An Outbreak of Diarrhea in Mandera, Kenya, due to *Escherichia coli* Serogroup O-Nontypable Strain That Had a Coding Gene for Enteraggregative *E. coli* Heat-Stable Enterotoxin 1

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Abstract. In an outbreak of gastroenteritis in December 2009, in Mandera, Kenya, *Escherichia coli* O-nontypable (ONT) strain was isolated from stool specimens of patients (18/24, 75%). The *E. coli* ONT organisms could not be assigned to any of the recognized diarrheagenic groups of *E. coli*. However, they possessed the enteraggregative *E. coli* heat-stable enterotoxin-1 gene. The cell-free culture filtrates of the *E. coli* ONT strain isolated from the outbreak cases induced considerable amount of fluid accumulation in suckling mouse intestine, indicating production of an enterotoxic factor(s). These results identify *E. coli* that did not have any diarrheagenic characteristics except *astA* as the etiological agent of the diarrheal outbreak in Mandera. It is however considered necessary to characterize the fluid accumulation factor(s) to determine whether any novel toxins were responsible for the fluid accumulation. Moreover, it is important to study dissemination of strains producing the enterotoxic factor(s) to assess their public health significance distribution in the environment.

INTRODUCTION

Escherichia coli is known as a harmless commensal of the gastrointestinal tract in warm-blooded animals.¹ However, it also has the pathogenic capacity to cause significant intestinal and extraintestinal diseases. Those strains causing intestinal infections can be divided into six pathotypes: enteraggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC). The pathotype to which a particular strain belongs is defined by the clinical manifestation of disease, the repertoire of virulence factors, epidemiology, and phylogenetic profiles.²

EAEC heat-stable enterotoxin 1 (EAST1) was originally found as an enterotoxin of EAEC.³ However, the role of EAST1 in human disease is still controversial. EAST1 has been reported to be produced by approximately half of the EAEC^{4,5}; consequently, it is not clear whether production of this toxin was relevant to the manifestation of diarrhea due to EAEC. Recently, it was reported that the EAST1 gene (*astA*), or its variants, were present not only in EAEC but in other diarrheagenic *E. coli* including some EPEC and ETEC.^{5–8}

Escherichia coli that does not exhibit any diarrheagenic characteristics other than EAST1 gene, termed EASTEC, was isolated from patients of a diarrheal outbreak in Japan.^{9,10} In a case-control study in Spain, it had been shown that EASTEC was associated with diarrheal disease rather than EAEC.¹¹ It is conceivable that EAST1 could contribute to diarrhea in EASTEC strains. However, the pathogenicity of EASTEC only has not been determined in an animal model and therefore the role of EAST1 in diarrhea remains an open question.^{12–14} The objectives of this study were to identify the source of the diarrheal outbreak in Mandera, Kenya, and to suggest recommendations to prevent the occurrence of similar outbreaks in the future.

MATERIALS AND METHODS

Description of the outbreak and epidemiological studies. An outbreak of gastroenteritis occurred in December 2009 in Laffey sublocation, Mandera District, Kenya, affecting 324 people. On December 15, 2009, a wedding party took place within Laffey location. At the party, a camel was slaughtered for the celebration. The slaughtered animal was among the animals that came back from the neighboring Somali. People of this area believe in a conventional therapy (called “kib”) that inserting a wounded body part into the intestinal content of the slaughtered animal results in healing of the wound. Among the participants in the party was a 3-year-old boy who had suffered some burns on his hand 3 months earlier. As reported by the father, the boy was advised to dip the wounded hand for around 5 hours into the intestinal bowel of the slaughtered camel and later wash with the soup of the boiled meat. The following day, the boy presented with vomiting and profuse diarrhea, and died while being taken to hospital. This was the first case of the entire outbreak. The following day, the brothers of the boy also presented with same symptoms but were treated. More cases were reported henceforth.

Members of the index group were interviewed since December 26. The control group consisted of people who lived in Laffey location and had not had diarrhea in the previous 2 weeks. Fecal specimens for bacterial culture were obtained between December 26 and 28. They were examined for the presence of *Salmonella*, *Shigella*, *Vibrio*, *E. coli*, *Campylobacter*, *Aeromonas*, and Norwalk-like viruses. Direct examination of culture plates of the patients showed heavy growth of lactose-fermenting colonies on deoxycholate hydrogen-sulfide lactose agar (Nissui Pharmaceutical, Tokyo, Japan).

Bacterial strains. A total of 75 strains (24 cases, 24 controls, 22 domestic animal stools, and five domestic animal milks) of *E. coli* were examined. *Escherichia coli* JM109, a strain of K-12 that does not cause diarrhea in humans and has no specific pattern in adherence assays, was used as a negative control. The presence of O antigens was determined by slide agglutination with the available O (O1–O165) antisera (Denka Seiken, Tokyo, Japan).

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Polymerase chain reaction. Polymerase chain reaction (PCR) was used to examine the presence of genes associated with diarrheagenicity. The bacteria were grown overnight at 37°C in Luria-Bertani (LB) broth (Difco, Sparks, MD). A sample (100 µL) of the culture was centrifuged and the pellet was resuspended in distilled water. The cell suspension was boiled, and the supernatant was used as a template for PCR. The primer sets used are shown in Table 1.^{15–24} For detection of PCR products, 10 µL of the amplification mixture and molecular weight markers were subjected to electrophoresis in 2% agarose gels. Amplified DNA fragments of specific sizes were located by ultraviolet (UV) fluorescence after staining with ethidium bromide.

Adherence to tissue culture cells. Adhesion tests to HEp-2 cells in culture were done in 3-hour periods as described previously.²² HEp-2 cells (ATCC CCL-23) were grown for 24 hours to 50–70% confluence on 8-well Laboratory-Tec chamber slides (Nunc, Roskilde, Denmark). One milliliter of Dulbecco's modified Eagle's medium without antibiotics or sera but containing 1% (w/v) D-mannose (Sigma-Aldrich, St Louis, MO) was added to each well. Overnight bacterial culture (10 µL) was inoculated into each well and the chamber slides were incubated at 37°C for 2 hours. The HEp-2 cells were washed three times with phosphate-buffered saline and fixed with absolute methanol and stained with 10% (v/v) Giemsa.

Susceptibility to antimicrobial agents. Sensitivity of the *E. coli* strains to the following antibiotics was examined using Sensi Disk according to the manufacturer's instructions (Becton-Dickinson Microbiology Systems, BD Diagnostics, Sparks, MD): ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, cefepime, meropenem, amikacin, gentamicin, tobramycin, norfloxacin, nitrofurantoin, sulfamethoxazole/trimethoprim, tetracycline, and trimethoprim.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed as previously described by Izumiya and others.²⁴ *NotI* and *XbaI* were used as the restriction endonucleases. Digested DNA was electrophoresed in a 1% agarose gel in 0.5× Tris-borate-ethylenediamine tetraacetic acid buffer by using a CHEF Mapper apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) at 12°C at 200 V. A linearly ramped switching time of 2.91–63.8 seconds was applied for 27 hours 12 minutes.

Plasmid banding pattern. *Escherichia coli* cultures were grown in LB for 18 hours at 37°C with vigorous agitation. Samples of broth culture (5 mL) were centrifuged, and the pelleted organisms were then subjected to the rapid alkaline lysis method of Kado and Liu.²⁵ Plasmid DNA was then separated by horizontal electrophoresis through a 1.0% agarose gel, stained with ethidium bromide, and photographed under UV light.

TABLE 1
Genes or regions targeted in PCR amplification and primers used in this study

Target	Primer (5'-3')	Product size	Reference
<i>aggR</i>	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	254 bp	21
<i>aat</i> (CVD432)	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630 bp	22
<i>elt</i>	AGCAGGTTTCCCACCGGATCACCA GTGCTCAGATTCTGGGTCTC	132 bp	23
<i>estIa</i>	TCCGTGAAACAACATGACGG ATAACATCCAGCACAGGCAG	244 bp	17
<i>estII</i>	GCCTATGCATCTACACAATC TGAGAAATGGACAATGTCCG	278 bp	17
<i>eae</i>	GCTTAGTGCTGGTTAGGAT CTCTGCAGATTAACCTCTGC	591 bp	21
<i>bfpA</i>	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACTGGTA	326 bp	20
EAF	CAGGGTAAAAGAAAGATGATAA TATGGGGACCATGTATTATCA	397 bp	18
<i>invE</i>	ATATCTCTATTTCCAATCGCGT GATGGCGAGAAATTATATCCCG	382 bp	23
<i>Stx1/2</i>	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAAT	518 bp	24
<i>afaC</i>	CGGCTTTTCTGCTGAAGTGGCAGGC CCGTCAGCCCCACGGCAGACC	672 bp	16
<i>astA</i>	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	111 bp	7
<i>hlyA</i>	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1,177 bp	22
<i>set</i> (ShET-1)	TCACGCTACCATCAAAGA TATCCCCCTTTGGTGTA	309 bp	22
<i>sen</i> (ShET-2)	ATGTGCCTGCTATTATTTAT CATAATAAAGCGGTCAGC	799 bp	22
<i>sat</i>	ACTGGCGGACTCATGCTGT AACCCTGTAAGAGACTGAGC	387 bp	22
<i>pet</i>	ACTGGCGGACTCATTGCTGT GCGTTTTTCCGTTCCCTATT	832 bp	22
<i>pic</i>	GGGTATTGTCCGTTCCGAT ACAACGATACCGTCTCCCG	1,175 bp	19

bp = base pairs; PCR = polymerase chain reaction.

DNA sequencing. DNA purified from the *E. coli* cultures were used as template DNA for PCR. The PCR product of the *astA* with 100– base pair (bp) upstream and 30-bp downstream flanking region was purified by using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). The purified PCR products were directly sequenced with ABI BigDye v3.1 cycle sequencing kit (Foster City, CA).

Enterotoxigenicity. The suckling mouse assay was carried out by the method of Giannella.²⁶ The test organism was propagated in tryptic soy broth (TSB) overnight at 37°C, and the supernatant obtained by centrifugation (15,000 × *g*, 1 minute) was filtered through a 0.22-μm-pore-size sterile membrane filter (Millipore Products, Bedford, MA). Newborn suckling mice (five suckling mice Jcl:ICR each 3–4 days of age and weighing between 2.0 and 3.0 g) were then administered intragastrically with a total volume of 100 μL of heated and unheated control fluid (CF) or control plus 2 μL of a 2% Evans blue to ascertain the proper administration of the sample.

The mice were killed 3 hours later having been left at room temperature, and the G/B ratio (the ratio of the weight of the gut to the remaining body weight) was measured for the individual animals, and the average and standard error (SE) were calculated for each test group. The test sample was heated for 15 minutes at 65°C or 100°C and subjected to the same test to assess the effect of heat on the enterotoxin factor(s). Results are shown in Table 2. EASTEC strains 0081, 0093, 0151, 0153, and 0231 were isolated from the patients of the outbreak; strains 0041 and 0123 were isolated from the control group. Values represent means ± SE for four to five experiments. Samples with ratios greater than 0.083 were considered enterotoxigenic.

Statistical methods. Differences between the numbers of microbial findings from persons with and without diarrhea were compared by using Fisher's exact test. A *P* value of < 0.05 indicated statistical significance.

Ethics statement. This study was approved by the Ethical Review Board of the Kenya Medical Research Institute under review number KEMRI/SSC-1323. All experiments carried out in mice in this study followed the Guidelines for the Management of Laboratory Animals in the Fujita Health University which adheres to the "Act on Welfare and Management of Animals, Japan."

RESULTS

Epidemiology. Epidemic curves are shown in Figure 1. Outbreak of diarrhea started at the beginning of December.

A number of cases among patients exhibited three peaks at December 17, 20, and 23. Although the majority of cases of patients were children 0–5 years of age, adults of over 20 years of age also developed diarrhea.

In the period of the outbreak of diarrhea, the patients did not eat any common foods except camel milk, one of their staple foods. The raw milk was suspected to be the contaminated food. Symptoms were effortless diarrhea (24/24, 100%), abdominal pain (24/24, 100%), nausea (24/24, 100%), and watery vomiting (24/24, 100%). There was neither fever nor headache in any patient. Of the 24 diarrheal patients in Laffey local hospital, diarrhea occurring in 20 was characterized by greenish and/or mucoid stools. Eighty percent of the stool specimens were greenish. The stool specimens collected from 24 patients and 24 controls during December 26–28 were examined by bacteriological, biochemical, and multiple PCR tests for the presence of *E. coli*, *Vibrio cholerae*, *Shigella* spp., *Salmonella* spp., *Aeromonas* spp., and *Campylobacter jejuni*. The rates of detection of bacterial pathogens in patients and controls are shown in Table 3. All laboratory tests for other bacterial pathogens were negative. We classified *E. coli* isolated from the stool specimens of patients and controls into six main pathotypes on the basis of their specific virulence-associated gene(s).² To differentiate between the six categories of diarrheagenic *E. coli*, we selected the target virulence-associated genes: *aggR* and *aat* for EAEC; *elt*, *estIa*, and *estII* for ETEC; *eae*, *bfpA*, and *EAF* for EPEC; *invE* for EIEC; *stx1*, *stx2*, and *eae* for EHEC; *afaC* for DAEC; and *astA* and *hlyA* distributed over the categories of diarrheagenic *E. coli*, and carried out PCR assay for *E. coli* isolated from the specimens. *Escherichia coli* isolated from the specimens was classified into one putative diarrheagenic *E. coli* and two diarrheagenic *E. coli* pathotypes: EASTEC, EAEC, and ETEC. EASTEC was isolated at high frequency of 75% (18 of 24 cases), but at low frequency of 8% (two of 24 cases) in controls (Table 4). EASTEC was significantly associated with diarrhea (Fisher's exact test, *P* < 0.001). The difference in the incidence of EAEC and ETEC between patients and controls was not statistically significant (Table 4).

No pathogenic bacteria were detected in two water samples (Table 3). *Escherichia coli* were isolated from all (100%) of 22 fecal samples and five (42%) of 12 milk samples from domestic animals (Table 3). Other bacterial pathogens were not isolated from these animal samples. The presence of diarrheagenic *E. coli* in animal stools and milk was

TABLE 2
Gut weight/carcass weight ratio obtained for the Mandera's EASTEC strains of mice

<i>Escherichia coli</i> strain	Enterotoxin gene			Gut weight/carcass weight heat treatment		
	<i>elt</i>	<i>est</i>	<i>astA</i>	Nontreatment	65°C	100°C
Control (TSB)				0.055 ± 0.001	0.055 ± 0.001	0.056 ± 0.001
JM109	–	–	–	0.058 ± 0.001	0.056 ± 0.001	0.058 ± 0.001
EASTEC 0081	–	–	+	0.104 ± 0.004	0.121 ± 0.007	0.126 ± 0.005
EASTEC 0093	–	–	+	0.112 ± 0.005	0.111 ± 0.00	0.119 ± 0.003
EASTEC 0151	–	–	+	0.133 ± 0.002	0.137 ± 0.001	0.126 ± 0.003
EASTEC 0153	–	–	+	0.143 ± 0.003	0.123 ± 0.008	0.142 ± 0.006
EASTEC 0231	–	–	+	0.110 ± 0.004	0.121 ± 0.004	0.117 ± 0.002
EASTEC 0041	–	–	+	0.057 ± 0.004	0.057 ± 0.002	0.056 ± 0.001
EASTEC 0234	–	–	+	0.058 ± 0.001	0.057 ± 0.001	0.058 ± 0.001

TSB = tryptic soy broth.

FIGURE 1. Epidemic curve of diarrheal disease outbreak. The epidemic curve was drawn based on the number of diarrheal cases clinically confirmed between December 1 and 28, 2009, by date of onset. Arrow indicates the dates warning of diarrhea prevalence was emitted, outbreak information was offered, and surveillance was conducted.

assessed. EPEC, EIEC, and EHEC were not detected from the animal samples (Table 3). ETEC was isolated from the chicken stool, three (60%) of five cow stools, and one of the two camel milk samples (Table 4). EAEC was isolated from four (80%) of cow stool samples (Table 4). However, EASTEC strain was not recovered from 20 fecal and 12 milk samples from animals in Laffey sublocation (Table 3).

Recently, three novel toxins, *Shigella* enterotoxin 1 (ShET-1), *Shigella* enterotoxin 2 (ShET-2) and the secreted auto-transporter toxin (Sat), have been described in *Shigella flexneri*.^{27–30} Furthermore, two virulence factors, plasmid-encoded toxin (Pet) and autotransporter protein involved in colonization (Pic) have been described also for EAEC.³¹ We examined whether there were these additional virulence genes other than *astA* in the EASTEC outbreak strains in Mandera. All EASTEC outbreak strains were not detected the additional virulence genes in PCR test.

PFGE and DNA sequencing analysis. We analyzed the EASTEC outbreak strains by PFGE. PFGE patterns from five randomly selected EASTEC outbreak strains (0081, 0093, 0151, 0153, and 0231) showed the similar DNA banding pattern in PFGE after treatment with the restriction enzymes *NotI* and *XbaI*, and the pattern was distinct from those of the two EASTEC strains (0041 and 0234) isolated from the specimen of controls (Figure 2). Similarly, the EASTEC outbreak strains showed the same plasmid banding pattern, and it was distinct from that of the two EASTEC strains from controls (Figure 3).

DNA sequence of *astA*. EAEC strain 042 is a prototypical EAEC strain. Recombinant EAST1 from the strain 042 induces an increase in short circuit current in Ussing cham-

bers indicative of a net anion secretion.¹⁴ We compared DNA sequences through alignments of *astA* in Mandera's EASTEC strains with *astA* of the EAEC strain 042 which has been deposited in the GenBank (accession no.: FN554767). The nucleotide sequences of the *astA* with 100-bp upstream and 30-bp downstream flanking region of the outbreak strains were identical to that of EAEC strain 042, resulting in no deduced amino acid changes (data not shown). Similarly, the *astA* region of strain 0041 was identical to that of EAEC strain 042 (data not shown).

The EASTEC outbreak strains were all sensitive to the 19 kinds of antibiotics examined. We performed serotyping of the strain with the antisera. However, the serotypes of all outbreak strains were O-nontypable (ONT) with the tested antisera.

Adherence to HEP-2 cells. Toshima and others reported that adherence to HEP-2 cells was observed in many of EASTEC strains isolated from the fecal specimens of diarrheal patients.³² We examined the EASTEC outbreak strains in Mandera for adherence to HEP-2 cells. All EASTEC isolates were not observed to adhere to HEP-2 cells, just like JM109 that was used as a negative control (data not shown).

Examinations for enteropathogenicity. In vivo enterotoxicity assay using the suckling mice model was used to evaluate the biological activity of filtrates of the EASTEC outbreak strains in Mandera. Most investigators define a positive response as a G/B ratio of 0.083 or greater.^{12,14,26} The culture filtrate of *E. coli* JM109 and sterile culture medium (TSB) were used as negative controls (G/B ratio of ≤ 0.059 and ≤ 0.058 , respectively). CF from EASTEC outbreak strain (0081) was inoculated into suckling mice and gave a G/B ratio of 0.104 ± 0.004 ($N = 6$) (Table 2). The EASTEC outbreak strains (0081, 0093, 0151, 0153, and 0231) other than the 0081 strain also produced an extra-cellular factor(s) in the test media and thus stimulated considerable fluid accumulation in the intestines of the suckling mice (G/B ratio, > 0.083). Two strains (0041 and 0234) of EASTEC isolated from control group did not induce fluid accumulation in the suckling mice. To determine the effect of heat on the activity, CF of the outbreak strains were preheated at 65°C or 100°C for 15 minutes before the suckling mouse assay. The factor(s) causing intestinal fluid accumulation in the suckling mice was heat stable (Table 2).

DISCUSSION

The EASTEC ONT organisms were isolated from 18 (75%) of 24 stool specimens collected from patients during the previous 3 days (December 26–28) of the outbreak. The epidemic curve exhibited three peaks in one big peak. The epidemic curve was consistent with a common source

TABLE 3
Frequency of isolated enteropathogens

<i>Escherichia coli</i> or enteropathogens	No. (%) of isolates in human stools (cases, $N = 24$)	No. (%) of isolates in human stools (Controls, $N = 24$)	No. (%) of isolates in stool from domestic animals ($N = 22$)	No. (%) of isolates in milk from domestic animals ($N = 22$)	No. (%) of isolates in water ($N = 2$)
<i>Escherichia coli</i>	24 (100)	24 (100)	22 (100)	5 (42)	0
<i>Vibrio cholerae</i>	0	0	0	0	0
<i>Shigella</i> spp.	0	0	0	0	0
<i>Salmonella</i> spp.	1 (4)	0	0	0	0
<i>Aeromonas</i> spp.	0	0	0	0	0
<i>Campylobacter jejuni</i>	0	0	0	0	0

TABLE 4
Frequency of diarrheagenic *Escherichia coli* from human, animal stool, and animal milk samples

<i>E. coli</i> categories	No. (%) of isolates for									
	Human stools		Animal stools					Animal milk		
	Case	Control	Camel	Chicken	Cow	Donkey	Goat	Camel	Cow	Goat
	(<i>N</i> = 24)	(<i>N</i> = 24)	(<i>N</i> = 4)	(<i>N</i> = 1)	(<i>N</i> = 5)	(<i>N</i> = 5)	(<i>N</i> = 5)	(<i>N</i> = 4)	(<i>N</i> = 4)	(<i>N</i> = 4)
EASTEC	18 (75)	2 (8)	0	0	0	0	0	0	0	0
EAEC	2 (8)	2 (8)	0	0	4 (80)	0	0	0	0	0
ETEC	5 (21)	4 (16)	0	1 (100)	3 (60)	0	0	1 (50)	0	0
EPEC	0	0	0	0	0	0	0	0	0	0
EIEC	0	0	0	0	0	0	0	0	0	0
EHEC	0	0	0	0	0	0	0	0	0	0

EAEC = enteroaggregative *E. coli*; EHEC = enterohemorrhagic *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*.

outbreak. The outbreak isolates were recognized to be one clone considering epidemiological information, antibiotic sensitivity, plasmid banding, and PFGE patterns. The isolated EASTEC ONT organisms were sensitive to all antimicrobial drugs tested. This not only suggests that they are a single clone but also the possibility that these organisms originated from a nonhuman source such as domesticated/wild animals or are environmental strains. In our outbreak investigation, we have described a cultural practice by the Somali people called "kib" during local celebrations in which a camel is slaughtered. This is very supportive of our data that the camel was the likely source of the EASTEC ONT organisms. It was assumed that these organisms were the causative agent of the diarrhea. However, they did not have any diarrheagenic traits except the *astA*. Consequently, they could not be assigned to any of the recognized diarrheagenic groups of *E. coli*.

EAST1 is reported to be a heat-stable enterotoxin of EAEC.⁴ The toxin is a 38-amino acid homologue of the ETEC heat-stable enterotoxin a (STa) and is present in many EAEC strains. Furthermore, it has been demonstrated that the purified recombinant EAST1¹² and a synthetic peptide consisting of amino acid residues 8–29 of EAST1⁴ produced an increase in potential difference and short-circuit current in Ussing chambers with mounted rabbit ileal tissue. Previously, the results of Vila and others have suggested an

association between EAST1-positive strains and diarrhea in children.¹¹ In addition, Zhou and others reported a gastroenteritis outbreak caused by a strain of EASTEC, strain O166:H15, in Osaka, Japan.¹⁰ However, *astA* is widely found in different categories of diarrheagenic *E. coli*, and EASTEC was found to be highly prevalent in healthy individuals, and to a similar extent as in diarrheal patients.^{5,33,34} This lack of clarity around EASTEC as a diarrheagenic *E. coli* may be due to the fact that only strains that carry other pathogenic factors in addition to EAST1 are diarrheagenic in humans. Several novel virulence genes apart from typical pathogenic determinants have been reported for diarrheagenic *E. coli* strains.²⁹ There are no reports on the presence of these virulence genes in EASTEC. We examined the presence of nontypical virulence genes (*ShET-1*, *ShET-2*, *Sat*, *Pet*, and *Pic* genes) in the EASTEC outbreak strains. However, the EASTEC strains did not harbor any of these virulence genes.

ETEC strains produce two kinds of classical virulence factors.^{35,36} One is an enterotoxin and the other is a colonization factor. Colonization factors should be essential for EASTEC as well as for ETEC. Adherence to HEp-2 tissue culture cells has been used to detect virulence among diarrheagenic *E. coli*.³⁷ We examined the EASTEC outbreak strains for adherence to HEp-2 cells. The EASTEC strains did not adhere to HEp-2 cells. Adherence to HEp-2 cells by

FIGURE 2. Pulsed-field gel electrophoresis (PFGE) patterns of *NotI*- and *XbaI*-digested genomic DNA of EASTEC isolates. (A) *NotI* digestion patterns; (B) *XbaI* digestion patterns. EASTEC strain 0081 (lane 2), 0093 (lane 3), 0151 (lane 4), 0152 (lane 5), 0153 (lane 6), 0154 (lane 7), and 0231 (lane 8) were isolated from the patients of the outbreak; strain 0041 (lane 9) and 0123 (lane 10) were isolated from the control group in Mandera. Lane 1, molecular size marker (48.5-kb lambda DNA ladder).

FIGURE 3. Plasmid profile of EASTEC isolates from the patients of the outbreak and control group in Mandera. EASTEC strain 0081 (lane 2), 0093 (lane 3), 0151 (lane 4), 0153 (lane 5), and 0231 (lane 6) were isolated from the patients of the outbreak; strain 0041 (lane 7) and 0123 (lane 8) were isolated from the control group. Lane 1, molecular size marker (λ HindIII).

EAEC and DAEC strains is associated with the expression of fimbrial and nonfimbrial adhesins, designated aggregative adherence fimbriae (AAF), and afimbrial adhesin/Dr (Afa/Dr) adhesions.² A nonadherent phenotype of the EASTEC outbreak strains to HEP-2 cells is compatible with the result of PCR analysis showing that the outbreak strains do not possess the genes (*aggR*, *aat*, and *afaC*) associated with fimbrial and nonfimbrial adhesion. Our data indicate that the outbreak strains do not express AAF and Afa/Dr adhesins on the outer surface of the organisms.

Zhou and others¹⁰ showed no expression of ETEC colonization factors (CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCFO159, PCFO166, and CFA/III) on EASTEC isolated from patients in a gastroenteritis outbreak. EASTEC may have colonization factors different from those which were well established in these diarrheagenic *E. coli*.

Recently, a new putative category of diarrheagenic *E. coli*, termed cell-detaching *E. coli* (CDEC) was identified.³⁸ CDEC causes detachment of HEP-2 cell monolayers from glass coverslips and lacks the virulence factors found in the six well-recognized categories of diarrheagenic *E. coli* strain.³⁸ Alpha-hemolysin (HlyA) produced by CDEC is considered to be the main factor responsible for detachment of cells cultured in vitro.³⁹ We validated the presence of *hlyA* by PCR in the EASTEC outbreak strain, but it was negative. The strains were also tested for hemolytic activity. The strains were not hemolytic on blood agar plates (data not shown).

Based on the findings obtained in a case-control study, Villa and others had suggested that EASTEC strains are associated with diarrheal diseases rather than EAEC.¹¹ However, EASTEC has not been for now accepted as an etiological agent in diarrheal diseases. One of the reasons is that the pathogenicity of EASTEC only has not been determined in an animal model. We chose a suckling mouse system as an animal model because of its value in simulating the actual course of diarrhea. The results indicate that the EASTEC outbreak strains are diarrheagenic to suckling mice and contain the factor(s) causing intestinal

fluid accumulation in the culture supernatant. However, there are a number of limitations to the interpretation of this study. A factor(s) that induces fluid accumulation in suckling mice was not shown to have been produced by EASTEC. Further study to establish a purification method for the factor(s) is needed. Purified factor(s) will facilitate the investigation of the diarrheagenic effect. The results of these investigations will lend support for our data that the EASTEC behaves as a diarrheagenic *E. coli*. The pathological role of EAST1 could have been emphasized if purified EAST1 functionally active as a positive control for the in vivo analysis was studied alongside the outbreak strains. Moreover, the Ussing chamber method which has been widely applied to the EAST1 determination, since its pathophysiological role was first proposed by Savarino and others in 1993,⁴ would have provided definitive results of the pathological role of EAST1 in the outbreak strains.

Despite these limitations, diarrheagenicity of the EASTEC strains isolated from the outbreak patients was for the first time elucidated by a suckling mouse assay for fluid accumulation in the intestinal tracts. More detailed characterization of the fluid accumulation factor(s) of the EASTEC strains (e.g., the relationship between the enterotoxin factor(s) of the EASTEC and those of related diarrheagenic *E. coli*) and studies of the dissemination of the EASTEC strains producing the enterotoxin factor(s) are needed to assess the public health significance of the enterotoxin EASTEC strains distributed in the environment.

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