Virulence characteristics and the molecular epidemiology of enteroaggregative *Escherichia coli* isolates from travellers to developing countries

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Enteroaggregative *Escherichia coli* (EAEC) is associated with diarrhoea among travellers to developing countries. EAEC virulence properties predisposing to illness are not clear. Sixty-four EAEC strains identified by a HEp-2 cell assay and isolated from faecal samples from US and European travellers to developing countries were studied for the prevalence of 11 putative virulence genes by PCR: 49 EAEC strains from adults with acute diarrhoea and 15 EAEC strains from adults without diarrhoea. *E. coli* strains from the stools of healthy travellers to the same region were used as controls. EAEC carrying *aggR*, *aap*, *astA* and *set1A* were identified individually more often in the stools of subjects with diarrhoea compared with those without diarrhoea ($P<0.05$). EAEC isolates with two or three of these genes were associated with diarrhoea compared with EAEC isolates without the presence of these genes ($P<0.05$). Subjects with diarrhoea who shed EAEC isolates positive for these genes were more likely than subjects shedding EAEC negative for these genes to pass stools with gross mucus (57 vs 14 %) and faecal leukocytes (40 vs 7 %) ($P<0.05$). This study shows the heterogeneity of gene profiles of EAEC strains found in the stools of international travellers and suggests that the presence of *aggR*, *aap*, *astA* or *set1A*, the number of genes present and stool characteristics may be markers for more virulent EAEC strains.

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INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) are important emerging enteric pathogens of travellers' diarrhoea and are widely distributed worldwide (Huang *et al.*, 2004a). A recent study showed that EAEC was the only enteric pathogen identified among 19 % of people with diarrhoea while visiting Goa, India, 26 % of visitors to Montego Bay, Jamaica, and 33 % of students during a short time in Guadalajara, Mexico (Adachi *et al.*, 2001).

The pathogenesis of EAEC diarrhoea is now being defined and is related to host- and pathogen-specific properties. A major obstacle in identifying the EAEC pathogenic mechanisms lies in the heterogeneity of gene profiles of EAEC strains. Although many putative virulence genes have been identified for EAEC, confusion regarding the

role of EAEC in acute diarrhoeal illness derives from the lack of clearly implicated virulence factors and mechanisms. One study has suggested that infection with EAEC that contains virulence factors is associated with production of increased levels of faecal cytokines (Jiang *et al.*, 2003). It is unknown whether other faecal inflammatory markers such as faecal leukocytes are associated with virulent strains of EAEC.

The prevailing model of the pathogenesis of EAEC infection includes adherence to the terminal ileum and colon by aggregative adherence fimbriae, production of cytotoxins such as the plasmid-encoded toxin (encoded by *pet*), *E. coli* heat-stable enterotoxin 1 (EAST1) and *Shigella* enterotoxin 1 (encoded by *set1A*), and probably a package of genes, including genes on a chromosomal island, that are regulated by *AggR*. The primary objective of this study was to determine the distribution of putative virulence genes in EAEC isolates identified from travellers with and without diarrhoea. An association of the studied genes with

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; ETEC, enterotoxigenic *E. coli*.

resultant inflammatory stool characteristics including leukocytes and gross mucus was also sought. We hypothesized that EAEC carrying certain virulence genes would be more likely to cause acute diarrhoea in travellers than EAEC not carrying these genes. Additionally, we hypothesized that stools from travellers with diarrhoea caused by EAEC carrying certain genes would be more likely to contain inflammatory markers than EAEC isolates not carrying these genes.

METHODS

The following study design was a nested case-control study of a cohort of US travellers to different regions of the developing world. On arrival at their destination, all travellers completed a daily diary documenting enteric symptoms and signs, and recorded the form and timing of all stools passed. When diarrhoeal illness occurred, subjects would submit a stool sample to the laboratory for processing. In a subset of subjects without diarrhoea, a stool sample was obtained from participants at enrolment, within approximately 72 h of arrival in the developing country. Participation in this study was voluntary and required written informed consent of participants prior to enrolment. This study was approved by the Committee for the Protection of Human Subjects of the University of Texas at Houston Health Science Center and written informed consent was obtained from each subject.

Study population and collection of EAEC isolates. Stools samples were collected during 1999–2004 from travellers from industrialized countries with ($n=49$) and without ($n=15$) diarrhoea during short-term stays in Mexico (47 isolates), India (10 isolates) and Guatemala (seven isolates) (Jiang *et al.*, 2002a). EAEC isolates from travellers with and without diarrhoea in Mexico were from stool samples from the same population. EAEC isolates from travellers with diarrhoea in India and Guatemala were from stool samples from a cohort similar to travellers to Mexico. Each EAEC isolate came from a different traveller, and only EAEC isolates identified as the sole pathogen in the stool sample of travellers were included in this study (Jiang *et al.*, 2002a, b). Acute diarrhoea was defined as the passage of three or more unformed stools within a 24 h period plus one or more signs or symptoms of enteric infection (e.g. nausea, vomiting, abdominal pain or cramping, excessive gas, faecal urgency, bloody or mucus stool, or tenesmus) (DuPont & Ericsson, 1993). Seventeen *E. coli* isolates from travellers without diarrhoea in Mexico that did not demonstrate HEP-2 adherence and that did not carry genes for enterotoxigenic *E. coli* (ETEC) heat-labile toxin and/or heat-stable toxin production were used in the present study as non-diarrhoeagenic *E. coli* controls (Jiang *et al.*, 2002a, b). Travellers who received an antibacterial agent with activity against enteric pathogens (e.g. fluoroquinolone, macrolide, azalide or trimethoprim-sulfamethoxazole) or who received anti-diarrhoeal medication (e.g. loperamide, bismuth subsalicylate or kapectate) within the week prior to enrolment were excluded.

Microbiological studies. The presence of conventional pathogens was assessed prospectively in each case using published methods: stools were studied for the presence of *Shigella* spp., *Salmonella* spp., *Vibrio* spp., *Campylobacter jejuni*, *Yersinia enterocolitica*, *Aeromonas* spp. and *Plesiomonas shigelloides* (Jiang *et al.*, 2002b). *Entamoeba histolytica*, *Cryptosporidium* spp. and *Giardia lamblia* were identified by enzyme immunoassays (Jiang *et al.*, 2002b). Five lactose-positive colonies were retrieved from MacConkey agar plates from each stool sample and inoculated into individual peptone stabs that were transported to Houston, TX, for identification of known *E. coli* pathotypes. *E. coli* colonies negative for heat-labile and heat-stable

enterotoxins of ETEC by oligonucleotide probes (Murray *et al.*, 1987) were examined further for the aggregative phenotype diagnostic of EAEC using the HEP-2 cell adherence assay (Donnenberg & Nataro, 1995). *E. coli* isolates that did not demonstrate the aggregative phenotype and did not carry genes for ETEC heat-labile toxin and/or heat-stable toxin production were used as non-diarrhoeagenic *E. coli* controls. Enteropathogenic *E. coli*, enterohemorrhagic (or Shiga toxin-producing) *E. coli* and enteroinvasive *E. coli* were not screened for, based on the low prevalence of these pathogens among travellers with diarrhoea to the same regions in earlier studies (Vargas *et al.*, 1998).

Identification of EAEC. *E. coli* in each stool sample were tested for the presence of EAEC on the basis of a characteristic pattern of adherence to cultured HEP-2 cells (Donnenberg & Nataro, 1995). Briefly, a chamber slide (Dynatek) was seeded with HEP-2 cells (ATCC) grown at 37 °C in 5% CO₂ in minimal essential medium (Gibco) supplemented with 10% fetal calf serum. *E. coli* isolates were grown overnight at 37 °C without shaking in trypticase soy broth (BBL Microbiology Systems) with 1% D-mannose. Bacterial culture (25 µl) was added to each chamber and incubated at 37 °C for 3 h. The chamber slide was washed five times with PBS, fixed with 100% methanol, and stained with crystal violet (Difco). A positive-control EAEC strain (042) isolated in Peru from a child with diarrhoea and a negative-control non-adherent *E. coli* strain (HS) from a healthy subject were included in each assay. Each *E. coli* strain was tested twice in a blind fashion. A sample was interpreted as positive for EAEC if it showed the characteristic 'stacked-brick' aggregative appearance described by Donnenberg & Nataro (1995).

EAEC isolates found in the presence of conventional pathogens, as described above, were excluded from analysis.

Molecular studies. DNA from *E. coli* isolates was isolated from an individual colony suspended in 25 µl PCR H₂O subjected to 95 °C for 5 min. The oligonucleotide primers for the 11 genes assessed in this study are listed in Table 1. Positive and negative controls for each gene were used in each PCR assay. Each PCR assay was performed in a final reaction volume of 10 µl containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 100 µM each of dATP, dCTP, dGTP and dTTP, 2.5 U AmpliTaq polymerase (Perkin-Elmer), 10 pmol each primer and 2 µl boiled bacterial cell lysate as the DNA template. The sequences of primers, the sizes of the amplicons and the reaction conditions are listed in Table 1. Amplifications were performed in a DNA thermal cycler (Perkin-Elmer). The amplified PCR products were analysed on a 1% agarose gel.

Stool characterization studies. Occult blood in the stool was tested by use of guaiac reagents (Haemocult; SmithKline Diagnostics). Stools were characterized as containing gross mucus by laboratory personnel examining the sample. The presence of faecal leukocytes was determined microscopically by counting the number of cells present in a faecal smear stained with methylene blue (Jiang *et al.*, 1994). The presence of 10 or more leukocytes per high-power field was considered to be positive (Jiang *et al.*, 1994).

Statistical analysis. Multiple comparisons of the distribution of genes carried by EAEC isolates and the stool characteristics of travellers among groups were performed using χ^2 or Fisher's exact test for categorical data. Data were examined in a two-tailed fashion to estimate the *P* value.

RESULTS

The distribution of genes is shown for the three groups in Table 2. The genes *aggR*, *aap*, *astA* and *set1A* were each

Table 1. PCR primer pairs for the detection of putative virulence genes

Target gene	Description of encoded protein	Primer sequence (5'→3')	Amplicon size (bp)	PCR conditions (30 cycles)*	Reference
<i>aggR</i>	AAF/I and AAF/II transcriptional activator	CTAATTGTACAATCGATGTA ATGAAGTAATTCTTGAAT	308	1 min 94 °C, 1 min 42 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>aggA</i>	AAF/I fimbrial subunit	TTAGTCTTCTATCTAGGG	450	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>aafA</i>	AAF/II fimbrial subunit	AAATTAATTCCGGCATGG ATGTATTTTATAGAGGTTGAC	518	1 min 94 °C, 1 min 60 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>aap</i>	Anti-aggregation protein (dispersin)	TATTATATTGTCACAAGCTC CTTTTCTGGCATCTTGGGT	232	1 min 94 °C, 1 min 52 °C, 1 min 72 °C	Sheikh <i>et al.</i> (2002)
<i>set1A</i>	<i>Shigella</i> enterotoxin 1 mucinase	GTAACAACCCCTTTGGAAGT TCACGCTACCATCAAAGA	309	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Fasano <i>et al.</i> (1995)
<i>astA</i>	Aggregative heat-stable toxin 1 (EAST1)	TATCCCCCTTTGGTGGA CCATCAACACAGTATATCCGA	111	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Yatsuyanagi <i>et al.</i> (2003)
<i>irp2</i>	Yersiniabactin biosynthesis gene	GGTCGCGAGTGACGGCTTTGT AAGGATTCGCTGTTACCGGAC	264	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>pet</i>	Plasmid-encoded toxin	TCGTCGGGCAGCGTTTCTTCT GACCATGACCTATACCGACAGC	832	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>shf</i>	Cryptic ORF	CCGATTTCTCAAACCTCAAGACC ACTTTCTCCGAGACATTC	613	1 min 94 °C, 1 min 50 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>agg-3A</i>	AAF/III fimbrial subunit	CTTTAGCGGGAGCATTTCAT GTATCATTGCGAGTCTGGTATTCAG	462	1 min 94 °C, 1 min 50 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)
<i>cdt</i>	Cytolethal distending toxin	GGGCTGTTATAGAGTAACTTCCAG CTGTCAGAGCGGCAAAACCA GTCTACAGGGGCATTGTT	1029	1 min 94 °C, 1 min 55 °C, 1 min 72 °C	Czeczulin <i>et al.</i> (1999)

*For all PCR conditions, a final extension step, 7 min 72 °C, was performed.

identified individually significantly more frequently among the EAEC strains derived from the diarrhoea group compared with the non-diarrhoea group ($P<0.05$). Table 3 shows that many of the EAEC isolates in the diarrhoea group had two or three of these genes compared with the absence of these genes from the non-diarrhoea group ($P<0.05$). EAEC isolates negative for all four genes were identified more frequently from the non-diarrhoea group ($P=0.002$). EAEC strains with the combination *aggR* and *set1A*, with or without additional factors, were particularly strongly associated with diarrhoea; these genes were found in 18/49 travellers (37%) with diarrhoea compared with 1/32 travellers (3%) without diarrhoea ($P<0.001$).

The presence of the *aggR*, *aap*, *astA* and *set1A* genes and stool characteristics of the three groups are listed in Table 4. Travellers with diarrhoea who shed EAEC isolates carrying *aggR*, *aap*, *astA* or *set1A* more commonly passed stools

with gross mucus (57%) and faecal leukocytes (40%) compared with EAEC isolates negative for these genes (14 and 7%, respectively) ($P<0.05$).

The relative frequencies of EAEC carrying the studied genes and the distribution of genes were similar across the regions of the world and during the years the isolates were collected.

DISCUSSION

EAEC are a heterogeneous group of *E. coli* that display variation in causing clinical illness. The factors associated with virulence are poorly understood. In this case-control study, we determined the distribution of 11 genes in isolates identified from travellers with and without diarrhoea. Our study showed significant heterogeneity in gene profiles among the EAEC isolates. None of the genes

Table 2. Distribution of genes among *E. coli* strains (EAEC and non-diarrhoeagenic *E. coli*) from travellers with and without diarrhoea

Percentage values are given in parentheses.

Gene	EAEC diarrhoea group (n=49)	Non-diarrhoea group		P value
		EAEC non-diarrhoea group (n=15)	Non-diarrhoea <i>E. coli</i> controls (n=17)	
<i>aggR</i> *	19 (39)	1 (7)	0 (0)	0.002
<i>aggA</i>	14 (29)	5 (33)	2 (12)	0.19
<i>aafA</i>	3 (6)	4 (27)	3 (18)	0.05
<i>aap</i> †	13 (27)	1 (7)	0 (0)	0.01
<i>set1A</i> *	25 (51)	1 (7)	2 (12)	0.0001
<i>astA</i> †	22 (45)	4 (27)	2 (12)	0.02
<i>pet</i>	20 (41)	4 (27)	3 (18)	0.095
<i>agg-3A</i>	1 (2)	0 (0)	0 (0)	1.0
<i>cdt</i>	4 (8)	2 (13)	1 (6)	1.0
<i>irp2</i>	28 (57)	8 (55)	7 (41)	0.49
<i>shf</i>	14 (29)	2 (13)	2 (12)	0.12

* $P < 0.005$, EAEC diarrhoea group vs non-diarrhoea group.

† $P < 0.05$, EAEC diarrhoea group vs non-diarrhoea group.

Table 3. Presence of *aggR*, *aap*, *astA* and *set1A* among *E. coli* strains (EAEC and non-diarrhoeagenic *E. coli*) from travellers with and without diarrhoea

Percentage values are given in parentheses.

Isolate/genes	EAEC diarrhoea group (n=49)	Non-diarrhoea group		P value
		EAEC non-diarrhoea group (n=15)	Non-diarrhoea <i>E. coli</i> controls (n=17)	
EAEC isolates with only one gene	10 (20)	3 (20)	4 (24)	0.87
<i>aggR</i> only	0	0	0	
<i>aap</i> only	0	0	0	
<i>astA</i> only	8	3	2	
<i>set1A</i> only	2	0	2	
EAEC isolates with two genes*	10 (20)	0 (0)	0 (0)	0.01
<i>aggR</i> , <i>set1A</i>	5	0	0	
<i>aap</i> , <i>astA</i>	1	0	0	
<i>set1A</i> , <i>astA</i>	4	0	0	
EAEC isolates with three genes*	11 (22)	0 (0)	0 (0)	0.004
<i>aggR</i> , <i>set1A</i> , <i>astA</i>	3	0	0	
<i>aggR</i> , <i>set1A</i> , <i>aap</i>	6	0	0	
<i>aggR</i> , <i>aap</i> , <i>astA</i>	1	0	0	
<i>set1A</i> , <i>aap</i> , <i>astA</i>	1	0	0	
EAEC isolates with four genes	4 (8)	1 (7)	0 (0)	0.04
<i>aggR</i> , <i>set1A</i> , <i>aap</i> , <i>astA</i>	4	1	0	
EAEC isolates negative for all genes†	14 (29)	11 (73)	13 (76)	0.0001

* $P < 0.05$, EAEC diarrhoea group vs non-diarrhoea group.

† $P = 0.002$, EAEC diarrhoea group vs non-diarrhoea group.

Table 4. Presence of *aggR*, *aap*, *astA* and *set1A* among *E. coli* strains (EAEC and non-diarrhoeagenic *E. coli*) and stool characteristics of adult travellers with and without diarrhoea

Percentage values are given in parentheses.

Stool characteristic	EAEC diarrhoea group (n=49)		EAEC non-diarrhoea group (n=15)		Non-diarrhoea <i>E. coli</i> controls (n=17)	
	No. gene positive (n=35)	No. gene negative (n=14)	No. gene positive (n=4)	No. gene negative (n=11)	No. gene positive (n=4)	No. gene negative (n=11)
Gross mucus	20 (57)*	2 (14)	0 (0)	0 (0)	0 (0)	0 (0)
Occult blood	3 (9)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)
White blood cells	14 (40)*	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)

* $P<0.05$, EAEC diarrhoea group vs non-diarrhoea group.

studied was present in all of the EAEC isolates. However, this study identified *aggR*, *aap*, *astA* and *set1A* more often among EAEC isolates from travellers with diarrhoea compared with matched travellers without diarrhoea.

The genes *aggR*, *aap*, *astA* and *set1A* have been described previously. *aggR* is a shared transcriptional activator for both *aggA* (AAF/I fimbrial subunit) and *aafA* (AAF/II fimbrial subunit). Other EAEC genes encoding possible virulence factors have also been shown to be under *aggR* control, such as *aap*, *aat* (CVD432), a chromosomal island of the *pheU* locus, and several enterotoxins (Dudley *et al.*, 2006), and these genes may travel as a package of virulence genes (Jenkins *et al.*, 2005). In our study, 27% of EAEC isolates from travellers with diarrhoea had the dispersin gene (*aap*). Dispersin is a secreted low-molecular-mass protein (10.2 kDa) that coats the bacterial surface and promotes dispersal of EAEC on the intestinal mucosa. *aap* lies immediately upstream of *aggR* in EAEC strain 042 (a prototype EAEC isolate) (Grover *et al.*, 2001; Sheikh *et al.*, 2002). There is remarkable conservation of the *aap-aggR* locus among EAEC strains, as *aap* and *aggR* are separated by less than 1 kb (Czczulin *et al.*, 1999). In our study, 12/14 *aap*-positive strains (86%) were positive for *aggR*. These findings are consistent with the findings of other studies, which show that EAEC isolates carrying *aggR* and *aap* may be phylogenetically or pathogenetically linked (Cerna *et al.*, 2003; Lopez-Saucedo *et al.*, 2003). *astA* encodes a heat-stable enterotoxin (EAST1). EAST1 is a 4.1 kDa protein originally discovered in EAEC but now known to be found in other diarrhoeagenic *E. coli* (Menard & Dubreuil, 2002; Vila *et al.*, 1998; Yatsuyanagi *et al.*, 2003; Zamboni *et al.*, 2004; Zhou *et al.*, 2002). This toxin is often compared with *E. coli* STa enterotoxin from ETEC, as they share physical and mechanistic similarities. *set1A* encodes *Shigella* enterotoxin 1. Similar to *astA*, *set1A* is also found in other *E. coli* pathotypes (Fasano *et al.*, 1995).

Although this study did not test for gene products, the results identified genes of EAEC that appeared to predict virulent strains. EAEC isolates carrying two or three of the

genes examined were frequently identified from travellers with diarrhoea and not from travellers without diarrhoea. We were particularly interested in the combination of *aggR*, a plasmid-borne master virulence regulator, and *set1A*, which is encoded on a chromosomal island partially under *aggR* control. Interestingly, *Shigella* enterotoxin 1, encoded by the *set1A* gene, is an oligomeric toxin that was first identified in *Shigella flexneri* 2a strains (Fasano *et al.*, 1995). EAEC strains carrying specific genes under *aggR* control may be important alone or in combination with other virulence factors and, when present, may indicate pathogenic strains of EAEC. Recent data have shown that the AggR regulon controls both plasmid-borne virulence factors and chromosomal genes associated with clinical illness (Dudley *et al.*, 2006).

Among travellers with diarrhoea, EAEC isolates carrying the genes of interest (*aggR*, *aap*, *astA* and *set1A*) were more likely to be associated with faecal mucus and faecal leukocytes than those from travellers with diarrhoea who shed EAEC isolates not carrying these genes. These findings are consistent with other studies, which suggest that EAEC can cause inflammatory enteritis (Bouckennooghe *et al.*, 2000; Greenberg *et al.*, 2002; Harrington *et al.*, 2006; Wanke, 1995). These studies found that travellers who developed EAEC diarrhoea had higher stool levels of inflammatory markers, including interleukin (IL)-8, lactoferrin and leukocytes, than healthy volunteers with no identified enteric pathogen in their stool (Bouckennooghe *et al.*, 2000; Greenberg *et al.*, 2002; Huang *et al.*, 2004b). IL-8 is thought to play an important role in EAEC pathogenesis. This chemokine recruits neutrophils to the intestinal epithelial mucosa, causing epithelial destruction and fluid secretion (Madara *et al.*, 1993; Sansonetti *et al.*, 1999). An intestinal faecal IL-8 response to EAEC infection may explain our findings of faecal leukocytes in many of these patients.

In summary, a limited number of genes (*aggR*, *aap*, *astA* and *set1A*) appeared to be commonly associated with EAEC diarrhoea. The identification of these genes, both qualitatively and quantitatively, along with characterization

of stool inflammatory markers from travellers, may permit earlier diagnosis of this infection and improve the epidemiological understanding of this emerging enteric pathogen.

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