

An outbreak of gastroenteritis in Osaka, Japan due to *Escherichia coli* serogroup O166:H15 that had a coding gene for enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1)

Z. ZHOU^{1*}, J. OGASAWARA¹, Y. NISHIKAWA^{1,2†}, Y. SETO¹, A. HELANDER³,
A. HASE¹, N. IRITANI¹, H. NAKAMURA¹, K. ARIKAWA², A. KAI⁴,
Y. KAMATA⁵, H. HOSHI⁵ AND K. HARUKI¹

¹ Department of Health and Epidemiology, Osaka City Institute of Public Health and Environmental Sciences, 543-0026, Japan

² Graduate School of Human Life Science, Osaka City University, 558-8585, Japan

³ Department of Medical Microbiology and Immunology, Göteborg University, Sweden

⁴ Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, 169-0073, Japan

⁵ Laboratory of Veterinary Public Hygiene, Osaka Prefecture University, 599-8570, Japan

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SUMMARY

In an outbreak of gastroenteritis on 23 July 1996, in Osaka, Japan, 54 of 91 persons who had attended a meeting the previous day became ill. *Escherichia coli* O166:H15 was isolated from stool specimens of patients (29/33, 88%). Laboratory tests for other bacterial pathogens and viruses were negative. The *E. coli* O166 organisms did not adhere to HEp-2 cells in a localized, diffuse, or enteroaggregative manner. The organisms did not express known enterotoxigenic *E. coli* (ETEC) colonization factors. In polymerase chain reaction tests, the bacteria did not have coding genes for shigatoxin of enterohemorrhagic *E. coli* (EHEC), heat-labile, or heat-stable enterotoxin of ETEC, attachment and effacement (*eaeA*) of EPEC, or invasion (*invE*) of enteroinvasive *E. coli* (EIEC). Consequently, they could not be assigned to any of the recognized diarrhoeagenic groups of *E. coli*: EPEC, ETEC, EHEC, EIEC, enteroaggregative *E. coli* (EAggEC), or diffusely adhering *E. coli*. However, the organisms possessed the EAggEC heat-stable enterotoxin (EAST1) gene. To our knowledge, this is the first report of an outbreak caused by *E. coli* that did not have well-characterized virulence genes other than EAST1. The isolates showed the same DNA banding pattern in pulsed-field gel electrophoresis after digestion with the restriction enzymes *Xba*I or *Not*I. Three O166:H15 strains isolated from two sporadic cases and another outbreak during 1997–8 were distinct, indicating that multiple clones have spread already. We propose that diarrhoeal specimens should be examined for *E. coli* possessing the EAST1 gene.

INTRODUCTION

Enteroaggregative *Escherichia coli* (EAggEC) heat-stable enterotoxin 1 (EAST1) was originally found as an enterotoxin of EAggEC [1]. However, the role of EAST1 in human disease is still controversial. EAST1

has been reported to be produced by approximately half of the EAggEC [2, 3]; consequently, it is not clear if production of this toxin was relevant to the manifestation of diarrhoea due to EAggEC. Recently, it was reported that the EAST1 gene, or its variants, were present not only in EAggEC but in other diarrhoeagenic *E. coli* including some EPEC and ETEC [4–6]. Actually, Hedberg and colleagues found that an outbreak of gastrointestinal illness in 1991 had

* Author for correspondence.

† Present address: Changchun University of Agriculture and Animal Sciences, Changchun, China.

been caused by *E. coli* O39:NM which possessed the EPEC gene locus (*eaeA*) for enterocyte effacement and the EAST1 gene [7]; however, it was not apparent if the EAST1 played an important role in the pathogenesis since *eaeA* is a well-known enterovirulent gene.

In the investigations of the *E. coli* O166 isolates from the outbreak of diarrhoeal illness that occurred in Osaka, Japan, in 1996 we found that the organisms possessed the EAST1 gene [8]. Now, we would like to report our epidemiological findings and the properties of the bacteria, and discuss the possibility that *E. coli* with the EAST1 gene but possessing no other identifiable pathogenic properties may compose a new group of diarrhoea-associated *E. coli* or a new subgroup of ETEC.

MATERIALS AND METHODS

Description of the outbreak and epidemiological studies

An outbreak of gastroenteritis occurred on 23 July 1996 in Osaka, Japan, and 54 of 91 people at risk were affected. The patients attended a meeting held in an office on 22 July 1996, and ate lunch served by a caterer. Members of the index group were interviewed from 23 July. Faecal specimens for bacterial culture were obtained between 23 July and 30 July.

Environmental health inspections of kitchen facilities of the caterer were conducted on 23 July. Food preparation procedures were reviewed and food-handlers were interviewed if they had diarrheal illness recently. Illegally, no portions of the lunch had been kept. Stool samples were collected from all food-handlers on 24 July.

Faecal specimens were examined for the presence of salmonella, shigella, vibrios, *Escherichia coli*, campylobacter, *Bacillus cereus*, *Clostridium perfringens*, coagulase-positive staphylococci, aeromonad, plesiomonad, and Norwalk-like viruses. Direct examination of culture plates of the patients showed heavy growth of lactose-fermenting colonies on desoxycholate hydrogen sulfide lactose agar (Nissui Pharmaceutical, Tokyo).

Bacterial strains

A total of 32 strains of *E. coli* O166:H15 was examined (Table 1). Twenty-nine strains of *E. coli* O166:H15 were isolated from the outbreak patients. Two sporadic strains, V-255 and V-471, were isolated in our laboratory in December 1997 and October

1998, respectively. One strain of *E. coli* O166:H15 was kindly provided by Dr F. Ishiguro at the Fukui Prefecture Institute of Public Health. It was isolated from a patient of an outbreak that occurred in Fukui Prefecture, in September 1997.

PCR

PCR was used to examine the presence of genes associated with diarrhoeagenicity. The bacteria were grown overnight at 37 °C in nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan). A sample (100 µl) of the culture was centrifuged and the pellet was resuspended in distilled water. The suspension was boiled, and the supernatant was used as a template for PCR with a Takara PCR Thermal Cycler MP (Takara Shuzo, Otsu, Shiga, Japan). The primer sets and amplification conditions used are shown in Table 2 [6, 9–12]. For detection of PCR products, 10 µl of the amplification mixture and molecular weight markers (Biomarker low, BioVentures Inc., Murfreesboro, TN, USA) were subjected to electrophoresis in 2% or 3% agarose gels. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide.

Reverse transcription-PCR (RT-PCR)

A RT-PCR was used to examine the transcription of EAST1 gene (*astA*) with the oligonucleotide primers and total bacterial RNA. As a positive control, expression of the β -galactosidase gene (*lacZ*) was also tested by RT-PCR [13]. Total RNA was isolated from the bacteria grown to exponential or stationary phase in nutrient broth using an RNeasy total RNA kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by digestion with DNaseI (Boehringer Mannheim Corp., Indianapolis, IN, USA). RNA (5 µg) was reverse transcribed and amplified using Ready To Go RT-PCR (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instruction. For each pair of RT reactions, one tube was preheated 10 min at 95 °C to inactivate reverse transcriptase and to test for DNA contamination. The RT-PCR products were analysed with agarose gel electrophoresis as described above.

Tissue culture adhesion tests

Adhesion tests to HEp-2 cells in culture were done in 6 h periods as described previously [14]. Monolayers of HEp-2 cells grown on coverslips (13 mm in diameter) in 24-well plates were prepared. One ml of

Basal Eagle's medium without antibiotics or sera but containing D-mannose (1% w/v) was added to each well. Overnight bacterial culture (20 μ l) was inoculated into each well and the plates were incubated at 37 °C for 3 h. The monolayers were washed three times with PBS and 1 ml of medium was added to each well. After a further 3 h incubation period, the monolayers were washed thoroughly three times with PBS, fixed with absolute methanol and stained with 10% (v/v) Giemsa.

Susceptibility to antimicrobial agents

Sensitivity of the *E. coli* O166 strains to the following antibiotics was examined using Sensi Disk according to the manufacturer's instructions (Becton–Dickinson Microbiology Systems): ampicillin, chloramphenicol, cefotaxime, ciprofloxacin, fosfomycin, gentamycin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole/trimethoprim, tetracycline, and trimethoprim. The plates were incubated at 37 °C and assayed for inhibition of bacterial growth.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed by the method of Izumiya et al. [15] at the National Institute of Infectious Diseases, Japan. *Xba*I and *Not*I were used as the restriction endonucleases. Digested DNA was electrophoresed in a 1% agarose gel in 0.5 \times TBE (Tris-borate-EDTA) buffer by using a CHEF DR II apparatus (Bio–Rad Laboratories) at 10 °C at 200 V. A linearly ramped switching time of 4–8 sec was applied for 11 h and then a linearly ramped switching time of 8–50 sec was applied for 9 h.

Plasmid banding pattern

E. coli cultures were grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, MI) for 18 h at 37 °C with vigorous agitation. Samples of broth culture (1.5 ml) were centrifuged, and the pelleted organisms were then subjected to the rapid alkaline lysis method of Kado and Liu [16]. Plasmid DNA was then separated by horizontal electrophoresis through a 0.8% agarose gel, stained with ethidium bromide, and photographed under UV light.

DNA hybridization

A 393 bps fragment was amplified from strain H10407 using primer set EAST13a and EAST13b, simultaneously labelled with digoxigenin (PCR DIG Probe

Synthesis Kit, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction, and subsequently used as a probe. Southern blotting and hybridization were performed according to the standard methods by using a nylon filter membrane (Hybond-N⁺, Amersham Pharmacia, Bucks, UK). DNA hybrids on the membrane were treated with alkaline phosphatase-conjugated antidigoxigenin antibody, and with 3-(2'-spirodamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane by the DIG luminescent detection kit (Roche Diagnostics). The resultant chemiluminescence was detected by exposing the membrane to X-ray film.

DNA cloning and sequencing

After extraction of DNA of *E. coli* O166:H15 strain 96-127-23, plasmid and chromosomal DNA were separated by agarose gel electrophoresis. Each of plasmid and chromosomal DNA was excised from the agarose gel and used as template DNA for PCR. The PCR product of the EAST1 gene, amplified from plasmid or chromosomal DNA with the primer set (EAST13a,b), was purified by using a QIAquick PCR Purification Kit (Qiagen Inc.). Ten μ g of purified PCR products were ligated with 50 ng of cloning vector pGEM-T (Promega Corp., Madison, WI). Ligated DNA was transformed into DH5 α competent cells (Life Technologies, Inc., Gaithersburg, MD), selecting for ampicillin resistance on agar containing IPTG and Blugal. White colonies were picked and cultured in LB containing ampicillin. After incubation, the plasmids were extracted by lysing bacterial cells according to the alkaline method using a plasmid isolation kit (High Pure Plasmid Isolation Kit, Boehringer Mannheim). The PCR products amplified from recombinant plasmids were used for DNA sequence analysis of EAST1 gene of either plasmid or chromosomal origin. Nucleotides sequencing of both strands of the products were performed using ABI PRISM dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin–Elmer Co., Foster City, CA) on an automated sequencer (ABI PRISM 310 model, Perkin–Elmer).

Haemagglutination

A 3% (v/v) suspension of human blood group A or bovine erythrocyte in Dulbecco's PBS was used for the haemagglutination (HA) assays [17]. The bacteria were cultured on CFA agar plates, with [18] or without [19] bile salts, respectively at 37 °C overnight. The harvested bacteria were suspended in PBS to a

density of 40 mg/ml (approximately 5×10^{10} c.f.u./ml). Both suspensions were chilled in flaked ice. The slide agglutination test was performed by mixing 10 μ l of erythrocytes with an equal volume of bacterial suspension on a slide and shaking the slide on ice for 5 min with a rotator. Mannose-resistant HA (MRHA) was examined by mixing an erythrocyte suspension with bacteria suspended in PBS containing 2% (w/v) α -methyl-D-mannopyranoside.

Dot-blot test for colonization factor antigens

The isolates were analysed for reactivity with MAbs specific for CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCFO159, PCFO166, and CFA/III [20–22] in a dot blot test [23]. Nitro-cellulose membranes (Sartorius AG, Göttingen, Germany) were soaked in PBS (phosphate buffered saline, pH 7.2) and allowed to air-dry. Thereafter, whole bacteria at concentrations of approximately 1×10^{11} bacteria/ml PBS were applied in 2 μ l volumes on the membranes, allowed to dry, and blocking was performed with 1% (w/v) bovine serum albumin (BSA, Sigma Chemical Company, St Louis, MO) in PBS for 30 min with gentle agitation. All incubations were performed at room temperature. Following washing twice in PBS the nitro-cellulose membranes were incubated with the respective MAb, diluted 1/30 or 1/50 in 0.1% (w/v) BSA-PBS-0.05% (v/v) Tween, for 3 h with gentle agitation. The membranes were washed thrice in PBS-0.05% Tween and an anti-mouse IgG HRP-conjugate (Jackson Immuno Research Laboratories, West Grove, PA) diluted in 0.1% BSA-PBS-0.05% Tween was added. After incubating for 2 h the membranes were washed twice in PBS-Tween, once in PBS and then developed for approximately 10 min using hydrogen peroxide (0.0012%) as substrate and 4-chloro-naphthol (Bio-Rad Laboratories, Richmond, CA) as chromogen in Tris-buffered saline (20 mM Tris, 0.5 M NaCl, pH 7.5). Stained dots on a white background indicated positive results.

RESULTS

Epidemiology

The patients did not have any common foods except the lunch served at the office. Boiled vegetables were suspected as the contaminated food ($\chi^2 = 4.086$, $P < 0.05$). Symptoms were as follows: diarrhoea (52/54, 96%), abdominal pain (32/54, 59%), nausea (8/54, 15%), vomiting (5/54, 10%), and fever (8/54, 15%). The mean incubation period was 17 h.

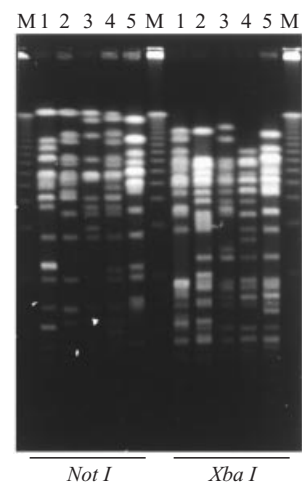


Fig. 1. Pulsed field gel electrophoresis of DNA of *E. coli* O166:H15 strains after digestion with *NotI* or *XbaI*. Lanes: M, size marker; 1, strain 96-127-23 isolated from a patient of the outbreak; 2, strain V-255 isolated from a sporadic case in 1997; 3, *E. coli* O166:HUT strain V-406 having no EAST1 gene; 4, strain V-471 isolated from a sporadic case in 1998; 5, a strain isolated from a patient of outbreak occurred in Fukui Prefecture, 1997.

The stool specimens collected from 33 patients during 23–26 July were examined and *E. coli* O166:H15 was isolated from 29 patients. Five specimens collected on 29 and 30 July were negative for the organisms. All laboratory tests for other bacterial pathogens and viruses were negative. The outbreak strains showed the same DNA banding pattern in PFGE after treatment with the restriction enzymes *XbaI* or *NotI*, and the pattern was distinct from those of the other three O166:H15 strains isolated during 1997–8 (Fig. 1). Similarly, the outbreak strains showed the same plasmid banding pattern, and it was distinct from that of the O166 strains (Fig. 2). The outbreak strains were all sensitive to the 12 kinds of antibiotics examined; the strain V-471 isolated from sporadic case in 1998 was resistant to tetracycline.

The kitchen facilities and hands of the food-handlers had been widely (13 positive out of 15) and heavily (2×10^2 to 1×10^6 c.f.u./swab) contaminated with coliform bacteria. However, *E. coli* O166:H15 were not detected in stool samples from food-handlers, nor were they isolated from any swab samples.

Examinations for enteropathogenicity

In PCR tests, the specific DNA fragments were not amplified with the primer sets for shigatoxin of enterohaemorrhagic *E. coli* (EHEC), heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) of ETEC,

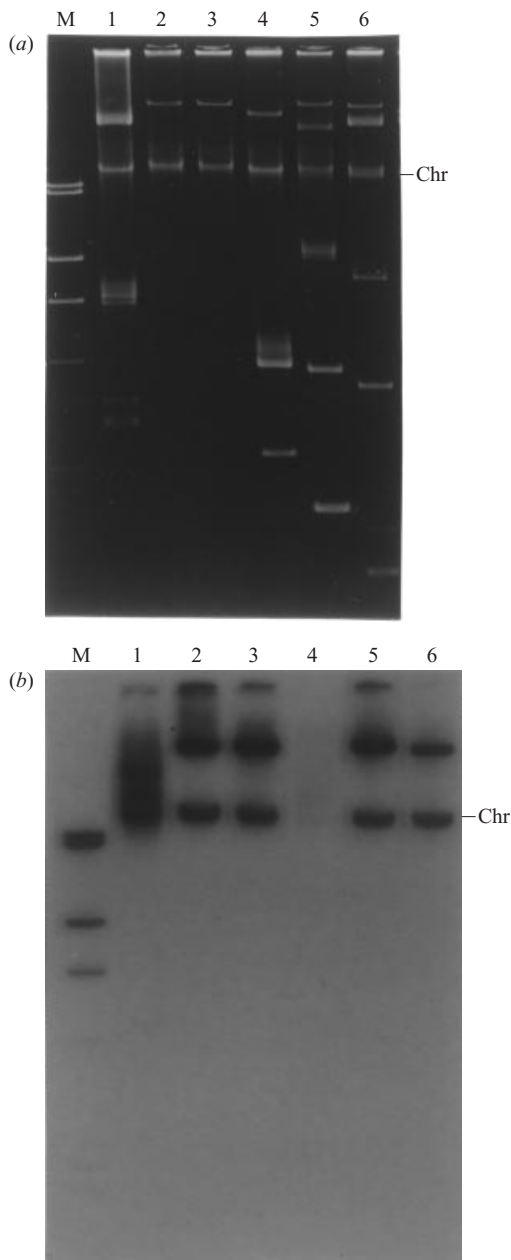


Fig. 2. Plasmid banding patterns and the Southern blotting with EAST1-gene probe. (a) Agarose gel electrophoresis of plasmids of *E. coli* O166:H15 strains; (b) Southern hybridization with the EAST1-gene probe. Lanes: M, size marker; 1, strain H10407 as positive control; 2, strain 96-127-23 isolated from a patient of the outbreak; 3, strain 96-127-28 isolated from a patient of the outbreak; 4, strain V-255 isolated from a sporadic case in 1997; 5, strain V-471 isolated from a sporadic case in 1998; 6, a strain isolated from a patient of outbreak occurred in Fukui Prefecture, 1997. Chr, chromosomal DNA.

attachment and effacement (*eaeA*) of EPEC, invasion (*invE*) of enteroinvasive *E. coli* (EIEC), or fimbria for aggregative adhesion of EAaggEC. The *E. coli* O166:H15 organisms did not adhere to HEp-2 cells in a localized, diffuse, or enteroaggregative manner, and

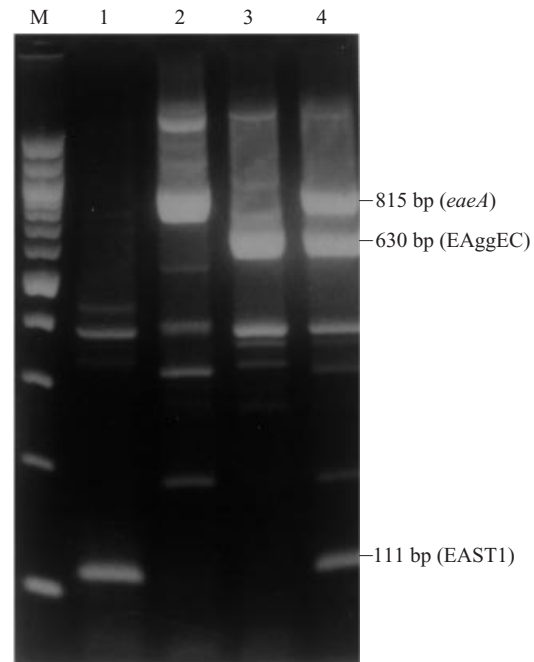


Fig. 3. Multiplex PCR of DNA from *E. coli* O166:H15 strains with oligonucleotide primer pairs for *eaeA*, EAST1 and enteroaggregative adhesion genes. Lanes: M, size marker; 1, strain 96-127-23 isolated from a patient of the outbreak; 2, enteropathogenic *E. coli* O127:H6; 3, enteroaggregative *E. coli*; 4, positive control.

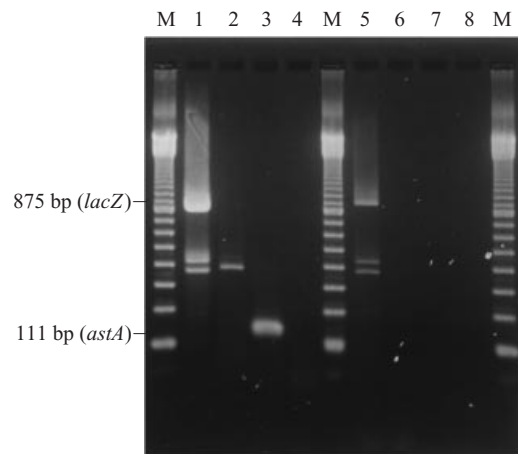


Fig. 4. RT-PCR of RNA from *E. coli* O166:H15 strain 96-127-23 with oligonucleotide primer pairs for β -galactosidase (Lanes 1, 2, 5, 6) and EAST1 (Lanes 3, 4, 7, 8). Although the *lacZ* was amplified from RNA sample that was not digested by DNase (lane 1), it was not produced from RNA sample that was digested by DNase (lane 2). On the other hand, *astA* was amplified from the DNase-treated RNA sample (lane 3), and it was not produced once the reverse transcriptase was inactivated (lane 4). Bacteria cultured in lactose-added broth were used to extract the RNA sample for lanes 5–8. The *lacZ* was amplified from RNA sample digested with DNase (lane 5); however, the *astA* was not amplified from the RNA sample (lanes 7, 8). The *lacZ* was not produced when the reverse transcriptase was inactivated (lane 6). Lane M, size marker.

				5					10					15
Met	Pro	Ser	Thr	Gln	Tyr	Ile	Arg	Arg	Pro	Ala	Ser	Ser	Tyr	Ala
ATG	CCA	TCA	ACA	CAG	TAT	ATC	CGA	AGG	CCC	GCA	TCC	AGT	TAT	GCA
...
...
...
...
				20					25					30
Ser	Cys	Ile	Trp	Cys	Ala	Thr	Ala	Cys	Ala	Ser	Cys	His	Gly	Arg
TCG	TGC	ATA	TGG	TGC	GCA	ACA	GCC	TGC	GCT	TCG	TGT	CAT	GGA	AGG
...	A
...	A
...	C	A
...	G
				35				38						
Thr	Thr	Lys	Pro	Ser	Leu	Ala	Thr							
ACT	ACA	AAG	CCG	TCA	CTC	GCG	ACC							
...							
...							
...	...	G							
...	A							
...	A							

Fig. 5. Comparison of nucleotide sequences of the EAST1 genes of *E. coli* O166:H15 strains (strain number is shown in block letter) with those of other diarrhoeagenic *E. coli* reported by Yamamoto et al. (4). The deduced amino acid sequence of EAST1 is shown above the nucleotide sequence. Nucleotides that are identical to those for strain H10407 are shown as dots.

did not give MRHA of human or bovine red blood cells. Further, dot-blot assays using specific monoclonal antibodies revealed no expression of ETEC colonization factors (CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCFO159, PCFO166, CFA/III).

EAST1 gene

It was clearly shown that all of the O166:H15 strains possessed the EAST1 gene by PCR (Fig. 3). The *astA*

was amplified by RT-PCR from RNA sample after the digestion with DNase. However, it was not produced once the reverse transcriptase was inactivated (Fig. 4). The RT-PCR did not amplify *lacZ* from the RNA sample for *astA*, although it showed positive results once the bacteria were cultured in the broth containing lactose (Fig. 4). The DNA probe for EAST1 gene hybridized with both plasmid and chromosomal DNA on the nylon membrane (Fig. 2). A strain V-255, that was isolated from a sporadic case in 1997, showed a weak signal of hybridization

with the probe. The sequence data are summarized in Fig. 5. The nucleotide sequence of the chromosomal EAST1 gene of the outbreak strain (96-127-23) differed from that of ETEC strain H10407 by three bases at the 20th, 26th and 34th codon positions, resulting in two deduced amino acid changes (Cys20Arg, Pro34Gln). Similarly, the EAST1 gene of the plasmid of strain 96-127-23 differed by two bases at 30th and 34th codon as compared to that of strain H10407 resulting in two deduced amino acid changes (Arg30Gly, Pro34Gln).

DISCUSSION

The *E. coli* O166:H15 organisms were isolated from 29 (89%) of 33 patients stool specimens collected from patients during the first 4 days (23–26 July) of the outbreak. Five specimens collected on 29 and 30 July were negative for the organisms since the patients had received antibiotics. The outbreak isolates were recognized to be one clone considering epidemiological information, antibiotic sensitivity, plasmid banding patterns, and PFGE patterns. It was assumed that these organisms were the causative agent of the gastroenteritis. However, they did not have any diarrhoeagenic traits except the EAST1 gene: the organisms had not reacted to PCR primers for the well-established enterovirulent genes. Consequently, they could not be assigned to any of the recognized diarrhoeagenic groups of *E. coli*: EPEC, ETEC, EHEC, EIEC, or EAggEC. They did not show diffuse adhesion to HEp-2 cells, either. To our knowledge, this is the first report of an outbreak caused by *E. coli* that did not have other well-characterized virulence properties except the EAST1 gene.

Two strains of *E. coli* O166:H15 were isolated from sporadic cases in Osaka City during 1997–8; further, another clone of the organism caused an outbreak in Fukui Prefecture in 1997. It is obvious that there are multiple clones of the *E. coli* O166:H15 in Japan since they showed marked differences in plasmid banding patterns and PFGE patterns. The role of EAST1 in diarrhoeagenicity has not been demonstrated, although EAST1 clones yield net increases in short circuit current in the rabbit mucosal Ussing chamber model [1]. The outbreak caused by the O166 organisms that had no well-known virulence genes except the EAST1 gene provided circumstantial evidence that the EAST1 plays a role in human disease. Based on the findings obtained in a case-control study, Vila and colleagues had also suggested that EAST1-

producing *E. coli* (EAST1EC) strains are associated with diarrhoeal diseases rather than EAggEC [24].

Yamamoto et al. found two variant types (EAST1v1 and EAST1v2) of the EAST1 gene sequence in an EAggEC strain and an EPEC-related strain of a non-EPEC serotype, respectively [6]. The current study revealed that the outbreak strain of *E. coli* O166:H15 had two new variant genes: EAST1v3 was on the chromosome and EAST1v4 was on the plasmid. The multiple presence of *astA* gene is likely due to that the gene is located on an insertion sequence element as McVeigh et al. reported [25]. Although 54 of 91 people at risk suffered from enteritis due to the strain of *E. coli* O166:H15, it is not apparent yet which toxin, EAST1v3 or EAST1v4, played the more important role in pathogenesis. RT-PCR showed the presence of mRNA that was transcribed from *astA*, but it is not determined yet if either EAST1v3 or EAST1v4 gene was transcribed to produce the mRNA. It is also possible that both toxins were elaborated equally and were as important in causing disease. The *astA* seems to receive catabolite repression since it was not transcribed under the presence of lactose [14]. EAggEC strain O42 that had an intact EAST1 gene was reported to be more virulent than strain 17-2 possessing EAST1v1 [6, 26]. Since the *E. coli* O166:H15 strains caused outbreaks, it is likely that the toxigenicity of either EAST1v3 or EAST1v4 is stronger than the EAST1 or EAST1v1.

It seems that the production of EAST1 alone is insufficient to cause disease in adults. In a volunteer study where four EAggEC strains were examined, Nataro and colleagues found that one strain that induced diarrhoea, as well as one that did not, secreted EAST1 [26]. Therefore, colonization factors must be essential for EAST1EC as well as for ETEC. It is likely that EAST1EC has unidentified new colonization factors, because the O166 strains did not show reactions with monoclonal antibodies to well-established colonization factors.

In conclusion, we consider that the EAST1EC, that does not have any well-established enterovirulent genes except the EAST1 gene, should be assigned to a new subgroup of ETEC for further research. Such strains would not be detected in most current surveys for diarrhoeagenic *E. coli* as tests for EAST1 are rarely included. In order to get and analyse bacteriological information (serotype, colonization factors and so on) of the EAST1EC that is diarrhoeagenic for humans, and to determine the distribution of these organisms worldwide we propose that it is essential to

examine diarrhoeal specimens for EAST1EC irrespective of their serotypes. Methods that make it easier to assay EAST1 itself are necessary to examine if the EAST1 gene-positive bacteria actually elaborate the toxin. A competitive enzyme-linked immunosorbent assay, such as exists for ST, could presumably be useful for EAST1 [27].

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