RESEARCH LETTER

Clonal relations of atypical enteropathogenic *Escherichia coli* O157:H16 strains isolated from various sources from several countries

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Abstract

Atypical enteropathogenic *Escherichia coli* (aEPEC) is comprised of a large heterogeneous group of strains and serotypes that carry the intimin gene (*eae*) but no other EPEC virulence factors. In a previous study, we examined a few aEPEC strains of O157:H16 serotype from the U.S. and France and found these to be nearly homologous, and speculated that the same strain had been disseminated or perhaps they are part of a large clonal group that exists worldwide. To test that hypothesis, we examined additional 45 strains isolated from various sources from 4 other countries and determined that although there are a few *eae*-negative O157:H16 strains, most are aEPEC that carried *eae* and specifically, the ε-*eae* allele. Analysis by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing showed that as a whole, O157:H16 strains are phylogenetically diverse and have different sequence types and PFGE profiles. But the aEPEC strains within the O157:H16 serotype, regardless of the *eae* allele carried, are a highly conserved and homologous group of sequence type (ST)-171 strains that shared similar PFGE profiles. These aEPEC strains of O157:H16 serotype are not closely related to any of the major EPEC and enterohemorrhagic *E. coli* clonal lineages and appear to be part of a large clonal group that are prevalent worldwide.

Introduction

Aside from the well-recognized enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 strains, the O157 serogroup contains a large diversity of strains that include many non-H7 strains that are often nonpathogenic, as well as O157:H45 strains that are enteropathogenic *E. coli* (EPEC). EPEC is a common cause of infantile diarrhea in developing countries (Trabulsi *et al.*, 2002). EPEC pathogenesis begins with localized adherence to epithelial cells, probably via the bundle-forming pilus (BFP), which triggers signal transduction activity encoded by genes on the locus of enterocyte effacement (LEE) pathogenicity island. Also present on LEE are the *tir* and *eae* genes that encode for the translocated intimin receptor (Tir) and intimin, respectively. Tir is secreted and inserted into the membrane of epithelial cells to serve as the receptor for intimin. The latter enables intimate adherence of EPEC to epithelial cells and the resulting attaching and effacing (A/E) lesions cause the accumulation of polymerized actin at the site of attachment (Trabulsi *et al.*, 2002; Torres *et al.*, 2005). Intimin is a major EPEC virulence factor, and there are reported to be at least 30 *eae* alleles. Typical EPEC strains have *eae* and exhibit the A/E phenotype and also have the EPEC adherence factor (EAF) plasmid that carries *bfpA* that encodes for bundlin, the major structural subunit of the BFP. However, as *eae* is also a virulence gene of EHEC, the absence of Shiga toxin (Stx) production is used to distinguish EPEC from EHEC. Although some domestic animals have been found to

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carry typical EPEC (Krause *et al.*, 2005), the main reservoir for typical EPEC is humans and most EPEC strains reside within several well recognized or classical serotypes (Kaper, 1996). There are also many strains of *E. coli* that carry *eae* but not the EAF plasmid, and these are commonly designated as atypical EPEC (aEPEC; Kaper, 1996). Strains of aEPEC are prevalent in both children with and without diarrhea and are also found in animals and in various environmental sources. Although aEPEC are suspected to be less virulent, possibly due to lack of the EAF plasmid (Trabulsi *et al.*, 2002), the pathogenicity of aEPEC for humans remains uncertain. Some studies do not support the implication of aEPEC as a diarrheal agent, but others have found aEPEC strains to be associated with endemic infantile diarrhea and diarrheal outbreaks in some countries (Gomes *et al.*, 2004; Robins-Browne *et al.*, 2004; Bando *et al.*, 2009).

Several studies have shown that aEPEC is comprised of a large heterogeneous group of strains and serotypes (Vieira *et al.*, 2001; Trabulsi *et al.*, 2002; Afset *et al.*, 2008; Bando *et al.*, 2009). Aside from *eae*, some aEPEC have been found to possess virulence factors associated with other groups of pathogenic *E. coli* and are even closely related to these other groups. A multilocus sequence typing (MLST) study from Brazil showed that aEPEC strains of the same serotype clustered in the same sequence type (ST) but the strains had very distinct pulsed field gel electrophoresis (PFGE) profiles (Bando *et al.*, 2009), suggesting that there are many diversity within serotypes. The aEPEC strains of O157:H16 serotype may be an exception as our previous analysis of isolates from water, clinical and food sources in the US and from meats in France showed these *eae*-positive O157:H16 strains to be fairly homogeneous and a few even had nearly identical PFGE profiles (Feng *et al.*, 2010). At that time, it was uncertain if the same strain had been disseminated to the two countries or perhaps, these aEPEC of the O157:H16 serotype are part of a conserved clonal group. To test this hypothesis, we examined other O157:H16 strains isolated from clinical and environmental sources from four other countries to determine their phylogenetic relationships.

Materials and methods

Bacterial strains and characterization

The 45 additional O157:H16 strains examined consisted of 21 from Germany (clinical), 11 from Argentina (animals), 10 from The Netherlands (environmental), and 3 from Norway (clinical; Table 1). Although these strains had been fully serotyped at the time of isolation, they were confirmed to have O157 antigen by latex agglutination (RIM O157:H7, Remel, Lenexa, KS), and selected strains were also tested by H genotyping to confirm the presence of the H16 gene. In the latter procedure, the flagellar structural gene *fliC* was amplified by PCR, sequenced and analyzed by BLAST to identify and match with *fliC* sequences of various H types in GenBank (Lacher *et al.*, 2007). All strains were tested for the presence of *stx* and *eae* genes by multiplex PCR (Monday *et al.*, 2007). Those strains that were found to carry *eae* were further tested by PCR with *eae* allele-specific primers (unpublished) and also for the presence of *bfpA* (Gunzburg *et al.*, 1995).

Pulsed field gel electrophoresis

XbaI-digested genomic DNA was analyzed on a 1% SeaKem Gold agarose gel in 0.5× TBE buffer, pH 8.2 at 14 °C

Table 1. Isolation source and results of analysis of O157:H16 strains

Country	Source	*n*	*stx*	*bfpA*	*eae*	ST	Reference
Argentina	Dogs	11	–	–	ε	171	Bentancor *et al.* (2010)
Germany	Clinical	14	–	–	ε	171	Kozub-Witkowski *et al.* (2008)
		2	–	–	ß	171	
		4	–	NT	–	344	
		1	–	NT	–	83	
The Netherlands	Environmental	9	–	–	ε	171	
		1	–	NT	–	344	
Norway	Clinical	3	–	–	ε	171	Afset *et al.* (2008)
France*	Meat	2	–	–	ε	171	
USA*	Water	6	–	–	ε	171	
	Clinical	2	–	–	ß	171	
	Clinical	1	–	NT	–	344	
		1	–	NT	–	344 v1	
	Meat	1	–	NT	–	344	
		1	–	NT	–	344 v2	
	Total	59					

NT, Strains without *eae* not tested for *bfpA*.
*Data from previous study (Feng *et al.*, 2010).

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using CHEF MAPPER (BioRad, Hercules, CA; Ribot *et al.*, 2006). The run time was 18.5 h at 6 V cm^{-1}, with initial and final switch times of 2.16 and 54.17 s, respectively. The gel was stained with 1 μg mL^{-1} ethidium bromide, visualized on the Gel Doc XR system (BioRad), and analyzed with the BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium).

Multilocus sequence typing

The MLST protocol is described at http://www.shigatox.net/ecmlst/cgi-bin/index. The assay uses primers to amplify and sequence internal segments of seven housekeeping genes [aspartate aminotransferase (*aspC*), caseinolytic protease (*clpX*), acyl-CoA synthetase (*fadD*), isocitrate dehydrogenase (*icdA*), lysine permease (*lysP*), malate dehydrogenase (*mdh*), and ß-D-glucuronidase (*uidA*)]. Each unique sequence is given an allele number, and the combinations of alleles from the seven genes are used to generate an allelic profile or sequence type (ST), which is then compared with those of other *E. coli* strains in the ECMLST database (Qi *et al.*, 2004). Based on MLST data, a neighbor-joining tree was constructed using the Kimura two-parameter model of nucleotide substitution with the MEGA3 software (Kumar *et al.*, 2004), and the inferred phylogeny was tested with 500 bootstrap replications.

Results

All isolates were serologically confirmed to be O157 and H genotyping of selected strains verified that they carried the H16 *fliC* allele (data not shown). None of the strains had *stx* genes but 39 of the 45 strains had *eae*. Except for two German strains that had the *β-eae* allele, 37/39 strains had the ε-*eae* allele (Table 1). All 39 *eae*-positive strains were negative for *bfpA* by PCR, suggesting the absence of the EAF plasmid and therefore appeared to be aEPEC. For comparative purposes, Table 1 also includes data from the O157:H16 strains examined previously (Feng *et al.*, 2010).

Analysis by PFGE showed that the six *eae*-negative O157:H16 strains had diverse XbaI profiles and were distinct from those of *eae*-positive strains (Fig. 1). On the other hand, the profiles of the *eae*-positive O157:H16 strains were similar, and the profiles of some German and US strains were nearly identical (Fig. 1). MLST analysis indicated that all the *eae*-negative O157:H16 strains had ST344, except for a German strain that was a ST83. In contrast, all the *eae*-positive O157:H16 strains, regardless as to whether they had the *β*- or ε-*eae* allele, were ST171 (Table 1). A neighbor-joining tree constructed from MLST data for the O157:H16 strains examined from this, and the previous study (Feng *et al.*, 2010) showed that all the O157:H16 strains clustered together except for CB7926, the *eae*-negative ST83 strain (Fig. 2). We had initially suspected that perhaps CB7926 may not be an O157:H16 strain; however, it was serologically confirmed to have the O157 antigen and the H16 *fliC* allele. The results in Fig. 2 demonstrate a high level of diversity among the *eae*-negative O157:H16 strains but, all the *eae*-positive strains examined are homogeneous and within the ST171 group.

Discussion

The aEPEC group is large and comprised of heterogeneous strains and serotypes. In addition to *eae*, some aEPEC strains can carry virulence genes associated with other pathogenic *E. coli* groups, leading to speculations that aEPEC may represent different *E. coli* pathotypes that have acquired LEE by horizontal gene transfer (Bando *et al.*, 2009). Other aEPEC strains are closely related to typical EPEC strains and are thought to be EPEC that have lost the EAF plasmid. Still, some aEPEC have been found to be more closely related to EHEC and may be strains that have lost the ability to produce Stx. The *stx* genes are phage encoded and EHEC strains can lose the phage during culturing or infection (Feng *et al.*, 2001; Friedrich *et al.*, 2007), resulting in strains that only have *eae*. In addition to pathotype differences, there is considerable genetic diversity even within specific serotypes. Analysis of aEPEC strains of serotype O26:H11 and O55:H7 in Brazil showed that although strains within the same serotype clustered in the same sequence type, their PFGE profiles were very distinct (Bando *et al.*, 2009). An exception, however, may be the aEPEC strains of O157:H16 serotype, which have been isolated worldwide. In a previous study, we examined *eae*-positive and *eae*-negative O157:H16 strains isolated from the US and France and observed that the XbaI profiles of the French and a few of the US isolates were nearly identical, suggesting that the O157:H16 aEPEC strains are a homogeneous group (Feng *et al.*, 2010). To determine whether these strains are part of a larger, conserved clone that exists worldwide, we examined 45 additional O157:H16 strains isolated from various sources from four other countries. The results obtained were entirely consistent with our previous findings from the 14 US and French O157:H16 strains (Table 1). In both studies, not all O157:H16 strains had *eae*, but the majority did carry *eae* and of those, except for a few that had ß-*eae*, most had the ε-*eae* allele. None of these strains had other virulence genes, including *bfpA*, suggesting the absence of the EAF plasmid and therefore they appeared to be aEPEC. Analysis by PFGE showed that the *eae*-negative O157:H16 strains had diverse XbaI profiles but the *eae*-positive strains shared similar profiles. For example, strains

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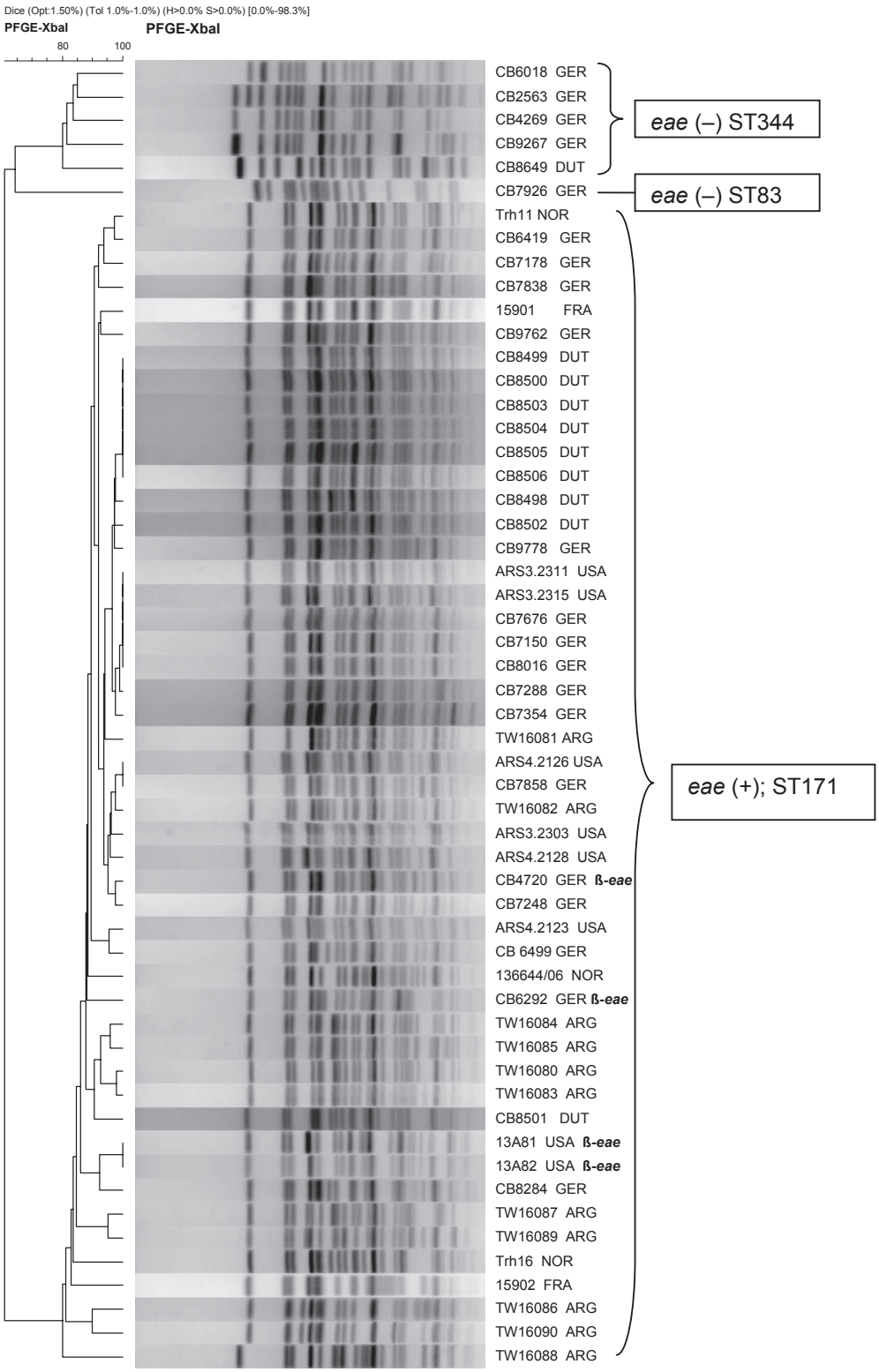

Fig. 1. PFGE of O157:H16 strains isolated from various countries around the world. The country designations are as follows: ARG, Argentina; DUT, the Netherlands; FRA, France; GER, Germany; NOR, Norway; and USA. The presence or absence of *eae* and the ST data for the strains are shown on the right. The six isolates on top are *eae*-negative strains and are ST344 except for CB7926 that was ST83. All other strains carried ε-*eae* allele, except for the four strains that carried ß-*eae* (shown in bold). All *eae*-positive strains are ST171.

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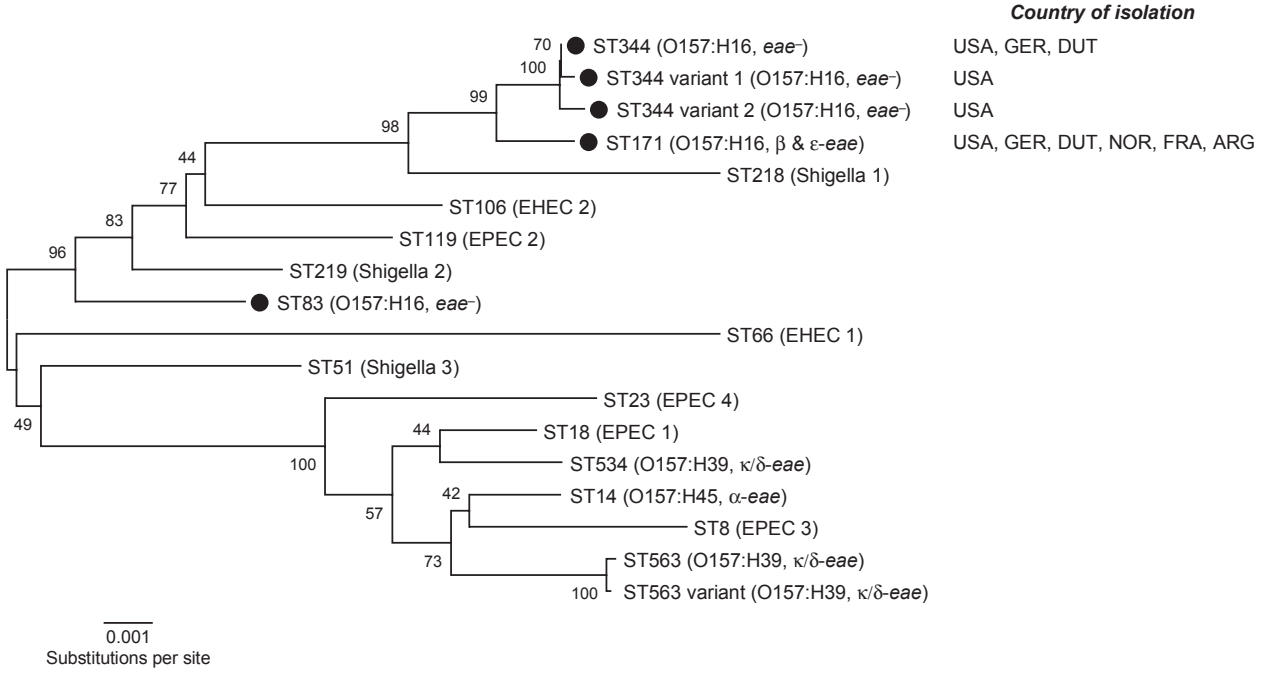

Fig. 2. Phylogenetic relationships of O157:H16 strains examined. This neighbor-joining tree was constructed from MLST data for the O157:H16 strains (shown with black dots) and displays their relationships to the major EHEC lineages, the four EPEC lineages and the three *Shigella* groups. All of the *eae*-negative O157:H16 strains, except for the German ST83 isolate, were ST344 or ST344 variant types and clustered closely together. All of the *eae*-positive O157:H16 strains, regardless of *eae* allele, were ST171.

CB4720 and CB7248 that carried ß and ε-*eae* allele, respectively, had similar profiles, and some German strains (CB7858) had nearly identical profiles as the US strains (ARS4.2126; Fig. 1). Analysis by MLST showed that the *eae*-negative strains clustered apart from the *eae*-positive strains and exhibited greater ST diversity, with most strains being ST344 and one ST83 strain. On the other hand, all the *eae*-positive strains, regardless of the *eae* allele carried, had ST171 and formed a highly conserved group. This finding is consistent with the results obtained by a study that examined the distribution of the O157-antigen biosynthesis gene among O157 serogroup strains and observed that the *eae*-positive and negative O157:H16 strains clustered in separate groups (Iguchi *et al.*, 2011). Phylogenetically, these O157:H16 aEPEC strains are distantly related to the two major EHEC lineages, the four EPEC lineages, and the three *Shigella* groups (Fig. 2). Therefore, they do not appear to be EPEC strains that have lost the EAF plasmid nor are they EHEC strains that have lost *stx*. The fact that the O157:H16 strains examined in both studies were isolated from various clinical, animal, and environmental sources from 6 different countries, suggests it is unlikely that the same strain had been broadly disseminated but rather supports the assumption that the O157:H16 aEPEC strains belong to a highly conserved clonal group that exists worldwide.

Many studies report that aEPEC are pathogenic and cause diarrhea (Trabulsi *et al.*, 2002; Gomes *et al.*, 2004; Robins-Browne *et al.*, 2004). Some aEPEC have also been implicated in bloody diarrhea, but many of these also produced enterohemolysin and genetic analysis has shown that these were most likely EHEC strains that had lost *stx* (Vieira *et al.*, 2001; Bielaszewska *et al.*, 2008). Adherence studies showed that unlike typical EPEC, which exhibit localized adherence on HEp-2 cells, aEPEC strains exhibit a localized adherence-like (LAL) pattern that is also mediated by intimin (Trabulsi *et al.*, 2002). Analysis of O157:H16 aEPEC from Argentina showed that these strains also exhibited LAL on HEp-2 cells (Bentancor *et al.*, 2010). Still, the fact that the aEPEC strains are prevalent in both patients with and without diarrhea (Gomes *et al.*, 2004), and that two of the Norwegian strains we examined, Trh11 and Trh16, were isolated from children without diarrhea, but the third was from a 15-month-old diarrhea patient that also had norovirus (Afset *et al.*, 2008), the pathogenicity of these O157:H16 aEPEC strains we examined remains uncertain. One should also bear in mind that many pathogenic *E. coli* virulence factors reside on mobile genetic elements and can be transferred. An example is the phage-encoded *stx* gene, which has been found and expressed in other enteric bacteria. So, it is not entirely inconceivable that some aEPEC strains of O157:H16 serotype, which already carry the ε-*eae* attachment factor,

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may acquire the *stx* phage via transduction and possibly become pathogenic STEC strains.

In conclusion, the O157:H16 serotype contains both *eae*-positive and *eae*-negative strains and as a whole is phylogenetically diverse and comprised of strains that have different ST and PFGE profiles. However, the aEPEC strains within the O157:H16 serotype, regardless of the *eae* allele carried, belong to a highly conserved and homogeneous group of ST-171 strains that are prevalent worldwide.

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