RESEARCH LETTER

Prevalence, characterization and clonal analysis of *Escherichia coli* O157: non-H7 serotypes that carry *eae* alleles

Peter C.H. Feng[1], Christine Keys[1], David Lacher[2], Steven R. Monday[1], Dan Shelton[3], Christine Rozand[4], Marta Rivas[5] & Thomas Whittam[6,*]

[1]Division of Microbiology, FDA, College Park, MD, USA; [2]Division of Molecular Biology, FDA, Laurel, MD, USA; [3]Agriculture Research Service, USDA, Beltsville, MD, USA; [4]Division Industrie et Environnment, bioMerieux, Marcy-l'Etoile, France; [5]Servicio Fisiopatogenia, INEI-ANLIS, Buenos Aires, Argentina; and [6]National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI, USA

Correspondence: Peter C.H. Feng, Division of Microbiology, HFS-711, FDA, 5100 Paint Branch Parkway, College Park, MD 20740, USA. Tel.: +1 301 436 1650; fax: +1 301 436 2644; e-mail: peter.feng@fda.hhs.gov

*Deceased

Received 19 November 2009; revised 8 March 2010; accepted 9 April 2010.
Final version published online 10 May 2010.

DOI:10.1111/j.1574-6968.2010.01990.x

Editor: Ian Henderson

Keywords
Escherichia coli; O157:non-H7; *eae* alleles; clonality.

Abstract

We examined O157:non-H7 strains isolated from various sources and geographical locations and found 15/57 strains to carry *eae* alleles, including α, β, ε and κ/δ, suggesting that these strains may be prevalent. All strains were serologically and genetically confirmed to be O157, but none were the H7 serotype or carried any trait virulence factors of the *Escherichia coli* O157:H7 serotype. Genetic H typing of the *eae*-positive strains showed that the α-*eae*-bearing strain was H45, while the β- and ε-*eae* strains were H16 and the κ/δ-*eae* strains were H39. The β- and ε-*eae*-bearing O157:H16 strains shared ∼90% pulsed-field gel electrophoresis (PFGE) similarity and were distinct from the other strains that had other *eae* alleles. Interestingly, an ε-*eae* O157:H16 strain isolated from meat in France shared PFGE similarity to the O157:H16 strains from water in the United States. Multilocus sequence typing showed that there is clonal diversity within the O157 serogroup, as some O157:non-H7 strains clustered with EPEC clonal groups, while others clustered within the ST-171 group of diverse strains and serotypes that had not previously included any strains from the O157 serogroup. Clonal analysis also showed that none of the *eae*-positive O157:non-H7 strains we examined were closely related to the pathogenic O157:H7 serotype.

Introduction

The O157 serogroup is best known for serotype O157:H7, the prototypic enterohemorrhagic *Escherichia coli* (EHEC) that causes food-borne illness worldwide. However, the O157 serogroup is a large and diverse group that includes many non-H7 serotypes that are commonly found in animals, foods or clinical samples. Because these strains carry the O157 antigen, they are commonly mistaken for O157:H7 during analysis. However, once they have been determined not to be O157:H7 strains, no further testing is carried out and they are either discarded or kept in the collections as partially serotyped or characterized strains.

Strains of O157:non-H7 serotypes seldom carry EHEC virulence factors. Previously, an O157:H45 strain has been reported (Machino *et al.*, 1999) to carry the *eae* gene that encodes for intimin, a virulence factor of both enteropathogenic *E. coli* (EPEC) and EHEC. However, for the most part, O157:non-H7 strains are regarded as nonpathogenic and analogous to generic *E. coli*. Recently, several O157:non-H7 strains were isolated from surface waters in Maryland (Shelton *et al.*, 2006) and found to carry the *eae* gene, suggesting that O157:non-H7 strains that carry virulence traits may be more prevalent than anticipated. In this study, we examined several O157:non-H7 strains isolated from various countries for the prevalence of virulence genes. In addition, as many of these strains were only partially characterized, we also genetically serotyped their H antigen and examined their clonal relatedness to O157:H7 as well as to other pathogenic *E. coli* groups.

Materials and methods

Bacterial strains and characterization

A total of 57 O157:non-H7 strains isolated from animals, foods, surface water and clinical samples were obtained from various countries around the world. Isolates were plated on

Sorbitol MacConkey agar with ColiComplete (BioControl, Belleview, WA) to test for sorbitol fermentation, β-galactosidase and β-glucuronidase (GUD) activity. The isolates were serotyped for the O157 and H7 antigens by latex agglutination (RIM O157:H7, Remel, Lenexa, KS) and screened for virulence factors by PCR. One multiplex PCR (Feng & Monday, 2000) tested for the presence of EHEC genes encoding shiga toxin 1 (*stx1*), *stx2*, *ehxA* (enterohemolysin) and the γ-*eae* allele. The PCR also detected the presence of the +93 *uidA* (GUD) single nucleotide polymorphism (SNP) that is found exclusively in O157:H7. Strains were also tested by multiplex PCR (Monday *et al.*, 2007) for the O157 antigen gene and other *eae* alleles. There are over 15 known *eae* alleles, of which γ-*eae* is found mostly in O157:H7, O55:H7 and a few other serotypes, while other EHEC and EPEC strains are known to carry various other *eae* alleles. Those strains that were found to carry *eae* were further evaluated by PCR with *eae* allele-specific PCR primers (unpublished) and for the presence of the *bfpA* gene (Gunzburg *et al.*, 1995) that encodes for the bundle forming pilus, a virulence factor in EPEC. Genetic H serotyping was performed by PCR amplification, sequencing and comparative BLAST analysis at GenBank of *fliC* (Lacher *et al.*, 2007), the structural gene that encodes for flagella.

Pulsed-field gel electrophoresis (PFGE)

XbaI-digested genomic DNA was analyzed on a 1% SeaKem Gold agarose gel in 0.5 × TBE buffer, pH 8.2, at 14 °C 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 using the BIONUMERICS fingerprinting software (Applied Maths, St-Martens-Latem, Belgium).

Multilocus sequence typing (MLST)

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

Results

Strain characterization

All the isolates exhibited β-galactosidase activity indicative of coliforms with 55 of 57 strains having GUD activity that is typical for *E. coli*. All strains reacted with anti-O157 latex reagent and were genetically confirmed to have O157 genes, but no strains reacted with the anti-H7 latex reagents. None of the strains had *stx1* or *stx2*, and so they were not Shiga toxigenic *E. coli* (STEC) nor did they have enterohemolysin (*ehxA*). Similarly, none of the strains had the +93 *uidA* SNP or the γ-*eae* allele characteristic of O157:H7. However, 15/57 strains had other *eae* alleles, which were determined to be of the α, β, ε and κ/δ isotypes. Only one strain had the *bfpA* gene (Table 1). The 15 *eae*-positive strains, consisting of six strains from water in Maryland, three from clinical samples in the United States, two from meat in France and four from food and clinical samples from Argentina, were further characterized. The H type of a few of these strains had been determined previously, but most were unknown (Table 1). Genetic H typing confirmed the H serotype of the known strains and identified the H type of all the isolates. The numbers of strains carrying respective H types are: 10, H16; 4, H39 and 1, H45 (Table 1). There were eight O157:H16 strains, six from water in Maryland and two from ground meats in France that had identical traits, including the ε-*eae* allele (Table 1).

Molecular subtyping

The 15 *eae*-positive strains were subjected to molecular subtyping. The eight ε- and two β-*eae*-bearing O157:H16 strains shared ~90% similarity in PFGE profiles, which were distinct from those of other *eae*-carrying O157 strains (Fig. 1). The profile of the O157:H45 strain that carried α-*eae* shared little similarity to the other O157:non-H7 strains. Similarly, some diversity was also observed among the four κ/δ-*eae*-positive O157:H39 strains, except for strains 7797 and 7798, which shared ~90% profile similarity (Fig. 1).

There were four other *eae*-negative O157:H16 strains, but, because this was the predominant serotype among the isolates examined, they were also included in the subtyping studies. The PFGE profiles of the *eae*-positive O157:H16 strains shared only ~70% similarity to the four strains that did not carry *eae* (Fig. 2). Interestingly, the profiles of the six ε-bearing O157:H16 strains from water in Maryland shared ~90% similarity to one of the ε-bearing O157:H16 strains isolated from ground meats in France (Fig. 2).

Analysis by MLST showed that the α-*eae*-bearing O157:H45 strain had ST-14 and the four κ/δ-bearing

Table 1. Summary of the analytical results of O157 non-H7 strains

Source	Strain	Sor	GUD	O157	H7	*uidA*	stx_1	stx_2	*ehxA*	H	[h]	eae	ST
USA – water	3.2303	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – water	3.2311	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – water	3.2315	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – water	4.2123	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – water	4.2126	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – water	4.2128	+	+	+	–	–	–	–	–	NT	H16	ε	171
FRA – meat	15 901	+	+	+	–	–	–	–	–	NT	H16	ε	171
FRA – meat	15 902	+	+	+	–	–	–	–	–	NT	H16	ε	171
USA – clinical	3003	+	+	+	–	–	–	–	–	H45	H45	α	14
USA – clinical	3006	–	+	+	–	–	–	–	–	H16	H16	–	New2
USA – clinical	13A80	–	+	+	–	–	–	–	–	H16	H16	–	344
USA – clinical	13A81	–	+	+	–	–	–	–	–	H16	H16	β	171
USA – clinical	13A82	–	+	+	–	–	–	–	–	H16	H16	β	171
USA – meat	P11	–	+	+	–	–	–	–	–	H16	H16	–	344
USA – meat	7123	–	–	+	–	–	–	–	–	NM	H16	–	New3
ARG – HC	7793	+	+	+	–	–	–	–	–	NT	H39	κ/δ	534
ARG – AS	7796	+	+	+	–	–	–	–	–	NT	H39	κ/δ	563
ARG – AS	7797	–	+	+	–	–	–	–	–	NT	H39	κ/δ	New1
ARG – D	7798	–	+	+	–	–	–	–	–	NT	H39	κ/δ	New1

FRA, France; ARG, Argentina; HC, hemorrhagic colitis; AS, asymptomatic; D, diarrhea.
Column headings are: Sor, sorbitol fermentation; GUD, β-glucuronidase activity; O157 and H7, O157 and H7 latex agglutination; *uidA*, +93 *uidA* SNP; stx_1 and stx_2, shigatoxin 1 and 2; *ehxA*, enterohemolysin; H, original H type; [h], H genotype; *eae*, intimin allele; ST, multilocus sequence type.
NT, not serotyped.

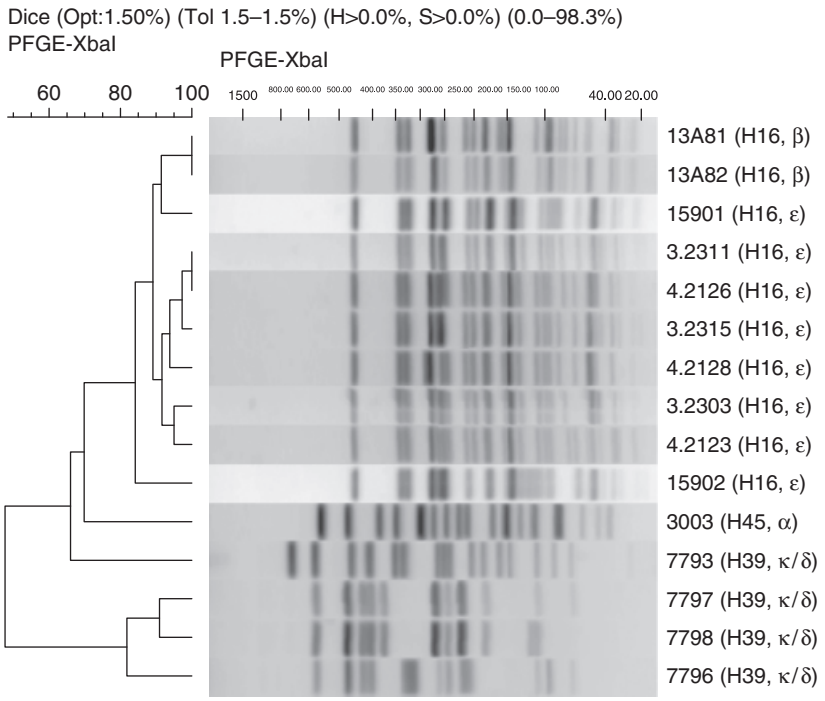

Fig. 1. Pulsed-field gel electrophoresis of XbaI-digested DNA from O157:non-H7 strains with various *eae* alleles. Strain designation with the H genotype and *eae* alleles are shown in parentheses. The unweighted pair-group method with an arithmetic mean dendrogram was generated in BIONUMERICS software using the Dice coefficient with a 1.5% lane optimization and 1.5% band position tolerance. The scale above the dendrogram indicates percent similarity.

O157:H39 strains were ST-534, ST-563 or a new ST that was a variant of ST-563. The eight ε-*eae* and two β-*eae*-positive O157:H16 strains all had ST-171, while the four *eae*-negative O157:H16 strains were either ST-344 or had new ST that are variants of ST-344 (Table 1). Using the MLST data, we examined the clonal relationship between these O157:non-H7 strains, the pathogenic O157:H7 serotype and other reference EHEC, EPEC and *Shigella* groups. The neighbor-

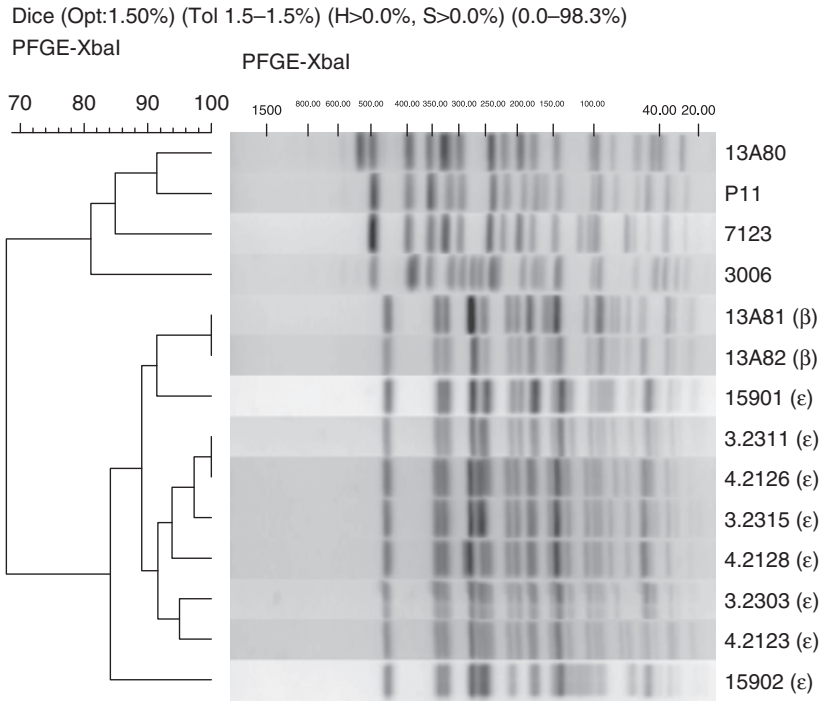

Fig. 2. Pulsed-field gel electrophoresis of XbaI-digested DNA from *eae*-positive and -negative O157:H16 strains. Strain designations with the *eae* alleles, if present, in parentheses are shown on the right.

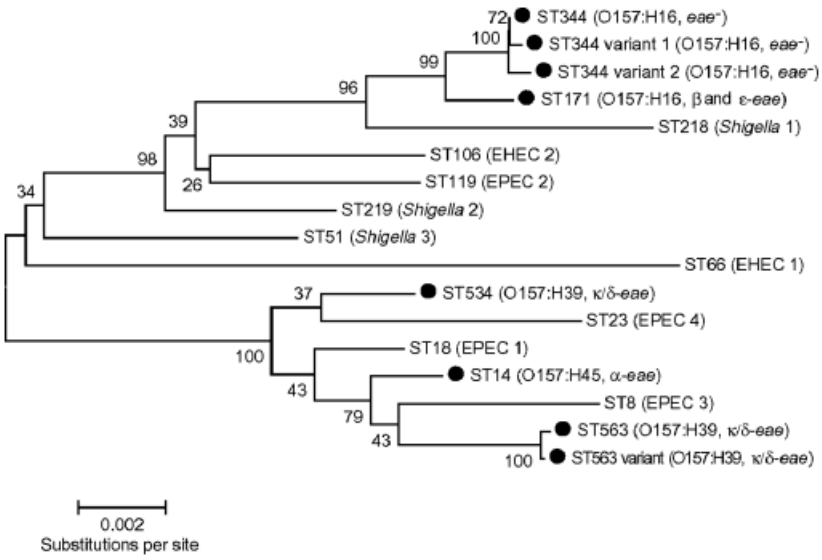

Fig. 3. Neighbor-joining tree constructed from MLST data obtained from the O157:non-H7 strains (black circle) shown in Table 1 and their relation to the representative STs for the two main EHEC lineages, the four EPEC lineages and the three *Shigella* groups. Bootstrap confidence values based on 500 replications are given at the internal nodes.

joining tree showed that the O157:H16 strains, including the *eae*-negative strains, clustered together and that the eight ε-*eae*- and two β-*eae*-positive strains are very closely related, if not identical (Fig. 3). All O157:H16 strains, however, are very distant to the prototypic O157:H7 strains that are in the EHEC 1 clonal group. Similarly, the other *eae*-positive O157:non-H7 strains were not related to the EHEC clonal groups, but instead clustered, not closely, with the EPEC clonal groups.

Discussion

Although strains of the O157:non-H7 serotype do not usually carry virulence genes, we examined several strains isolated from different sources and geographical areas worldwide and found that 15/57 strains of different H types carried various *eae* alleles. The *eae* gene is located on the Locus for Enterocyte and Effacement (LEE) pathogenicity island that is found mostly in EPEC and EHEC strains. However, it has been

shown that LEE or components of LEE can become involved in horizontal transfer events (Castillo *et al.*, 2005); hence, it is conceivable that *eae* genes can be laterally transferred from these pathogenic groups to other *E. coli* strains.

Strains of *E. coli* that carry *eae*, but no other EPEC virulence factors such as *bfpA* are often designated as atypical EPEC and some of these have been found in association with endemic diarrhea in children in developing countries. One study examined 43 atypical EPEC strains and found huge genetic diversity among these strains, but the study did not include any strains from the O157 serogroup (Bando *et al.*, 2009). We have found that atypical EPEC of O157 serotype with various H types also exists and to carry various *eae* alleles. Among the 15 *eae*-positive O157:non-H7 strains isolated, eight carried the ε-*eae* allele, which was originally found in O103:H2 (Oswald *et al.*, 2000), an STEC serotype that has been associated with infections in Europe (Karama *et al.*, 2008). The ε-*eae* allele has since been found in strains of the O8, O11, O45, O121, O165 (Nielsen *et al.*, 2004) serogroups, and, more recently, in the O157 serogroup. One study (Kozub-Witkowski *et al.*, 2008) examined stool samples from children with diarrhea in Germany and found two strains of O157:H16 that carried ε-*eae*. Another study (Afset *et al.*, 2008) showed that atypical EPEC strains that carry *eae*, but not *bfpA* or other virulence factors are frequently isolated from both healthy and children with diarrhea. Two such O157:H16 strains isolated from nondiarrhea fecal samples carried ε-*eae* and shared 90% similarity in PFGE profiles. Consistent with those findings, many of the O157:H16 strains we examined also carried ε-*eae* and had similar PFGE profiles, suggesting that some strains within this serotype may be conserved.

The great similarity in PFGE profiles among the *eae*-bearing O157:H16 strains is supported by the MLST data, which showed all these strains to be ST-171 and, therefore, in the same clonal group (Fig. 3). The *eae*-negative O157:H16 strains showed more diversity in PFGE profiles that also differed from those of *eae*-positive O157:H16 strains. This is also reflected in MLST data, as these *eae*-negative strains were either ST-344 or ST-344 variants. Although ST-344 is a rare ST, it nevertheless clustered in the vicinity of ST-171 with high bootstrap support (Fig. 3). In the *Ec*MLST database (STEC Center, Michigan State University), strains with ST-171 are fairly common and include the *E. coli* K-12 strain MG1655; however, it had not previously included any strains from the O157 serogroup. Moreover, clonal analysis demonstrated that strains with ST-171 are distant from both the EHEC 1 clonal group that consists of the prototypic O157:H7 strains or the EHEC 2 clonal group that includes other prominent EHEC pathogens of O26 and O111 serotypes (Fig. 3).

The PFGE of the α-*eae*-bearing O157:H45 strain (3003) was distinct from that of the other O157 strains. This difference was also reflected in MLST, as this strain was the only isolate that had ST-14 (Fig. 3). Strains with ST-14 have been observed previously (Lacher *et al.*, 2007) and included EPEC strains of the O157:H45 serotype that carried α-*eae* and *bfpA* and was implicated in a large EPEC outbreak in Japan (Machino *et al.*, 1999).

Strain 3003 in our study had similar virulence traits and ST, suggesting that it is an EPEC strain.

The four κ/δ-positive O157:H39 strains showed more diversity in PFGE profiles and ST. The three strains that shared ~80% similarity in PFGE profiles (Fig. 2) were ST-563 or a variant of ST-563 (Table 1) and clustered together (Fig. 3). Strain 7793 had a distinct PFGE profile, had ST-534 and did not cluster with the other three strains (Fig. 3). All four of these strains were very distant from the EHEC clones and, instead, scattered among the various EPEC clonal groups, suggesting that they are more related to EPEC.

These results show that even though all these *eae*-positive O157:non-H7 strains are within the O157 serogroup, the fact that some clustered with the common ST-171 clonal group, while others clustered with EPEC groups, indicates that a large clonal diversity also exists within the O157 serogroup. This is consistent with the genetic diversity reported for the other atypical EPEC strains (Bando *et al.*, 2009). Similarly, and in agreement with the findings of Toth *et al.*, 2008, none of the *eae*-positive O157:non-H7 strains we examined were closely related to the best-known representative of the serogroup, namely the O157:H7 serotype. The latter observation also supports the existing concept that O157:H7 strains are in a unique clonal group, which evolved distinctively from other *E. coli* and pathogenic *E. coli* groups (Feng *et al.*, 1998).

Lastly, it was puzzling that the six ε-*eae*-bearing O157:H16 strains isolated from surface waters in Maryland and the two ε-*eae*-bearing O157:H16 strains isolated from ground meats in France had identical phenotypic traits, had ST-171 and shared similar PFGE profiles. This may be coincidental or it is possible that these ε-*eae*-positive O157:H16 strains may be representatives of a widespread clone that has simply gone unreported. Alternatively, there is evidence to support that bacterial pathogens can be dispersed to new geographical locations by migratory birds (Koehler *et al.*, 2008; Tsiodras *et al.*, 2008). Studies showed that wild birds may become infected from farm animals or vice versa as evidenced by the isolation of STEC strains from starlings that had identical traits and PFGE profiles with cattle isolates from the same farms (Nielsen *et al.*, 2004). Similarly, a survey of the microbial flora of birds in Japan found 39 bird isolates of *E. coli* that were deemed atypical EPEC because they only carried *eae*, including ε-*eae*, but no other virulence factors. These isolates also had many *E. coli* O serotypes, but did not include any O157 strains (Kobayashi *et al.*, 2009). These findings show that wild birds can be carriers of STEC and

EPEC, and so, it is perhaps plausible that the ε-*eae*-bearing O157:H16 strains in Maryland may have been disseminated by migratory birds to France or vice versa.

In conclusion, 15/57 strains of O157:non-H7 serotypes isolated from different sources and geographical regions were found to carry various *eae* alleles, suggesting that these strains may be fairly prevalent. Many of the O157:H16 strains found, including strains that were isolated from water in the United States and from meat in France, carried the ε-*eae* allele, shared similar PFGE profiles and had ST-171, a common type in the *Ec*MLST database that, until now, had not included any strains from the O157 serogroup. Clonal analysis also showed that none of these *eae*-positive O157:non-H7 strains were closely related to the pathogenic O157:H7 serotype and that there is a large genetic diversity within the O157 serogroup.

Dedication

The authors would like to dedicate this work to the memory of Dr Thomas S. Whittam.

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