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Epidemiology and properties of heat-stable enterotoxin-producing *Escherichia coli* serotype O169:H41

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SUMMARY

Enterotoxigenic *Escherichia coli* (ETEC) serotype O169:H41 organisms have become the most prevalent ETEC in Japan since the first outbreak in 1991. It was assumed that the outbreaks were due to clonal spread of this new ETEC serotype. The relationship of 32 strains isolated from 6 outbreaks were examined for biotype, antibiotic susceptibility, enterotoxigenicity, protein banding pattern, lipopolysaccharide banding pattern, plasmid analysis, and ribotyping. Further, the strains were examined by haemagglutination, surface hydrophobicity, and the ability to adhere to HEp-2 cells. The present study suggests that the outbreaks were caused by multiple clones of STp-producing O169:H41 since they showed differences in ribotype and outer membrane protein banding patterns. The strains did not agglutinate human or bovine red blood cells in a mannose-resistant manner. They adhered to HEp-2 cells in a manner resembling enteroaggregative *E. coli*. Five strains were examined by dot-blot tests for the colonization factor antigens CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, PCFO159, PCFO166 and CFA/III. Although four strains expressed CS6, no structure for CS6 was identified. A strain that the anti-CS6 MAbs did not react with could adhere to HEp-2 cells in mannose resistant manner; thus, it is unlikely that CS6 play an important role in the adhesion to the cells. Electron microscopy studies of the O169:H41 strains suggested that curly fimbriae, a possible new colonization factor, may be playing an important role in the adhesion of the bacteria to HEp-2 cells. In conclusion, outbreaks due to ETEC O169:H41 were caused by multiple clones, and the strains should be examined in detail for a possible new colonization factor.

INTRODUCTION

Escherichia coli is a normal inhabitant of the intestinal tract of humans and warm-blooded animals; certain strains, however, possess virulence factors that enable the organisms to cause disease in its host. It is therefore important in diagnostic work to distinguish between the *E. coli* strains belonging to the normal enteric flora and possible pathogenic strains. Enterotoxigenic *E. coli* (ETEC) cause diarrhoea by adhering to the intestinal mucosa with their unique colonization factors and producing either a heat-labile enterotoxin (LT), a heat-stable enterotoxin (ST), or both. Consequently, ETEC organisms can be identified either by detection of the enterotoxins by immunological assays or by detection of enterotoxin-encoding genes with DNA probes or PCR amplification. In many clinical laboratories, however, serological typing is used to judge if the isolates are diarrhoeagenic.

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Although *E. coli* serotype O169:H8 has been well recognised as one of the ETEC strains [1], serotype O169:H41 is not yet established worldwide as one of the diarrhoeagenic *E. coli* of the ETEC group [2]. In Japan, O169:H41 has become the most prevalent ETEC serotype since the first outbreak in 1991 [3, 4]. Judging from the data officially reported in 'Infectious Agents Surveillance Report', ETEC of serotypes O6:H16 and O148:H28 are the most common after O169:H41. During the period 1991–4 strains of O169:H41 caused 15 outbreaks while O6:H16 and O148:H28 caused 10 and 8 outbreaks respectively.

In this study, *E. coli* isolates of serotype O169:H41 were collected and their relationship was investigated epidemiologically to clarify if the outbreaks were due to clonal spread of this new ETEC serotype. In addition, we examined these new organisms for colonization factors.

MATERIALS AND METHODS

Bacterial strains

Thirty-two strains of *E. coli* O169:H41, from six outbreaks, were examined (Table 1). Strains YN 1 and YN 2 were isolated from patients stool during a family outbreak, in Osaka, in June 1991 where imported Korean food (kimchi) was implicated. Seven strains (KK 1–7) isolated from patients during an outbreak which occurred at Kitakyushu City in August 1992 were kindly provided by Dr Kano of the Kitakyushu Municipal Institute of Environment and Public Health. Strain TT 1 was isolated from a patient, who suffered from diarrhoea during a tour in Korea, in July 1993 and was kindly provided by Dr Honda of Tottori Prefecture Institute of Public Health. Strains '93-81-1 and '93-81-2 were isolated from patients during an outbreak which occurred in Saga Prefecture in July 1993. Five strains (FO 1–5) isolated from patients of the same outbreak were kindly provided by Dr Hiwaki and Dr Oda of Fukuoka Municipal Institute of Public Hygiene. Seven strains (HK 1–7) were isolated from diarrhoeal stools of patients involved in an outbreak in Hokkaido Prefecture in August 1993 and Dr Hiwaki and Dr Oda of Fukuoka Municipal Institute of Public Hygiene kindly provided these strains. Seven strains ('93-95-4 to 9 and '93-95-12) were recovered from patients during an outbreak that occurred in a wedding reception in Osaka Prefecture, September 1993, Strain '93-97-1 was isolated from frozen ready-to-eat seafood

Table 1. Escherichia coli *O169:H41 food poisoning outbreaks and the strains studied in this work*

Outbreaks	Date	Prefecture	Number of patients/ number of people involved in the incident	Number of O169 positive/ number of specimens	Strains
A	Jun. 1991	Osaka	3/7	2/3	YN 1 & 2
B	Aug. 1992	Kitakyushu City	8/22	7/7	KK 1–7
C	Jul. 1993	Tottori	21/48	3/19	TT 1
D	Jul. 1993	Saga	406/857	29/302	'93-81-1 and 2, FO 1–5
E	Aug. 1993	Hokkaido	35/291	7/8	HK 1–7
F	Sep. 1993	Osaka	776/1242	21/53	'93-95-4–9, 12. '93-97-1

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that was taken from a shop which delivered foods for the wedding party.

Biotyping

All 32 isolates were biotyped according to the scheme of Crichton and colleagues [5]. Fermentation of raffinose, sorbose, dulcitol, 2-deoxy-D-ribose, and decarboxylation of ornithine were examined as primary tests. Fermentation of rhamnose, lysine decarboxylation and motility were examined as secondary tests.

Susceptibility to antimicrobial agents

A loop of overnight culture (1 μl) was spotted on each of Müller–Hinton agar plates containing 8 μg/ml (A1) or 128 μg/ml (A2) of ampicillin, chloramphenicol (C, 8 μg/ml), cefazolin (Cf, 32 μg/ml), 4 μg/ml (G1) or 32 μg/ml (G2) of gentamicin, kanamycin (K, 16 μg/ml), nalidixic acid (Nx, 16 μg/ml), 16 μg/ml (S1) or 128 μg/ml (S2) of streptomycin, sulphamethoxazole (Su, 32 μg/ml), 8 μg/ml (T1) or 128 μg/ml (T2) of tetracycline, or trimethoprim (Tm, 2 μg/ml). The plates were incubated at 37 °C and examined for bacterial growth.

PCR and ELISA for enterotoxin

PCR was used to examine the presence of enterotoxin genes. The bacteria were grown overnight at 37 °C in nutrient broth (Eiken Chemical Co., Ltd, Tokyo, Japan). A sample of 100 μl of the culture was centrifuged and the pellet was resuspended in distilled water. After the suspension was boiled for 10 min, the supernatant was used as a template for PCR. EC Nucleotide Mix (Nippon Shoji Co., Osaka, Japan) including four sets of primers was used to detect genes for LT, ST, Verocytotoxin and enteroinvasion simultaneously [6] according to the manufacturer's instruction. Two sets of primers (ESH-1, 2 and ESP-1, 2, Shimazu Co., Kyoto, Japan) were used to distinguish STh and STp genes [7, 8]. For detection of PCR products, amplification mixtures were subjected to electrophoresis in a 2% agarose gel. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. ETEC O6:H16 strain '94-SK which produce STh and LT, and a strain of *Shigella dysenteriae* serovar 1 that have genes for verocytotoxin and enteroinvasion were used as positive controls for the PCR tests.

ST production was examined by using the COLI ST EIA (Denka Seiken Co., Ltd, Tokyo, Japan), that is a competitive ELISA for ST I [9], according to the manufacturer's instruction.

Whole cell protein analysis

Bacterial cells were harvested from 10 ml of nutrient broth (Eiken Chemical Co., Ltd) by centrifugation. The cells were washed and resuspended in 0·5 ml of lysis buffer (70% (v/v) 0·5 M Tris-HCl, pH 6·8, 20% (v/v) glycerol, 8% (v/v) distilled water, 2% (v/v) mercaptoethanol, 0·8% (w/v) SDS), and the lysate heated at 95 °C for 5 min. Distilled water (0·5 ml) was added and the tubes were heated again for a further 5 min. The lysate was centrifuged and proteins in the supernatant were separated by SDS–PAGE using the buffer system described by Laemmli [10], and the gels were stained with Coomassie blue or silver as described by Wray and colleagues [11].

Outer membrane protein (OMP) analysis

OMPs were isolated by the method of Chart & Griffiths [12]. Bacterial pellets harvested from 150 ml of nutrient broth (Eiken Chemical Co., Ltd) were resuspended in 5 ml of lysis buffer (25 mM Tris-HCl, pH 7·4, 1 mM EDTA) and sonicated at 300 W for 30 sec three times. Unbroken bacteria were sedimented by centrifugation at 5000 ***g*** for 20 min, then total membranes were recovered from the supernatant by centrifugation for 1 h at 40000 ***g*** at 4 °C. The resulting pellets were resuspended in 20 ml of 25 mM Tris-HCl, pH 7·4 containing *N*-lauroylsarcosine, and were incubated for 30 min at RT with vigorous agitation. Outer membranes were sedimented by centrifugation for 1 h at 40000 ***g*** at 4 °C. The OMP extracts were analysed with SDS–PAGE as described above.

Extraction of lipopolysaccharide

About 10 mg of bacterial cells were suspended at 1 mg per 30 μl of solubilization buffer (62·5 mM Tris-HCl, pH 6·8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, 0·01% (w/v) bromophenol blue). After the lysate was heated at 100 °C for 10 min, an equal volume of solubilization buffer containing proteinase-K (1 mg/ml) was added, and the lysate was incubated at 60 °C for 1 h to digest proteins [13]. LPS molecules were separated by SDS–PAGE, and the gels were stained with silver as

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described by Tsai & Frasch [14]. A strain of *E. coli* O111:H− was included as a control to show typical ladder of LPS.

Plasmid banding pattern

Bacterial strains were cultured overnight at 37 °C in 1·5 ml of Brain Heart Infusion (BHI) broth (Difco, Detroit, MI, USA). The pelleted organisms were then subjected to the rapid alkaline lysis method of Kado & Liu [15] with slight modification [16]. Plasmid DNA was then separated by horizontal electrophoresis through a 0·8% agarose gel, stained with ethidium bromide, and photographed under UV light.

Ribotyping

Cultures were grown overnight at 37 °C on tryptic soy agar containing 5% sheep blood and DNA was extracted and purified using the procedure described by Graves & Swaminathan [17]. *E. coli* DNA (2 μg) was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I or *Sma*I in accordance with the protocol established by the enzyme supplier (New England BioLabs, Beverly, MA, USA). Restricted DNA was electrophoresed in 0·04 M Tris-acetate, 0·001 M EDTA buffer, pH 8·0, overnight on 1% agarose gels at 1 V/cm and transferred to nylon membranes (Micron Separations, Inc., Westboro, MA, USA) by Southern blotting. Plasmid pKK3535 DNA was digested with *Eco*RI and labelled with digoxigenin by a randomly primed labelling method (Genius System, Boehringer–Mannheim Biochemicals, Indianapolis, IN, USA). The probe was allowed to hybridize with the Southern blots of *E. coli* DNA and the resulting hybridization patterns were visualized according to the methods described in the Genius System User's Guide for Filter Hybridization (Boehringer–mannheim Biochemicals, Indianapolis, IN, USA). Digital membrane images were produced using a flat-bed scanner and the banding patterns were analysed visually.

Tissue culture adhesion tests

Adhesion tests to HEp-2 cells in culture were done in the presence of 1% D-mannose in 6-h periods as described by Scotland and colleagues [18]. Monolayers of HEp-2 cells were grown to near confluence on coverslips (diameter 13 mm) in 24-well plates in 1 ml of Basal Eagle's medium. The monolayers were infected with bacterial strains (20 μl) grown overnight at 37 °C in 1% casitone water. After 3 h incubation at 37 °C, the monolayers were washed three times with PBS and 1 ml of the 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 Giemsa.

Haemagglutination

A 3% (v/v) erythrocyte suspension in Dulbecco's PBS was used for the haemmagglutination (HA) assays [19]. Bacteria were grown on CFA agar [20] for 24 h at 37 °C. The bacteria were suspended in PBS to a density of 40 mg/ml (approximately 5×10^{10} c.f.u./ml). The slide agglutination test was performed by mixing 10 μl 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) were examined by mixing a erythrocyte suspension with bacteria suspended in PBS containing 2% (w/v) α-methyl-D-mannopyranoside.

Microplate HA assays were carried out using 96-well U-bottom microtitre plate. Serial doubling dilutions of the bacterial suspension (5×10^{10}/ml) in PBS were made in 25 μl volumes, after which an equal volume of erythrocyte suspension was added to each well. The plate was incubated at 4 °C for 4 h, and the results were read.

Strain H10407 (O78:H11, CFA/I), ETEC O6:H16 strains '94-SK which produce STh and LT, and a strain of enteroaggregative *E. coli* O92:H33 were used as controls throughout the HA tests and the following salting out tests.

Salting out test for hydrophobicity

The test was performed as previously described by Lindahl and colleagues [21]. Bacterial suspensions (25 μl) were mixed with different concentrations of ammonium sulphate in 0·002 M PBS (pH 6·8). The test was performed on glass slides and read after 2 min. Presence of bacterial clumps in the mixture was read as a positive test at that particular ammonium sulphate concentration, and scored as the relative cell-surface hydrophobicity. The lower value thus corresponded to greater cell-surface hydrophobicity. Strains 50R502 (CFA/I negative) and 50R503 (CFA/I positive), cloned from strain H10407, were kindly provided by Dr H. R. Smith at Central Public Health Laboratory, London, and were used as controls.

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Dot blot test

A selection of the isolates were analysed for reactivity with MAbs specific for CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, PCFO159, PCFO166 and CFA/III [22–25] in a dot blot test [22].

Inhibition ELISA

The capacity of the different strains tested to inhibit binding of anti-CS6 antibodies to whole CS6-positive bacteria (E11881/14, O25:H42, $ST^{-}LT^{-}$) was evaluated by an inhibition ELISA [22, 23]. Strains that inhibited binding of the anti-CS6 MAb by $> 50\%$ were regarded as CS6-positive.

Immunoblot

Bacterial heat extracts, obtained by heating the bacteria in a water bath at 60 °C for 30 min [26], were separated in 16% polyacrylamide slab gels as described [27] using the Laemmli buffer system, except that 2-mercaptoethanol was omitted in the sample buffer. Protein profiles were immobilized onto nitrocellulose membranes (0·45 μm pore size; Sartorius AG) and either stained with amido black (0·1% w/v) or reacted with the anti-CS6 MAb 20:11:9 [22] as described for the dot blot.

Electron microscopy

Bacteria were cultured on CFA-agar plates with [20] and without bile salts [28], respectively, at 37 °C overnight. Bacteria were suspended in a drop of 2% phosphotungstic acid (pH 7·0), then a carbon-coated grid was floated, sample side down, on the bacterial suspension for 1 min. Grids were removed from the drops, blotted, washed twice with distilled water and examined in a JEOL transmission electronmicroscope (JEM-1200 EXII).

For immunoelectron microscopy, the anti-CS6 MAbs 20:11:9 and 2A:14, respectively, were used as previously described [22].

RESULTS

Properties of the O169:H41 strains

The organisms were identified as the biotype 6a according to the biotyping scheme of Crichton and colleagues [5]: raffinose positive, sorbose positive, ornithine decarboxylase negative, dulcitol positive, 2-deoxy-ribose negative, rhamnose positive, lysine decarboxylase positive and motile. All isolates were resistant to tetracycline and weakly resistant to sulphamethoxazole; however, they were susceptible to all other antibiotics used in this study.

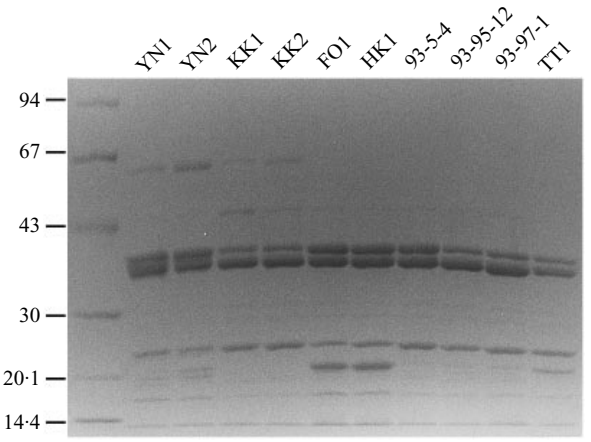

Fig. 1. SDS–PAGE profiles of outer membrane proteins of *E. coli* O169:H41 strains. Lane 1, size markers; lanes 2, 3, strains YN 1, 2 from outbreak A; lanes 4, 5, KK 1, 2 from outbreak B; lane 6, FO 1 from outbreak D; lane 7, HK 1 from outbreak E; lanes 8–10, 93-95-4, 93-95-12, and 93-97-1 from outbreak F; lane 11, TT 1 from outbreak C.

OMP of the isolates showed four banding patterns (Table 2, Fig. 1). Isolates from each of outbreaks A, B and F showed different banding patterns A, B and D, respectively. Strains of the outbreaks C, D and E had a similar OMP banding pattern, i.e. pattern C. Differences were not found among the O169 strains in whole cell protein banding patterns. The lipopolysaccharide of O169 organisms did not show a clear ladder pattern unlike the O111 strains, and differences were not found among strains in the banding patterns. Only one large plasmid (approx. 100 MDa) was extracted from the ST-producing *E. coli* O169:H41 strains, and this was missing in the ST-negative strains.

Typical strains from outbreaks A–F were identical by ribotyping when chromosomal DNA was digested with restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I or *Sma*I. When chromosomal DNA was digested with *Pvu*II, two ribotyping patterns were discernible, patterns A and B (Table 2, Fig. 2). Strains with ribotyping pattern B were isolated from outbreaks A and B, while strains with ribotyping pattern A were isolated from outbreaks C–F.

PCR and ELISA for enterotoxin

All isolates except one strain (93-95-12) carried ST genes but not LT genes (Fig. 3*a*). A group of strains

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Table 2. *Typing of* Escherichia coli *O169:H41 strains studied in this work and the properties associated with entero-adhesion*

Outbreaks	Strains	Outer membrane protein banding patterns	Ribotyping	Mannose-resistant hemagglutination*						Hydrophobicity†	Adhesion to HEp-2 cells
				Hum.	Bov.	Equ.	Ovi.	Rab.	Gui.	Rat		
A	YN 1 and 2	A	B	−	−	−	−	+	−	+	0·4	+
B	KK 1–7	B	B	−	−	−	−	+	−	+	0·4	−
C	TT 1	C	A	−	−	−	−	+	−	+	0·4	−
D	93-81-1 and 2, FO 1–5	C	A	−	−	−	−	+	−	+	0·4	+
E	HK 1–7	C	A	−	−	−	−	+	−	+	0·4	+
F	93-95-4–9, '93-97-1	D	A	−	−	−	−	+	−	+	0·4	+
	ETEC O78:H11 (ST+)			+	+	−	−	−	−	−	0·12	−
	ETEC O6:H16 (LT+,ST+)			−	+	−	−	−	−	−	0·4	−
	EAggEC O92:H33			+	+	+	+	+	+	+	2·0	+

* Mannose-resistant agglutination of human, bovine, equine, ovine, rabbit, guinea pig and rat erythrocytes on slide glass.
† Hydrophobicity is indicated by results of the agglutination test, in terms of the molar concentration of ammonium sulphate.

(KK 1–7) and strain TT 1 did not show any production of ST in this study, although their ST production had been confirmed at the time of isolation by Dr Kano and colleagues of Kitakyushu Municipal Institute of Environment and Public Health and by Dr Honda of Tottori Prefecture Institute of Public Health, respectively. PCR with two sets of primes showed that the ST gene of these *E. coli* O169 strains was STp (Fig. 3*b*). ST production of the strains that showed positive results in PCR were confirmed by the ELISA.

Tissue culture adhesion tests

Only ST-producing *E. coli* O169:H41 strains adhered to HEp-2 cells with an aggregative pattern (Fig. 4). Strains, that lost the ability to produce ST and were PCR negative with the ST primers, did not show any adhesion to the epithelial cells. Although *E. coli* O6:H16 or H10407 were used as representatives of well-known ETEC organisms, they did not adhere to HEp-2 cells. Scanning electron microscopy showed fimbria-like strands connecting the organisms to each other and to HEp-2 cells (Fig. 5).

Haemagglutination

In slide haemagglutination tests, ETEC H10407 organisms used as a positive control agglutinated human and bovine red blood cells in mannose-resistant manner in contrast to the O169 strains. On the other hand, O169 strains showed weak agglutination of rabbit and rat erythrocytes irrespective of their abilities to produce ST or to adhere to HEp-2 cells and they showed mannose-sensitive haemagglutination of guinea-pig or equine red blood cells.

In microplate haemagglutination tests, H10407 organisms agglutinated human and rabbit erythrocytes, and the EAggEC strain agglutinated erythrocytes of all animals species used in this study. The O169 strains, however, caused agglutination of rabbit and rat erythrocytes regardless of their abilities to produce ST or to adhere to HEp-2 cells comparable to in the slide haemagglutination tests.

Dot blot test, inhibition ELISA, immunoblot and immunoelectron microscopy

A selection of the strains, i.e. HK-3, FO-5, 93-81-1, 93-95-5 and 93-95-9, were analysed for expression of known ETEC colonization factors using specific

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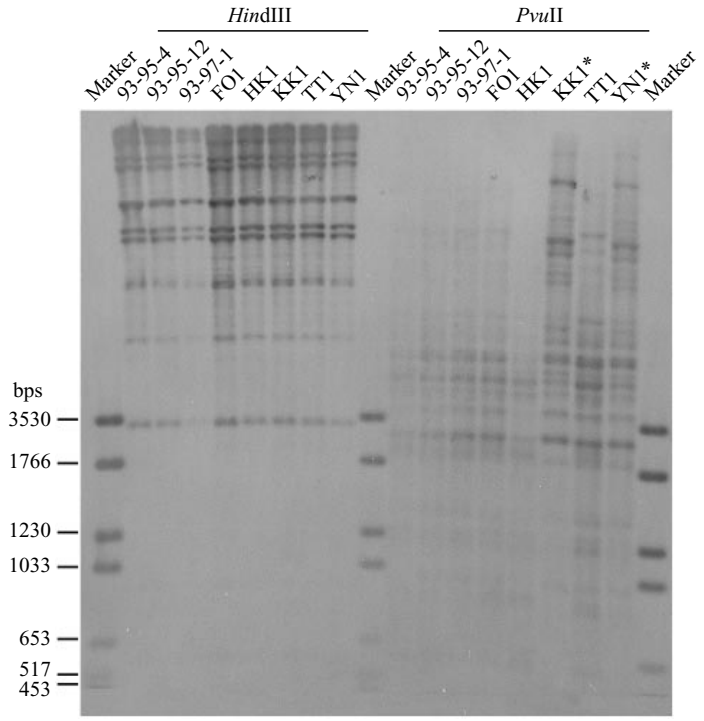

Fig. 2. Ribosomal RNA gene restriction patterns of *E. coli* O169:H41 strains. Lanes 1, 10, and 19, control showing DNA bands of 3530, 1766, 1230, 1033, 653, and 517 base pairs; lanes 2, 11, strain 93-95-4 from outbreak F; lanes 3, 12, 93-95-12 from outbreak F; lanes 4, 13, 93-97-1 from outbreak F; lanes 5, 14, FO 1 from outbreak D; lanes 6, 15, HK 1 from outbreak E; lanes 7, 16, KK 1 from outbreak B; lanes 8, 17, TT 1 from outbreak C; lanes 9, 18, YN 1 from outbreak A. Chromosomal DNA digested with *Hin*dIII or *Pvu*II were used in each of lanes 2–9 and lanes 11–18, respectively. *KK 1 and YN 1 show different pattern (type B) in lanes 16, 18.

MAbs in a dot blot assay. It was found that four of the strains, i.e. HK-3, FO-5, 93-81-1 and 93-95-9, expressed CS6 whereas strain 93-95-5 did not express this CS factor; none of the strains tested expressed CFA/I, CS1, CS2, CS3, CS4, CS5, CS7, CS17, PCFO159, PCFO166 or CFA/III. The expression of CS6 by the four strains that were positive in dot blot was confirmed by inhibition ELISA and immunoblot using the anti-CS6 MAbs. Large numbers of rigid fimbriae and curly fimbriae were observed on the surface of the *E. coli* O169 strains with the electron microscopy (Fig. 6). Immunoelectron microscopy using the anti-CS6 MAbs did not show any fimbriae labelled by the CS6-MAbs.

Hydrophobicity

Strain 50R503 (CFA/I) was agglutinated at 0·12 M or more of ammonium sulphate solution, while strain 50R502 (CFA/I negative) was agglutinated at 3·6 M as a minimum. In contrast, the O169 strains were agglutinated with 0·4 M or more of ammonium sulphate solution independently of their ability to adhere to HEp-2 cells. The EAggEC strain was agglutinated at 2·0 M or more.

DISCUSSION

Since the first report of an outbreak due to ETEC O169:H41 in 1991, this serotype has been one of the most prevalent ETEC in Japan. It was assumed that a clone of O169:H41 was spreading over Japan because soon after emergence they became prevalent. All isolates were classified into biotype 6a [5]. Our present investigations, however, suggest that outbreaks were caused by multiple clones of O169:H41. Although the same ribotype was observed among strains from the outbreak A and B, they showed differences in the OMP banding patterns. ST-producing O169:H41 strains were detected from frozen ready-to-eat seafoods in the epidemiological investigations for the outbreak F in 1993. Since frozen foods could be delivered nationwide and consumed at different times in different places, it was possible that several outbreaks were caused by one lot of polluted frozen foods. Isolates from the four outbreaks C, D, E and F

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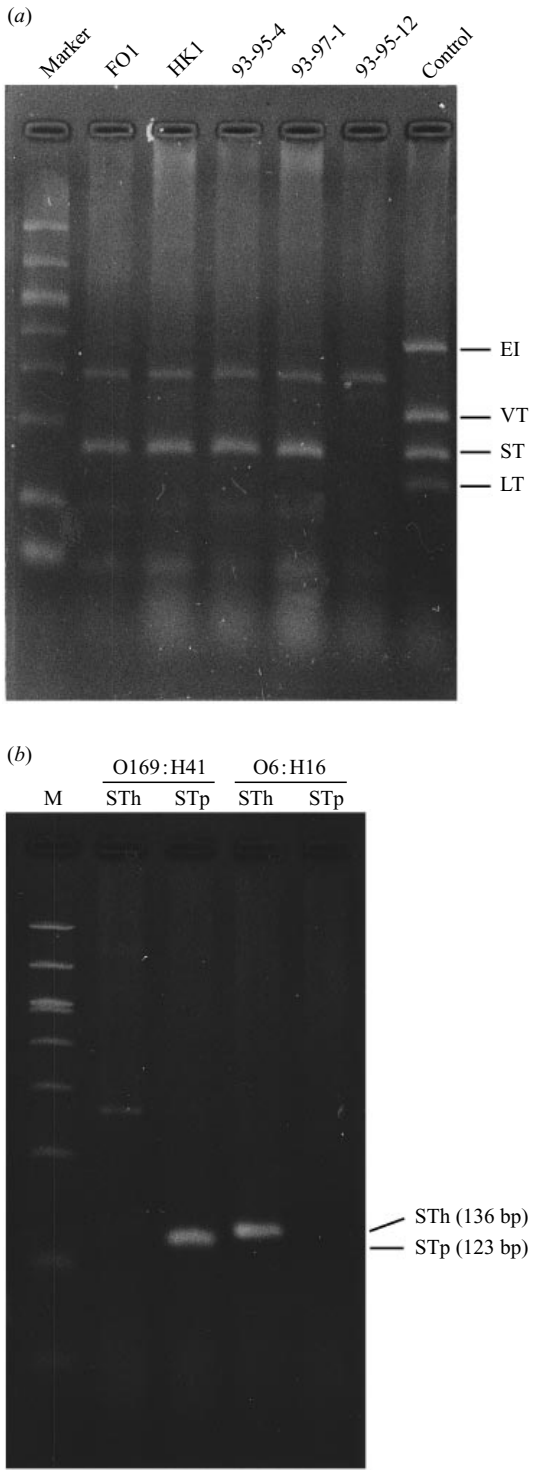

Fig. 3. (*a*) Tetraplex amplification of DNA from *E. coli* O169:H41 strains with oligonucleotide primer pairs for LT, ST, VT, and enteroinvasion (EI). Lane 1, DNA size markers of 1000, 700, 525, 500, 400, 300, 200, 100 and 50 base pairs; lane 2, ST-producing *E. coli* O169:H41 strain FO 1; lane 3, HK 1; lane 4, 93-95-4; lane 5, 93-97-1; lane 6, ST-non-producer (strain 93-95-12); lane 7, positive control show specific PCR products of EI (382 bp), VT (228 bp), ST (171 bp), and LT (132 bp). (*b*) Agarose gel electrophoresis of PCR products amplified with oligonucleotide primer pairs for STh and STp. Lane 1, size markers are the same as those in Fig. 1; lanes 2, 3, STp-specific DNA band was amplified from *E. coli* O169:H41; lanes 4, 5, STh-specific DNA band was produced from *E. coli* O6:H16.

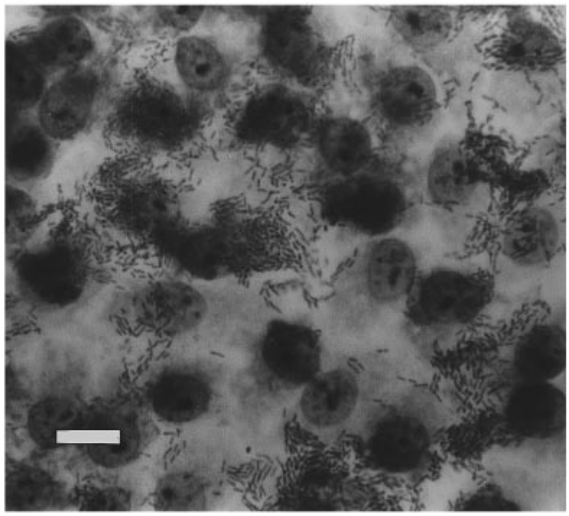

Fig. 4. Light micrograph of HEp-2 cells infected by ST-producing *E. coli* O169:H41 (strain 93-81-1). Bar, 10 μm.

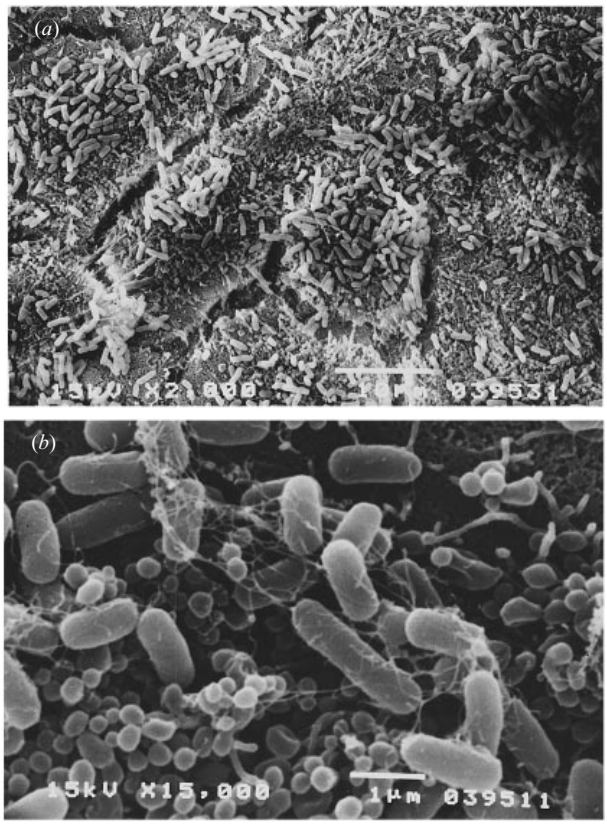

Fig. 5. Scanning electron photomicrographs of Hep-2 cells infected by ST-producing *E. coli* O169:H41 (strain 93-81-1). (*a*) numerous bacteria are observed. (*b*) Fimbria-like structure connecting the *E. coli* organisms to each other and to HEp-2 cells are seen. Bar, 1 μm.

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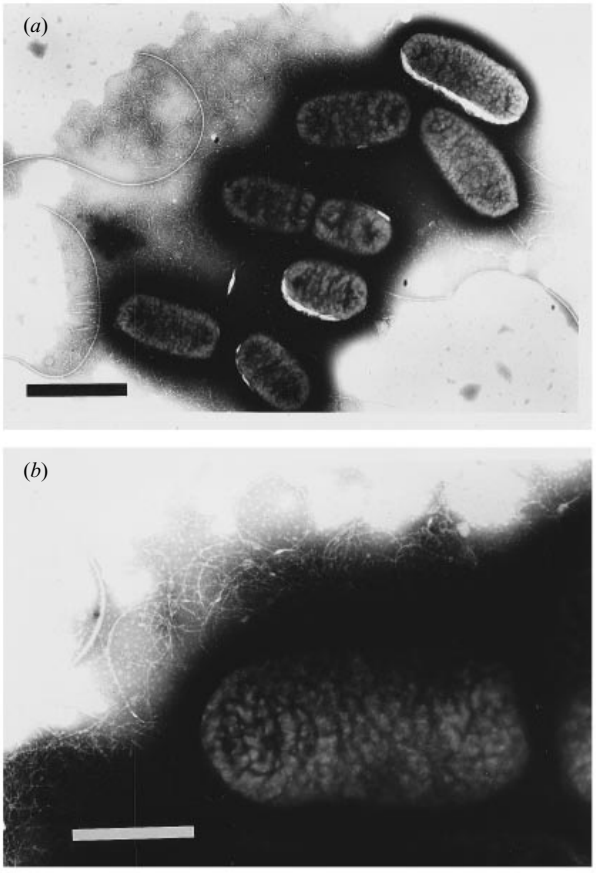

Fig. 6. Transmission electron photomicrographs of ST-producing *E. coli* O169:H41 (strain 93-81-1). (*a*) Straight rigid pili and curly fimbria are observed. Bar, 1 μm. (*b*) Curly fimbriae are shown. Bar, 0·5 μm.

in 1993 showed the same ribotype. However, the banding pattern of OMP extracted from strains of outbreak F differed from those of strains from the other outbreaks; although OMP banding patterns of the isolates from outbreaks C, D and E were similar to each other. The clone that caused outbreaks C, D and E seemed to be different from the bacteria of outbreak F.

Interestingly, isolates in 1991 and 1992 appeared to have the same ribotype B, and the newer strains isolated in 1993 were allotted to another ribotype A. Although OMP profiling subdivided the strains of each of the ribotypes, it might be possible that the organisms which caused outbreaks A and B were originally from the common ancestor. The bacteria isolated from outbreaks C–F also might be from another common ancestor.

The reasons why several clones of ETEC O169:H41 have emerged and spread rapidly are not clear. It is likely that the organisms were present in Japan before the first report of 1991, although they had not been detected. Serological typing was the only convenient method for local laboratories to detect ETEC elaborating only ST before the commercial kits to detect ST became available in 1988 in Japan. However, some batches of commercially available O169 antisera did not agglutinate *E. coli* O169. This unexpected fault in antisera might have allowed the O169 organisms to evade our surveillance for years.

Plasmid extraction showed one large plasmid common to ST-producing strains. Loss of the plasmid resulted in loss of ability to produce ST and to adhere to HEp-2 cells, although we have not performed experiments to show that ST-coding gene was on the plasmid. The O169 strains appeared to lose this plasmid spontaneously at a high rate. An isolate of outbreak F had already lost the plasmid when it was isolated from the patient faecal specimen (Fig. 3a). Isolates of outbreaks B and C had lost the plasmids when we received the strains.

HEp-2 cells or INT407 cells have been used to study the adhesion of diarrhoeagenic *E. coli* to epithelial cells *in vitro*. However, no cell line has been described that would enable study of the adhesion of ETEC strains, excepting Caco-2 cells [29]. In previous studies, therefore, mannose-resistant haemagglutination tests have been used to identify the colonization factors, and several colonization factor antigens (CFAs) have been found. Among these, CFA/I, CFA/II and CFA/IV are well known [30–36]. In addition, 8786, CS7, PCFO166 and CS17 have been reported as putative colonization factors causing mannose-resistant haemagglutination [28, 37–39]. The unusual property of the O169:H41 strains was the adhesion to HEp-2 cells. Although they adhered in an aggregative pattern, their haemagglutination reactions and cell-surface hydrophobicity were different from the EAggEC strain and the ETEC strains used as controls.

Although several antigens have been reported as new colonization factors which did not cause mannose resistant haemagglutination [40–45], none of them was reported to adhere to HEp-2 cells. Yano and colleagues reported that ETEC isolated from piglets could adhere to HeLa cells with a probable new adhesive factor F42. Although the photograph in their paper revealed that the adhesins was similar to that of O169:H41 to HEp-2 cells, the F42 was apparently different from adhesins of O169:H41 since it caused mannose resistant agglutination of human, guinea pig, horse, sheep and chicken erythrocytes [46]. Present studies showed that four strains of

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O169:H41 possessed CS6. It has not been reported that CS6 mediates adhesion to HEp-2 cells, although Helander and colleagues recently showed that CS6-expressing bacteria bind to isolated human small intestinal enterocytes [47]. In addition, a strain which did not react with the anti-CS6 MAbs adhered to HEp-2 cells as well as the CS6-positive strains. It appears that the O169:H41 strains may have a new adhesin.

The factor responsible for the adhesion to HEp-2 cells remains to be elucidated. It is possible that the bacteria adhere to HEp-2 cells by means of fimbriae or non-fimbrial factors [37], and scanning electron-microscopy supported the view that fimbrial structures are playing an important role in the adhesion to HEp-2 cells. Transmission electron microscopy showed many rigid fimbriae and curly fimbriae on the organisms. The rigid fimbriae, however, can be the type 1 pili since they showed mannose-sensitive haemagglutination. Although wiry strands have been reported as possible colonization factors [35, 42], we cannot exclude the possibility that the curly fimbriae observed in this study were the curli [48]. Further investigations are in progress to clarify this possible new colonization factor. In conclusion, ETEC O169:H41 organisms, that emerged in 1991 and became the most prevalent ETEC serotype in Japan, are composed of multiple clone and seem to possess a new colonization factor.

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REFERENCES

1. Orskov I, Orskov F, Rowe B. Six new *Escherichia coli* O groups O165, O166, O167, O168, O169 and O170. Acta Pathol Microbiol Immunol Scand Sect B 1984; **92**: 189–93.
2. Wolf MK. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. Clin Microbiol Rev 1997; **10**: 569–84.
3. Ando K, Itaya T, Aoki A, Saito A, Masaki H, Tokumaru Y. An outbreak of food poisoning caused by enterotoxigenic *Escherichia coli* O169:H41. Jpn J Food Microbiol 1993; **10**: 77–81.
4. Nishikawa Y, Hanaoka M, Ogasawara J, Moyer NP, Kimura T. Heat-stable enterotoxin-producing *Escherichia coli* O169:H41 in Japan. Emerging Infect Dis 1995; **1**: 61.
5. Crichton PB, Logan JMJ, Old DC. A miniaturized biotyping system for strain discrimination in *Escherichia coli*. Epidemiol Infect 1982; **111**: 81–8.
6. Itoh F, Ogino T, Itoh K, Watanabe H. Differentiation and detection of pathogenic determinants among diarrheagenic *Escherichia coli* by polymerase chain reaction using mixed primers. Jpn J Clin Med 1992; **50**: 343–7.
7. Moseley SL, Hardy JW, Huq MI, Echeverria P, Falkow S. Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. Infect Immun 1983; **39**: 1167–74.
8. So M, McCarthy BJ. Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains. Proc. Natl Acad Sci USA 1980; **77**: 4011–5.
9. Scotland SM, Willshaw GA, Said B, Smith HR, Rowe B. Identification of *Escherichia coli* that produces heat-stable enterotoxin ST_A by a commercially available enzyme-linked immunoassay and comparison of the assay with infant mouse and DNA probe tests. J Clin Microbiol 1989; **27**: 1697–9.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227**: 680–5.
11. Wray W, Boulikas T, Wray P, Hancock A. Silver staining of proteins in polyacrylamide gels. Analyt Biochem 1982; **118**: 197–203.
12. Chart H, Griffiths E. Antigenic and molecular homology of the ferric enterobactin receptor protein of *Escherichia coli*. J Gen Microbiol 1985; **131**: 1503–9.
13. Fitzgerald SP, Rogers HJ. Bacteriostatic effect of serum: role of antibody to lipopolysaccharide. Infect Immun 1980; **27**: 302–8.
14. Tsai, CM, Frasch CE. A sensitive silver strain for detecting lipopolysaccharide in polyacrylamide gels. Analyt Biochem 1982; **119**: 115–19.
15. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981; **145**: 1365–73.
16. Sasakawa C, Kamata K, Sakai T, Murayama SY, Makino S, Yoshikawa M. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and congo red binding activity in *Shigella flexneri*. Infect Immun 1986; **51**: 470–5.
17. Graves LM, Swaminathan B. Universal bacterial DNA isolation procedure. In: Persing DH, Smith TF, Tenover FC, White TJ, eds. Diagnostic and molecular biology: principals and applications. Washington DC: American Society for Microbiology, 1993: 617–21.
18. Scotland SM, Willshaw GA, Smith HR, Rowe B.

Properties of strains of *Escherichia coli* O26:H11 in relation to their enteropathogenic or enterohemorrhagic classification. J Infect Dis 1990; **162**: 1069–74.
19. Old DC. Haemagglutination methods in the study of *Escherichia coli*. In: Sussman M, ed. The virulence of *Escherichia coli*. London: Academic Press, 1985: 287–313.
20. Evans DG, Evans DJ Jr, Tjoa W. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect Immun 1977; **18**: 330–7.
21. Lindahl M, Faris A, Wadström T, Hjertén S. A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. Biochim Biophys Acta 1981; **677**: 471–6.
22. Helander A, Grewal HMS, Gaastra W, Svennerholm A-M. Detection and characterisation of the coli surface antigen 6 of enterotoxigenic *Escherichia coli* using monoclonal antibodies. J Clin Microbiol 1997; **35**: 867–72.
23. Lopez-Vidal Y, Klemm P, Svennerholm A-M. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin-producing *Escherichia coli*. J Clin Microbiol 1988; **26**: 1967–72.
24. Lopez-Vidal Y, Svennerholm A-M. Monoclonal antibodies against the different subcomponents of CFA/II of enterotoxigenic *Escherichia coli*. J Clin Microbiol 1990; **28**: 1906–12.
25. Viboud GI, Binsztein N, Svennerholm A-M. Characterization of monoclonal antibodies against putative colonization factors of enterotoxigenic *Escherichia coli* and their use in an epidemiological study. J Clin Microbiol 1993; **31**: 558–64.
26. Orskov F, Orskov I, Jann B, Jann K. Immunoelectrophoretic patterns of extracts from *Escherichia coli* O and K antigen strains: correlation with pathogenicity. Acta Pathol Microbiol Scand 1971; **79**: 142–52.
27. Jonson G, Svennerholm A-M, Holmgren J. *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during *in vitro* culture. Infect Immun 1989; **57**: 1809–15.
28. McConnell MM, Chart H, Field AM, Hibberd M, Rowe B. Characterization of a putative colonization factor (PCFO166) of enterotoxigenic *Escherichia coli* of serogroup O166. J Gen Microbiol 1989; **135**: 1135–44.
29. Darfeuille-Michaud A, Aubel D, Chauviere G, et al. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. Infect Immun 1990; **58**: 893–902.
30. Cravioto A, Scotland SM, Rowe B. Hemagglutination activity and colonization factor antigens I and II in enterotoxigenic and non-enterotoxigenic strains of *Escherichia coli* isolated from humans. Infect Immun 1982; **36**: 189–97.
31. Evans DG, Silver RP, Evans DJ Jr, Chase DG, Gorbach SL. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect Immun 1975; **12**: 656–67.
32. Evans DG, Evans DJ Jr. New surface associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect Immun 1978; **21**: 638–47.
33. Knutton S, McConell MM, Rowe B, McNeish AS. Adhesion and ultrastructural properties of human enterotoxigenic *Escherichia coli* producing colonization factor antigens III and IV. Infect Immun 1989; **57**: 3364–71.
34. Smyth CJ. Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype O6.K15.H16 or H^- isolated from travellers and infantile diarrhoea. J Gen Microbiol 1982; **128**: 2081–96.
35. Thomas LV, Rowe B. The occurrence of colonization factors (CFA/I, CFA/II and E8775) in enterotoxigenic *Escherichia coli* from various countries in South East Asia. Med Microbiol Immunol 1982; **171**: 85–90.
36. Thomas LV, McConnell MM, Rowe B, Field AM. The possession of three novel coli surface antigens by enterotoxigenic *Escherichia coli* strains positive for the new putative colonization factor PCF8775. J Gen Microbiol 1985; **131**: 2319–26.
37. Aubel D, Darfeuille-Michaud A, Joly B. New adhesive factor (antigen 8786) on a human enterotoxigenic *Escherichia coli* O117:H4 strain isolated in Africa. Infect Immun 1991; **59**: 1290–9.
38. Hibberd ML, McConnell MM, Field AM, Rowe B. The fimbriae of human enterotoxigenic *Escherichia coli* strain 334 are related to CS5 fimbriae. J Gen Microbiol 1990; **136**: 2449–56.
39. McConnell MM, Hibberd M, Field AM, Chart H, Rowe B. Characterization of a new putative colonization factor (CS17) from a human enterotoxigenic *Escherichia coli* of serotype O114:H21 which produces only heat-labile enterotoxin. J Infect Dis 1990; **161**: 343–7.
40. Darfeuille-Michaud A, Forestier C, Joly B, Cluzel R. Identification of a nonfimbrial adhesive factor of an enterotoxigenic *Escherichia coli* strain. Infect Immun 1986; **52**: 468–75.
41. Heuzenroeder MW, Elliot TR, Thomas CJ, Halter R, Manning PA. A new fimbrial type (PCFO9) on enterotoxigenic *Escherichia coli* O9:H^-LT^+ isolated from a case of infant diarrhea in Central Australia. FEMS Microbiol Lett 1990; **66**: 55–60.
42. Honda T, Arita M, Miwatani T. Characterization of new hydrophobic pili of human enterotoxigenic *Escherichia coli*: a possible new colonization factor. Infect Immun 1984; **43**: 959–65.
43. Knutton S, Lloyd DR, McNeish AS. Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotype O148:H28 which adheres to human intestinal mucosa: a potentially new human ETEC colonization factor. Infect Immun 1987; **55**: 86–92.
44. Tacket CO, Maneval DR, Levine MM. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic *Escherichia coli* O159:H4. Infect Immun 1987; **55**: 1063–9.
45. Viboud GI, Binsztein N, Svennerholm A-M. A new

fimbrial putative colonization factor, PCFO20, in human enterotoxigenic *Escherichia coli*. Infect Immun 1993; **61**: 5190–7.
46. Yano T, Leite DS, Barsanti de Camargo IJ, Pesta de Castro AF. A probable new adhesive factor (F42) produced by enterotoxigenic *Escherichia coli* isolated from pigs. Microbiol Immunol 1986; **30**: 495–508.
47. Helander A, Hansson GC, Svennerholm A-M. Binding of enterotoxigenic *Escherichia coli* to isolated enterocytes and intestinal mucus. Microbial Pathogenesis 1997; **23**: 335–46.
48. Olsén A, Jonsson A, Normark S. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature 1989; **338**: 652–5.

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