Enteroaggregative *Escherichia coli*

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Contents

Abstract Enteroaggregative *Escherichia coli* (EAEC, formerly known as "EAggEC") cause acute or persistent watery diarrhoea (with or without mucus) in children, predominantly in low-income countries, and are associated with travellers' diarrhoea in children and adults in middle and high income countries. The diverse nature of EAEC is such that not all strains cause disease. Conversely, certain strains of EAEC possess additional virulence determinants associated with the ability to cause severe diarrhoea and other symptoms, which might be life-threatening in vulnerable patients. The EAEC virulence factors described to date are either encoded on the large virulence plasmid of EAEC (plasmid of aggregative adherence) or on pathogenicity islands on the chromosome. Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. The variability of the plasmid structure and virulence gene sequences and the possibility that this mobile genetic element may be lost has necessitated the inclusion of chromosomal markers in the molecular screening assays. There is evidence in the literature of foodborne transmission of EAEC, but

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currently no evidence of a zoonotic reservoir. Fimbriae-mediated adhesion and biofilm formation are likely to be involved in both clinical manifestations of infection and attachment to foodstuffs. Multidrug resistance appears to be common in EAEC and geographically widespread. Whole-genome sequencing has revealed the mosaic genomic structure of EAEC and provided evidence that horizontal gene transfer and recombination are the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. This has significant public health implications in terms of the diversity and pathogenesis of EAEC and its ability to colonise and cause disease in the human host.

1 Introduction

Enteroaggregative *Escherichia coli* (EAEC, formerly known as "EAggEC") cause acute or persistent watery diarrhoea (with or without mucus) in children, predominantly in low-income countries (Okeke and Nataro 2001), and are associated with travellers' diarrhoea in children and adults in middle- and high-income countries (Wilson et al. 2001). Other symptoms include nausea and vomiting, anorexia, borborygmi and tenesmus (Huang et al. 2006). In low-income countries, the propensity of EAEC to cause persistent diarrhoea for more than two weeks is associated with significant morbidity.

The diverse nature of EAEC is such that not all strains cause disease. Conversely, certain strains of EAEC possess additional virulence determinants associated with the ability to cause severe diarrhoea and other symptoms, which might be life-threatening in vulnerable patients. EAEC were first described by Nataro et al. in 1987 and were identified by their ability to aggregately adhere to tissue culture cells in a distinct stacked-brick pattern (Fig. 1). The ability to aggregate in this way is mediated by aggregative adherence fimbriae (AAF), of which there are at least five variants (I, II, III, IV and V). Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR (Dudley et al. 2006). More recent studies use the term "typical" EAEC to refer to strains of EAEC harbouring aggR, and strains without EAEC are referred to as "atypical".

A study of infectious intestinal disease (IID) in the UK in 1993–96 showed that EAEC were the most commonly isolated diarrhoeagenic *E. coli* in patients with symptoms of gastroenteritis presenting to a doctor (5.1%) (Wilson et al. 2001). There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies. However, relatively little is known about the burden of EAEC in IID or about the reservoir(s) and transmission pathways.

This chapter presents an overview of EAEC with respect to clinical presentation, the pathogenicity mechanisms associated with this group and interrelationships with other *E. coli* pathotypes and provides an update of the methods for the detection, identification and characterisation of EAEC. The public health risk of EAEC infections arising from the presence of EAEC in the food chain and antimicrobial

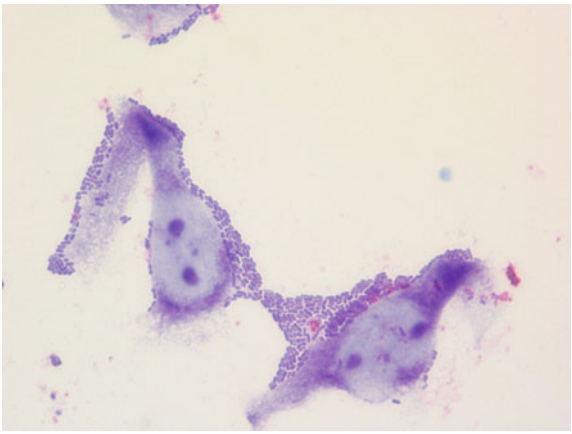

Fig. 1 EAEC were first identified by their ability to aggregately adhere to tissue culture cells in a distinct stacked-brick pattern (Courtesy of Marie Chattaway, Gastrointestinal Bacterial Reference Unit, Public Health England, London, UK)

resistance is assessed, and recent insights into this emerging gastrointestinal pathogen from the analysis of whole-genome sequencing data are summarised.

2 Pathogenicity Mechanisms

Pathogenesis of EAEC is complex as strains are heterogeneous. Case-control studies have documented the prevalence of putative virulence genes but, for the most part, have been unable to correlate the presence of specific genes to disease. The current model of EAEC pathogenesis comprises three steps (Fig. 2):

- Adherence to the intestinal mucosa via aggregative adherence fimbriae,
- Increased mucus production leading to extensive biofilm formation on the surface of the enterocytes, and
- Secretion of toxins and induction of the inflammatory response.

The EAEC virulence factors described to date are either encoded on the large virulence plasmid of EAEC, designated plasmid of aggregative adherence (pAA) or on pathogenicity islands on the chromosome (Table 1). The key virulence regulator of EAEC is AggR, a member of the AraC/XylS family of bacterial transcriptional regulators, and the defining factor for typical EAEC strains. *aggR* is located on the pAA plasmid and controls a number of genes encoding putative virulence factors located on the pAA and additional factors located on the chromosome. Expression of the aggregative adherence fimbriae (AAF), dispersin, the dispersin translocator Aat, and the Aai type VI secretion system, is all regulated by AggR (Morin et al. 2013).

Initial attachment of EAEC to the intestinal mucosa is mediated by AAFs. AAFs are regarded as the principle adhesin of EAEC and are found exclusively in this pathotype (Jønsson et al. 2015). AAFs were first described with respect to their role in the formation of the characteristic stacked-brick aggregative pattern on HEp-2

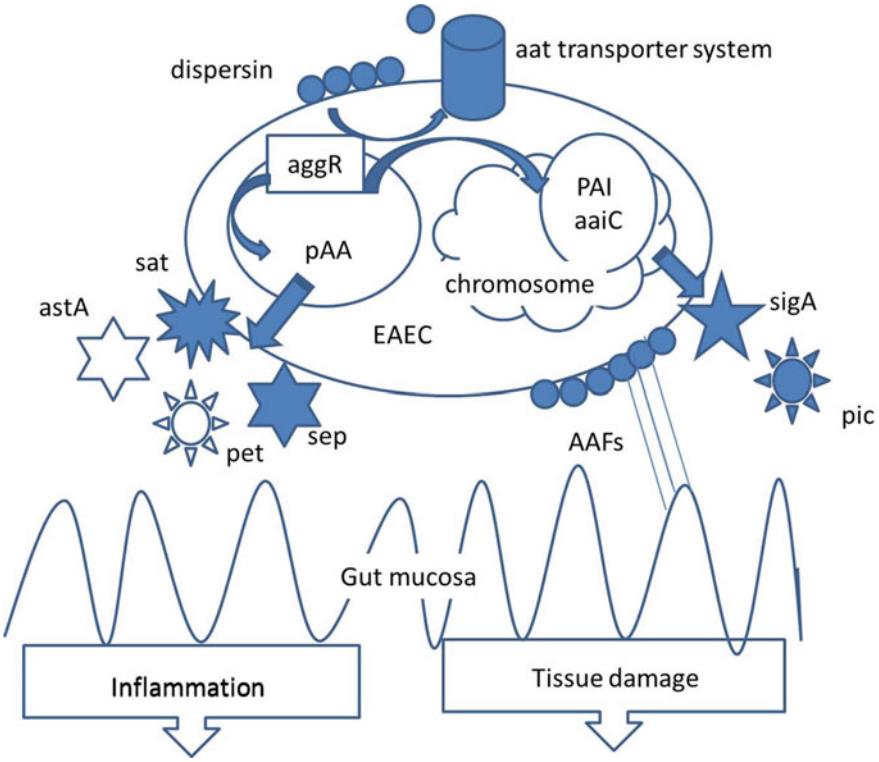

Fig. 2 Current model of EAEC pathogenesis (Adapted from a figure by Erik Juncker Boll, Department of Microbiological infection and Control, Statens Serum Institute, Copenhagen, Denmark)

cells (Nataro et al. 1987). Following adhesion to the epithelial surface, the AAFs have also been associated with epithelial inflammation in vitro, such as interleukin secretion, disruption of epithelial junctions and triggering migration of polymorphonuclear leucocytes (Harrington et al. 2005; Boll et al. 2012). Currently, five different AAF variants have been identified (AAF I–V), all showing a high level of conservation of their accessory genes, despite low level of amino acid identity among the pilin subunits (Jønsson et al. 2015).

The AAFs are members of the chaperone–usher fimbrial group, common to many Gram-negative bacteria. The operon consists of four proteins: the usher, the chaperone, the micro-pilin subunit and major pilin subunit. AAFs have a high isoelectric point (pI 8.9–9.4) relative to other adhesins of the chaperone–usher family. In the gut, where the pH ranges from 6 to 7.4, the AAFs carry a high positive charge, which may play a role in binding (Jønsson Ph.D. Thesis, 2017).

The gene encoding dispersin (aap) is located on the pAA lying immediately upstream of the AggR transcriptional activator and is under AggR control (Sheikh et al. 2002). Dispersin is a positively charged small protein that binds

Table 1 Genes and toxins often found in the EAEC pathotype

Common EAEC factor	Description	Location
aggR	Master regulator for EAEC plasmid virulence genes, including aggregative adherence factors, fimbriae AAF/I-AAF/V, and a large cluster of chromosomal genes inserted on a pathogenicity island at the PheU locus	pAA
aatA-P	Encodes proteins responsible for transporting the dispersin protein out of the outer membrane of EAEC	pAA
aap	Encodes a 10 kDa secreted protein named dispersin and is responsible for "dispersing" EAEC across the intestinal mucosa	pAA
aggA	Encodes AAF/I mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aafA	Encodes AAF/II, mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg3A	Encodes AAF/III haemagglutination of erythrocytes	pAA
agg4A	Encodes AAF/IV mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg5A	Encodes AAF/V mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aaiA-Y	PAI encoding a type VI secretion system (T6SS)	chromosome
pet	A 108 kDa autotransporter protein that functions as a heat-labile enterotoxin and cytotoxin	pAA
sigA	IgA protease-like homologue, enterotoxin and cytotoxin	Chromosome
pic	Mucinase, immunomodulation, colonisation, lectin-like haemagglutinin	Chromosome
sepA	*Shigella* extracellular enterotoxin	pAA
sat	Secreted autotransporter toxin. Enterotoxin and cytotoxin, impairment of tight junctions, autophagy	pAA
astA	*astA* encodes the enteroaggregative heat-stable toxin (EAST-1), which has physical and mechanistic similarities to *E. coli* STa enterotoxin	pAA

non-covalently to the lipopolysaccharide of the outer membrane of EAEC. It participates in formation of a surface coat that acts to disperse the bacteria, partially counteracting aggregation mediated by aggregative adherence fimbriae permitting the AAFs to extend from the surface of the bacterium (Jønsson Ph.D. Thesis, 2017).

In addition to the virulence genes on the pAA, a number of pathogenicity islands (PAIs) have been identified on the chromosome of EAEC. One of these islands consists of 25 contiguous genes (aaiA-Y), activated by AggR and located on a 117 kb PAI inserted at pheU in EAEC (Dudley et al. 2006). Many of these genes have homologues in other Gram-negative bacteria and were recently proposed to constitute a type VI secretion system (T6SS). Distribution studies indicated that aaiA and aaiC are commonly found in EAEC isolates worldwide, particularly in strains defined as typical EAEC. These data support the hypothesis that AggR is a

global regulator of EAEC virulence determinants on both the chromosome and the plasmid, and builds on the hypothesis that T6SS is an important mediator of pathogenesis (Dudley et al. 2006).

Another PAI is designated SHE (also found in *Shigella flexneri*) and encodes the Serine Protease Autotransporter Pic and ShET1 enterotoxins (Jønsson Ph.D. Thesis, 2017). Serine Protease Autotransporters of Enterobacteriaceae (SPATEs) are a family of extracellular proteases thought to play a role in EAEC pathogenesis. The SPATEs are named for their serine protease motif that confers proteolytic capability and are secreted via a type V secretion system. SPATEs are implicated in immune evasion, mucosal damage and colonisation. The most commonly found SPATEs in EAEC include: plasmid-encoded toxin (Pet), protein involved in intestinal colonisation (Pic), secreted autotransporter toxin (Sat), *Shigella* IgA-like protease homology (SigA) and *E. coli*-secreted protein (EspP) (Boisen et al. 2009). All SPATEs found in EAEC are located on the chromosome, except for Pet which is located on the pAA.

EAEC strains often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-encoded *astA* genes and haemolysin E (HlyE), but like ShET1, these toxins are not specific to EAEC (Harrington et al. 2006).

3 Interrelationships with Other *E. Coli* Pathotypes

EAEC are one of the six diarrhoeagenic *E. coli* (DEC) pathotypes defined by their pathogenicity gene profiles (Tozzoli and Scheutz 2014). These are enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), Shiga toxin-producing *E. coli* (STEC), and EAEC. *E. coli* can also cause extra-intestinal (ExPEC) infections in humans, primarily urinary tract (caused by uropathogenic *E. coli*) and sepsis/meningitis (caused by neonatal meningitis *E. coli*).

In recent years, the more widespread use of molecular techniques has revealed that many strains of *E. coli* harbour virulence genes associated with more than one pathogenic group. Most of the *E. coli* virulence factors are encoded by genes carried on mobile genetic elements (e.g. plasmids, phages and pathogenicity islands), and the horizontal gene transfer of such elements is the driver for the continuous emergence of new pathotypes (Tozzoli et al. 2014).

The Stx-producing EAEC O104:H4 strain that caused the large outbreak of HUS in Germany in 2011 outbreak carried the EAEC genes *aggR*, *aggA*, *set1*, *pic* and *aap* as well as a prophage encoding the *stx2* gene (Bielaszewska et al. 2011). This outbreak highlighted the threat to public health associated with strains of *E. coli* comprising more than one single pathotype; however, strains of *E. coli* comprising multiple pathotypes had been described previously. Such strains were first reported as the causative agent of a small HUS outbreak that occurred in France at the beginning of the 1990s (Morabito et al. 1998), where patients were infected with an *E. coli* O111:H2 strain showing the ability to adhere to cultured cells with the stacked-brick

adhesion mechanism (Nataro and Kaper 1998) and able to produce Stx2 (Morabito et al. 1998). Furthermore, sporadic cases of infection with Stx-producing EAEC strains of serotype O104:H4 were retrospectively described in the time period 2000–2010 soon after the German outbreak (King et al. 2012). Subsequently, a sporadic HUS case caused by a Stx-producing EAEC O111:H21 and a small outbreak of infection with a Stx-producing EAEC O127:H4 occurred in Northern Ireland in 2012 (Dallman et al. 2012) and in Italy in 2013 (Tozzoli et al. 2014), respectively.

The observation that the genomic backbone of Stx-producing EAEC is similar to that of non-Stx-producing EAEC, indicates that these strains may emerge following the acquisition of an Stx-carrying phage from a ruminant reservoir by strains of EAEC from human sewage (Tozzoli et al. 2014). Countries where EAEC infections are endemic and treatment of human sewage is limited may represent a source for the emergence of the Stx-producing EAEC pathotype. It has been proposed that the occurrence of the EAEC/STEC pathotype *E. coli* may be an ongoing, low-frequency event. The occurrence of outbreaks probably relates primarily to epidemiological opportunities for propagation and dissemination of the organisms in food or infected carriers.

Other combinations of EAEC pathotypes have been detected, such as those present in isolates possessing EAEC-associated genes together with ExPEC-associated traits as described in the *E. coli* serotypeO78:H10 responsible for causing an outbreak of UTI in Denmark (Olesen et al. 2012). The outbreak strain carried a range of virulence genes including *fimH* (type I fimbriae; ubiquitous in *E. coli*); *fyuA*, *traT* and *iutA* (associated with extra-intestinal pathogenic *E. coli*); and *sat*, *pic*, *aatA*, *aggR*, *aggA*, ORF61, *aaiC*, *aap* and ORF3 (associated with EAEC). In a study of ESBL-producing *E. coli*, eight multidrug-resistant ESBL-producing EAEC were isolated from urine specimens and one from a blood culture (Chattaway et al. 2014a, b). The multidrug-resistant EAEC isolates belonged to sequence type (ST) 38, predominantly associated with urinary tract infections. It is clear that the spectrum of pathogenic *E. coli* types is continuous rather than a rigid list of separated groups.

4 Methods for the Detection, Identification and Characterisation

Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from these matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. Following the outbreak of Stx-producing EAEC O104:H4 in 2011, the STEC European Union Reference Laboratory (EU-RL) developed a molecular methodology to screen food samples and faecal specimens for the presence of EAEC by the detection of *aggR* and *aaiC* (http://www.iss.it/vtec/index.php?lang=2&anno=2017&tipo=3).

In the 1980s, EAEC were described as exhibiting a characteristic "stacked-brick" pattern on adhesion to HEp-2 cells monolayers (Nataro et al. 1987). Since then the HEp-2 adhesion assay has been considered the gold standard for the identification of the EAEC. Although regarded as a sensitive and specific assay for the identification of this *E. coli* pathogroup, this approach is cumbersome and requires experienced personnel, specialised facilities making it an unsuitable assay for a routine testing. Molecular methods have largely replaced the phenotypic adhesion assay for the identification and characterisation of EAEC. A number of different PCR protocols are available, targeting a wide variety of genes. Given the recognised heterogeneity of EAEC, the different PCR assays produce variable results when compared to the phenotypic adhesions assay.

Early studies established evidence that the aggregative adhesion properties of EAEC were associated with the pAA plasmid, and the design of molecular screening tools was directed towards the use of sequences from this plasmid (Vial et al. 1988). Baudry et al. developed a DNA probe, CVD432, which showed a high degree of correlation with the phenotypic assay (Baudry et al. 1990), although a number of subsequent studies conducted using the CVD432 probe for screening EAEC strains isolated from cases of diarrhoea in different geographic locations showed more variable results (Okeke and Nataro 2001).

In 1995, the first PCR tool was developed based on the sequence of the *Eco*RI/*Pst*I fragment of pCVD432 plasmid, later found to correspond to a gene encoding the aggregative autotransporter, *aat* (Schmidt et al. 1995). A number of subsequent studies showed limited correlation between the molecular hybridisation and PCR assays suggesting that, in spite of the initial strong association of the presence of the plasmid with the ability to induce the stacked-brick pattern of adhesion, there was a certain degree of variability in the plasmid structure (Dutta et al. 1999; Tsai et al. 2003). More recent studies have been aimed at a more complete characterisation of the plasmid itself, and assays based on the detection of more than one marker have been deployed (Czeczulin et al. 1999; Cerna et al. 2003; Jenkins et al. 2006; Scheutz et al. 2014).

The variability of the plasmid structure and sequence, and the possibility that this mobile genetic element may be lost, has led to the conclusion that chromosomal markers should be included in the molecular screening assays (Jenkins et al. 2006; Scheutz et al. 2014). Following extensive genotyping of EAEC in different studies (Jenkins et al. 2006; Boisen et al. 2012), it was recognised that, similarly to the plasmid-associated genes, no chromosomal markers are present in 100% of EAEC. Some markers have been identified as being significantly associated with EAEC isolated from symptomatic cases, such as the SPATE toxin SepA (Boisen et al. 2012). As described above, the STEC EU-RL PCR assay for screening food samples and faecal specimens targets the pAA-encoded *aggR* and *aaiC* which is located on the chromosome. This assay is recommended for clinical diagnostic use.

An increasing number of diagnostic microbiology laboratories are implementing a multiplex gastrointestinal (GI) PCR approach for the detection of GI pathogens in clinical cases and foods, including target for EAEC. These assays provide a rapid, standardised, cost-effective pan-pathogen approach for the detection of bacteria

associated with GI infection and, moving forward, will improve the surveillance of EAEC disease.

5 Clinical Symptoms and Burden of Disease

EAEC are commonly associated with acute and chronic diarrhoeal illness among children in both developing and developed and/or industrialised regions and travellers with diarrhoea. The incubation period of diarrhoeagenic EAEC is typically between 8 and 18 h (Harrington et al. 2006). Infection with EAEC usually presents clinically as watery diarrhoea, often with mucus, nausea and vomiting, with or without fever (Huang et al. 2003). Other less common symptoms include anorexia, borborygmi and tenesmus. Additionally, there is evidence to suggest that the odds of developing post-infectious irritable bowel syndrome (IBS) are dramatically increased after acute infectious gastroenteritis with EAEC has been discussed (Sobieszczańska et al. 2007). A predominant feature of EAEC infection in low-income countries is the propensity to cause persistent diarrhoea for more than 2 weeks, making these bacteria a significant cause of mortality (Huang et al. 2006). The most significant public health concern stemming from EAEC infections in children in low-income countries is malnourishment, as persistent EAEC infections lead to chronic inflammation, which damages the intestinal epithelium and reduces its ability to absorb nutrients.

Studies suggest EAEC are a major cause of diarrhoeal disease, and it has been estimated that between 2 and 68% of patients with diarrhoea are infected with EAEC (Nataro et al. 1998; Wilson et al. 2001; Kahali et al. 2004). In the UK IID study in 1993–96, EAEC were the most commonly isolated enterovirulent *E. coli* in patients with symptoms of gastroenteritis presenting to a doctor (5.1%) (Wilson et al. 2001). In the second IID study in 2008–09, EAEC were isolated from more than 1.9% of cases in the population and 1.4% of cases presenting to a doctor (Tam et al. 2012). Data from the IID studies confirmed previous conclusions that concluded that the current definition of EAEC by plasmid gene detection includes true pathogens and non-pathogenic variants (Chattaway et al. 2013).

6 The Zoonotic Potential of EAEC and Contamination of the Environment

Reports of animals being a reservoir of EAEC are often based on the presence of genes that are not specific for EAEC, such as *astA*, in specimens from both healthy and sick animals. Most reports originate from parts of the world where pollution by human faecal waste is common (Table 2). Studies using EAEC-specific targets have found no evidence of EAEC in animals (Cassar et al. 2004).

Following the outbreak of Stx-producing EAEC O104:H4 in Germany in 2011, 2000 colonies from faecal samples of 100 cattle from 34 different farms, all located in the HUS outbreak region of Northern Germany, were screened for genes associated with the O104:H4 HUS outbreak strain (*terD*, *rfb*(O104), *fliC*(H4)), STEC (*stx1*, *stx2*, *escV*), EAEC (*pAA*, *aggR*, *astA*) and ESBL production (*bla*(CTX-M), *bla*(TEM), *bla*(SHV)) (Wieler et al. 2011). No EAEC were detected. In a similar study undertaken in France after the 2011 outbreak, 1468 cattle were analysed for faecal carriage of the Stx-producing *E. coli* O104:H4 outbreak strain by PCR assays targeting *stx2*, *wzxO104*, *fliCH4* and *aggR* genetic markers. None of the faecal samples contained the four markers simultaneously, indicating that cattle in France were not likely to be a reservoir of O104:H4, but results of the test for *aggR* were not reported (Auvray et al. 2012). In a recent study in Japan, no EAEC isolates, as assessed by the presence of *aggR*, were detected (Akiyama et al. 2015). To date, there is no evidence that EAEC have a zoonotic reservoir.

Contamination of the environment by EAEC, particularly watercourses, can occur in parts of the world where human sanitary systems are insufficient, and there is a high incidence of EAEC in people (Table 2). Prolonged survival of EAEC for at least several weeks in wet and dry substrates appears to be possible, and environmental contamination may also be a pathway for EAEC on salads and other vegetable produce (Table 2).

7 Foodborne Transmission

There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies (Table 3). In Japan, a major outbreak caused by EAEC O untypeable:H10 in 1993 involving up to 2500 cases mainly in schoolchildren was associated with school lunches (Itoh et al. 1997). In the UK in the 1990s, four EAEC outbreaks associated with restaurants, a charity Christmas dinner and a conference were reported but no specific food vehicle was identified in any of these outbreaks (Smith et al. 1997). The 2011 German outbreak of EAEC O104:H4 was epidemiologically linked to contaminated fenugreek seeds (Frank et al. 2011). In June 2013, a foodborne outbreak was caused by EAEC isolated from kippered trotters mixed with vegetables, 22 cases and four asymptomatic food handlers, who probably contaminated the food (Shin et al. 2015) (Table 3).

In two further foodborne outbreaks of gastroenteritis that occurred 10 days apart among individuals who had meals at the restaurant of a farm holiday resort in Italy in 2007, an EAEC strain of serotype O92:H33 was isolated from six participants and one member of staff. A retrospective cohort study indicated a pecorino cheese made with unpasteurised sheep milk as a possible source of infection (Scavia et al. 2008), but since the outbreak EAEC strain was only isolated from food handlers, cross-contamination of the food product cannot be excluded, nor can contamination of food by asymptomatic excretors.

Table 2 Reports of evidence of EAEC detected in animals, food and the environment

Animal	Country	Findings	Reference
• Cattle (n = 304) • Chickens (n = 350) • Pigs (n = 263)	Burkina Faso	*astA* in 7% of cattle, 6% of chicken and 3.2% of pig samples	Kagambega et al. (2012)
• Pigs (n = 50)	South African	20.5% of *E. coli* from pigs had *astA*	Mohlatlole et al. (2013)
• Antelopes • Cattle	Zambia	*astA* was detected more frequently in antelopes (83.3%) than in cattle (33.3%)	Kuroda et al. (2013)
• Diarrhoeic dog	Germany		Breitwieser (1999)
• Dogs and cats • Poultry manure	Brazil		Puno-Sarmiento et al. (2013, 2014)
Food	Country	Findings	Reference
100 poultry carcases	Burkina Faso	*aggR* detected in 13 isolates of *E. coli*	Kagambega et al. (2012)
120 samples of beef and edible intestines	Korea	5 (4%) isolates	Kagambega et al. (2012)
Okra		ESBL-producing strain of EAEC	Zurfluh et al. (2015)
Environment	Country	Findings	Reference
Village-wide outbreak in 1996	India	Epidemiologically associated with the consumption of water from open well	Pai et al. (1997)
Survey of natural water	Bangladesh	ETEC, EPEC and STEC pathotypes were detected consistently, but genes from the EIEC and EAEC pathotypes were only found occasionally, and never in the rainy season or in winter	Akter et al. (2013)
Longitudinal study in a high population density, urban setting sampling domestic rainwater harvest tanks and in river water samples	South Africa	EAEC was found in 16% of 80 samples	Dobrowsky et al. (2014)

(continued)

Table 2 (continued)

Environment	Country	Findings	Reference
Pre-treated water in a drinking water treatment plant	Taiwan	EAEC-associated genes were found in 3.6% of 55 water samples, alongside high levels of other potentially pathogenic *E. coli*	Huang et al. (2011)
Urban floodwater	Australia	*astA* (69%) and *aggR* (29%) genes, carried by EAEC, were frequently detected in *E. coli* isolates	Sidhu et al. (2013)

Table 3 Outbreaks of EAEC and the AMR profile of the outbreak strain

Outbreak	Resistance profile	Comments	Reference
Urinary tract infection of multiresistant *E. coli* O78:H10, Denmark, 1991	Ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim		Olesen et al. (2012)
Shiga toxin (Stx)-producing EAEC O104:H4 outbreak, EU, USA and Canada, 2011	Ampicillin, amoxicillin/clavulanic acid, piperacillin/sulbactam, piperacillin/tazobactam, cefuroxime, cefotaxime, cefpirome and ceftazidime, and also was resistant to streptomycin, nalidixic acid, tetracyclines and trimethoprim and the sulphonamides but was susceptible to the carbapenems	The strain contained an 88.5-kb IncI1-ST31 plasmid —pESBL-EA11— that encoded bla-CTX-M-15 and bla-TEM. Although not considered important in treatment of affected persons in this outbreak, the presence of resistance genes may have contributed to the development and spread of the causative organism	Bielaszewska et al. (2011), Rasko et al. (2011), Scheutz et al. (2014), EFSA (2011)
Multipathogen foodborne outbreak, UK, 2013	Of 20 EAEC isolates characterised, a range of resistance profiles were identified, ranging from nalidixic acid alone through to ampicillin, sulphonamides, streptomycin, nalidixic acid, ceftazidime, cefataxime, ceftiofur and cefpirome	Ten EAEC serotypes were identified in faecal samples recovered from patients in the large and complex multipathogen foodborne outbreak in the UK in February/March 2013	Dallman et al. (2014)
Outbreak of *E. coli* O untypeable: H10 in Japan in 1993 associated with school lunches, in which over 2600 children were affected	All isolates were susceptible to nalidixic acid, chloramphenicol, streptomycin, kanamycin and cephalothin but were resistant to ampicillin		Itoh et al. (1997)

In an outbreak of gastrointestinal foodborne illness associated with a Street Spice festival in the UK in 2011 and involving over 400 persons, 29 cases of *Salmonella* infection were confirmed. As most cases had reported symptoms characteristic of EAEC infection, such as abdominal cramps and persistent diarrhoea, further investigations were carried out retrospectively using a GI PCR assay. A high proportion of specimens were positive for the aggR target, and EAEC were cultured from 20 cases (Dallman et al. 2014). Risk factors associated with illness included eating foods from one particular vendor and eating a food item containing uncooked curry leaves. Although the *E. coli* count in colony forming unit (cfu) per ml from the curry leaves associated with the outbreak was high (>1000 cfu/ml), the testing algorithm at that time did not include tests specific for EAEC and EAEC were not cultured from the food samples. Strains of EAEC were detected in the food handlers, and contamination of the food by the food handlers was thought to be the most likely source (Table 3).

The infection status of food handlers, including asymptomatic carriage of EAEC, and hygienic conditions applied during the handling and processing of foodstuffs in some countries appears to be an important factor in contamination of foods at retail, catering or household level (Oundo et al. 2008). Multiple EAEC adherence factors are involved in the interaction of EAEC with leaves, and similar colonisation factors are used to bind such to the gut mucosa and leaf surfaces (Berger et al. 2009). It is thought that prolonged survival of organisms on dry fenugreek seeds may have been involved in the Stx-producing EAEC O104:H4 outbreak (EFSA BIOHAZ Panel 2011).

8 Biofilm Formation

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix (consisting of proteins, exopolysaccharide and nucleic acid) attached to biological and non-biological surfaces. Biofilms allow bacteria to survive and thrive in hostile environments as well as being associated with chronic or persistent infections. Bacteria within biofilms can withstand host immune responses and are less susceptible to antimicrobials and disinfectants.

EAEC form thick biofilms on the intestinal mucosa, and most EAEC strains form a biofilm on glass or plastic surfaces when grown in cell culture medium with high sugar and osmolarity. AAFs bind extracellular matrix proteins and show species specificity in terms of erythrocyte agglutination, suggesting that this binding specificity could impact on the efficiency and selectivity of biofilm formation. Transposon mutagenesis confirmed the involvement of genes known to be required for AAF/II expression, as well as the *E. coli* chromosomal *fis* gene, a DNA-binding protein that is involved in growth phase-dependent regulation, in biofilm formation (Sheikh et al. 2001). The incompatibility group (Inc) I1 plasmid of EAEC C1096 encodes a type IV pilus that contributes to plasmid conjugation, epithelial cell adherence and adherence to abiotic surfaces, including via biofilm formation (Dudley et al. 2006).

When subjected to low iron conditions, an EAEC strain (042) showed a decrease in biofilm formation. Conversely, an increase in biofilm formation was observed for clinical EAEC strains cultured in restricted iron conditions, but the reduction of iron concentration inhibited the aggregative adherence to HEp-2 cells of all EAEC strains tested. Low iron availability may therefore modulate biofilm formation and adhesive properties of EAEC as a result of redox stress (Alves et al. 2010).

AAF-mediated adhesion and biofilm formation are likely to be involved in both clinical manifestations of infection and attachment to foodstuffs, such as lettuce after irrigation or washing using water that has become contaminated with human faecal waste (Berger et al. 2009; Castro-Rosas et al. 2012). Uropathogenic strains in particular may make use of biofilm formation to persist on epithelial surfaces and canulae (Boll et al. 2013). A high proportion of EAEC strains associated with travellers' diarrhoea produce biofilms, as well as being highly antimicrobial-resistant (Mohamed et al. 2007; Mendez Arancibia et al. 2009).

9 Antimicrobial Resistance

Although gastrointestinal symptoms associated with EAEC may persist for weeks, infection is usually self-limiting and the standard recommended treatment is oral rehydration therapy. However, the symptoms can be debilitating and have a high socio-economic impact, especially in low-income settings, and treatment may be sought if the diarrhoea and abdominal pain are severe and/or prolonged. Multidrug resistance appears to be common in EAEC and geographically widespread.

Isolates of EAEC exhibiting high incidence of resistance to co-trimoxazole, ampicillin and tetracyclines were detected in studies carried out in Africa and Asia (Oundo et al. 2008; Chen et al. 2014). During a study in India between 2006 and 2007, an increase in isolates with resistance to quinolones was observed (Raju and Ballal 2009). Resistance to ampicillin, cefotaxime (encoded by a CTX-M-15 β-lactamase), gentamicin, co-trimoxazole, nalidixic acid and ciprofloxacin has been reported in EAEC isolates from travellers from India returning to Spain (Vila et al. 2001; Guiral et al. 2011). In studies in Central and South America from 2006 to 2007, the most common *E. coli* pathogens in cases of diarrhoea were EAEC (14%), of which greater than 90% of isolates were resistant to antimicrobials (Ochoa et al. 2009).

In Europe, of 160 strains of *E. coli* identified as EAEC isolated from patients in the UK with infectious intestinal disease or gastroenteritis between 1993 and 1996, over 50% were resistant to one or more of eight antimicrobials, and 30 (19%) were resistant to four or more drugs with one strain being resistant to eight antimicrobials (Wilson et al. 2001). Multidrug-resistant isolates of EAEC have been described elsewhere in Europe, notably in Poland and Spain (Sobieszczańska et al. 2003; Mendez Arancibia et al. 2009)

The most frequently used first-line antimicrobials which have traditionally been used for the treatment of travellers' diarrhoea are ampicillin, co-trimoxazole,

tetracyclines (doxycycline) and quinolones, due to their ready availability and inexpensive cost (Kong et al. 2015). As EAEC have become increasingly resistant to various antibiotics, selection of an appropriate antibiotic should take into account the region of the world where the infection was acquired, as there are different antimicrobial susceptibility patterns for each geographical region. EAEC infections have been successfully treated with ciprofloxacin and other fluoroquinolones, although this group of antimicrobials is not in general regarded as suitable for use in children. The emergence of multiple antimicrobial-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment in some regions (Kong et al. 2015). The use of antimicrobials to eliminate carriage of Stx-producing strains from patients or food handlers is still considered a controversial treatment because of the risk of promoting the development of HUS by stimulating Stx production (Siefert and Tarr 2012).

Of note for EAEC is the high occurrence of resistance to antimicrobials in comparison with other *E. coli* pathotypes associated with food production animals, specifically STEC. Although AMR has been identified in STEC from both human infections (Day et al. 2017) and from cattle and beef products (Ennis et al. 2012), resistance does appear to be less common than in EAEC isolates from cases of human infection. Possible explanations for this anomaly may be related to either differences in the innate propensity of STEC and EAEC strains to acquire and maintain plasmids encoding for AMR, or to antimicrobial selective pressure, with patients with EAEC infections more likely to have been exposed to antimicrobials than cattle, the major reservoir of STEC.

10 Whole-Genome Sequencing

Whole-genome sequencing analysis has provided further evidence that EAEC are a heterogeneous group of pathogens with respect to their genotypic characteristics. This high level of genetic diversity is apparent at every level from the population structure, to the genomic architecture of the pAA plasmid, and the presence and absence of putative virulence genes and their variants on the plasmid and the chromosome (Jenkins et al. 2005; Rasko et al. 2008; Dallman et al. 2014).

MLST and WGS data provide evidence that prevailing "successful" EAEC lineages have evolved independently many times and are dispersed throughout the entire *E. coli* population (Fig. 3). Pupo et al. (2000) suggested that strains of *E. coli* act as genetic repositories with the ability to acquire DNA from multiple sources and the ability to act as donors. The successful lineages, as defined by MLST complex, appear to be globally distributed. There is some evidence that certain lineages may be more pathogenic than others (Chattaway et al. 2014a, b). ClonalFrame analysis showed that EAEC mutation and recombination rates vary across the lineages and that both events play an important part in the evolution of EAEC. Although the dataset was limited, Chattaway et al. (2014a, b) showed that recombination rate was higher in the STs associated with disease. Analysis of WGS

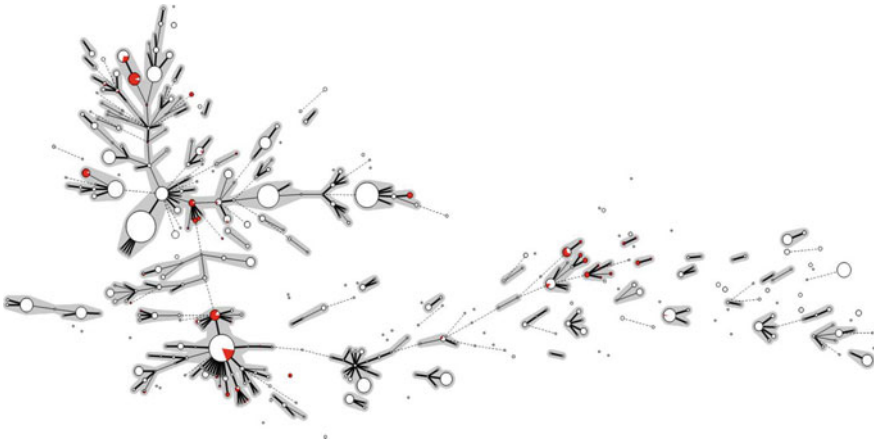

Fig. 3 Minimum spanning tree illustrating that EAEC lineages (highlighted in red) has evolved independently many times and is dispersed throughout the *E. coli* population (Courtesy of Marie Chattaway, Gastrointestinal Bacterial Reference Unit, Public Health England, London, UK)

data indicates that prophage and phage elements play a significant role in the evolution of certain *E. coli* pathovars (Rasko et al. 2008).

The pAA is regarded as a defining feature of EAEC, but recent WGS analysis has shown the pAA is associated with a wide range of plasmid replicon types and that it has a diverse genomic architecture (Dallman et al. 2014). WGS data can also be used to determine the presence or absence of all the major putative EAEC virulence genes, including *aggR, aat, aap, sepA, sigA, pic,* aggregative adherence fimbrial (AAF) types I–V and, more recently, a putative isopentenyl isomerise (IDI) enzyme (Rasko et al. 2011). WGS data have also been used to determine the integrity of the chromosomally encoded AAI operon and to provide information on antibiotic resistance (Dallman et al. 2014).

As yet, WGS is not used routinely for the detection of EAEC either from human faecal samples or from foods; however, the technology is progressing rapidly and there is potential of WGS to be used for such purposes (Loman et al. 2013). Multilocus sequence typing (MLST) and whole-genome sequencing (WGS) data have made a significant contribution to our understanding of the evolution and pathogenic potential of enteroaggregative *E. coli* (EAEC). The mosaic genomic structure of EAEC facilitates horizontal gene transfer, and recombination is the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification. This has significant public health implications in terms of the diversity and pathogenesis of EAEC and its ability to colonise and cause disease in the human host.

11 Summary

1. EAEC are a heterogeneous group of pathogens with respect to both phenotypic and genotypic characteristics. The current model of EAEC pathogenesis involves the initial adherence to the intestinal mucosa via aggregative adherence fimbriae under the control of the transcriptional regulator, AggR, biofilm formation on the surface of the enterocytes, secretion of toxins and induction of the inflammatory response. Key virulence factors are encoded on the pAA or PAI located on the chromosome.
2. Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from these matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes using PCR. The STEC EU-RL PCR assay for screening food samples and faecal specimens targets the pAA-encoded *aggR* and *aaiC* which is located on the chromosome, and is recommended for clinical diagnostic use.
3. EAEC are commonly associated with acute and chronic diarrhoeal illness among children in both developing and developed and/or industrialised regions and travellers with diarrhoea. Studies suggest EAEC are a major cause of diarrhoeal disease. Increasing number of diagnostic microbiology laboratories are implementing a PCR approach for the detection of EAEC in clinical cases and foods, and this will improve the surveillance of EAEC disease.
4. There is no evidence that EAEC have a zoonotic reservoir but contamination of the environment can occur in parts of the world where human sanitary systems are insufficient and there is a high incidence of EAEC.
5. There is evidence in the literature of foodborne transmission of EAEC, and the infection status of food handlers, including asymptomatic carriage of EAEC, and hygienic conditions applied during the handling and processing of foodstuffs in some countries may be an important factor in contamination of foods at retail, catering or household level.
6. The ability to form biofilms is linked to the severity of human disease and is likely to be involved in environmental survival.
7. Multidrug resistance appears to be common in EAEC and geographically widespread. The emergence of multiple antimicrobial-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment in some regions.
8. Whole-genome sequencing analysis has provided evidence that EAEC exhibit a high level of genetic diversity and that prevailing “successful” EAEC lineages have evolved independently many times and are dispersed throughout the entire *E. coli* population.
9. The mosaic genomic structure of EAEC facilitates horizontal gene transfer, and recombination is the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. The emergence of mixed EAEC/STEC pathotype *E. coli* is likely to be an ongoing low-frequency event and has significant public health implications.

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