Prevalence and genetic characteristics of Shigatoxigenic Escherichia coli from patients with diarrhoea in Maasailand, Kenya

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Abstract

Introduction: Shigatoxigenic *Escherichia coli* strains are food-borne bacterial pathogens that may cause haemorrhagic colitis (HC) in humans which can lead to life-threatening systemic complication, including haemolytic uremic syndrome (HUS).
This study aimed to characterize and analyze virulence properties of pathogenic *E. coli* isolates among patients with diarrhoea from a Maasai community in Kenya.
Methodology: Stool samples from 380 patients of all ages from the Kajiado and Narok districts of Kenya were investigated for the presence of enteric bacterial pathogens by conventional and molecular methods.
Results: Bacterial diarrhoea was diagnosed in 141/380 (37.1%) cases, of which enterotoxigenic *E. coli* (ETEC) compromised 29.8%, shigatoxigenic *E. coli* (STEC) 24.1%, enteroaggregative *E. coli* (EAEC) 14.2%, enteroinvasive *E. coli* (EIEC) 12.8% and enteropathogenic *E. coli* (EPEC) 3.5%. Gene analysis for STEC virulence factors showed that 52.9% isolates carried *stx1*, 29.4% possessed *stx2*, 14.7% carried both *stx1* and *stx2,* and 2.9% had *stx2e*. 23.5% isolates carried enterohaemolysin and 20.5% isolates possessed the Intimin gene. From 9 strains that exhibited adherence, 7 contained both Intimin and Haemolysin genes. Infections with Intimin-positive STEC strains (46%) were more frequent in patients with bloody diarrhoea, especially in children under 5 years of age, whereas Intimin-negative STEC infections dominated in adults.
Conclusion: Although STEC infection as a cause of bloody diarrhoea has not attracted much attention as a medical problem in Kenya, our findings indicate that this is a problem that must be investigated. The 24.1% isolation rate of STEC among the Maasai is one of the highest reported rates worldwide.

Key words: STEC; ETEC; EAEC; EIEC; EPEC; Intimin; Haemolysin; stx1; stx2; stx2e

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Introduction

Diarrhoea is a significant health problem worldwide, especially in the developing world where adequate sanitation facilities are lacking [1]. Globally diarrhoeal diseases account for almost a fifth of all deaths of children below five years of age, with an estimated 2.2 million deaths annually [2]. Epidemiological studies of diarrhoea have been reported from several African countries including South Africa [3], Gabon [4], Egypt [5] and Kenya [6]. A study conducted on travellers' diarrhoea that occurred in Europeans touring Mombasa, Kenya, reported an ETEC attack rate of 35% [7].

In developed countries, STEC strains are the major food-borne bacterial pathogens that have been implicated in diarrhoea, HUS and HC [8-10]. One serotype, O157:H7, is the dominant serotype associated with disease in many parts of the world, but other serotypes, notably O26, O103, O111, O121, O145, and O113 are also frequently implicated in disease [11]. Cattle have long been regarded as the principal reservoir of STEC strains, including those belonging to serotype O157:H7. Several outbreaks and sporadic cases of human illness caused by infection with *E. coli* O157:H7, O157: H- or the other STEC isolates from dairy cattle emphasize the role of raw milk as an important vehicle of transmission [12].

The first reported cases of *E. coli* O157:H7 hemorrhagic colitis in Africa were made in South Africa [13] and in Kenya [14].The aim of the present study was to characterize and analyze virulence properties of pathogenic *E. coli* isolates among

Table 1. Sequences of multiplex (m) PCR primers; forward (fp) and reverse (bp), (R = A and G) product and sizes

Gene	Ampli	Primers	Sequence (5'-3')
MEinv a MEinv b	140	invasive	fp: TGG AAA AAC TCA GTG CCT CTG CGG bp: TTC TGA TGC CTG ATG GAC CAG GAG
mVT1 a mVT1 b	121	Verotoxin-1	fp: ACG TTA CAG CGT GTT GCA GGG ATC bp: TTG CCA CAG ACT GCG TCA GTG AGG
mVT2a mVT2b	102	Verotoxin-2	fp: TGT GGC TGG GTT CGT TAA TAC GGC bp: TCC GTT GTC ATG GAA ACC GTT GTC
mVT2ea mVT2eb	322	Verotoxin-animal	fp: CCA GAA TGT CAG TAT ACT GGC GAC bp: GCT GAG GAC TTT GTA ACA ATG GCT G
mEagga mEaggb	194	aggregative	fp: AGA CTC TGG CGA AAG ACT GTA TC bp: ATG GCT GTC TGT AAT AGA TGA GAA C
mST1a mST1b	160	Heat-stable toxin 1	fp: TTT CCC CTC TTT TAG TCA GTC AAC TG bp: GGC AGG ATT ACA ACA AAG TTC ACA G
mST2a mST2b	423	Heat-stable toxin 2	fp: CCC CCT CTC TTT TGC ACT TCT TTC C bp: TGC TCC AGC AGT ACC ATC TCT AAC CC
mEaeA mEAEAb	241	Attaching and effacing	fp: TGA GCG GCT GGC ATG AGT CAT AC bp: TCG ATC CCC ATC GTC ACC AGA GG
mLT1a mLT1b	360	Heat-labile toxin 1	fp: TGG ATT CAT CAT GCA CCA CAA GG bp: CCA TTT CTC TTT TGC CTG CCA TC
mCNF1a mCNF1b	552	Cytotoxic necrotizing-1	fp: GGC GAC AAA TGC AGT ATT GCT TGG bp: GAC GTT GGT TGC GGT AAT TTT GGG
mCNF2a mCNF2b	839	Cytotoxic necrotizing-2	fp: GTG AGG CTC AAC GAG ATT ATG CAC TG bp: CCA CGC TTC TTC TTC AGT TGT TCC TC

* Ampli- Amplicon Source: [Ref 17]

patients with diarrhoea from the Maasai community who sought treatment in the Entosopia clinic, Kajiado district, and in the Narok District Hospital. These two districts are dominated by the pastoralist Maasai community and have previously reported high incidents of bloody diarrhoea. We hypothesized that STEC plays a significant role as a pathogen of bloody diarrhoea within the Maasai community.

Methodology

Specimen collection

The protocol for this study was granted approval by both the KEMRI Scientific Steering Committee as well as the National Ethical Review Committee.

After obtaining informed consent, stool samples were collected between August 2004 and July 2005 from 380 outpatients of all ages presenting with diarrhoea who were not on antibiotics in the last 72 hours. In total, 318 samples were obtained from the Narok District Hospital and 62 from the Entosopia clinic. Diarrhoea was defined as at least 3 loose stools in 24 hours, or any number of watery stools.

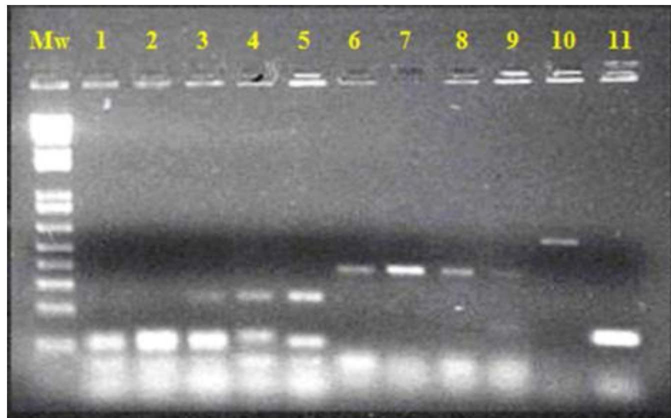
Collection was done in sterile plastic containers. The specimens were transferred into Cary-Blair transport media (MML Diagnostics Inc, Troutdale, Oregon, USA), labeled only with a unique study number, and then placed in an insulated box with ice packs and transported to the Kenya Medical Research Institute laboratory where they were processed within six hours of collection.

All stool samples were plated onto MacConkey agar, Xylose-Lysine-Deoxycholate agar (XLD), Sorbitol-MacConkey agar and *Campylobacter* blood-free agar. Initially selenite broth was used for enrichment purposes. The plates were incubated aerobically at 37°C for 18 to 24 hours, with the exception of *Campylobacter* plates, which were incubated at 42°C in microaerophilic conditions for 48 hours [15].

After overnight growth at 37°C, one to two suspect colonies each of *Shigella* and *Salmonella* and five to ten single colonies with typical *E. coli* morphology were selected and characterized on the basis of their biochemical reactions using BBL Enterotubes II (Becton Dickson Microbiology Systems, Sparks, Maryland USA).

The strains identified as *Salmonella, Shigella*, or *E. coli* by their colonial morphology and biochemical properties were further serotyped using O-antigen and H-antigen antisera (Denka Seiken Co LTD, Tokyo, Japan) by slide agglutination assays as previously described [16].

Figure 1. Multiplex PCR for detection of *E. coli* virulence factors. Agarose gel electrophoresis of the PCR representative DNA fragments amplified by 11 multiplex PCR products of *E. coli* pathotypes



Identification of virulence markers by multiplex PCR

A multiplex PCR assay allowed the detection of eleven trait genes or virulence markers in enteropathogenic *E. coli* serotypes. Primers for amplifying segments of genes of the Shiga toxins (stx1, stx2, and stx2e), Cytotoxin necrotising factors (CNFI and CNF2), attaching and effacing mechanisms (eaeA), enteroaggregative (EAEC), enteroinvasive mechanism (EIEC), and heat-labile (LT) and heat-stable (ST1 and ST2) toxins were used as described previously [17].

Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair are given in Table 1. PCR was performed in 0.2 ml Eppendorf tubes in a PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA) in a reaction volume of 25 µl. The DNA template, (2 µl of bacterial suspension) was added to a 25 µl reaction mixture containing 2.0 µl of 10 mM mix deoxynucleotide triphosphate (dNTPs), 2.5 µl of $MgCl_2$ (25 mM), 2.5 µl 10X buffer solution, and 1.25 µl of each of the PCR primer with a concentration of 0.5 pmol/µl (Bioserve Biotechnologies, Laurel, MD.USA). Finally, 0.3 µl of *Taq* Polymerase (5U/µl), (Applied Biosystems, Roche Molecular, Inc, and Branchbury, New Jersey, USA) was added to this reaction mix. Base sequences and predicted sizes of amplified products for the specific oligonucleotide primers were used as controls in the study.

Detection of Vero cytotoxin (VT or Stx)

The production of Shiga toxin by *stx* PCR-positive strains was checked by using the Vero cell cytotoxicity assay with slight modifications [18]. Vero cells (African green monkey kidney cells; ATCC CRL 1587) were grown at 37°C in EMEM (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Seromed), 1% L-glutamine (Life Technologies, Paisley, Scotland), 100,000 U of penicillin per liter, 100 mg of streptomycin per liter, 25 µg of amphotericin B per liter, and 1% minimal essential medium vitamin solution (Life Technologies) in an atmosphere of 5% CO_2. Briefly, the bacterial strains were inoculated into 10 ml of trypticase soy broth (Difco laboratories, Detroit, Michigan, USA) and incubated at 37°C overnight. After centrifugation at 12,000 × g for five minutes, supernatants were filtered through a Millipore filter (pore size 0.45 µm) (PolyLabo, Molsheim, France) and screened for Verocytotoxicity. Twofold serial dilutions of the culture filtrates were done in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) (100 µl per well; 12 wells per strain; dilutions from 1/2 to 1/2,048). A total of 20 µl of the test filtrate containing 10^5 Vero cells in suspension were added to each well. The culture plates were incubated for 24 hours at 37°C in a 5% CO_2. After 24 hours, cell monolayers were washed with phosphate-buffered saline (pH 7.2) and the cells fixed with methanol for five minutes and thereafter stained with Giemsa (5% w/v in phosphate buffer). After 45 minutes the monolayer was washed three times with distilled water and dried. The cells were examined microscopically. *E. coli* K-12 C600 was used as a negative control.

The toxin titers were expressed as the reciprocals of the highest dilution that caused cytotoxicity in 50% of Vero cells after 4 days of incubation, as judged by the dye intensity and microscopic observation.

Results

Isolation of bacteria

Stool samples from a total of 380 patients were tested by culture for the presence of enteric bacterial pathogens. These patients came from two districts, Narok and Kajiado, which are occupied mainly by the Maasai community. The study was conducted at the Narok District Hospital where 318 patients were examined and at the Entosopia clinic in Kajiado where 62 patients were recruited.

Bacterial diarrhoea was observed in 141 of 380 patients (37.1%). Diarrhoeagenic *E. coli* including ETEC, STEC, EAEC, EIEC, and EPEC were detected. The distribution of these 141 cases among the different *E. coli* pathotypes and other bacterial pathogens is shown in Table 1. ETEC comprised 42/141 (29.8%),

Table 2. Prevalence of enteric bacterial pathogens from 380 patients

Enteric pathogen	Number of patients	% of infected patients
ETEC	42	11.1%
STEC	34	8.9%
EAEC	20	5.3%
Shigella	19	5.0%
EIEC	18	4.7%
EPEC	5	1.3%
Salmonella	3	0.78%

STEC 34/141 (24.1%), EAEC 20/141 (14.2%), EIEC 18/141 (12.8%), and EPEC 5/141 (3.5%). Other enteric pathogens such as *Shigella* and *Salmonella* comprised 19/141 (13.5%) and 3/141 (2.1%), respectively.

Age distribution of diarrhoeal patients from whom STEC was isolated

The age distribution of patients with diarrhoea ranged between 2.4 months and 54 years. The median age was eight years while the mode was 45 years. The samples were divided into three distinctive groups based on the ages of the participants as follows: under five years; between five and 18 years; and above 18 years. STEC was isolated from 56% of females and 44% of males in all groups. The distribution of STEC infected patients per age groups were as follows: 50% were under five years of age, 18% were between 5 and 18 years and 32% were over 18 years (Table 2).

Multiplex PCR

Multiplex PCR analysis of 380 stool samples from diarrhoeal patients detected 11 of the major virulence genes of *E. coli* with four multiplex PCRs. The following four combinations of primers gave distinct and adequate amplification of their respective targets: VT1, VT2, VT2e and eaeA; CNF1 and EAEC; CNF2 and EIEC; and LTI, ST1 and ST2. The O157:H7 positive control strain yielded all three specific gene products in question or expected (*Stx1*, *Stx2* and *eaeA*), while no products were obtained from the *E. coli* negative control.

The combination of the different primers used in the multiplex reaction to amplify more genes in a single reaction is represented in Figure 1. With respect to the *stx* genes, analysis showed that all STEC (EHEC) strains examined had PCR products for VT1, VT2 or both; and seven among them harbored the *eaeA* gene that codes for Intimin (Table 3).

The prevalence of STEC virulence factors within diarrhoeal cases

A total of 34 patients were infected with STEC bacteria serogroups: O86, O6, O55, O28, O27, O25, O164, O151, O111, O148, O128, O126 and O78. The distributions of STEC infection with various virulence traits were as follows: 18 (52.9%) of 34 patients harbored *E. coli* containing *stx1* toxin genes, and all of them were 100% positive for Vero cell cytotoxic effect but had no other virulence factors tested (Table 3). The 10 isolates (29.4%) that harbored the *stx2* toxin gene only were 100% positive for Vero cell cytotoxicity assay and 40% positive for Haemolysin gene. They were also 30% positive for Intimin gene. The other five (14.7%) carried both *stx1* and *stx2* toxin genes and were also 100% positive for cytotoxicity and 80% positive for both Haemolysin and Intimin genes, while one (2.9%) which had *stx2e* toxin gene had no virulence factors detected (Figure 2).

Discussion

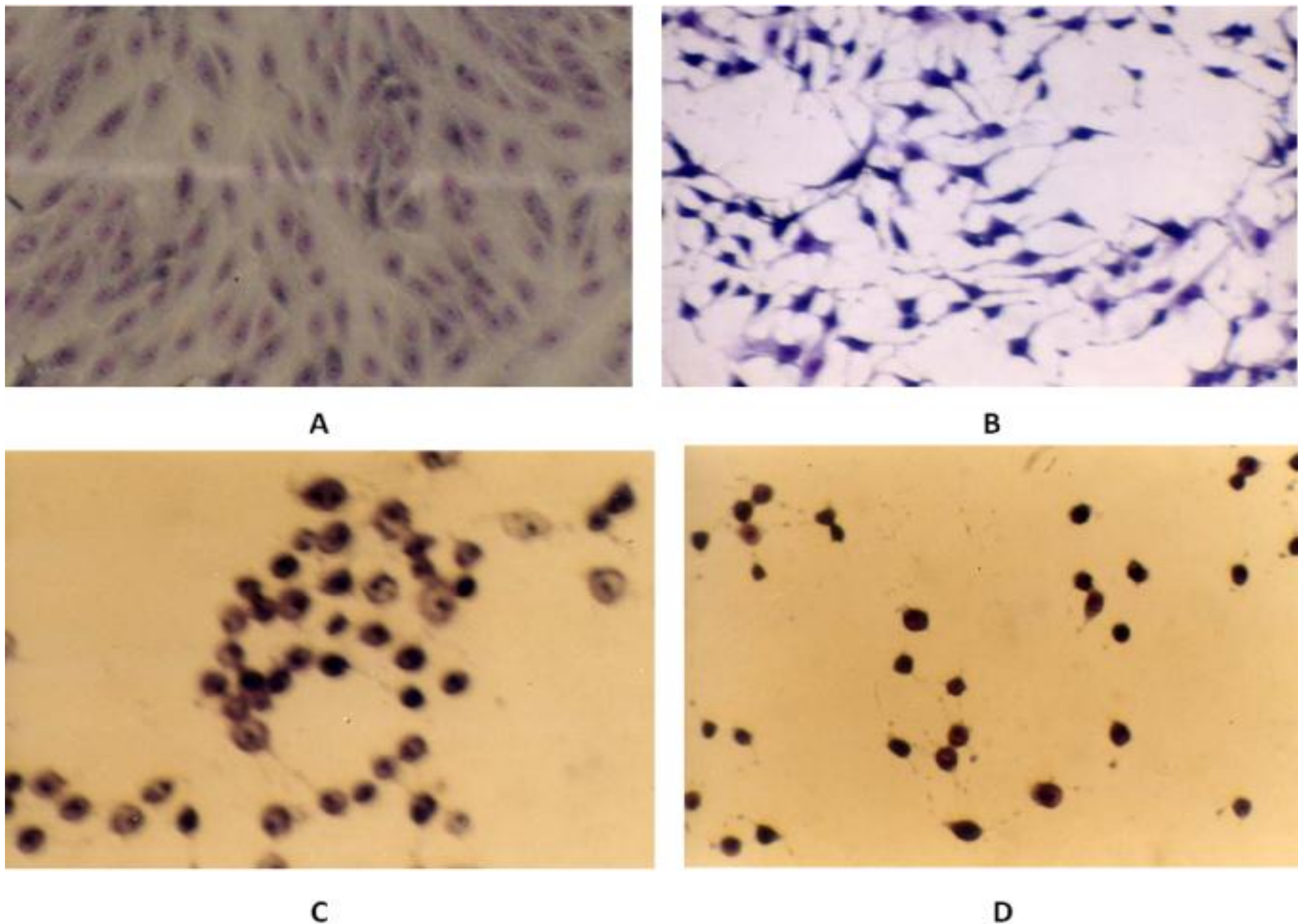
Previous studies in Kenya have documented the prevalence of some traditionally recognized agents of

Table 3. Prevalence of virulence factors in STEC bacteria

Virulence factors						
	Cytotoxic		**Haemolysin**		**eaeA**	
STEC type	N	%	N	%	N	%
VT1 alone	18	100.00%	0	0.00%	0	0.00%
VT2 alone	10	100.00%	4	40.00%	3	30.00%
VT1+VT2	5	100.00%	4	80.00%	4	80.00%
VT2e	1	100.00%	0	0.00%	0	0.00%

N = number

Figure 2. Test for Vero cytotoxic effect using different titers



A - The Vero cell line monolayer used as control 20×
B - Cytotoxic effect of Vero cell line at dilutions 1:64, 20×
C - Cytotoxic effect of Vero cell line at dilutions 1:32, 40×
D - Cytotoxic effect of Vero cell line at dilutions 1:4, 40×

diarrhoea such as *Shigella, Salmonella* and ETEC [6,14,19]. This study is the first to address the prevalence of STEC in stool samples from the Maasai community where poor hygiene conditions in the homesteads are common. Furthermore, this study correlated the presence of specific recognized or putative virulence factors with disease or severity of disease.

In the present study serotype O157:H7 or its non-motile variant O157:H- was not isolated. The STEC strains that were isolated comprised the following serogroups: O86, O6, O55, O28, O27, O25, O164, O151, O111, O148, O128, O126 and O78. Several of these isolates, 8/34 (23.5%), possessed some of the characteristics of virulence factors similar to those of the O157:H7 which cause serious diseases in humans. It is noteworthy that such a wide spectrum of different STEC serotypes can exist within a particular region.

The overall results show that ETEC and STEC are major bacterial pathogens in the community studied with a prevalence of 29.8% and 24.1% respectively. The large proportion of diarrhoeal cases in our study (62.9%) from whom no recognized pathogen was identified raises a number of issues. First, many different organisms may cause diarrhoea and it is not possible in a study such as this to screen specimens for the entire range of potential enteropathogens. Second, even when specifically sought, some pathogens may not be detected by conventional microbiological methods because of excretion in insufficient numbers. A study by Joyce *et al*. showed a prevalence of 13% enteropathogenic *E. coli* among Maasai children under five years of age [22]. However, their study did not characterize *E. coli* into their pathotypes.

Studies from different countries have also shown that humans can be infected with a large spectrum of

serologically different STEC types [20]. STEC is associated with a broad spectrum of illnesses in humans including non-bloody and bloody diarrhoea, haemorrhagic colitis (HC) and the often deadly haemorrhagic uremic syndrome (HUS) [9].

Finding the newly described diarrhoeal pathogens, especially of varying STEC serotypes, in our patients in a relatively higher proportion (24.1%) than those which have been reported in many countries including Switzerland 1% [21], Germany 1% [22], Belgium 0.2% [23], and 3% [24] France, is of great clinical importance. Notably, some of the serotypes detected (O86, O28) in this study were not those typically known to be associated with STEC and nearly all the strains carry genes known as potential virulence factors associated with STEC infections. The O25 and O111 serogroups observed here have been reported at higher frequencies varying from 9% to12% in Chile and 3% to 15% in Argentina among HUS patients [25,26].

Although STEC infection has not attracted much attention as a medical problem in Kenya, the findings from this study have indicated that this is an existing problem that needs to be investigated.

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