two strains (AK33 and AK36) that were isolated from human and cow stool samples showed an identical RAPD profile (Fig. 2, panel III, lanes 2 and 3). The remaining strains showed RAPD patterns that were different from each other.

The phylogenetic analysis of STEC strains reveals seven major clusters (clusters A through G in Fig. 3). Even though the RAPD banding patterns were not identical, the phylogenetic analysis combined many human and cow isolates of STEC. Except for cluster B, the rest comprised human and cow STEC strains. Even though there is no complete homology, three out of four STEC strains isolated from beef samples are combined with strains of human origin at cluster C through E,

TABLE 2. Virulence gene profiles of STEC strains

Virulence gene(s)	No. of strains with indicated virulence gene(s)			
	Human	Cow	Beef	Total (%)
<i>stx</i> ₁	6	5	0	11 (17.5)
<i>stx</i> ₂	3	4	0	7 (11.0)
<i>stx</i> ₁ and <i>stx</i> ₂	4	9	2	15 (23.8)
<i>stx</i> ₁ and <i>hlyA</i>	0	1	0	1 (1.6)
<i>stx</i> ₂ and <i>hlyA</i>	0	3	0	3 (4.8)
<i>stx</i> ₁ , <i>stx</i> ₂ , and <i>hlyA</i>	3	9	0	12 (19.0)
<i>stx</i> ₁ and <i>eae</i>	1	2	1	4 (6.4)
<i>stx</i> ₁ and <i>katP</i>	1	1	0	2 (3.2)
<i>stx</i> ₁ , <i>katP</i> , and <i>eae</i>	0	1	0	1 (1.6)
<i>stx</i> ₂ , <i>katP</i> , and <i>hlyA</i>	0	2	0	2 (3.2)
<i>stx</i> ₁ , <i>stx</i> ₂ , <i>katP</i> , and <i>hlyA</i>	0	0	1	1 (1.6)
<i>stx</i> ₁ , <i>eae</i> , <i>katP</i> , and <i>hlyA</i>	1	0	0	1 (1.6)
<i>stx</i> ₁ , <i>eae</i> , and <i>etpD</i>	0	1	0	1 (1.6)
<i>stx</i> ₁ , <i>eae</i> , <i>katP</i> , <i>etpD</i> , and <i>hlyA</i>	0	2	0	2 (3.2)

FIG. 2. RAPD-PCR results for STEC strains that exhibited identical DNA banding patterns. In panel I, lanes 2 and 3 contain strains SD2 and SD5; lanes 4 to 6 contain strains SD8, SD10, and SD12, respectively. In panel II, lanes 2 and 3 contain SD4 and SD7; lanes 4 and 5 contain SD1 and SD3, respectively. In panel III, lanes 2 and 3 contain AK33 and AK36, respectively. In panel IV, lanes 2 and 3 contain AK48 and AK54, respectively. Lane 1 in each panel contains a 1-kb DNA ladder marker.

indicating some degree of relatedness. To confirm the RAPD-PCR results, we performed PFGE with representative strains that exhibited close relatedness in the assay.

PFGE profile of STEC strains. We analyzed the *Xba*I pattern by PFGE to determine the clonal relatedness among STEC strains that gave identical or nearly similar DNA fingerprints in RAPD-PCR and had similar virulence genotypes (Fig. 4). PFGE of 25 strains produced 12 to 23 fragments ranging in size from approximately <48.5 to 450 kb. SD2 and SD5, SD4 and SD7, and AK48 and AK54 (strain pairs which gave the same RAPD profiles) also showed similar PFGE results (Fig. 4, panel II, lanes 3 and 6; panel II, lanes 5 and 7; and panel IV, lanes 2 and 3). SD8, SD10, and SD12 had identical RAPD profiles, but SD10 showed a one-band difference compared to SD8 and SD12 in PFGE (Fig. 4, panel I, lanes 2, 3, and 4). However, strains AK33 and AK36, which were isolated from human stool and cow stool samples, respectively, and possess the same RAPD profile, showed different PFGE profiles (Fig. 4, panel III, lanes 2 and 3). Likewise, SD1 and SD3 also showed identical RAPD profiles but showed different PFGE profiles (Fig. 4, panel II, lanes 2 and 4).

DISCUSSION

In less than 20 years, strains of *E. coli* that produce Shiga toxin have emerged as a cause of serious human gastrointestinal disease and HUS. The most common serotype of STEC implicated worldwide is O157:H7. In addition to *E. coli* O157:H7, a large number of other STEC strains have been isolated from human patients (11). The significance of non-O157 STEC strains in human disease is less understood than that of the STEC O157:H7 strains and is probably underesti-

mated. Among the clinical STEC strains that have been isolated, a subset of enterohemorrhagic *E. coli* strains has been found which carry a common set of virulence genes that encode the factors for attachment to host cells, elaboration of effective molecules, and production of two different types of Shiga toxins. The sets of virulence genes are found in the LEE, lamboid phages, and a large virulence-associated plasmid (9, 10, 21, 23, 24, 25, 36, 38).

In the present study, we characterized the virulence gene profile of the STEC strains isolated in Calcutta, India. STEC strains with different combinations of virulence genes were observed. The dominant combinations of virulence factors present in the strains studied were *stx*₁ and *stx*₂ (44.5% of strains) and *stx*₁, *stx*₂, and *hlyA* (enterohemorrhagic *E. coli* hemolysin gene) (19% of strains). This pattern of distribution of virulence genes was similar to the virulence gene profiles of STEC strains isolated in New South Wales, Australia, where 47.5% of the STEC harbored *stx*₁, *stx*₂, and *hlyA* (8). Such a high presence of *stx*₁ and *stx*₂ genes in STEC strains is, however, in contrast to the report from Germany, where STEC strains usually possessed *stx*₁ and only rarely contained the *stx*₂ gene (35, 40). In France, the prominent toxin genotype was that of STEC strains carrying *stx*₂ (32).

Intimin is the only intestinal adherence factor identified to date for STEC, and the *eae* gene, which encodes intimin, is widely distributed in STEC strains (17). Previous studies suggest that *eae* may be the single most important accessory factor correlated with severe disease (3, 15). In this study, only 6.4% of the STEC strains harbored *eae*. Interestingly, a case of HUS reported recently was associated with a strain of STEC O5:H⁻, which lacked the *eae* gene (39). *eae*-lacking STEC strains belonging to serogroups O91, O104, or O113 have also been

FIG. 3. Phylogenetic analysis of STEC strains. The unrooted neighbor-joining method was used to summarize the similarity of RAPD-PCR profiles of STEC strains in a dendrogram. Seven major clusters (A through G) are shown near the nodes of each cluster.

isolated from patients with HUS and hemorrhagic colitis (20, 30). The absence of *eae* in STEC strains indicates that there are additional adherence factors yet to be discovered for STEC. Unlike *E. coli* O157:H7, in which the production of Stx2 toxin

is more common than Stx1, only 19% of STEC possessed the *stx*₂ gene in this study. No *eae* and *etpD* gene sequences were observed among the STEC strains carrying the *stx*₂ gene sequence. From these results, it appears that STEC strains con-

FIG. 4. PFGE profiles of *Xba*I digest of STEC strains. PFGE results for STEC strains that exhibited identical profiles by RAPD-PCR (panels I to IV) and differences upon virulence gene analysis (panels V and VI) are shown. Lanes 1 in all panels and lane 7 in panel V contain λ ladder molecular size markers.

taining the *stx*₁ gene are more frequently encountered than those carrying *stx*₂. This also contrasts with the situation observed in O157:H7, in which the possession of *stx*₂ is associated with a higher likelihood of causing HUS (35). Another noteworthy feature was that two strains (AK38 and P-33-2-26) isolated from two different cow stool samples at different time periods possessed all the putative virulence genes searched for in this study. Only one isolate (AK47) from a human diarrhea stool sample carried four virulence genes (*stx*₁, *eae*, *katP*, and *hlyA*), while 52.4% of STEC strains did not carry any virulence marker genes except the toxin gene(s). Thus, it appears that the STEC in this part of the world lacks some factors that are essential for it to become a frequent cause of diarrhea.

E. coli O157:H7 is reportedly susceptible to many antibiotics (34, 42). However, non-O157 strains isolated from humans and animals have developed antibiotic resistance and many are resistant to multiple antimicrobials commonly used in human and veterinary medicine (12, 14, 37). Approximately 49.2% of the present strains showed antibiotic resistance to one or more of the antimicrobial agents used in this study. Some of the strains exhibited multidrug resistance.

We used different subtyping methods to principally understand the relatedness between the STEC strains isolated from different sources since we were unable to serotype these strains. Both RAPD and PFGE showed that the STEC strains were genetically heterogeneous except for some sets of strains (such as SD2 and SD5, SD4 and SD7, and AK48 and AK54) which gave identical RAPD and PFGE profiles. One of the aims of our study was to determine whether the non-O157 STEC strains isolated from human patients were related to those in cow or beef isolates. Except for two strains (AK33 and AK36) that showed an identical RAPD profile but different PFGE profile, there was no match in the RAPD and PFGE profiles between strains of STEC isolated from cows and humans. Phylogenetic analysis, however, revealed the clustering of strains from human and cow origins, indicating an evolutionary relationship. A high level of heterogeneity was also found among STEC strains isolated in France, where non-O157 STEC strains isolated from HUS patients were related, but not

identical, to those isolated from cattle and food (32). Our study indicates that clones present in bovine sources are not transmitted to humans in the Calcutta setting. This may be related to food habits in this part of the world, where beef consumption is relatively low due to religious connotations. It should be mentioned that the non-O157 STEC strains isolated in this study form a small population of organisms that have been randomly isolated, and it would be quite easy to miss the clonal strains. Human STEC has still not become a major problem in India.

AK33 and AK36 had the same RAPD profile but were isolated from human and cow stool samples, respectively. SD1 and SD3 had different virulence gene profiles but yielded identical RAPD profiles. However, these two sets of strains exhibited different restriction enzyme profiles upon PFGE. The incongruity in the results between RAPD and PFGE typing reflects the difference in the molecular bases of RAPD and PFGE, since PFGE explores the whole length of the chromosome whereas RAPD explores only randomly selected parts of it (33). As a consequence, the clustering of isolates can vary considerably for RAPD and PFGE. However, in general, agreement between the results of the two methods was good for strain differentiation, as most strains emerged as a single lineage by both typing methods.

The standardization of the RAPD-PCR and PFGE methods and computer-based submission of the genomic profiles enable discriminative and rapid comparison of STEC strains among laboratories. In the United States, a national network (PulseNet) of public health laboratories that performs DNA fingerprinting has been developed for this purpose (16). However, at the European level, the genomic profiles of STEC isolates can, at present, be compared only on an intralaboratory basis. In this part of the world, standardization of the RAPD-PCR and PFGE method would be needed for efficient international epidemiologic research of the vast number of non-O157 STEC strains. The fact that STEC has not become a major problem in India also gives an opportunity to investigate the natural history of this organism should it emerge as an enteric pathogen in the near future.

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REFERENCES

- Beutin, L., D. Geier, H. Steinruck, S. Zimmermann, and F. Scheut. 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J. Clin. Microbiol.* **31**:2483–2488.
- Birch, M., D. W. Denning, and D. Law. 1996. Rapid genotyping of *Escherichia coli* O157 isolates by random amplification of polymorphic DNA. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**:297–302.
- Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles. 1999. Association between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* **37**:497–503.
- Bopp, C., K. Greene, F. Downes, E. Sowers, J. Wells, and I. Wachsmuth. 1987. Unusual verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis. *J. Clin. Microbiol.* **25**:1486–1489.
- Brunner, W., H. Schmidt, and H. Karch. 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol. Microbiol.* **24**:767–778.
- Brunner, W., H. Schmidt, and H. Karch. 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* **142**:3305–3325.
- Challmers, R. M., H. Aird, and F. J. Bolton. 2000. Water borne *Escherichia coli* O157. *J. Appl. Microbiol.* **88**:124S–132S.
- Djordjevic, S. P., M. A. Hornitzky, G. Bailey, P. Gill, B. Vanselow, K. Walker, and K. A. Bettelheim. 2001. Virulence properties and serotypes of Shiga toxin-producing *Escherichia coli* from healthy Australian slaughter-age sheep. *J. Clin. Microbiol.* **39**:2017–2021.
- Donnenberg, M. S., S. Tzipori, M. L. McKee, A. D. O'Brien, J. Alroy, and J. B. Kaper. 1993. The role of the *eae* gene of enterohaemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. *J. Clin. Invest.* **92**:1418–1424.
- Dytoc, M., R. Soni, F. Cockerill III, J. De Azavedo, M. L. Louie, J. Brunton, and P. Sherman. 1993. Multiple determinants of verotoxin-producing *Escherichia coli* O157:H7 attachment effacement. *Infect. Immun.* **61**:3382–3391.
- Eklund, M., F. Scheut, and A. Siitonen. 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. *J. Clin. Microbiol.* **39**:2829–2834.
- Farina, C., A. Goglio, G. Conedera, F. Minelli, and A. Caprioli. 1996. Antimicrobial susceptibility of *Escherichia coli* O157 and other enterohaemorrhagic *Escherichia coli* isolated in Italy. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**:351–353.
- Galland, J. C., D. R. Hyatt, S. S. Crupper, and D. W. Acheson. 2001. Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. *J. Clin. Microbiol.* **67**:1619–1627.
- Gonzalez, E. A., and J. Blanco. 1989. Serotypes and antibiotic resistance of verotoxigenic (VTEC) *Escherichia coli* strains isolated from calves with diarrhoea. *FEMS Microbiol. Lett.* **51**:31–36.
- Gyles, C., R. Johnson, A. Gao, K. Ziebell, D. Pierard, S. Aleksic, and P. Boerlin. 1998. Association of enterohaemorrhagic *Escherichia coli* hemolysin with serotypes of Shiga-like toxin-producing *E. coli* of human and bovine origins. *Appl. Environ. Microbiol.* **64**:4134–4141.
- Hilborn, E. D., J. H. Mermin, P. A. Mshar, J. L. Hadler, A. Voetsch, C. Wojtkunshi, M. Swartz, R. Mshar, M. A. Lambert-Fair, J. A. Farrar, M. K. Glynn, and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch. Intern. Med.* **159**:1758–1764.
- Kaper, J. B., S. Elliott, V. Sperandio, N. T. Perna, G. F. Mayhew, and F. R. Blattner. 1998. Attaching and effacing intestinal histopathology and the locus of enterocyte effacement, p. 163–182. In J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
- Karmali, M. A. 1989. Infection by verocytotoxin-producing *Escherichia coli*. *Clin. Microbiol. Rev.* **2**:15–38.
- Khan, A., S. Yamasaki, T. Sato, T. Ramamurthy, A. Pal, S. Datta, N. R. Chowdhury, S. C. Das, A. Sikdar, T. Tsukamoto, S. K. Bhattacharya, Y. Takeda, and G. B. Nair. 2002. 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. *Emerg. Infect. Dis.* **8**:54–62.
- Law, D. 2000. Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *J. Appl. Microbiol.* **88**:729–745.
- McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci. USA* **92**:1664–1668.
- National Committee for Clinical Laboratory Standards. 1997. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A6, 6th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Newland, J. W., N. A. Stockbrine, F. F. Miller, A. D. O'Brien, and R. K. Holmes. 1985. Cloning of Shiga like toxin genes from a toxin converting phage of *Escherichia coli*. *Science* **230**:179–181.
- Nicole, T. P., G. F. Mayhew, G. Pósfai, S. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner. 1998. Molecular evolution of a pathogenicity island from enterohaemorrhagic *Escherichia coli* O157:H7. *Infect. Immun.* **66**:3810–3817.
- O'Brien, A. D., L. R. Marques, C. F. Kerry, J. W. Newland, and R. K. Holmes. 1989. Shiga-like toxin converting phage of enterohaemorrhagic *Escherichia coli* strain 933. *Microb. Pathog.* **6**:381–390.
- O'Brien, A. D., and R. K. Holmes. 1987. Shiga and Shiga-like toxin. *Microbiol. Rev.* **1**:206–220.
- O'Brien, A. D., V. L. Tesh, and A. D. Rolfe. 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Curr. Top. Microbiol. Immunol.* **180**:65–94.
- Oswald, E., H. Schmidt, S. Morabito, H. Karch, O. Marches, and A. Caprioli. 2000. Typing of intimin genes in human and animal enterohaemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect. Immun.* **68**:64–71.
- Pal, A., S. Ghosh, T. Ramamurthy, S. Yamasaki, T. Tsukamoto, S. K. Bhattacharya, Y. Takeda, and G. B. Nair. 1999. Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi-urban community in Calcutta, India. *Indian J. Med. Res.* **110**:83–85.
- Paton, A. W., M. C. Woodrow, R. M. Doyle, J. A. Lanser, and J. C. Paton. 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J. Clin. Microbiol.* **37**:3357–3361.
- Paton, J. C., and A. W. Paton. 1998. Pathogenesis and diagnosis of Shiga toxin producing *Escherichia coli* infections. *Clin. Microbiol. Rev.* **11**:450–479.
- Pradel, N., K. Boukhors, Y. Bertin, C. Forestier, C. Martin, and V. Livrelli. 2001. Heterogeneity of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic-uremic syndrome patients, cattle, and food samples in central France. *Appl. Environ. Microbiol.* **67**:2460–2468.
- Radu, S., O. W. Ling, G. Rusul, M. I. A. Karim, and M. Nishibuchi. 2001. Detection of *Escherichia coli* O157:H7 by multiplex PCR and their characterization by plasmid profiling, antimicrobial resistance, RAPD and PFGE analysis. *J. Microbiol. Methods* **46**:131–139.
- Ratnam, S., S. March, R. Ahmed, G. Bezanson, and S. Kasatiya. 1988. Characterization of *Escherichia coli* serotype O157:H7. *J. Clin. Microbiol.* **26**:2006–2012.
- Schmidt, H., C. Geitz, I. T. Phillips, F. Matthias, and H. Karch. 1999. Non-O157 pathogenic shiga toxin producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. *J. Infect. Dis.* **179**:115–123.
- Schmidt, H., B. Henkel, and H. Karch. 1997. A gene cluster related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohaemorrhagic *Escherichia coli* O157 strains. *FEMS Microbiol. Lett.* **148**:265–272.
- Schmidt, H., J. von Maldegem, M. Frosch, and H. Karch. 1998. Antibiotic susceptibilities of verocytotoxin-producing *Escherichia coli* O157 and non-O157 strains isolated from patients and healthy subjects in Germany during 1996. *J. Antimicrob. Chemother.* **42**:548–550.
- Schmidt, H., L. Beutin, and H. Karch. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun.* **63**:1055–1061.
- Starr, M., V. Bennett-Wood, A. K. Bigham, T. F. de Koning-Ward, A. M. Bordun, D. Lightfoot, K. A. Bettelheim, C. L. Jones, and R. M. Robins-Browne. 1998. Hemolytic-uremic syndrome following urinary tract infection with enterohaemorrhagic *Escherichia coli*: case report and review. *Clin. Infect. Dis.* **27**:310–315.
- Strockbine, N. A., J. G. Wells, C. A. Bopp, and T. J. Barrett. 1998. Overview of detection and subtyping methods, p. 331–356. In J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
- Swerdlow, D. L., B. A. Woodruff, R. C. Brady, P. M. Griffin, S. Tippet, H. P. Ponnell, E. Geldreich, B. Payne, A. Meyer, and J. G. Wells. 1992. A water borne outbreak in Missouri of *Escherichia coli* O157:H7, associated with bloody diarrhea and death. *Ann. Intern. Med.* **117**:812–819.
- Tarr, P. I. 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. *Clin. Infect. Dis.* **20**:1–10.
- Yu, J., and J. B. Kaper. 1992. Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **6**:411–417.
- Zhao, S., D. G. White, B. Ge, S. Ayers, S. Friedman, L. English, D. Wagner, S. Gaines, and J. Meng. 2001. Identification and characterization of integrin-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. *Appl. Environ. Microbiol.* **67**:1558–1564.