Gastroenterology and Hepatology From Bed to Bench

REVIEW ARTICLE

Enteroaggregative *Escherichia coli*, a heterogenous, underestimated and under-diagnosed *E. coli* pathotype in Iran

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ABSTRACT

The main features of enteroaggregative *Escherichia coli* (EAEC) pathogenesis include attachment of bacteria to the intestinal mucosa, production of various toxins and cytotoxins, and stimulation of mucosal inflammation. 'Virulence' genes encode these features. Comparison of different EAEC isolates has shown that the virulence gene content of these isolates varies considerably. The heterogeneity of EAEC strains was concluded from the results obtained from the volunteer as well as other studies. Although the underlying mechanism behind the apparent increase in O104:H4 virulence is not known, several bacterial factors have been implicated. In this review, the known virulence factors involved in pathogenesis of EAEC pathotype are summarized.

Keywords: *Escherichia coli*, Enteroaggregative *Escherichia coli*, *E. coli* pathotype, Heterogeneity.

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Introduction

Escherichia coli is a common human intestinal microrganism. It is a versatile gram-negative microorganism capable of causing both intestinal and extra-intestinal diseases (1). In both developed and developing countries pathogenic *E. coli* are a major cause of diarrhea leading to high morbidity and mortality especially among children in developing world. Due to the lack of data from large epidemiological studies the true world-wide burden of diarrheagenic *E. coli* (DAEC) is unknown (2).

Based on their clinical association, phenotypic assays, and virulence factors, these pathogens are divided into enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), diffusely adhering *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC).

Association of EAEC with acute diarrhea of children and adults and persistent diarrhea in children of developing world has been documented in various studies in both developing and developed countries (3). Moreover, this microorganism has been identified as an agent of travelers' diarrhea as well as an emerging food-borne pathogen (4, 5).

The defining characteristic of EAEC strains is their ability to produce a "stacked-brick" bacterial network when incubated with epithelial cells such as HEp-2 or HeLa. This pattern of adhesion was described by Nataro *et al.* (6) and termed "aggregative adherence" (AA) in a report on the

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etiology of infantile diarrhea following the protocol established by Cravioto *et al.* (7). Strains adhere to each other to epithelial cells as well as cover slip and tissue culture plates, but variants adhering predominantly to cover slip or epithelial cells have also been described (8, 9). Adult volunteer studies, performed by Nataro *et al.,* (10) using several EAEC strains showed that the O42 strain isolated from a Peruvian child suffering from diarrhea caused the illness in the majority of the challenged participants. Volunteers challenged with the other strains (such as 17-2 an EAEC isolate from a Chilean patient) did not manifest diarrheal symptoms. The data obtained established the O42 as the prototypic strain for virulence factors, pathogenicity, genetic studies and genome characterization (2, 10-13).

The main features of EAEC pathogenesis include attachment of bacteria to the intestinal mucosa, production of various toxins and cytotoxins, and stimulation of mucosal inflammation (2, 3, 14-16). 'Virulence' genes encode these features. Comparison of different EAEC isolates has shown that the virulence gene content of these isolates varies considerably. The heterogeneity of EAEC strains was concluded from the results obtained from the volunteer as well as other studies. Results were recently confirmed by molecular phylogenetic analysis using multilocus sequence typing of EAEC isolates identified by HEp-2 assay (10, 17-22). The heterogeneity is thought to be the result of the acquisition as well as the deletion of genetic elements and localization of many virulence-associated genes on bacteriophages, plasmids, transposons and pathogenicity islands makes gain or loss of pathogenic attributes possible (23). The most recent example of *E. coli* genome plasticity is the emergence of an uncommon pathogen containing a rare combination of virulence attributes of EAEC and EHEC that manifested itself in adherence to intestinal epithelial cells in the stacked-brick pattern and Shiga toxin production respectively (24). This newly emerged strain caused the highest frequency of hemolytic uremic syndrome (HUS) and death ever recorded. The number of HUS cases and deaths was 2.4 and 1.4 times higher in the *E. coli* O104:H4 outbreak than the 350 outbreaks reported for O157:H7 between 1982 and 2001 in U.S. The 350 *E. coli* O157:H7 outbreaks resulted in 8,598 cases, 354 cases of HUS and 40 deaths in the US, while over 3,816 cases were reported in the 2011 *E. coli* O104:H4 outbreak, with 845 cases of HUS and 54 deaths (25, 26). Although the underlying mechanism behind the apparent increase in O104:H4 virulence is not known, several bacterial factors have been implicated (27). In this review, the known virulence factors involved in pathogenesis of EAEC pathotype are summarized.

EAEC in Iran

There is a paucity of data concerning this pathogen in Iran due to the unavailability of a simple, easy to perform and cost-effective test for its detection. Tissue culture adherence assay is the only reliable test, which is only available in a limited number of research laboratories. Therefore of the few articles that have dealt with diarrheagenic *E. coli*, almost all have used PCR for EAEC detection, either targeting the AA or *aggR* sequences (22, 28-32). Using these sequences leads to the identification of isolates that have come to be known as typical EAEC (33) leaving the atypical EAECs unaccounted for. Furthermore none of these articles except Bouzari *et al.* and Aslani *et al.* (19, 22) have dealt with the virulence factors of these isolates and no article has been published that have assessed the role of any of these genes in pathogenesis of these isolates. Moreover, no study dealing with the ability of the Iranian EAEC isolates causing diarrhea has been published although some studies have shown significant association between these isolates and diarrhea by comparing the isolation rate in controls and patients (19, 28, 34). A

geographic distribution for association of EAEC with diarrhea has been postulated (11) and among the few published studies in Iran only one has reported the EAEC isolates as the leading aetiological pathogen (28).

Diarrheal samples however, are rarely referred to the laboratories for *E. coli* detection except in the case of neonates but these laboratories can only detect the EPEC pathogens leaving other pathogens undetected. Moreover, despite the fact that in many regions EAEC is a common diarrheal isolate, identification of the truly pathogenic strains is difficult since at the molecular level strains demonstrating the aggregative phenotype are heterogenous. Recently the National *E. coli* Reference Laboratory (NERL, Pasteur Institute of Iran) has organized a number of workshops to raise awareness among the public health authorities, clinicians as well as the technician concerning the importance of stool culture and the need for *E. coli* isolation and pathogen typing, emphasizing the fact that this subject should not be limited to a number of small research studies.

EAEC pathogenesis

Adhesins

The first step in production of disease by EAEC is adherence to and colonization of the intestinal mucosa. This is manifested by the characteristic AA pattern observed both in vitro and in vivo where a biofilm composed of bacterial aggregates is associated with a thick mucus layer (3). Fimbrial and afimbrial adhesins are responsible for this phenomenon, but the genes coding for this trait have been in low prevalence which is indicative of a high diversity of the adhesins responsible for the AA pattern (34-41).

An expanding repertoire of nonstructural outer membrane proteins ranging from 18 to 58 kDa have been demonstrated in different serotypes of EAEC isolates that contribute to colonization, autoaggregation and biofilm formation. The general characteristics of these afimbrial adhesins is the mannose-resistant hemagglutination of erythrocytes and antibodies raised against these adhesins abolishes the AA phenotype and the diversity of OMP profiles observed among EAEC strains is indicative of the heterogeneity of adhesins in this pathotype (36, 39, 42-44).

The structural adhesins so far identified in different EAEC strains are collectively known as aggregative adherence fimbriae (AAFs) and belong to the Dr superfamily of adhesins. This superfamily of adhesins is also detected on uropathogenic strains. The genes encoding these fimbriae are located on two regions of a high-molecular plasmid termed pAA and their biogenesis employs the chaperone-usher secretion pathway (35, 37-38). The genetic organizations and ultrastructures of AAFs I, II, and III are described but the ultrastructure of a new adhesin named Hda shown to be responsible for AA in EAEC strains isolated in Denmark and proposed as AAF/IV has not been shown (41). Furthermore, a type IV pili located on an IncI1 incompatibility group plasmid is shown to be responsible for the aggregative adherence of atypical EAEC strains to the epithelial cells and abiotic surfaces (40). These genes however, are not detected in a large number of EAEC strains delineating the involvement of a number of different adhesins in the production of AA pattern. The full genome sequencing of the O42 EAEC prototype revealed the presence of 11 fimbrial operons on the chromosome of this strain in addition to the AAF/II that are located on its pAA2 plasmid (13). Other factors such as type 1 pili and the *E. coli* common pilus (ECP) are also shown to be involved in the establishment of the AA in some EAEC strains (45, 46).

Biofilm formation on biotic and abiotic surfaces is an important characteristic of the EAEC strains, a multifactorial phenotype distinct from that of non-pathogenic *E. coli* and the involvement of AAF/I and II, type 1 fimbriae, Fis, YafK and Hra 1 in this process has been demonstrated (2, 40, 47). Expression of AAF/I and

biofilm formation are considered the contributing factors to the high virulence of the 2011 *E. coli* O104:H4 German outbreak strain (27).

Some EAEC strains express a protein named antiaggregation protein (aap) or dispersin which allows the bacteria to escape the constraints of the biofilm by decreasing bacterial autoaggregation and thus allowing the bacteria to move along the intestinal mucosa. The role of this protein in EAEC pathogenesis is unclear, since the *aap* encoding gene is not present in all EAEC isolates and has been detected in commensal *E. coli* as well (48-49).

Toxins

Recently the complete genome sequence of the O42 prototypic EAEC strain was published consisting of a circular chromosome of 5,241,977 bp and a plasmid named pAA of 113,346 bp (13). Genes for enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1) and plasmid-encoded toxin (Pet) are carried by this plasmid, whereas the genes for other cytotoxins and enterotoxins are located on the chromosome.

EAST-1 is an enterotoxin composed of 38 amino acids (Mr=4.1 kDa) with 4 cysteines forming two disulfide bridges with a C1-C2 and C3-C4 conformation (3, 50) and is encoded by the *astA* gene located adjacent to *pet* gene on the pAA plasmid (37). Despite the fact that the enterotoxicity of this toxin has been demonstrated by Sevarino *et al.* (51) in an Ussing chamber assay its presence in EAEC 17-2 strain did not produce diarrhea even though its activity was identical to the EAST-1 elaborated by the O42 strain in the in vitro toxicity models (10, 51). Although EAST-1 and heat stable enterotoxin a (STa) from enterotoxigenic *E. coli* which causes watery diarrhea have often been compared, these two toxins are immunologically different since no cross-neutralization with polyclonal anti-STa has been observed (52). EAST-1 is not specific for EAEC isolates and has been detected in different *E. coli* pathotypes as wells in commensals (3).

Plasmid-encoded toxin (Pet) of EAEC is a 104 kDa cytoskeleton-altering protein that induces the loss of actin filament structure leading to cell rounding and detachment. This could lead to the loss of epithelial integrity and a potentially life-threatening diarrhea (53). Pet is a serine protease autotransporter that was originally identified in the culture supernatants of EAEC strains causing diarrheal outbreaks in Mexican hospitals (54). The cytotoxicity and enterotoxicity of this toxin depends on its protease activity and serine protease inhibitors such as phenylmethylsulfonyl fluoride abolished both activities (55). This protein is highly immunogenic and both IgG and IgM antibody isotopes specific for Pet have been detected in convalescent children (56). Although the gene encoding this toxin has not been detected in all the EAEC isolates, but no serine protease autotransporter of *Enterobacteriaceae* (SPATE) has yet been identified in nonpathogenic bacteria (3).

SepA, another SPATE toxin, originally identified in *Shigella* has also been found strongly associated with diarrhea in children aged between 0-59 months (57).

Protein involved in intestinal colonization (Pic) is also a SPATE member present in some EAECs but unlike Pet it is a chromosomal gene and is identical to the mucinase protein from Shigella termed Shemu (*Shigella mucinase*), which is localized on *Shigella she* PAI (58). Pic is a 109.8 kDa protein and is implicated in serum resistance, hemagglutination and similar to Pet in biofilm formation. Pic is also immunogenic and induces intestinal mucus hypersecretion, one of the pathophysiologic features of the diarrhea mediated by EAEC (58).

In silico analysis of *pic* genetic organization has revealed that this gene resides on the positive strand and the two genes (*set1*A and *set1B*) encoding ShET1 (*Shigella* enterotoxin 1) protein

are carried by the complementary strand completely within the *pic* gene and therefore have the same prevalence and disease association as *pic* (60). ShET1 is a member of AB_5 family of toxins consisting of a single 22 kDa SetA protein associated with a pentamer of five 7 kDa B subunits which appears to induce intestinal secretion although the exact mechanism of action and detailed biochemistry is still unknown (2).

In addition the presence of the *hlyE* gene, which encodes a hemolytic pore-forming protein, and was first identified in *E. coli* K12 has been shown in the genome of O42 strain (13). HlyE is a 34 kDa protein, that through oligomerization forms a pore-forming toxin causing cytolytic and cytopathic effects on cultured cells. The role of this toxin in the pathogenesis of EAEC is unclear, but it should be noted that like other EAEC virulence factors, *hlyE* has not been detected in all the EAEC isolates and its occurrence among non-pathogenic bacteria makes its role in mediating disease a minor one (2).

Moreover, EAEC has been associated with irritable bowel syndrome (IBS) resulting from its invasive capability and in a study conducted in 2007 an isolation rate of more than 80% for this pathogen has been reported from individuals with diarrheal form of IBS. Subsequently, the invasive capability of typical and atypical EAEC isolates from IBS, non-IBS, inflammatory bowel disease (IBD) and acute diarrhea were assayed using the embryonic intestinal epithelial cell line Int407. Results obtained showed that invasion efficiency of these strains was not correlated with the presence of any of the genes encoding the putative invasins (61).

Recently this pathotype attracted a great deal of attention when the EAEC isolate serotype O104:H4 acquired the pathogenic traits of STEC pathotype and caused a devastating outbreak of diarrhea and HUS. Similar to typical EAECs the O104:H4 strain carried pAA plasmid. This expressed AAF/I fimbriae and adhered to intestinal epithelial cells with a stacked-brick pattern. The genes for dispersin (*aap*), protein involved in intestinal colonization (*pic*) and *Shigella* enterotoxin (*set*1) were also detected in this strain. In addition through the acquisition of various mobile genetic elements the outbreak strain had obtained new traits including the phage-mediated Shiga toxin 2a, tellurite and mercury resistance colicin, and resistance to all penicillins, cephalosporins, and co-trimoxazole, but lacked the LEE pathogenicity island (62-64). These strains illustrate the genome plasticity and evolution of *E. coli* and that any isolate has the potential to acquire novel virulence factors and emphasize the importance of being able to detect and characterize *E. coli* pathotypes.

Conclusion

Data accumulated from both developed and developing countries have shown EAEC as a cause of acute and persistent diarrhea in children, adults, HIV-infected patients and travelers' to developing countries. Diagnosis of EAEC depends on the observation of the characteristic 'stacked-brick'-like adherence pattern when co-cultured with HEp-2 cells. At the molecular level, strains demonstrating the aggregative phenotype are heterogeneous and no virulence factor has been identified as common to all EAEC strains. A large number of virulence factors and combinations have been associated with clinical illness in epidemiologic studies, and it is possible that either, the principal determinants of pathogenicity vary by site and population or the true determinants have not yet been identified. Isolates identified by HEp-2 assays have been classified as typical or atypical depending on the presence or absence of the *aggR* regulator respectively. The capacity of atypical EAEC strains to cause diarrheal illness has been documented, but characterization of the atypical EAEC strains suggests that they are even more heterogeneous than typical EAEC. Therefore, identification of

molecular markers for these strains will be even more challenging.

The importance of EAEC in Iran is poorly understood, because large multicenter case-control studies with well defined criteria for inclusion or exclusion of participants has not been published. Moreover, for such a divergent pathogen reliance on detection of *aggR* gene alone only detects a portion of the EAEC isolates thereby underestimating and under-diagnosing the pathogen.

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