The prevalence and virulence characteristics of enteroaggregative *Escherichia coli* at an urgent-care clinic in the USA: a case–control study

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This case–control study examined the prevalence of enteroaggregative *Escherichia coli* (EAEC), its genes and elicited inflammatory response, and the stool characteristics of adult patients with and without acute diarrhoeal illness presenting to an urgent-care clinic in the USA. A total of 1004 individual stool specimens (253 from patients with acute diarrhoeal illness and 751 from patients without diarrhoeal illness) were collected between 1 June 2003 and 30 June 2008. EAEC was identified as the sole cause of acute diarrhoeal illness in 6% ($n=15$) of patients and in 2% ($n=15$) without diarrhoeal illness. Control patients ($n=15$) were similar to case patients ($n=15$) for age, gender and co-morbidities. The EAEC genes *aggR*, *aap*, *aat*, *astA* and/or *set1A* were identified more frequently in case patients compared with control patients ($P<0.05$). *aggR*-positive EAEC elicited higher levels of interleukin (IL)-1 α , IL-6, IL-8 and tumour necrosis factor- α compared with *aggR*-negative EAEC during co-incubation with HCT-8 cells. Patients with EAEC diarrhoea and isolates with the genes *aggR*, *aap*, *aatA*, *astA* or *set1A* had stools characterized by gross mucus and the presence of faecal leukocytes ($P<0.05$). These results indicate that EAEC is a potential cause of acute diarrhoeal illness affecting patients presenting to an acute-care clinic in the USA and suggest that *aggR*, *aap*, *aatA*, *astA* and *set1A* may be markers for virulence.

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INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) has increasingly been recognized as a cause of acute diarrhoea in developing and industrialized countries. Adults and children are affected and travellers to endemic regions are at particular risk (Huang *et al.*, 2006).

Although EAEC has commonly been identified in developing regions, EAEC has also been implicated in diarrhoeal illness in the USA (Nataro *et al.*, 2006; Cohen *et al.*, 2005). EAEC is defined by its characteristic aggregative or 'stacked-brick' pattern of adherence to HEp-2 cells.

However, not all EAEC infections cause diarrhoeal illness. Recent work has focused on the genetics of EAEC responsible for pathogenesis and virulence. A number of factors determine the virulence of EAEC. Many are dependent on or are enhanced by *aggR*, a transcriptional activator. This gene has been associated with the control of adherence factor expression, dispersin protein synthesis, formation of biofilm and other virulence markers (Huang

et al., 2007; Mohamed *et al.*, 2007; Nataro, 2005; Vila *et al.*, 2000).

Most epidemiological studies of EAEC have been conducted in developing regions of the world (Adachi *et al.*, 2001; Huang *et al.*, 2007) and few studies have been conducted in industrialized countries. In a 3-year survey of the causes of acute diarrhoea in urban and rural South Korea, EAEC was identified in 0.3–3.7% of samples (Cho *et al.*, 2008). EAEC has also been identified as a cause of acute diarrhoeal illness in patients presenting to emergency departments in the USA (Nataro *et al.*, 2006) and has been associated with food-borne gastroenteritis in Italy (Scavia *et al.*, 2008). To date, no prospective studies have examined the prevalence of EAEC in the USA among adults who present to urgent-care clinics with acute diarrhoeal illness. The objective of this prospective, case–control study was to determine the prevalence of EAEC and its genes and to determine the expression of inflammatory cytokines in the presence of *aggR*-positive and *aggR*-negative EAEC isolates among adult patients presenting to an urgent-care clinic in the USA.

METHODS

Subjects. A total of 1004 patients seen at an urgent-care clinic (Newark, NJ, USA) between 1 June 2003 and 30 June 2008 voluntarily

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; GM-CSF, granulocyte–monocyte colony stimulating factor; IL, interleukin; IP-10, interferon-inducible protein 10; MCP-1, monocyte chemotactic protein-1; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF- α , tumour necrosis factor- α .

participated: 253 consecutive patients with acute diarrhoeal illness and 751 consecutive patients without diarrhoeal illness. Control patients were similar to case patients for age, gender and comorbidities. Neither case nor control patients had a recent history of travel outside the USA within the past 3 months. Control patients, similar to case patients, presented to the same urgent-care clinic during the same time period for a non-infectious illness (e.g. hypertension, diabetes) and all had an absence of a history of diarrhoea in their household for at least 3 months. Acute diarrhoeal illness was defined as three or more watery stools per day for less than a week. Subjects were excluded if an antimicrobial agent had been received in the month preceding stool sampling. All subjects who met the inclusion criteria described above and who gave informed consent participated in this study. This study was approved by the University Institutional Review Board.

Collection and study of stool specimens. Fresh stool samples were collected from case and control patients at the clinic and immediately submitted to the hospital Microbiology Department. The presence of conventional enteropathogens was determined in submitted stools using published methods. These pathogens included *Shigella* spp., *Salmonella* spp., EAEC, *Campylobacter jejuni* and *Yersinia enterocolitica* (Nataro *et al.*, 2006). The presence of rotavirus and adenovirus was examined using commercial ELISA kits (Premier Rotacalone and Adenoclone; Meridian Diagnostics). Noroviruses were identified by RT-PCR from viral RNA extracted from 10% faecal suspensions in PBS using a QIAamp viral RNA extraction kit (Qiagen) using two sets of primers for the capsid region of the target viral genome (G1-SKF/F1-SKR and G2-SKF/G2-SKR), as described previously (Kojima *et al.*, 2002).

Identification of EAEC isolates. Five lactose- and indole-positive *E. coli* colonies were retrieved to determine the presence of EAEC isolates by a HEp-2 cell adherence assay (Donnenberg & Nataro, 1995). EAEC strain 042 served as a positive control as this strain elicits numerous inflammatory mediators from intestinal epithelial cells, and a non-pathogenic *E. coli* strain (isolated from a healthy volunteer; negative for genes associated with EAEC or other diarrhoeagenic *E. coli* strains) served as a negative control as this strain does not elicit inflammatory mediators from intestinal epithelial cells *in vitro* (Huang *et al.*, 2004). An isolate was interpreted as positive for EAEC if it showed the characteristic 'stacked-brick' aggregative appearance on co-culture with HEp-2 cells.

Identification of EAEC genes. Oligonucleotide primers for genes were selected based on existing sequences in databases and in the literature (Huang *et al.*, 2007).

The presence of the EAEC plasmid-borne genes *aggR*, *aggA*, *aafA*, *aap*, *aatA*, *astA*, *pet* and *set1A* was assessed among all EAEC isolates with respective positive and negative controls. DNA from EAEC isolates was purified using a ReadyAmp DNA purification system (Promega). The final amplification mix contained 98 µl PCR mix [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 100 µM each dATP, dCTP, dGTP and dTTP, 2.5 U AmpliTaq polymerase (PerkinElmer)], 25 pmol each primer and 2 µl DNA template. The reaction mixture was heated to 94 °C for 5 min and then subjected to 35 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min, in a DNA thermal cycler (PerkinElmer). Four microlitres of each of the amplified PCR products, together with a 1 kb DNA molecular mass marker (Gibco-BRL), was analysed by electrophoresis on a 1.5% agarose electrophoresis gel. The gels were stained with ethidium bromide and photographed under UV illumination. A positive reaction was defined as the presence of a PCR product of the expected size.

HCT-8 cell-culture assay. HCT-8, a human intestinal adenocarcinoma cell line, has been used as a tool to differentiate pro-inflammatory enteropathogens from non-inflammatory isolates (Huang *et al.*, 2004). Monolayers of HCT-8 cells (ATCC CCL-244) were grown in a 24-well culture plate (Corning Costar) with RPMI 1640 (Sigma) supplemented with 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 0.25 µg amphotericin B ml⁻¹ and 10% fetal bovine serum (HyClone Logan). The cells were maintained until confluent with approximately 4 × 10⁵ cells per well.

Bacterial infection. HCT-8 cells were infected with EAEC isolates in antibiotic- and serum-free RPMI 1640 and incubated in a humidified incubator at 37 °C in an atmosphere containing 5% CO₂. After 3 h, supernatants were collected and frozen at -80 °C. The cytokine supernatant concentration for each well was determined using a commercially available 23-multiplex immunoassay (LINCOplex; Linco Research). The results of each cytokine concentration were expressed as pg ml⁻¹. Each study run included a well with no bacteria in the HCT-8 cell-culture assay and a negative and positive control of non-pathogenic *E. coli* and EAEC strain 042, respectively. All samples were analysed in triplicate.

Culture conditions. EAEC strains were grown in Luria broth at 37 °C overnight without shaking.

Stool characterization studies. Occult blood in the stool was tested by use of guaiac reagents (Haemoccult; SmithKline Diagnostics). Stools were characterized as containing gross mucus by laboratory personnel examining the samples.

The presence of faecal leukocytes was determined microscopically by counting the number of cells present in a faecal smear stained with methylene blue. The presence of ten or more leukocytes per high-power field was considered to be positive (Jiang *et al.*, 1994).

Statistical analyses. The frequency of EAEC genes from case and control patients in which EAEC was identified as the sole enteropathogen was compared by a two-tailed χ^2 test and Fisher's exact test. Cytokine levels of *aggR*-positive and -negative EAEC were compared by a two-tailed Student's *t*-test. A Bonferroni adjustment was used to account for multiple tests between groups. A *P* value of <0.05 was considered significant.

RESULTS

Among the 253 consecutive case patients presenting with acute diarrhoeal illness, 7% (*n*=17) were identified as having EAEC in their stool samples: 6% (*n*=15) had EAEC as the sole enteropathogen and 1% (*n*=2) had a co-pathogen (norovirus, *n*=1; adenovirus, *n*=1). Among the 751 consecutive control patients without diarrhoeal illness, 3% (*n*=19) were identified as having EAEC in their stool samples: 2% (*n*=15) had EAEC as the sole enteropathogen and 0.5% (*n*=4) had a co-pathogen (norovirus, *n*=2; rotavirus, *n*=1; adenovirus, *n*=1).

For all EAEC strains identified as the sole enteropathogens in case and control patients, the most frequent genes identified were *aggR* (40%), *aap* (37%), *aatA* (37%), *astA* (40%), *pet* (37%) and *set1A* (27%) (Table 1). The genes identified more frequently in case patients compared with control patients were *aggR* (*P*=0.0002), *aap* (*P*=0.0007), *aatA* (*P*=0.0007), *astA* (*P*=0.03) and *set1A* (*P*=0.01). Twelve EAEC strains from case patients were identified as

Table 1. EAEC genes identified in isolates from adults presenting to an urgent-care clinic with and without acute diarrhoeal illness

Gene	Diarrhoea (n=15) (%)	No diarrhoea (n=15) (%)
<i>aggR</i> *	11 (73)	1 (7)
<i>aggA</i>	2 (13)	1 (7)
<i>aafA</i>	3 (20)	1 (7)
<i>aap</i> *	10 (67)	1 (7)
<i>aatA</i> *	10 (67)	1 (7)
<i>astA</i> †	9 (60)	3 (20)
<i>pet</i>	7 (47)	4 (27)
<i>set1A</i> †	7 (47)	1 (7)

**P* < 0.005.†*P* < 0.05.

carrying *aggR*, *aap*, *aatA*, *astA*, *pet* and/or *set1A* alone or in combination compared with three EAEC strains from control patients that had these identified EAEC genes (*aggR*, *aap*, *aatA*, *astA* and/or *set1A*) (*P* = 0.001).

Table 2 lists the detectable and non-detectable chemokine and cytokine profiles from an intestinal epithelial HCT-8 cell line infected with EAEC determined using a commercially available 23-multiplex immunoassay. Table 3 lists the quantitative chemokine and cytokine profile from an HCT-8 cell line infected with *aggR*-positive and -negative EAEC. *aggR*-positive EAEC elicited increased interleukin (IL)-1 α , IL-6, IL-8 and tumour necrosis factor (TNF)- α from HCT-8 cells compared with *aggR*-negative EAEC (*P* < 0.05). Although not statistically significant, *aggR*-positive EAEC also elicited increased granulocyte-monocyte colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), interferon-inducible protein 10 (IP-10) and RANTES (regulated upon activation, normal T-cell expressed and secreted) from HCT-8 cells compared with *aggR*-negative EAEC.

Among patients infected with these 12 EAEC strains (i.e. strains carrying one or more of the genes *aggR*, *aap*, *aatA*, *astA*, *pet* and/or *set1A*), 33 % (*n* = 4) were found to have stools with gross mucus, 17 % (*n* = 2) with occult blood and 50 % (*n* = 6) with faecal white blood cells (Table 4). No subjects excreting EAEC strains without these genes (*aggR*, *aap*, *aatA*, *astA*, *pet* and/or *set1A*) had stools characterized by the presence of gross mucus, occult blood or faecal white blood cells.

DISCUSSION

Our study identified EAEC as the sole cause of acute diarrhoea in 6 % of adults and in 2 % of adults without diarrhoeal symptoms presenting to an urgent-care clinic in the USA. None of the adults participating in this study had a history of travel outside the USA within 3 months of participation. These results suggest that EAEC is endemic in the USA. Our results also support the fact that EAEC displays heterogeneity in its genes. The genes *aggR*, *aap*, *aatA*, *astA* and *set1A* were associated with diarrhoeal illness. *aggR* is a master gene activator. It is a pathogen-specific regulator and belongs to the AraC family of transcriptional activators. *aggR* controls a variety of putative virulence genes, including those encoding the aggregative adherence fimbriae, a surface coat protein called dispersin (*aap*), a dispersin translocator apparatus called Aat and several chromosomal loci (Nataro, 2005). *astA* encodes a heat-stable enterotoxin (EAST-1) and *set1A* encodes an oligomeric enterotoxin and is identical to ShET1 enterotoxin found in *Shigella* (Behrens *et al.*, 2002).

Subjects infected with EAEC containing the gene *aggR* in conjunction with *aap*, *aatA*, *astA*, *pet* and/or *set* were more likely to have markers of inflammation present in their stool examination. No patients with EAEC-associated diarrhoea without these genes exhibited blood, mucus or significant numbers of leukocytes in their stools. These findings suggest that one or more of these genes may be a marker for virulence and are associated with inflammation during diarrhoeal illness. Similar results have been described in studies of EAEC identified among international travellers (Huang *et al.*, 2007).

The *in vitro* data of EAEC infection of HCT-8 cells further support *aggR*-positive EAEC eliciting inflammatory markers compared with the identified *aggR*-negative EAEC.

Significant increases in IL-6, IL-1 α , TNF- α and IL-8 were demonstrated in the *aggR*-positive EAEC. Whilst not reaching statistical significance, there was also a trend for increased GM-CSF, MCP-1, IP-10 and RANTES elicited in *aggR*-positive EAEC infections. IL-6 is a potent inducer of acute-phase proteins and is also known to upregulate T- and B-cell maturation. Specifically, it can trigger B-cell maturation to plasma cells and immunoglobulin secretion. IL-6 also increases the production of IL-1 α (Borish & Steinke, 2003). IL-1 α is believed to act as a natural regulator modulating the effects of IL-1. IL-1 is a primary activator of T lymphocytes, and increases immunoglobulin synthesis and enhances B-cell proliferation. In the

Table 2. Detectable and non-detectable chemokine and cytokine profile in an HCT-8 cell line infected with EAEC determined using a commercially available 23-multiplex immunoassay

Detectable chemokines	IL-8, MCP-1, IP-10, RANTES
Detectable cytokines	IL-1 α , IL-6, GM-CSF, TNF- α
Non-detectable chemokines	Eotaxin, MIP-1 α
Non-detectable cytokines	IL-1 β , IL-2, IL-4, IL-5, IL-7, IL-12 (p70), IL-13, IL-15, IL-17, IL-1 α , IFN- γ , G-CSF, M-CSF

Table 3. Cytokine profiles (pg ml⁻¹) from an intestinal epithelial HCT-8 cell line infected with *aggR*-positive and -negative EAEC

Cytokine	<i>aggR</i> ⁺ (n=12)	<i>aggR</i> ⁻ (n=18)	Positive control	Negative control
IL-1ra*	235.0	58.0	213.0	8.0
IL-6*	143.0	47.0	106.0	10.0
IL-8*	188.0	76.0	110.0	15.0
GM-CSF	6.4	4.7	6.0	3.2
TNF- α *	16.5	7.8	35.0	3.2
MCP-1	7.3	5.6	10.0	8.1
IP-10	5.6	4.0	3.2	3.2
RANTES	6.3	5.0	5.6	5.3

*P < 0.05.

endothelial system, IL-1 and TNF- α increase leukocyte adherence through the stimulation of ICAM-1, VCAM-1 and E-selectin (Borish & Steinke, 2003). TNF- α is a potent activator of neutrophils and enhances their chemotaxis, degranulation and respiratory burst, which increase pathogen killing. These actions are very similar to those of IL-8 (Borish & Steinke, 2003). IL-8 has been shown previously to be elevated in faecal samples of patients with EAEC. IL-8 elaboration leads to neutrophil accumulation *in vivo* with resultant tissue damage and fluid secretion (Nataro, 2005). The aggregative adherence fimbriae, a product of *aggR* activation in EAEC, have also been shown to increase IL-8 production in cell cultures (Harrington *et al.*, 2005). Collectively, these data indicate that *aggR*-positive EAEC infection causes inflammatory diarrhoea where intestinal epithelial cells result in the production of cytokines, which are potent stimulators of neutrophil chemotaxis and activation. The increase in neutrophil activity could be responsible for the clinical inflammation noted in studies that have demonstrated increased faecal blood and leukocytes.

Past studies have demonstrated the association of EAEC infection and inflammatory responses. EAEC is associated with inflammation as measured by faecal lactoferrin in travellers with diarrhoea (Bouckennooghe *et al.*, 2000). Another study has shown that travellers from Europe to

India with symptomatic EAEC diarrhoea had increased levels of the inflammatory cytokines IL-8, IL-1 β and IL-1ra (Greenberg *et al.*, 2002). Interestingly, these inflammatory cytokines were not associated with EAEC infection in US travellers to Mexico. These data suggest heterogeneity of EAEC isolates by location.

Further work has characterized the role of virulence factors during EAEC infection. Aggregative adherence fimbriae, a product of *aggR* activation, have been shown to produce a significant inflammatory response characterized by IL-8 production in cell culture (Harrington *et al.*, 2005). Similarly, EAEC has been shown to stimulate copious mucus production from epithelial cells with resultant biofilm formation. Production of biofilm has been linked to *aggR*, *aatA* and *set1A* (Mohamed *et al.*, 2007). EAST-1, a heat-stable enterotoxin encoded by *astA*, has been associated with acute diarrhoea in Brazilian children with EAEC infection (Pereira *et al.*, 2007). Collectively, these data suggest that certain genes are markers of EAEC virulence.

Our observations suggest that EAEC isolates carrying a 'package' of genes are more likely to be isolated from patients with diarrhoeal illness compared with EAEC isolates without these genes, similar to observations previously reported in the literature (Huang *et al.*, 2007). A 'package' of genes, such as *aap*, *aatA*, *aggA*, *afaA* and others, may be seen because of the proximity of these genes to each other at this locus. These genes are regulated by the master regulator *aggR* either on a plasmid or as part of a collective chromosomal virulence factor (Nataro, 2005). Therefore, our study shows that certain genes define virulent from non-virulent EAEC strains. A previous study has shown that travellers with diarrhoeal illness and infected with EAEC carrying *aggR* and *aggR* in conjunction with other virulence genes are associated with increased levels of faecal cytokines IL-8, IFN- γ , IL-1 β and IL-1ra compared with travellers infected with EAEC that does not carry *aggR* (Jiang *et al.*, 2002). Studies to define other EAEC virulence genes besides *aggR* that are independently associated with an elevated cytokine response or that are linked to virulence through their association with *aggR* are currently underway and will help to elucidate and identify the genes that are important for EAEC virulence.

Table 4. EAEC genes and stool characteristics of adults presenting to an urgent-care clinic with and without acute diarrhoeal illness

Stool characteristics	EAEC with diarrhoea		EAEC without diarrhoea	
	Positive for virulence genes (n=12) (%)	Negative for virulence genes (n=3) (%)	Positive for virulence genes (n=3) (%)	Negative for virulence genes (n=12) (%)
Gross mucus	4 (33)*	0 (0)	0 (0)	0 (0)
Occult blood	2 (17)	0 (0)	0 (0)	0 (0)
White blood cells	6 (50)*	0 (0)	0 (0)	0 (0)

*P < 0.05.

In summary, EAEC was identified as a sole cause of acute diarrhoeal illness in 6% of adults and was found in 2% of individuals without diarrhoeal symptoms visiting an urgent-care clinic in the USA. The EAEC genes *aggR*, *aap*, *aat*, *astA* and/or *set1A* were identified more frequently in case patients compared with control patients, and patients with EAEC diarrhoea and at least one of the EAEC genes *aggR*, *aap*, *aatA*, *astA* or *set1A* had stools characterized by gross mucus and the presence of faecal leukocytes ($P < 0.05$). These results indicate that EAEC is a cause of acute diarrhoeal illness in the USA and suggest that *aggR*, *aap*, *aatA*, *astA* and/or *set1A* may be markers for virulence.

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