Enteroaggregative *Escherichia coli*: A Pathogen Bridging the North and South

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Abstract Enteroaggregative *Escherichia coli* (EAEC) is a heterogeneous emerging enteric pathogen identified during the 1980s when EAEC strains were isolated from cases of acute and persistent diarrhea among infants in developing countries as well as traveler's diarrhea. Subsequently, EAEC strains were linked with foodborne outbreaks and diarrhea illness in adults and children from industrialized countries and HIV-infected subjects, and with the stunting of malnourished children living in impoverished countries. Today, EAEC is increasingly recognized as a major cause of acute diarrhea requiring hospitalization in children and of traveler's diarrhea worldwide. The defining phenotype of EAEC strains is the aggregative adherence (AA) pattern on epithelial cells. The role of AggR, a transcriptional regulator of several EAEC virulence genes, is a key factor in both understanding EAEC pathogenesis and defining typical EAEC (tEAEC) strains. The distribution of EAEC virulence genes among these strains is highly variable, and the identification of key virulence genes and their function in the setting of enteric disease presents ongoing challenges.

Keywords Enteroaggregative *Escherichia coli* (EAEC) · *Escherichia coli* · Epidemiology · Identification · Tropical medicine · GI infection · Aggregative adherence (AA) · Pathogen

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Introduction

Diarrheagenic *Escherichia coli* (DEC) groups are perhaps one of the most interesting new enteric pathogens described in the last 40 years. In contrast to other enteric pathogens identified during this period, such as *Campylobacter jejuni* and norovirus, the role of DEC strains in disease has been difficult to prove [1, 2], largely because most strains are biochemically indistinguishable from commensal *E. coli* strains isolated from the intestinal tract. Therefore, it became necessary to determine their pathogenic capability in volunteer studies [3, 4]. DEC are classified within six groups based on clinical associations, phenotypic assays, and virulence factors: enterotoxigenic *E. coli* (ETEC); typical and atypical enteropathogenic *E. coli* (tEPEC, aEPEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC); diffusely adherent *E. coli* (DAEC); and Shiga-toxin-producing *E. coli* (STEC) [5]. Each DEC pathotype is clinically, epidemiologically, and pathogenically distinct. In this review we will summarize EAEC history, epidemiology, pathogenesis, host susceptibility, and clinical manifestations, as well as current limitations to routine identification in the clinical lab setting.

EAEC History and Epidemiology

The distinctive characteristic of EAEC strains is their ability to produce a “stacked-brick” adherence pattern on HEP-2 epithelial cells, as described in a protocol developed by Cravioto et al. in 1979 [6] (Fig. 1). During the 1980s, using Cravioto's protocol, two independent groups reported that *E. coli* strains isolated from adult travelers to Mexico [7] and Peruvian infants [8] with diarrheal illness adhered in a stacked-brick fashion. The authors of the latter study used the term “aggregative adherence” (AA) to describe the *E. coli* strains, which were later named “enteroadherent-enteroaggregative,” and

Fig. 1 Panel A, HEp-2 cells cultured in DMEM/tryptone; and Panel B, HEp-2 infected with EAEC 042 strain showing the characteristic aggregative adherence pattern (“stacked brick” adherence) [10••]

eventually shortened to “enteroaggregative” [8]. From these studies, the EAEC reference strains JM221 [7] and 042 [8] were identified. These EAEC isolates and others were tested in an adult volunteer study [4] in which it was observed that the 042 strain elicited diarrhea in the majority of challenged subjects, whereas the other EAEC strains did not. These early studies provided evidence of the strong heterogeneity of the EAEC strains as well as the host responses. The 042 isolate (serotype O44:H18) became the prototypic EAEC strain for the study of virulence factors, pathogenicity [9•, 10••], genetic studies [10••], and animal models [11]. The EAEC 042 genome, which was characterized in 2010, comprises a circular chromosome of 5,241,977 bp and one plasmid of 113,346 bp, known as the “aggregative adherence plasmid” (pAA) [12]. The other EAEC reference strain used in animal models is JM221, which has been particularly useful in understanding the EAEC immune response in malnourished mice [13].

Shortly after EAEC strains were first described, Bhan et al. [14, 15] reported an association between EAEC strains and persistent diarrhea in Indian children. In 1991, Knutton et al. [16] found that *E. coli* strains isolated from children admitted with diarrheal symptoms at a London hospital adhered in a stacked-brick fashion. A landmark study in 1992 by Lima et al. [17] reported that EAEC persistent diarrhea was linked to growth shortfalls, decreased intellectual development, and death in impoverished Brazilian children. Follow-up studies of these children [18] revealed that not only children with

EAEC persistent diarrhea, but also those with EAEC infection, had increased levels of fecal lactoferrin, IL-8, and IL-1 β . In the same year, EAEC was linked to diarrhea in children with perinatal HIV infection in Zaire [19]. Likewise, EAEC was associated with diarrheal illness in an adult HIV patient in the U.S. [20] and among HIV-infected adults in Africa [21].

The identification of the AggR transcription activator encoded by the *aggR* gene on the pAA plasmid was a major step toward understanding EAEC pathogenesis [9•, 22, 23]. The important role of AggR, a major EAEC transcriptional regulator of several plasmid- and chromosomal-borne EAEC virulence genes, led Nataro in 2005 to propose that 1) the presence of the AggR regulon may identify pathogenic EAEC strains (known as typical EAEC), and 2) epidemiologic and clinical investigations of diarrheal disease should include detection of typical EAEC. A hybrid *E. coli* strain (serotype O104:H4) was recently associated with a major foodborne outbreak in Europe [24, 25]. The strain was unique insofar as it carried several virulence genes characteristic of EAEC (including *aggR*), as well as STEC and extraintestinal *E. coli* (ExPEC). During the spring of 2011, this strain sickened more than 4,300 previously healthy individuals, mainly adults, from 16 countries. More than 900 patients developed hemolytic uremic syndrome (HUS), and over 50 people died [25].

Studies of diarrheal illness worldwide have demonstrated that EAEC epidemiology is very different from that described for tEPEC and ETEC. For instance, during the last 30 years, tEPEC has been almost exclusively isolated in children, mainly under the age of 1 year, from less developed regions of the world [26–28]. ETEC is considered the major pathogen associated with weanling diarrhea among children in the developing world [28]. In contrast, almost from the time that they were first described EAEC strains have been associated with diarrheal illness in previously healthy children and adults from industrialized countries without history of international traveling, as well as in children from the developing world [10••, 16, 29–32].

After the 2011 outbreak of the hybrid EAEC-STEC-EXPEC O104:H4 strain, an increased number of epidemiological studies sought both EAEC and EAEC hybrid strains in patients with diarrhea, particularly in children from less developed regions of the world [33–36]. The distribution of EAEC infection and illness affecting different populations worldwide is shown in Table 1 [10••, 37–39].

Diarrheal illness remains among the leading causes of morbidity among children less than 5 years of age worldwide, as well as a major cause of death among children younger than 2 years from Sub-Saharan Africa, South Asia, and Latin America [40]. EAEC is currently recognized as a major bacterial agent of acute diarrhea illness. Several studies conducted in children from Latin America, Asia, Africa, and former Eastern European countries have identified EAEC more frequently than any other bacterial pathogen [10••, 33–38].

Table 1 EAEC distribution and clinical presentation by geographic region

Affected population	Clinical presentation	Less developed regions	Industrialized regions
Infants and children <5 years	Acute diarrhea	Latin America, South and Southeast Asia, Africa, Eastern Europe	Europe, U.S.
Children <5 years	Persistent diarrhea	India, Brazil, Mexico, Ghana	UK, Germany, U.S.
HIV-infected patients (adults and children)	Acute and persistent diarrhea	Senegal, Central African Republic, Zambia, Peru, Uganda, Zaire, Brazil, South Africa	U.S.
Adults	Acute diarrhea*	India, Nigeria, Ghana	U.S., UK
Children and adults	Traveler's diarrhea		Canada, U.S., Europe
Older children (>5 years) and adults	Acute infections and foodborne outbreaks		U.S., Europe, Japan
Malnourished children	Persistent diarrhea	Brazil, Ghana	

* Distinct from traveler's diarrhea

Studies conducted in the U.S., Europe, and Israel have also associated EAEC with diarrheal illness in young children [as revised by 10•, 38]. In the U.S., EAEC strains are the most common bacterial cause of diarrheal illness in young children, surpassing *Campylobacter* and *Salmonella* [31, 41]. Importantly, several of these studies have shown that EAEC was the leading bacterial pathogen in children hospitalized for acute diarrhea in both less developed and industrialized regions [as revised by 10•, 41–43].

Conversely, EAEC is much more prevalent among adults with diarrhea in industrialized countries [31, 32, 44, 45] than those from developing regions. In a recent retrospective study of UK children and adults that included over 2,000 stool specimens, EAEC was identified in approximately 5 % of subjects [32]. Likewise, EAEC was the most common bacterium identified in studies of diarrhea etiology conducted in emergency departments and outpatient clinics among adults with diarrhea from the U.S., [31, 44]. In addition, several EAEC foodborne outbreaks in Japan have involved older children (>5) and adults [30]. A massive outbreak that occurred in Japan was linked with EAEC that was significantly associated with the consumption of school lunches [30]. Of the 6,636 children and adolescents who ate the school lunch, 2,697 developed gastroenteritis, with an attack rate of approximately 41 % and an incubation period of 40–50 hours. Reports on diarrheal disease in older children and adult residents from developing countries are scarce [46]. In 1997, Pai et al. reported an EAEC outbreak in India affecting all age groups, with an overall attack rate of 15 % [47]. EAEC has also been linked to adult diarrheal episodes in Nigeria [48] and Ghana [49]. The lower prevalence of EAEC in adult diarrheal disease in developing and less developed countries may be due to protective immunity, although it is also possible that it is due to the paucity of studies on adult diarrhea in these regions.

Large epidemiological studies worldwide have indicated a rate of approximately 3.2 episodes of diarrhea per child, and it has been estimated that between 3 % and 20 % of these episodes correspond to persistent diarrhea (>14 days) [50].

Since the first association of EAEC with persistent diarrhea in Indian children, this bacterium has been identified as a cause of chronic diarrhea in children from less developed countries as well as from Europe and the U.S. [as revised by 10•]. Whether EAEC is the major bacterial agent causing persistent diarrhea in these regions remains to be determined. From a public health standpoint, as mentioned previously, the most significant impact of EAEC persistent diarrhea and EAEC infection is on malnourished children living in impoverished regions and its association with stunting [17, 18, 51]. Stunting (length for age Z-score < -2) is associated with increased severity and duration of infectious disease episodes. The World Health Organization has estimated that 32 % of children younger than 5 years living in impoverished areas are stunted [52, 53•]. Unfortunately, linear growth deficits that occur in early life are not fully reversible, and these permanent deficits are a marker of an enduring loss of human potential. Stunting not only occurs in children from poorer developing countries, but also in those living in impoverished slums from middle-income countries [17].

Over the years, studies have linked diarrheal illness with EAEC infection in both HIV-infected adults and children in Africa and South America [10•, 39] and in adults in the U.S. [54]. It remains to be determined whether EAEC is also associated with diarrhea illness in HIV patients from other developing and industrialized countries.

Traveler's diarrhea (TD) is the most common illness affecting individuals traveling from industrialized countries to less developed areas of the world. EAEC strains have been implicated as second only to ETEC as the most frequently isolated pathogen from traveler's diarrhea patients returning from Latin America and Southeast Asia [55, 56]. TD occurs in 15–50 % of visitors from industrialized countries to low- and middle-income regions, and up to 40 million cases of TD are reported each year [57•, 58]. Depending upon length of stay, TD may affect up to 80 % of travelers to high-risk destinations (WHO). In addition, it has been reported that a portion of patients with TD developed irritable bowel

syndrome (IBS) and chronic gastrointestinal symptoms [57•, 59, 60]. The precise role of EAEC infection in IBS and chronic gastrointestinal symptoms has yet to be elucidated.

Pathogenesis

Data accumulated from several studies have suggested three major features of EAEC pathogenesis: 1) abundant adherence to the intestinal mucosa and biofilm formation over enterocyte surface, 2) production of enterotoxins and cytotoxins, and 3) induction of mucosal inflammation, intestinal secretion, and damage [10•, 61•], (Fig. 2).

As previously mentioned, most of our knowledge of EAEC pathogenesis has come from the characterization of virulence factors present in the prototype 042 EAEC strain that causes diarrhea in healthy volunteers. EAEC pathogenomic studies have focused on the transcriptional regulator AggR, a member of the AraC/XylS family of bacterial transcriptional activators. It has been shown that AggR activates the expression of at least 44 chromosomal- and plasmid-borne genes, including multiple EAEC virulence factors [9•]. As discussed previously, AggR has been proposed as the defining factor for typical

EAEC strains [22]. However, the genes encoding for numerous adhesins, toxins, and proteins associated with virulence are highly variable among strains [10•, 36], and this heterogeneity precludes a clear definition of EAEC virulence genes associated with illness and intestinal inflammation.

Adherence and Biofilm Formation

As is the case for most pathogenic enteric bacteria, attachment to the intestinal mucosa is an essential step in colonization and production of disease by EAEC. Since the defining feature of EAEC strains is their ability to produce the stacked-brick pattern, this results in an abundant adherence of these bacteria to the intestinal mucosa. EAEC adherence has been characterized as a biofilm composed of aggregates of bacteria in association with a thick mucus layer [62]. The AA pattern is associated with both fimbrial and afimbrial adhesins. The first fimbrial structures described in EAEC were the aggregative adhesion fimbria (AAF), of which at least four variants have been identified. All AAF are encoded in large pAA plasmids. AAF II has been shown to bind extracellular matrix cell components of the intestinal mucosa, and it appears to confer autoaggregation and biofilm formation as well. In EAEC

Fig. 2 Schematic representation of EAEC virulence factors and their targets on mucosal epithelium cells. Taken from Estrada-Garcia T and Navarro-Garcia F, FEMS Immunology and Medical Microbiology 2012 66 :281-298

strains lacking AAF II, the heat-resistant agglutinin (hrai) that is chromosomally encoded plays a role similar to AAF II in autoaggregation and biofilm formation. It has been shown that *shF* genes of *Shigella flexneri* and *Staphylococcus epidermidis* homologs are also required for biofilm formation [63, 64]. It has also been shown that the biofilm formation is a common phenomenon among EAEC isolates and that multiple genes associated with biofilm formation are regulated by *aggR* [65]. Another molecule involved in EAEC pathogenesis is dispersin, an antiaggregative secreted protein encoded by the *aap* gene [66]. Dispersin is translocated by the antiaggregative transporter (AaT) system, which acts by decreasing bacterial autoaggregation and allowing EAEC dispersion along the intestinal mucosa. Various studies have shown that EAEC isolates from patients may preferentially adhere to the jejunal, ileal, or colonic mucosae, another example of EAEC heterogeneity.

Production of Enterotoxins and Cytotoxins

After adherence, EAEC is capable of producing several toxins and cytotoxins that may cause microvillus vesiculation, enlarged crypt openings, and increased epithelial extrusion. Among these toxins are the *E. coli* heat-stable enterotoxin 1 (EAST1) that activates chloride channels; plasmid-encoded toxin (pet), a cytoskeleton-altering protein; *Shigella* enterotoxin 1 (SHET1) that also induces intestinal secretion; and the protein involved in intestinal colonization (pic) that has a mucinase activity and is involved in serum resistance. An uncharacterized toxin identified in genome sequencing and metabolic profiling screens includes a potential hemolysin encoded by the *hlyE* gene that has pore-forming hemolytic activity and cytolytic effects [67]. Additionally, the gene encoding for the *Shigella* extracellular protease (*sepA*), with IgA endopeptidase activity, which was originally described in *S. flexneri* [68] was recently identified among EAEC strains and was strongly associated with diarrhea [36]. Several other putative virulence factors will surely be characterized in the coming years, as our knowledge of this pathogen is increasingly very rapidly.

Almost simultaneous with adherence, EAEC induces a host inflammatory response. The initial inflammatory response to EAEC infection is dependent upon the host innate immune system and type of EAEC strain causing the infection. The role of putative virulence genes and clinical outcomes is not well understood. However, the presence of several EAEC virulence factors (such as flagellin, AggR, AAF fimbria, and dispersin) correlate with elevated levels of fecal cytokines such as interleukin (IL)-1 α , IL-1 β , IL-8, interferon- γ , and inflammatory markers that include lactoferrin, fecal leukocytes, and occult blood [69–71]. We have also shown that when 042 strains are cultured with colonic epithelial Caco-2 cells, they can induce the production

and early release not only of IL-8, a principal chemoattractant for polymorphonuclear (PMN) leukocytes, but also of several other proinflammatory cytokines (IL-1 β , IL-6, IL-12, and TNF), as well as IL-10, a regulatory cytokine (Bernal-Reynaga paper in preparation). In addition, in vitro studies have shown that EAEC induces the activation of mitogen-activated protein kinases (MAPK) on intestinal cells, which in turn activates the transcriptional factor NF- κ B, leading to the secretion of IL-8 and potentially other cytokines [72, 73]. EAEC also activates the production of eicosanoid-based PMN (including neutrophils) chemoattractant, which leads to the recruitment and transmigration of neutrophils to the gut mucosa, causing intestinal damage that may promote EAEC colonization [74]. Both cytokine production and PMN transmigration contribute to EAEC pathogenesis and are a hallmark of inflammatory infectious diarrhea.

Host Susceptibility

There is increasing evidence that EAEC strains are very diverse, and consequently, the host response is also very heterogeneous [10••]. A study conducted on the natural history of EAEC and ETEC diarrhea among U.S. adult travelers during a four-week period after arrival to Mexico revealed that 1) in contrast to ETEC infection, to which adult travelers are most susceptible during the first weeks of exposure, travelers remain susceptible to EAEC infection throughout their stay; 2) the number of subjects with EAEC colonization increased over time, whereas the number of cases of EAEC diarrhea decreased with the length of stay; and 3) none of the 16 patients that developed EAEC-diarrhea had a repeat episode due to this bacteria. These observations suggest that immunity to symptomatic EAEC diarrhea may have been acquired early during travel, but that resistance to asymptomatic enteric infection did not occur in this adult population. Genetic susceptibility to EAEC infection has been observed in adult subjects who develop TD, suggesting that a single-nucleotide polymorphism in the IL-8 gene promoter region is strongly associated with both EAEC diarrheal illness and greater levels of fecal IL-8 [70]. Additionally, polymorphisms in lactoferrin, osteoprotegerin, and CD14 promoter genes have been associated with increased susceptibility to TD caused by both ETEC and EAEC [75]. In order to further enhance our understanding of EAEC infection and illness, it is necessary to conduct studies of natural EAEC infection, host genetic susceptibility, and immunity in endemic populations.

Clinical Manifestations and Treatment

The clinical features of EAEC illness have been described in volunteer studies, outbreaks, and sporadic cases. The

characteristic clinical picture includes watery secretory diarrhea (often with mucus, with or without blood), low-grade fever, abdominal pain, nausea, and vomiting [4, 15, 38, 76, 77]. While bloody diarrhea is not a distinctive feature of EAEC illness, Cravioto et al. (1991) [78] reported that one-third of affected infants under 2 years of age had grossly bloody stools. In a recent study that we conducted among children hospitalized for diarrhea, EAEC was identified in approximately 7 %. Of those 20 patients in whom EAEC was the only etiological agent identified, 55 % had mucus in feces, 50 % had more than six stool movements per day, and 10 % had bloody stools (authors' unpublished data). Overall, EAEC diarrheal episodes have been frequently associated with the presence of mucus, PMNs, and lactoferrin in stools [44, 51, 69, 71, 77].

In children, treatment of bacterial gastroenteritis, including EAEC, is primarily supportive and directed toward maintaining hydration and electrolyte balance. Antibiotic therapy is rarely indicated and should be deferred until culture results are available. Oral rehydration therapy (ORT) is the preferred treatment for fluid and electrolyte losses caused by diarrhea in children with mild-to-moderate dehydration. Intravenous hydration is often administered for severe dehydration or when vomiting prevents ORT [79, 80]. EAEC antimicrobial therapy should be used in cases of severe diarrheal disease to reduce the duration of illness, particularly because of its association with persistent diarrhea in children. Because experts in travel medicine discourage the use of absorbable antimicrobial agents for TD prophylaxis, the poorly absorbed antibiotic rifaximin has been proposed for prevention of traveler's diarrhea [57••].

The progressive increase in antibiotic resistance among EAEC strains in developing countries is cause for concern [42, 81, 82]. Several investigators have suggested that lactoferrin may protect infants from gastrointestinal infections, including EAEC, and may be an alternative treatment for antibiotic-resistant EAEC strains [83].

Controversies in EAEC Identification

For years, the gold standard for EAEC identification relied on the recognition of the characteristic AA (stacked-brick) pattern on human epithelial cells (HEp-2-adherent assay) [6]. Since *E. coli* is part of the human intestinal microbiota, laboratory identification of DEC includes the testing of at least five colonies in order to increase the probability of isolating a pathogenic strain. Unfortunately, there are several inherent limitations in the HEp-2-adherent assay with respect to surveillance systems and large epidemiological studies of patients or food. It is labor-intensive, expensive, requires well-trained personnel, and can only be performed in laboratories that culture cell lines. Furthermore, it cannot distinguish

between pathogenic and non-pathogenic strains. The recognition of strains carrying the AggR regulon as typical EAEC (tEAEC) strains was undoubtedly a major step in EAEC identification [20]. The high prevalence of tEAEC strains among EAEC isolates previously identified by the HEp-2 cell assay was illustrated when studies revealed that 100 % of EAEC reference strains [84] and 80 % of isolates from subjects with [85] and 70 % from subjects without diarrhea [42] harbored the *aggR* gene. Furthermore, microarray DNA analyses of a set of EAEC strains confirmed that tEAEC isolates carrying *aggR* shared a package of common virulence genes comprising a large number of conserved plasmid and chromosomal loci [86]. It was also observed that the presence of gross mucus and fecal leukocytes in the stools of patients infected with EAEC were associated with strains carrying *aggR* in combination with other virulence genes [42, 44, 87, 88].

Although case-control studies have isolated tEAEC more frequently from ill subjects than healthy controls [87, 89], other studies have not observed significant differences between the two groups [43, 44]. Several studies have consistently shown that tEAEC isolates from subjects with diarrhea frequently carried additional virulence genes [9•, 36, 43, 82, 84, 90]. A current challenge, therefore, is to identify key virulence genes defining tEAEC strains associated with both illness and intestinal inflammation. Despite numerous tEAEC molecular genetics characterization studies, recognition of these key defining genes remains elusive [10••, 36]. In summary, the current evidence suggests that typical EAEC strains are widely distributed, that *aggR* is a good marker for their identification, that not all tEAEC strains are pathogenic, and that a set of currently undefined virulence genes, probably controlled by the AggR master regulon, are associated with pathogenic EAEC strains.

Conclusions

EAEC infections are a major cause of diarrhea in poor and middle-income populations in developing and industrialized countries alike. EAEC epidemiology is unique, as it differs from that of other DEC pathotypes (ETEC, tEPEC) and enteropathogens traditionally associated with poverty such as *Shigella* [57••, 61••, 91]. Data on EAEC is limited due to the constraints for routine identification in clinical laboratories. Although several studies have demonstrated that tEAEC strains are associated with unfavorable clinical outcomes, its true global public health implications have yet to be determined. Surveillance systems in developed countries should include tEAEC identification, particularly in light of the large EAEC foodborne outbreaks in Japan and Europe [25, 30]. The high prevalence of tEAEC in childhood diarrhea and the role of this bacterium in persistent diarrhea, intestinal

inflammation, and stunting in children living in poor countries and in impoverished conditions in middle-income countries support the need for more extensive studies in developing countries as well [17, 51]. One of the major challenges in the field today is in identifying a set of key virulence genes that can define those tEAEc strains associated with both illness and intestinal inflammation.

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Compliance with Ethics Guidelines

Conflict of Interest Teresa Estrada-Garcia, Iza Perez-Martinez, Rodolfo Bernal-Reynaga, and Mussaret B. Zaidi declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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