Prevalence and Genetic Profiling of Virulence Determinants of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolated from Cattle, Beef, and Humans, Calcutta, India

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We investigated the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in hospitalized diarrhea patients in Calcutta, India, as well as in healthy domestic cattle and raw beef samples collected from the city's abattoir. Multiplex polymerase chain reaction using primers specific for *stx1* and *stx2* detected STEC in 18% of cow stool samples, 50% of raw beef samples, and 1.4% and 0.6% of bloody and watery stool samples, respectively, from hospitalized diarrhea patients. Various virulence genes in the STEC isolates indicated that *stx1* allele predominated. Plasmid-borne markers, namely, *hlyA, katP, espP*, and *etpD*, were also identified. Bead enzyme-linked immunosorbent assay and Vero cell assay were performed to detect and evaluate the cytotoxic effect of the Shiga toxins produced by the strains. STEC is not an important cause of diarrhea in India; however, its presence in domestic cattle and beef samples suggests that this enteropathogen may become a major public health problem in the future.

The first documented U.S. outbreak of diarrhea due to infection by Shiga toxin-producing *Escherichia coli* (STEC) belonging to the serotype O157:H7 occurred in 1982 (1). Since then, STEC has been increasingly recognized as an important human diarrheal pathogen and as the predominant cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS). STEC is now being detected in 75% to 100% of episodes of sporadic HUS in Europe, North America, Canada, and Latin America, especially Argentina (2).

Multiple virulence factors contribute to the pathogenicity of STEC. Its main pathogenic property is the production of Shiga toxin (Stx), which inhibits the protein synthesis of host cells leading to cell death (3,4). *E. coli* Stxs are classified into two types, Stx1 and Stx2, and each type has several variants. Stx1 and Stx2 are encoded by alleles in the genome of temperate, lambdoid bacteriophages that remain integrated in the *E. coli* chromosome (5). The strains carry in their chromosome a 35-kb pathogenicity island called locus of enterocyte effacement (LEE), whose genes are responsible for generation of attachment and effacement lesions. LEE encodes a type III

secretion system, and some LEE genes are necessary for initiation of signal transduction events (6). However, not all STEC strains harbor the LEE, and it is not necessary to cause human infection. Moreover, STEC strains almost invariably harbor a 97-kb plasmid encoding possible additional virulence traits such as STEC hemolysin (which acts as a pore-forming cytolysin on eukaryotic cells [7]); the bifunctional catalase peroxidase KatP (8); a secreted serine protease (espP, which can cleave human coagulation factor V [9]); and the *etpD* gene cluster (which probably encodes a type II secretion pathway [10,11]).

Cattle and sheep are the primary reservoirs of STEC, but STEC has been isolated from deer, horses, dogs, and birds (12). Feces from any of these animals could serve as primary source for STEC. The dynamics of these pathogens in animals and environment is not well understood. Principally, STEC is transmitted through the consumption of contaminated foods such as raw or undercooked ground meat products and raw milk (13). Although cattle are the primary known reservoirs of STEC, humans may acquire STEC infections from other sources, possibly vegetables (14), fruit juice (15), or contaminated drinking water (16), or through direct contact with feces of infected persons (17).

In industrialized countries such as the United States, Japan, Germany, Australia, and United Kingdom, large outbreaks and many sporadic cases of STEC infections have been reported and have become a major health concern. More than 50 sero-

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types of STEC have been isolated from stool samples of patients with hemorrhagic colitis or HUS; however, many of these serotypes have not been as thoroughly or systematically characterized for virulence genes and properties as *Escherichia coli* O157:H7. To date, very few isolates of STEC from humans or animals have been reported in industrialized countries.

In India, there is a paucity of information on STEC. It has not been identified as an etiologic agent of diarrhea in India. Though a few strains of O157 serogroup have been isolated from sporadic cases of diarrhea, these strains have not been well characterized, and their origin is uncertain (18).

Serotyping of STEC alone is insufficient to assess the pathogenic properties of the strains because such organisms are quite variable in their repertoire of virulence determinants. Analysis of the genotypes of the STEC strains by the use of specific gene probes or polymerase chain reaction (PCR) provides more detailed information about genetic variability and subtypes (11).

In this study, we investigated the prevalence of STEC in healthy domestic cattle of a semi-urban community, in raw beef samples, and in hospitalized patients with diarrhea in Calcutta, India. Isolated STEC strains were characterized in detail to identify the predominant virulence genes and to understand how the Indian strains compare with STEC strains isolated elsewhere in the world.

Materials and Methods

Hospital Surveillance

Stool samples were collected from diarrheal patients enrolled in an ongoing active surveillance system at the Infectious Diseases Hospital (IDH) and from all the cases attending the B.C. Roy Hospital for Children, Calcutta, during a 1-year study period from January to December 1999. In the surveillance system, every fifth patient attending the hospital was included for sampling. Stool samples were collected in sterile McCartney bottles by using sterile catheters; sterile cotton-tipped swabs were used to take rectal swabs from patients from whom stool could not be obtained. Rectal swabs were placed in Cary-Blair medium, and stool samples were transported to the laboratory within 1 hour of collection. All samples were examined for STEC and other enteric pathogens such as other diarrheogenic *E. coli*, vibrios, *Salmonella* spp, *Shigella* spp, *Aeromonas* spp, rotavirus, parasites, and protozoans, following standard methods (19).

Cow Stool Samples

One hundred forty stool samples were collected from domestic cows of a semi-urban community near Calcutta; 66 stool samples were obtained from the state livestock farm at Kalyani, 71 kms away from Calcutta. These samples were collected at no defined periodicity during the year 1999.

Beef Samples

A total of 111 beef samples were collected from Calcutta Municipal Corporation abattoir, situated in Tangra. Collections were made twice a month during March to July 1999, the season when the maximum number of diarrhea patients is admitted to IDH and other Calcutta hospitals.

Enrichment of Samples

A loopful of human or cow stool samples was directly inoculated into 3 mL of Bacto EC Medium (Difco, MI, USA) for enrichment and incubated overnight at 37°C under shaking conditions. With beef samples, 50 mL of EC broth was aseptically transferred into a polythene bag and mixed well with the beef sample. After 2 hours, the broth was transferred into a sterile conical flask and incubated overnight at 37°C with constant shaking. After overnight incubation, STEC was screened by using a variety of screening methods as described below.

Screening Strategies

Enrichment Broth PCR

After incubation, enriched broth was directly examined by PCR using *stx1* and *stx2* primers under the conditions described in Table 1. Broth cultures that yielded positive PCR results for either *stx1* or *stx2* or both were serially diluted in 10 mM phosphate-buffered saline (PBS) (pH 7.0) and 100 μL volume of each dilution was spread on Luria agar (Difco) plate in duplicate. Randomly picked colonies were further screened for the presence of STEC by PCR or colony hybridization using DIG-labeled *stx1* and *stx2* gene probes as described below.

PCR Screening for Single-Cell Isolation

Single colonies were randomly picked from the dilution plates and spot inoculated on a master Luria agar plate and then inoculated in 3 mL EC broth. Four colonies were pooled for each EC broth and incubated overnight at 37°C in a shaker. Attempts were made to inoculate as many colonies as possible from each plate. Following overnight incubation, the inoculated EC broth culture was diluted 10-fold with sterile PBS and boiled for 10 minutes. This method was used as the template for a multiplex PCR using primers for *stx1* or *stx2* genes (Table 1). PCR was done as described previously (20). When a positive PCR result was obtained, the PCR was repeated to determine which of the four pooled colonies contributed to the positive result. Once an STEC strain was identified, it was preserved in Luria broth supplemented with 15% glycerol at -70°C.

Colony Hybridization for Single-Cell Isolation

An alternative approach adopted to identify STEC colonies from the dilution plates was the colony hybridization procedure, followed as described previously (11,23). Positive STEC colonies, if present, appeared as dark purple spots on the membrane. Colonies in the dilution plates were matched with the

Table 1. Polymerase chain reaction (PCR) primers and conditions used in this study

| Primer no. | Nucleotide sequence of primers | Target | PCR conditions[a] | | | Amplicon (bp) | References |
			Denaturing	Annealing	Extension		
EVT1/ EVT2	5'-CAACACTGGATGATCTCAG-3' 5'-CCCCCTCAACTGCTAATA-3'	*Stx1* family	94°C, 60s	55°C, 60s[b]	72°C, 60s	349	20
EVS1/ EVC2	5'-ATCAGTCGTCACTCACTGGT-3' 5'-CTGCTGTCACAGTGACAAA-3'	*Stx2* family	94°C, 60s	55°C, 60s[b]	72°C, 60s	110	20
hlyA1/ hlyA4	5'-GGTGCAGCAGAAAAAGTTGTAG-3' 5'-TCTCGCCTGATAGTGTTTGGTA-3'	EHEC *hlyA*	94°C, 30s	57°C, 60s	72°C, 90s	1,551	7
wkat-B/ wkat-F	5'-CTTCCTGTTCTGATTCTTCTGG-3' 5'-AACTTATTTCTCGCATCATCC-3'	katP	94°C, 30s	56°C, 60s	72°C, 15s	2,125	9
D1/ D13R	5'-CGTCAGGAGGATGTTCAG-3' 5'-CGACTGCACCTGTTCCTGATTA-3'	etpD	94°C, 30s	52°C, 60s	72°C, 70s	1,062	21
EAE1/ EAE2	5'-AAACAGGTGAAACTGTTGCC-3' 5'-CTCTGCAGATTAACCTCTGC-3'	eae	94°C, 60s	55°C, 90s	72°C, 90s	350	22

[a] Unless stated, PCR was done for 30 cycles.

[b] After 35 cycles, final extension step of 10 min at 72°C was performed.

spots on the membrane, and isolated STEC strains were reconfirmed by PCR, as described. Probes used included fragments of *stx1* (905 bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN501) (24) and *stx2*-A (860 bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN502) (25). These probes were also used for the dot blot assay, which employed PCR amplicons obtained from enrichment cultures of human stool, cow stool, and beef samples to confirm the PCR specificity.

Screening for Pathogenic Factors by PCR

PCR for detecting both chromosomal and plasmid virulence markers was performed by using a thermal cycler in a total volume of 20 µL containing 2.5 mM of each deoxynucleoside triphosphate, 30 FM of each primer, 2 µL of 10X PCR buffer, and 1 U of r-Taq DNA polymerase (both from Takara, Shuzo, Otsu, Japan). Primer sequences and PCR conditions are given in Table 1.

Screening for Pathogenic Factors by Colony Hybridization

A colony hybridization test for detecting pathogenic factors was carried out as described previously (26). DNA probes for

espP, *katP*, *eae*, and *hlyA* were prepared by PCR. Primer sequences and PCR conditions are given in Table 2. After PCR, the products were purified by QIA Quick PCR Purification Kit (QIAGEN GmbH, Germany) in accordance with the manufacturer's instructions. The DNA probes were labeled by random priming method using Multiprime DNA labeling system and [α^{32}P] dCTP, and the colony hybridization procedure was followed as described (23).

Serotyping

Serotypes of the STEC strains were determined by slide agglutination with either commercially available O (poly and monovalent antisera) and H (monovalent antisera) (Denka Seiken Co., Japan) or antisera prepared at the Osaka Prefectural Public Health Institute, Osaka, Japan.

Vero Cell Assay

Preparation of Cell-Free Culture Filtrates

STEC strains were cultured in L-broth (Difco), at 37°C overnight with constant shaking. Bacterial cells were pelleted

Table 2. Polymerase chain reaction (PCR) primers and conditions for preparing DNA probes used in the colony hybridization test

| Nucleotide sequence of primers | Target | PCR conditions[a] | | | Amplican (bp) |
		Denaturing	Annealing	Extension	
5-GAGAATTCACAGATGGATATTTCAAATTTC-3 5-TCTCCTCGAGCTAGTTGACCTCGTTCAGAAACG-3	*espP*	94°C, 10s	68°C, 30s	72°C, 90s	2,900
5-GAGAGAATTCTTCCTGTTCTGATTCTTCTG-3 5-TCTCCTCGAGTCAAAACTTATTTCTCGCATCATCATCC-3	*katP*	94°C, 10s	68°C, 30s	72°C, 60s	2,130
5-TCTCAAGCTTTTAGAAATAGTCTCGCCAGTATTC-3 5-GAGAGAATTCGTCAGGAGGATGTTCAGG-3	*eae*	94°C, 10s	68°C, 30s	72°C, 90s	880
5-GAGAGGATCCGGTGCAGCAGAAAAAGTTGTA-3 5-TCTCCTCGAGTCATCTCGCCTGATAGTGTTTGG-3	*hlyA*	94°C, 10s	68°C, 30s	72°C, 60s	1,560

[a]PCR was done for 30 cycles.

by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was filter-sterilized by using 0.22-µm filters (Millipore, USA). The culture pellet was resuspended in PBS and then sonicated by using a Handy sonic (Tomy Seiko Co, Ltd., Tokyo, Japan), which was again centrifuged to remove debris. Supernatant was filter-sterilized. Both the culture supernatant and cell lysate were used for the assay.

Cytotoxic Assay

The cytotoxic effect of STEC strains was assayed on Vero cells in 96-well flat-bottom tissue culture plates (NUNC, Intermade, Denmark), as previously described (24). The cells were observed microscopically for 72 hours and the cytotoxicity titers determined; the highest toxin dilution that caused lysis of 50% of the cell monolayer was taken as the titer.

Bead ELISA

The initial procedure for preparation of cell-free culture supernatant and cell lysate was the same as for the Vero cell assay. The procedure for the bead ELISA has been described (23).

Hemolysin Activity

Hemolytic activity of the STEC strains was investigated by streaking the strains on tryptic soy agar (Difco) plates containing 5% washed and unwashed O group human blood cells. The hemolytic activity was observed from 3 to 18 hrs of incubation at 37°C (11).

Results

Prevalence of STEC in Human Stool Samples

The age distribution of the 1,241 and 284 patients whose samples were examined from IDH and B.C. Roy Hospital for Children, respectively, is shown in Table 3. Of the specimens from IDH patients during the period of surveillance, eight were positive for STEC by PCR in the preliminary screening. However, only nine strains could be isolated from six of the eight PCR-positive samples. The strains AK-11 and AK-26 were isolated from the same stool sample; AK-27, AK-28, and AK-29 were isolated from another single stool sample. Four of the 284 bloody stool samples examined from B.C. Roy Memorial Hospital for Children were positive for STEC by PCR, but again STEC could be isolated from only 3 of the 4 PCR-positive samples. Of note, 7 (58%) of 12 diarrhea patients from whom STEC was isolated were also co-infected with other pathogens (Table 4). STEC was isolated as the sole pathogen from 5 (42%) of 12 patients with diarrhea. The relative frequency of other enteric pathogens detected in the study samples is shown in Table 5.

Prevalence of STEC in Beef Samples

Of the 111 raw beef samples examined, 55 samples were positive for STEC when examined by multiplex PCR for *stx1* or *stx2*. However, STEC was isolated from only 4 of the 55

Table 3. Age distribution of patients admitted to hospitals where stool samples were examined

Age (in years)	Infectious Diseases Hospital (%)	B. C. Roy Hospital for Children (%)
≤ 5	25.1	97.6
>5-15	10.1	2.4
>15-25	20.2	-
>25-35	18.0	-
>35-45	11.0	-
>45-55	7.6	-
>55	8.1	-

PCR-positive samples by using the techniques adopted in this study.

Prevalence of STEC in Cow Stool Samples

Eight of 140 stool samples from cows examined from the community were positive for STEC; 10 STEC strains were recovered from the 8 samples. In contrast, of the 66 cow stool samples collected from Kalyani farm, STEC was detected in 29 by PCR. Only 5 strains could be isolated from the 29 PCR-positive samples.

STEC Serotypes

Of the 30 STEC strains isolated in this study, 8 strains were O antigen untypable with somatic (O) antiserum. However, except for one strain (AK-32), all the O-untypable strains were typable with different H-types (Table 6). Of the strains typed, four belonged to serotypes that are not listed in the updated list of serotypes of non-O157 STEC isolated from humans worldwide (http://www.microbionet.com.au/frames/feature/vtec/brief01.html). These are O96:H19, ONT:NM, ONT:H18, and ONT:H14. There was no clustering of any particular serotype. The serotypes ONT:H19 and O159:H12 were isolated from both humans and cows; otherwise, there was no match between serotypes of STEC isolated from human, cow, and beef samples (Table 6).

Analysis of Chromosomal Markers

stx1 and *stx2*

In our study, 30 strains were positive for *stx* by PCR. Of the 12 human isolates, 7 were positive for only *stx1*, 2 for only *stx2*, and 3 for both *stx1* and *stx2* (Table 6). Of the 14 strains isolated from cow stool samples, 6 carried *stx1*; 3, *stx2*; and 5 were positive for both *stx1* and *stx2*. Three of the four strains isolated from raw beef harbored *stx1* and *stx2*; the fourth carried only *stx1*.

eae

As in the case of enteropathogenic *E. coli* (EPEC), the characteristic attaching and effacing (A/E) ability is encoded by 41 genes present on the LEE pathogenicity island (6,27).

Table 4. Clinical manifestation and other information on patients from whom Shiga toxin-producing *Escherichia coli* was isolated

STEC strain number	Stool characteristics	Age (years)	Sex	Religion	Vomiting	Fever	Dehydration	Other pathogen(s) present in the stool samples
AK-11[a] AK-26[a]	Watery	32	M	H	Negative	Negative	Severe	*Vibrio parahaemolyticus*
None	Watery	28	F	MU	Positive	Positive	No	None
None	Watery	17	F	H	Positive	Positive	Moderate	None
AK-18	Watery	30	M	H	Positive	Positive	Severe	*V. cholerae* O139
AK-27[a] AK-28[a] AK-29[a]	Watery	50	M	O	Positive	Positive	Severe	*V. cholerae* O1
AK-31	Watery	2	M	H	Positive	Negative	Severe	None
AK-32	Watery	40	F	O	Negative	Negative	Moderate	*V. cholerae* non-O1 non-O139
AK-33	Watery	27	M	MU	Positive	Positive	No	None
AK-14	Bloody	2½	M	MU	Negative	Positive	No	*V. parahaemolyticus, Shigella* spp.
AK-30	Bloody	2	F	MU	Negative	Positive	No	None
AK-40	Bloody	1½	F	MU	Negative	Positive	Moderate	*Shigella* spp.

[a] Strains isolated from one stool specimen but belonging to different serotypes.
H, Hindu; MU, Muslim; O, Other religion; M, male; F, female.

Pathogenicity of STEC is associated with the presence of LEE and in particular *eae*. We performed PCR with primers EAE-1 and EAE-2. An 863-bp PCR product was demonstrated for four strains (P-33-2-26, AK-16, AK-38, and AK-40), indicating the presence of *eae* (Table 6).

Analysis of Plasmid-Encoded Markers

E-hly, etpD, katP, and *espP* are plasmid-encoded markers. PCR with the hemolysin primers hlyA1 and hlyA4 showed that 12 of the 30 STEC isolates contained *E-hly* sequences. However, AK-1, although negative by PCR, hybridized with *hlyA*

Table 5. Other enteropathogens isolated from stool samples examined for Shiga toxin-producing *Escherichia coli*, India

Enteropathogens		Infectious Diseases Hospital (%)	B. C. Roy Hospital for Children
Vibrio cholerae	O1	9.5	0.6
	O139	5.2	-
	Non-O1 non-O139	3.7	3.1
Vibrio parahaemolyticus		2.8	0.4
Shigella	*flexneri*	0.4	0.7
	sonnei	0.1	-
	boydii	0.2	-
Salmonella		0.5	1.1
E. coli	EPEC	0.5	-
	ETEC	1.3	-

EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*.

probe (Table 6). The remaining strains were negative. However, P-33-2-26 and AK-38 produced a PCR product of 1062 bp, and primers D1 and D13R suggested the presence of *etpD* gene cluster. Seven strains possessed the *katP* gene cluster, as demonstrated by PCR with primers pair wkatB and wkatF as well as by colony hybridization. While *espP* could be detected in 11 strains when analyzed by probe complementary to *espP* sequence, only 2 strains generated the specific amplicon when PCR was carried out with *espP-specific* primer.

Thus, the analysis of virulence markers revealed that in the present collection of strains AK-38, a cow stool isolate not belonging to the O157 serogroup carried all the potential virulence genes.

Phenotypic Characterization of STEC Strains

Vero Cell Assay

Expression of Stx was examined by Vero cell cytotoxic assay. Of the 30 strains examined, all were cytotoxic to the Vero cells. AK-38, despite having *stx1* and the full repertoire of virulence genes, did not exhibit the cytotoxic effect (Table 6).

Bead ELISA

A highly sensitive bead-ELISA was applied to detect the presence of Stx1 and Stx2 in the cell-free culture filtrate and cell lysate of the isolates in this study. This assay identified Stx1 in 12 of 14 *stx1* PCR-positive strains but identified only 3 of the 5 *stx2* PCR-positive strains in both cell filtrate and lysate. Of the 11 strains positive for *stx1* and *stx2* by PCR, 8 were positive by Bead-ELISA for both toxins (Table 6).

Table 6. Serotype, phenotypic and genotypic traits of Shiga toxin-producing *Escherichia coli* strains, India

Strain no.	Serotype	Origin	Genotype (PCR/colony hybridization)							Phenotype				
			Chromosomal genes			Plasmid genes								
			stx1	stx2	eae	hlyA	etpD	katP	espP	Hemolysis	SF[a]	Vero[b]	Stx1	Stx2
AK-40	O11:H8	Human	+/+	-/-	+/+	+/+	-/-	+/+	-/+	E-hly	+	ND	ND	ND
AK-11	ONT:H18	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	-
AK-18	O156:H7	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-27	ONT:H19	Human	+/+	-/-	-/-	-/-	-/-	+/+	-/-	-	+	+	+	-
AK-30	O7:H6	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-32	ONT:NM	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	ND	ND	ND	ND
AK-33	O159:H12	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	-
AK-26	O159:H9	Human	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-	+	+	-	+
AK-29	O110:H16	Human	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-	+	+	-	+
AK-14	ONT:H14	Human	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
AK-28	O43:H2	Human	+/+	+/+	-/-	+/+	-/-	-/-	-/-	-	+	+	+	+
AK-31	O96:H19	Human	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-	ND	ND	ND	ND
P-33-2-26	ONT:H2	Cow	+/+	-/-	+/+	+/+	+/+	+/+	-/-	E-hly	+	+	+	-
AK-38	O103:H2	Cow	+/+	-/-	+/+	+/+	+/+	+/+	-/-	-	+	-	-	-
P-179-101	O146:H1	Cow	+/+	-/-	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	-
C-45-3-19	O149:HNT	Cow	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-35	O22:H16	Cow	+/+	-/-	-/-	-/-	-/-	+/+	-/-	-	+	+	+	-
AK-39	O9B:H5	Cow	+/+	-/-	-/-	-/-	-/-	-/-	+/+	E-hly	+	+	+	-
C-5-1-13	O82:H8	Cow	-/-	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	-	+
AK-1	O2:H25	Cow	-/-	+/+	-/-	-/+	-/-	+/+	-/-	-	+	+	-	-
AK-37	O22:NM	Cow	-/-	+/+	-/-	+/+	-/-	-/-	+/+	-	ND	ND	ND	ND
C-2-1-15	O28ac:H21	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
C-3-2-25	ONT:H34	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
C-3-3-42-6	ONT:H19	Cow	+/+	+/+	-/-	-/-	-/-	-/-	-/+	E-hly	+	+	+	+
P-33-2-4	O88:HNT	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
AK-36	O159:H12	Cow	+/+	+/+	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	+
AK-16	ONT:H21	Beef	+/+	-/-	+/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-12	O20:H12	Beef	+/+	+/+	-/-	-/-	-/-	-/-	-/-	ND	+	+	+	-
AK-13	O110:HNT	Beef	+/+	+/+	-/-	-/-	-/-	-/-	-/-	ND	+	+	+	-
AK-17	O172:NM	Beef	+/+	+/+	-/-	+/+	-/-	+/+	-/+	E-hly	+	+	+	+

[a]Fermentation of sorbitol.

[b]Vero cell cytotoxicity.

NT, Not typable; ND, Not determined; NM, nonmotile.

Hemolysin

Fourteen of the thirty STEC isolates showed a hemolytic phenotype that resembled alpha-hemolytic activity rather than the typical enterohemolytic phenotype (Table 6). The zone was clear, large, and could be visualized after 3 to 18 hours' incubation at 37°C. Of the 14 strains, 9 were positive by PCR using E-*hlyA* primer pair. However, three other STEC, which carried the *hlyA* gene, did not produce hemolysin.

Discussion

During the past decade, STEC has evolved from a clinical novelty to a global public health concern. STEC infections have been reported from over 30 countries on six continents, causing a spectrum of human illness ranging from symptom-free carriage to severe bloody diarrhea and even to life-threatening sequelae such as HUS. However, there is a paucity of reports on STEC in the developing world including India. A

previous study in India found no STEC in children with diarrhea in Delhi (28); a study from Bangladesh reported that no STEC were recovered from children with diarrhea (29). This is possibly related to lack of surveillance for this organism because of the difficulty in isolating STEC. However, in an outbreak of bloody diarrhea in Cameroon, half the patient specimens yielded STEC and half *Shigella* species (30).

We attempted to document the prevalence of STEC in Calcutta and provide information on the phenotypic traits, serotypes, and molecular characterization of the virulence genes. The reasons for the low prevalence of STEC-associated diarrhea in Calcutta and possibly other places in India are not well understood. Perhaps Indians acquire protective antibodies at an early age or use cooking practices that effectively eliminate STEC.

The isolation rate of STEC from hospitalized secretory and bloody diarrhea cases was very low in this study, and there was no evidence of a seasonal distribution of STEC-positive samples. Among STEC-identified diarrhea cases, 58% were found to be infected with other enteric pathogens. One case was positive for *Vibrio parahaemolyticus*, STEC, and *Shigella*. In such situations, it is difficult to conclude the role played by STEC in causing disease. We included religion in the data analysis since the food habits of Hindus and Muslims vary (e.g., Muslims eat beef) (Table 3). Indirect routes of transmission may, however, be more significant in India, where most of the population does not eat beef for religious reasons. Possibly in areas like this, STEC transmission could occur through exposure to vegetables, fruits, or drinking water contaminated by bovine feces or through direct contact with feces of infected persons (14-17). Only non-O157 STEC strains were isolated from human cases. In general, with the exception of North America and Japan, non-O157 STEC strains are isolated more frequently than O157, with a median of a fourfold higher isolation rate but with wide variation among studies (31). From our intense survey conducted over a 1-year period, it was clear that STEC are not currently a major diarrhea-causing etiologic agent in India. However, this study indicates that strains of *E. coli* with the O157 lipopolysaccharide and non-O157 LPS are present in the milieu, as are virulence genes, which are known to contribute to STEC virulence. Mixing and matching of genes in the environment or in the human intestine could lead to the evolution of pathogenic STEC.

The prevalence of STEC in bovine fecal flora and beef was high, according to PCR results. The occurrence of STEC in raw beef samples strongly indicates that unhygienic practices prevailed in slaughterhouses in Calcutta. Therefore, STECs are clearly present in beef and, as in other countries (12), there is a bovine reservoir of STEC in India. Isolation of twelve O157:H7 strains from 9 of 25 beef samples originally imported from India and sold in retail stores in Malaysia has recently been reported (32). However, STEC strains have not been able to cycle from contaminated food items or bovine reservoirs to humans. It is not certain whether the STEC strains in India lack some factor that is essential for them to become a frequent

cause of diarrhea or whether this phenomenon is related to an as-yet-unidentified host factor.

There was a disparity in the ability to culture STEC from PCR-positive specimens. STEC was cultured from 75% of PCR-positive human stools and 100% of community cows, but we isolated STEC from only 17.2% of PCR-positive cows from the livestock farm and 7.3% of PCR-positive raw beef samples. One reason for these varying results could relate to the initial numbers present in the sample. We presume that the number of strains present in human diarrhea cases (where the causative agent is amplified) would be higher than that found in fecal samples of cows and raw beef samples. However, we cannot account for the 100% isolation from community cows. We plan to investigate this further. One of the reasons for the failure to isolate STEC strains was the frequent presence of swarming colonies, which tended to obliterate all other colonies. This happened despite increasing the agar concentration (3%) as well as 0.15% bile salts (for the inhibition of swarming colonies) in the Luria agar medium. To avoid false-positive PCR results, we confirmed that the PCR amplicons were specific for *stx1* and *stx2* by dot blot assay. We immobilized the PCR product on N^+-nylon membrane and hybridized with *stx1*- and *stx2*-specific probes derived from pKTN501 and pKTN502, respectively, using the DIG-labeled kit. All PCR products gave positive signals in this hybridization assay, confirming that the PCR assay is specific for *stx1* and *stx2*.

The inability to isolate STEC strains from PCR-positive samples might be due to the presence of very low numbers of the target strain. This appears to be one key reason for the rather low isolation rate of STEC despite its being present in the sample. Several previous studies have also reported difficulties in isolating the organism from stools of patients with HUS and hemorrhagic colitis. In one such study of HUS cases in which 20 individual colonies of *E. coli* from primary stool cultures were tested, the proportion of Stx-positive colonies varied from 5% to 20% (33). Moreover, many cases had free fecal Stx but no STEC were isolated (34). In those studies, we usually encountered swarming colonies in the plates assayed for STEC even after the addition of 3% agar and bile salts (0.15%). In the present study, we used three different procedures to isolate the STEC and O157 strains. We usually had to screen approximately 500 colonies to yield a positive PCR result in an attempt to isolate STEC. But since the frequency of occurrence of STEC is very low and since PCR is an expensive and laborious method for screening, we later opted for the colony hybridization-DIG procedure, which allows a greater number of colonies to be initially screened and therefore increases the probability of obtaining positive isolates.

The systematic analysis of virulence markers indicates that the STEC in Calcutta mostly contain *stx1*, whereas the *eae* gene occurs at low frequency among strains of human and bovine origin. Such low prevalence of *eae* in Calcutta strains is in contrast to a report from Germany, where *eae* was found in most STEC strains examined (11). PCR results revealed that *hlyA* was the most prevalent (43.3%) plasmid-encoded marker

compared with *katP* (23.3%) and *etp*D (6.7%). With the *espP* gene, a considerable discrepancy was observed between the PCR and colony blot hybridization results. While 36.7% of strains were positive by colony blot hybridization, only 6.7% generated the desired amplicon with the *espP* primers. Comparison of the virulence profiles of Calcutta STEC strains isolated from different sources demonstrated the relative abundance of *katP* gene in cow and beef isolates (28.7% and 25%, respectively) compared with human strains (18.2%). Likewise, the percentage of *hlyA* PCR-positive STEC of bovine origin (64.3%) was more than twice that isolated from humans (27.3%). However, for the *espP* genes detected by colony hybridization, there was an appreciable variation in the distribution of this gene in strains isolated from cows (57.1%) and humans (18.2%). Two strains from cow stool samples were positive for *etpD*. Except for strains AK-40 and AK-38, nearly all Calcutta strains did not have the entire complement of virulence genes and therefore may not have been associated with diarrhea.

The observation that one *stx2*-positive strains showed cytotoxic effect on Vero cells but yielded negative Bead-ELISA result indicates that the toxin produced by this strain was antigenically different and may constitute a new variant of Stx2. Except for one strain, the other eight *hlyA*-positive STEC strains did not show the hemolytic phenotype when streaked on blood agar plates. One reason may be that the hemolysin of these strains remains cell associated because of a deficient transport system for secretion of hemolysin. Interestingly, one STEC strain AK-38 had all the known virulence markers of STEC but still gave a negative result in the Vero cell cytotoxicity assay. Based on the negative results obtained in Bead-ELISA and hemolysin assays, we presume that both *stx1* and *hlyA* genes of this strain (AK38) might be silent. Another possibility is that additional DNA is present in this isolate close to the *stx* gene, which would presumably prevent expression of *stx1* (11). Taken together, our analysis of non-O157 STEC strains suggests that pathogenicity is a consequence of lineal descent. The mere possession of *stx* genes is probably inadequate to render an *E. coli* pathogenic; an assortment of traits no doubt contributes to virulence, and the appropriate constellation of virulence traits appears to be present only in selected lineages.

In conclusion, this survey showed that STEC strains could not be implicated as a major causal agent of diarrhea but are present in the food chain in Calcutta. What exactly will trigger such strains to cause outbreaks is unclear. Given that STEC are present in the food chain in Calcutta and that STEC are not currently important human enteropathogens there, it would be useful to trace the natural history of the organism, should they become important enteropathogens in the not-too-distant future.

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