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Plasmid-coded DNA fragment developed as a specific gene probe for the identification of enteroaggregative *Escherichia coli*

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Summary. Enteraggregative *Escherichia coli* (EAggEC) is a recently discovered diarrhoeal pathogen implicated as a cause of persistent diarrhoea in children. EAggEC strains exhibit a characteristic pattern of adherence when incubated with HEp-2 cells. Because of the difficulty in identifying this group of bacteria, the epidemiological significance of this pathogen as a diarrhoeal agent has not been fully realised. A gene probe was developed from the 60-MDa plasmid associated with EAggEC strains that encodes the genes for adherence and fimbriae. The sensitivity of the gene probe was 93 % and the specificity 98 % for detecting EAggEC isolates and is potentially useful for diagnostic and epidemiological studies.

Introduction

Escherichia coli strains that cause diarrhoea in man are separated into five categories on the basis of their pathogenic mechanisms: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC) and enteroadherent (EAEC).¹ The last category, EAEC, has been defined on the basis of the adherent properties and phenotypic characteristics exhibited when the bacteria are incubated with HEp-2 or HeLa cell lines. They are called diffuse-adherent (DA) when the bacteria cover the cell surface uniformly and aggregative adherent (AA) when clumps of bacteria with a characteristic “stacked brick” appearance are found on the surface of HEp-2 cells.² The phenotypic characteristics are sometimes difficult to discern by this procedure. Unfortunately, there are as yet no other means of identifying these groups. Some strains of *E. coli* show localised adherence (LA) on tissue culture cells, but these have been found generally to belong to the EPEC category. While it has been proposed that some DA *E. coli* strains are diarrhoeagenic, other strains have not been shown to be consistently pathogenic in epidemiological^{3,4} or challenge studies.⁵ AA *E. coli* (EAggEC) have been isolated recently from infants and children suffering from persistent diarrhoea in several countries world-wide.^{3,6–8} This form of diarrhoea, where the

episode continues for more than 14 days,^{6,9} seems to account for disproportionately high diarrhoea-related morbidity and mortality among children in the developing world.^{10–12}

The EAggEC isolates carry a 60-MDa plasmid which is required for the expression of the aggregative phenotype.¹³ The adhesion properties of many of the EAggEC strains have been attributed to the mannose-resistant fimbriae that mediate adhesion of the bacteria to rabbit mucosa.¹⁴ Recently, electronmicroscopy of colonic mucosa demonstrated that the adhesion of the EAggEC strains in infected human patients is also mediated by fimbriae.¹⁵ The 60-MDa plasmid for the prototype EAggEC culture 17-2, has been shown to encode for bundle-forming fimbriae called aggregative adherence fimbriae (AAF/1)¹⁶ that manifest AA properties. AAF/1 is also associated with haemagglutination of human erythrocytes. Further subcloning of the region encoding the *aaf-1* gene indicated that the expression of the fimbriae requires two separate regions of the plasmid.¹⁷ The 60-MDa plasmid also encodes the gene for a heat-stable, low mol. wt toxin called EAST.¹⁸ The role of the toxin in the virulence of these bacteria has not yet been elucidated. A 1-kb gene probe (pCVD 432) was constructed from the 60-MDa plasmid associated with the representative strain (17-2) isolated from a Chilean patient.¹⁹ The probe was found to be *c.* 89 % sensitive for detecting EAggEC isolates from Chile. However, recently the probe was found to be < 50 % sensitive for detecting EAggEC isolates from Thailand.²⁰

In this report, the construction of a gene probe from

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the 60-MDA plasmid associated with one of the prototype EAggEC strains is described.

Materials and methods

Bacterial strains and serotyping

The EAggEC strains used in this study were from the faeces of infants and children suffering from persistent diarrhoea in India and were isolated after overnight culture on MacConkey and deoxycholate citrate agar plates and in selenite enrichment broth at 37°C. The isolates were identified by standard methods.²¹ The isolates from Mexico were kindly provided by Dr J. Mathewson, University of Texas Medical School, USA, and those from Brazil by Dr R. Guerrant, University of Virginia, USA. Strain 17-2 and gene probe pCVD 432 were obtained from Dr J. B. Kaper, Center for Vaccine Development (CVD), MD, USA. Clone pJPN 37 was obtained from Dr J. Nataro, CVD. The other *E. coli* strains were from the *E. coli* Reference Center at Pennsylvania State University, USA. The O:H serotypes of diarrhoeagenic EAggEC isolates were determined by the method of Ørskov *et al.*²² 200 O-type and 52 H-type antisera were used.

Adhesion assay

The isolates were tested for their adherence properties with HEP-2 cells.² Briefly, bacteria grown overnight in Luria broth (LB) were incubated with a monolayer of HEP-2 cells for 3 h at 37°C, washed and stained with Giemsa, and observed by light microscopy. The isolates that showed an aggregative adherence (AA) pattern were used for evaluating the sensitivity of the gene probe.

Plasmid curing

Plasmid DNA was cured from a representative strain (F03) with acridine orange (AO) or acriflavin (AF).²³ Bacteria grown for 6 h in LB medium were diluted 50-fold in LB medium containing AO (0, 8.5, 10 and 15 µg/ml) or AF (0, 1, 2, 2.5, 3, 4 and 5 µg/ml) and incubated at 37°C for 24 h without shaking. The cultures were diluted 2×10^5 times with saline and assayed for viable cells on nutrient agar (NA). Fifty colonies were picked from representative plates inoculated from LB cultures containing different concentrations of AO and AF and streaked on NA plates with and without antibiotics—ampicillin 100 mg/L, tetracycline 20 mg/L, chloramphenicol 30 mg/L, amoxycillin 30 mg/L and streptomycin 30 mg/L. Those that were sensitive to antibiotics were presumed to have lost the plasmids and they were checked for the presence of plasmids by the method of Birnboim and Doly.²⁴

Construction of the gene probe

Plasmid DNA (60–65 MDa) was extracted from an overnight culture of a representative EAggEC strain (F03-1) by an alkaline lysis procedure.²⁴ The DNA was digested with restriction endonucleases *Eco*RI or *Bam*HI or *Pst*I according to manufacturer's protocol. The digested fragments were ligated to the corresponding site in a pUC19 vector sample digested with the respective enzymes. DH5α cells were transformed with the recombinant plasmid according to the protocol supplied by Bethesda Research Laboratory (BRL), MD, USA. The DH5α transformants were grown overnight on LB agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and ampicillin 100 mg/L.²⁵ Plasmids were isolated from c. 300 white colonies representing the clones with inserts, digested with restriction endonucleases and electrophoresed on agarose 0.7% gels. The sizes of the DNA inserts were determined by comparing the bands with standard *Hind*III digested lambda DNA mol. wt markers from BRL. The inserts were used as candidate probes after radiolabelling with [³²P]dATP by nick translation according to the protocol supplied by the manufacturer (BRL). The specific activities of the probes (pVAP and pCVD 432) used for hybridisation were c. 10⁶dpm (disintegrations/min).

Colony blot hybridisation

E. coli isolates were grown on LB agar and the colonies were transferred to Whatman 541 filter papers. The colonies were lysed by laying the filters on 3 MM filters soaked with lysing solution (0.5 N NaOH, 1.5 M NaCl) in a petri dish.²⁶ The dishes were heated at 100°C for 5 min. The filters were then neutralised (1 M Tris-HCl pH 7, 2 M NaCl) for 5 min. The filter papers were dried in air and hybridised with ³²P-labelled DNA probes. The filter papers were placed in plastic bags containing the hybridisation solution—(formamide 50%, 10×SSC (1.5 M NaCl, 0.15 M sodium citrate), SDS 0.1%, Ficoll 0.1%, polyvinylpyrrolidone 0.1%, bovine serum albumin 0.1%, 2 mM EDTA—and the radioactive gene probe was denatured by heating at 100°C for 5 min. The filters were incubated at 37°C as described by Sambrook *et al.*²⁵ The filters were washed three times with 50 ml of 0.2×SSC and SDS 0.1%, each time at 37°C. The probe-positive colonies were detected by exposing the filter paper to X-ray film; they appeared as dark spots on the film.

Transmission electronmicroscopy (TEM)

Bacteria grown on nutrient agar overnight at 37°C were washed three times with phosphate-buffered saline (PBS) and resuspended in distilled water. They were stained with ammonium molybdate (pH 7) 2% and bacitracin 150 mg/L.¹⁵ The suspension was applied to carbon-coated grids, the excess fluid was removed, and the grid was examined in a Jeol 1200 EXII Transmission electronmicroscope.

Figure. Electronmicrographs of wild-type and transformed EAggEC strains: prototypic strain (F03) showing a fimbria ($\times 100000$); plasmid-cured strain (PC) showing a flagellum and fimbriae ($\times 50000$); non-pathogenic strain HB101, transformed with the 60-MDa plasmid (HBT) showing fimbriae in the form of fimbriar bundles ($\times 250000$).

Results

EAggEC strains from patients suffering from persistent diarrhoea were obtained from four countries—Brazil, Mexico, Chile and India. All the isolates exhibited the aggregative phenotype when incubated with the HEp-2 cell line. The O serotypes of the isolates varied considerably but O78 (six isolates) was the commonest (table I); 31 different serotypes were recorded and 17 isolates did not appear to belong to any known O serotype and were designed as giving a negative reaction. The H serotype of isolates was mostly non-modal and 16 isolates gave negative reactions with all the H antisera. Plasmid profiles of the EAggEC strains from India showed a common 60-MDa plasmid in addition to one or more plasmids of 4–22 kb (data not shown). The major characteristic of EAggEC, the AA phenotype, was considered an important factor for gene probe development. Therefore, attempts were made to identify the location of the AA determinant before constructing the gene probe. The prototype strain F03, isolated from an infant with diarrhoea in India, harboured two plasmids (60 MDa and 4 kb). Curing the 4-kb plasmid did not affect AA, whereas the bacteria that lost both the 4-kb and 60-MDa plasmids (PC) also lost AA properties. When a non-pathogenic *E. coli* strain, HB101, was transformed with the 60-MDa plasmid from F03, the transformed bacteria (HBT) exhibited the AA phenotype. These results indicated clearly that the 60-MDa plasmid conferred the AA phenotype to EAggEC strains from the Indian subcontinent.

The correlation of the adherence properties of the bacteria with the development of fimbriae was also examined. The wild-type strain (F03), the transformed culture (HBT) and the plasmid-cured strain (PC) were observed by transmission electronmicroscopy (TEM). Fimbriae were observed in the wild-type strain F03 and the transformed culture HBT, whereas PC showed long flagella and fimbriae (figure). HB101, the host for the transformation experiment, did not exhibit any fimbriae (data not shown). The genes for fimbriae formation were encoded by the 60-MDa plasmid

which further supported the construction of a gene probe from the plasmid DNA.

Of 300 clones constructed from the plasmid DNA, 20 with inserts of 0.5–1 kb were selected as candidate probes. Each one was tested by colony hybridisation with EAggEC and other diarrhoeagenic strains. Three of the *Eco*R1 fragments tested as probes exhibited very little specificity for EAggEC strains. The other fragments derived by digesting the DNA with *Bam*H1 and *Pst*I exhibited only moderate sensitivity (25–62%) for detecting EAggEC strains. Only one of the clones (pVAP), with a 730-bp *Eco*R1 insert, exhibited very high sensitivity as well as specificity for EAggEC isolates from India, Brazil, Mexico and Chile and, therefore, was selected as the candidate probe. Probe pVAP, under highly stringent conditions of hybridisation, hybridised with 57 of 61 isolates (93% sensitivity) (table I). Under similar optimal hybridisation conditions, probe pCVD 432,¹⁹ currently available for detecting EAggEC, hybridised with only 43 of the 61 isolates (70% sensitivity) (table I). Both probes, pVAP and pCVD 432, hybridised with prototypic strains F03 and 17-2, used as positive controls, but not with strains HB101 and PC that served as negative controls. Probes pVAP and pCVD 432, despite being constructed from the 60-MDa plasmid, did not hybridise with each other and, therefore, do not share complementary DNA sequences.

Probe pVAP was evaluated against isolates belonging to EPEC, ETEC, EHEC and EAEC (DA and LA) categories of diarrhoeagenic *E. coli* from 20 different countries world-wide. Of the 90 isolates tested, only two EPEC strains, (2708-78 and 2394-80) of serotype O111:H21, hybridised with pVAP. The specificity of probe pVAP was also evaluated against other diarrhoeagenic bacteria (table II) such as *Salmonella*, *Yersinia*, *Shigella*, *Klebsiella*, *Vibrio*, *Pseudomonas* and *Citrobacter* spp. None of the 14 strains tested exhibited any complementarity with pVAP. Of 104 strains tested for specificity, only two cultures reacted positively with pVAP. The specificity of the probe was thus calculated to be c. 98%.

Table 1. Sensitivities of gene probes for EAggEC strains

Isolate no.	Origin	Serotype		HEp-2 assay	Hybridisation with probe	
		O	H		pVAP	pCVD432
FO1	India	17	6	AA	+	+
FO2	India	51	11	AA	+	+
FO3	India	4	7	AA	+	+
FO4	India	51	—	AA	+	+
FO5	India	75	NM	AA	—	—
FO6	India	77	18	AA	+	+
FO7	India	162	NM	AA	+	+
HO8	India	111	32	AA	+	—
HO9	India	142	33,35	AA	—	—
HO10	India	7	NM	AA	+	+
HO11	India	6	—	AA	+	—
HO12	India	—	32	AA	+	+
HO13	India	68,73	NM	AA	+	+
HO14	India	—	NM	AA	+	—
HO15	India	—	—	AA	+	+
HO16	India	78	—	AA	+	+
HO17	India	78	—	AA	+	+
HO18	India	85	—	AA	+	+
HO19	India	7	NM	AA	+	+
HO20	India	—	NM	AA	+	+
17-2	Chile	3	42	AA	+	+
PDAS21-1	Brazil	—	10	AA	+	+
PDAS23-1	Brazil	78	NM	AA	+	+
PDAS34-5	Brazil	—	NM	AA	+	+
PDAS46-1	Brazil	68	NM	AA	+	+
PDAS48-1	Brazil	15	NM	AA	+	+
PDAS52-5	Brazil	—	—	AA	+	+
PDAS71-1	Brazil	20	NM	AA	+	—
PDAS113-2	Brazil	86	—	AA	+	+
PDAS116-1	Brazil	—	—	AA	+	+
PDAS121-1	Brazil	—	NM	AA	—	—
PDAS131-1	Brazil	91	NM	AA	+	+
PDAS150-1	Brazil	81	—	AA	—	—
PDAS160-1	Brazil	44	NM	AA	+	+
PDAS174-1	Brazil	—	NM	AA	+	+
PDAS175-1	Brazil	5	10	AA	+	—
PDAS176-1	Brazil	—	NM	AA	+	+
PDAS178-3	Brazil	—	—	AA	+	+
PDAS188-1	Brazil	21	NM	AA	+	—
PDAS98-1	Brazil	11	NM	AA	+	+
JM 24	Mexico	15	1	AA	+	+
JM 32	Mexico	—	10	AA	+	+
JM 45	Mexico	86	2	AA	+	+
JM 70	Mexico	92,78	—	AA	+	+
JM 71	Mexico	25	NM	AA	+	—
JM 72	Mexico	85	NM	AA	+	—
JM 77	Mexico	15	—	AA	+	+
JM 162	Mexico	52	27	AA	+	+
JM 190	Mexico	17	18	AA	+	—
JM 215	Mexico	92, 78	33, 35	AA	+	+
JM 221	Mexico	—	—	AA	+	+
JM 314	Mexico	133	NM	AA	+	—
JM 315	Mexico	151	49	AA	+	+
JM 323	Mexico	—	32	AA	+	+
JM 333	Mexico	113	21	AA	+	—
JM 366	Mexico	36	—	AA	+	—
JM 389	Mexico	78	NM	AA	+	+
JM 392	Mexico	—	NM	AA	+	+
JM 398	Mexico	—	—	AA	+	+
JM 417	Mexico	59	NM	AA	+	—
JM 459	Mexico	59	NM	AA	+	—
FO3-1 (+ve control)		—	—	AA	+	+
PC (-ve control)		—	—	—	—	—
HB101 (-ve control)		—	—	—	—	—
pVAP		—	—	—	+	—
pCVD432		—	—	—	—	+

O and H serotypes: —, negative reaction; multiple numbers, multiple reaction; NM, nonmotile. AA: aggregative adherence.

Table II. Specificity of the gene probe with other bacteria; none hybridised with pVAP

Strain	Isolate no.	Source	Origin
<i>Salmonella typhi</i>	4050	Human	India
<i>S. typhi</i>	3935	Human	India
<i>Shigella flexneri</i>	456 II	Human	India
<i>Sh. flexneri</i>	27188	Human	India
<i>Klebsiella</i> spp.	H850 A	Human	India
<i>Klebsiella</i> spp.	H891 I	Human	India
<i>Vibrio cholerae</i>	460 I	Human	India
<i>V. cholerae</i>	433 I	Human	India
<i>S. typhimurium</i>	688 II	Human	India
<i>S. typhimurium</i>	658 III	Human	India
<i>S. typhimurium</i>	638 III	Human	India
<i>Yersinia</i> spp.	43 I	Human	India
<i>Pseudomonas aeruginosa</i>	462	Human	India
<i>Citrobacter</i> spp.	632	Human	India

Discussion

Diarrhoeal diseases caused by *E. coli* remain a major public health problem world-wide. EAggEC strains that cause persistent diarrhoea have been recognised increasingly as an important problem in developing countries.^{6, 27} With the present population of the world travelling more than ever before, attention should be given to measures to detect and control this group of enteric pathogens. The development of effective gene probes that will distinguish EAggEC from other diarrhoeagenic *E. coli* strains that are common in the developing world is important for patient management and for controlling the disease. As reports for detection of EAggEC strains among patients with diarrhoea are coming to light, the diversity in genotypic characteristics of the organisms is also becoming more apparent. This is reflected by the fact that the only gene probe presently available is ineffective in detecting all the different genotypes of this group of bacteria.²⁰ Because of the difficulty in recognising EAggEC isolates, there is an urgent need for establishing additional methods of detection in order to comprehend the epidemiological significance and global importance of this group of pathogens as diarrhoeal agents. This will assist in identifying the risk factors, modes of transmission and the source and spectrum of clinical illnesses.

Gene probes have proved to be very effective diagnostic tools for identifying other diarrhoeagenic *E. coli* such as ETEC, EPEC, EHEC and EIEC.²⁸⁻³¹

The gene probes developed for stable toxins (St-Ap, St-B), labile toxin (LT), Shiga-like toxins I and II, the attaching and effacing gene (*eae*) or the EAF factor that are employed to identify ETEC, EHEC and EIEC and EPEC strains did not hybridise with any of the EAggEC strains in the present investigation (data not shown). Therefore, this group is distinct from the other categories of diarrhoeagenic *E. coli*. The EAggEC isolates from India, Brazil and Mexico, when serotyped with antisera to 200 O-types and 52 H-types, exhibited wide diversity (table I) and could not be categorised or grouped on the basis of serotype. Seventeen strains out of 61 did not react with any of the O antisera and 16 strains did not react with any of the H antisera. This indicated that the O and H antigens of some of the EAggEC strains were unique and have not been observed previously.

The number of plasmids associated with these strains varied considerably. Whereas some of the strains exhibited only one plasmid, others harboured three or four. The plasmids seem to be responsible for inducing expression in non-pathogenic strain HB101 and conferring resistance to antimicrobial agents. Whereas PC was sensitive to all antibiotics tested, all the wild type EAggEC strains from India were resistant to penicillin (10 units), bacitracin (10 units) and streptomycin (100 units) and 95% were resistant to tetracycline (30 mg/L) and erythromycin (15 mg/L) (data not shown).

Gene probe pVAP, although developed from the 60-MDa plasmid, did not have any sequence complementary to the aggregative adherence fimbriae gene (*aaf-I*)¹⁷ as was evident from a hybridisation experiment with clone pJPN-437 that encodes the *aaf-I* region. The probe appears to be from a different region of the plasmid. Although the specificity of the probe was found to be 98%, it hybridised with two EPEC strains of serotype O111:H21. Scotland *et al.*³² also found that the gene probe pCVD 432 hybridised with EPEC strains of serotype O111ab:H21 that exhibited aggregative adherence. Several strains from Mexico that were considered to be DA before AA was recognised as a distinct pattern, were found to hybridise with pVAP and pCVD 432 and exhibited AA properties. Therefore, more comprehensive studies are required to determine the correlation of this newly discovered pathogen with the other categories of diarrhoeagenic *E. coli*.

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