BACTERIAL PATHOGENICITY

Isolation from a sheep of an attaching and effacing *Escherichia coli* O115:H[−] with a novel combination of virulence factors

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Attaching and effacing (AE) lesions were observed in the caecum, proximal colon and rectum of one of four lambs experimentally inoculated at 6 weeks of age with *Escherichia coli* O157:H7. However, the attached bacteria did not immunostain with O157-specific antiserum. Subsequent bacteriological analysis of samples from this animal yielded two *E. coli* O115:H[−] strains, one from the colon (CO) and one from the rectum (RC), and those bacteria forming the AE lesions were shown to be of the O115 serogroup by immunostaining. The O115:H[−] isolates formed microcolonies and attaching and effacing lesions, as demonstrated by the fluorescence actin staining test, on HEp-2 tissue culture cells. Both isolates were confirmed by PCR to encode the epsilon (ε) subtype of intimin. Supernates of both O115:H[−] isolates induced cytopathic effects on Vero cell monolayers, and PCR analysis verified that both isolates encoded EAST1, CNF1 and CNF2 toxins but not Shiga-like toxins. Both isolates harboured similar sized plasmids but PCR analysis indicated that only one of the O115:H[−] isolates (CO) possessed the plasmid-associated virulence determinants *ehxA* and *etpD*. Neither strain possessed the *espP*, *katP* or *bfpA* plasmid-associated virulence determinants. These *E. coli* O115:H[−] strains exhibited a novel combination of virulence determinants and are the first isolates found to possess both CNF1 and CNF2.

Introduction

Pathogenic *Escherichia coli* may be subdivided according to the possession of virulence factors responsible for the varied clinical manifestations that characterise *E. coli* infections. Many virulence factors may have been acquired by evolutionary mechanisms involving the horizontal transfer of genetic material, such as the insertion of mobile elements. Evidence for this includes the locus for enterocyte effacement (LEE), which is associated with the formation of attaching and effacing (AE) lesions, and which has a significantly lower GC ratio than the majority of the *E. coli* genome, indicating a distant evolutionary source [1]. Other

factors such as the Shiga-like toxin genes (*stx1* and *stx2*) of Shiga toxin-producing *E. coli* (STEC) are normally encoded by bacteriophages [2, 3]. Furthermore, enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* encode many virulence factors within large plasmids. Often these gene clusters are flanked by insertion sequence (IS) elements associated with transposition [4] and may be located within disrupted genes. Thus, there is good evidence for gene transfer in the *E. coli* gene pool and therefore there is likely to be considerable genetic and phenotypic heterogeneity within and between the various pathotypes of *E. coli*. Several studies have noted that certain *E. coli* isolates are not readily classified on the basis of virulence factors alone. For example, eight *stx2*-positive O111:H2 strains isolated from an outbreak of haemolytic uraemic syndrome (HUS) in man showed atypical aggregative adhesion to HEp-2 cells, in contrast to the localised adhesion which is normally associated with EHEC isolates and which might have been anticipated [5]. EPEC isolates may also express

Received 6 Dec. 2001; accepted 16 May 2002.

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the heat-stable enterotoxin EAST1 (*astA*) or cytolethal-distending toxin (CLDT) in addition to causing the AE phenotype [6–8]. While novel combinations of virulence factors may be rapidly detected in established pathogenic serovars of *E. coli* isolated from symptomatic patients, when potentially pathogenic strains arise in serovars not normally associated with disease, they may be overlooked. This may be of some importance in animal reservoirs where a strain may not cause clinical signs in the healthy adult animal, but may be of significance as a possible pathogen in the compromised animal, or in another species such as man. A clear example of this is the recent emergence of the human pathogen *E. coli* O157:H7, with cattle and sheep as recognised reservoirs [9].

This report describes the recovery of novel attaching and effacing *E. coli* (AEEC) O115 strains from a lamb that had been experimentally inoculated with *E. coli* O157:H7 18 days previously, at 6 weeks of age. This is the first AEEC to be described that causes natural lesions in a weaned sheep. As there is concern regarding the emergence and dissemination of new types of *E. coli* as potential food-borne pathogens, the genotype and phenotype of these ovine AEEC isolates are described in detail.

Materials and methods

Bacterial strains

The strains used in this study, including those reported previously, are summarised in Table 1 [10–16].

Animal procedures

The procedures have been described in detail previously [17–19]. Briefly, the affected animal was one of four conventionally reared 6-week-old weaned cross-bred lambs, inoculated orally with 10^9 cfu of a single human-derived strain of *E. coli* O157:H7 (140065 Nal^r) [19]. All lambs were maintained in a closed pen and received water and pelleted complete feed *ad libitum*. Faecal samples were taken daily from each animal for bacteriological analysis. Tissues were taken under terminal anaesthesia for pathological examination and bacteriological analysis.

All procedures complied with the Animals (Scientific Procedures) Act 1986 and were performed under Home Office Licence 70/4987.

Light microscopy

Tissues were processed for staining as described in previous studies [17–19]. Briefly, tissues were fixed in neutral buffered formalin 10% immediately after excision and 4- μ m paraffin wax-embedded sections were prepared and stained with haematoxylin and eosin (H&E) for light microscopy. Selected sections were immunostained by an indirect peroxidase-antiperoxidase (PAP) stain, as described previously [18, 19], with polyclonal primary antisera (Veterinary Laboratories Agency, Weybridge) specific for *E. coli* O somatic antigens.

Transmission electron microscopy

From one AE lesion identified by light microscopy, adjacent tissue was excised from the wax embedded specimen. This was de-waxed, post-fixed with osmium tetroxide, embedded in epoxy resin, and thin sections were prepared and examined in a Philips 201 electron microscope.

Bacterial recovery and identification

Faecal and tissue samples were processed as described previously [17–19] for bacteriological analysis by direct plating and immunomagnetic separation (IMS). Enriched cultures and washed beads (Dynal) were spread on to CHROMagar O157 plates (CHROMagar) supplemented with nalidixic acid 15 μ g/ml. Lilac-coloured colonies were tested by O157-specific latex agglutination (Oxoid) and representative O157 and non-O157 (latex agglutination-negative) isolates were stored on Dorset's egg slopes for future analysis. Both serotyping and toxin typing of the isolates were performed at the *Salmonella* and *E. coli* Serotyping Section, Veterinary Laboratories Agency (Weybridge), with standard conditions and reagents.

PCR analysis

The PCR primers used in this study are defined in Table 2 [13, 20–23]. Template DNA was purified from

Table 1. Serotype, strain and genotype of isolates

Serotype	Strain	Genotype	Reference
O115:H ⁻	CO	<i>eaeA</i> (ϵ), CNF1, CNF2, <i>astA</i> , <i>ehxA</i> , <i>etpD</i>	Present study
O115:H ⁻	RC	<i>eaeA</i> (ϵ), CNF1, CNF2, <i>astA</i>	Present study
O157:H7	A84	<i>stx1</i> , <i>stx2</i> , <i>eaeA</i> (γ), <i>ehxA</i> , <i>espP</i> , <i>etpD</i> , <i>katP</i>	10
O103:H2	E42724	<i>eaeA</i> (ϵ)	11
O127:H6	E2348/69	<i>eaeA</i> (α), <i>bfpA</i>	12
O86:H34	ICC95	<i>eaeA</i> (δ)	13
O26:H11	EC1537	<i>eaeA</i> (β)	14
O149:K91	Abbotstown	<i>astA</i>	15
O2:H1	BM2-1	CNF1	16
O15:H21	S5	CNF2	16

Table 2. Primers used to identify *E. coli* virulence factors

Primer	Sequence	PCR product (bp)	Accession no./reference no.
stx1F	GCCAGATGGAAGAGTCCGTGGGATTACGC	178	Z36900
stx1R	CACAATCAGGCGTCGCCAGCGCACTTGCT		
stx2F	CCACATCGGTGCTGTATTAAACACACC	374	X61283
stx2R	GCAGAACTGCTCTGGATGCATCTCTGGTC		
eaeAF(mid)	TGCGGCACAACAGGCGA	628	Z11541
eaeAR(mid)	CGGTGCGCCGACCAGGATTC		
int- α F	CCTTAGGTAAGTTAAGT	539	[13]
int-Ru	TTTATTTGCAGCCCCCAT		
int- δ F	TACGGATTTTGGGGCAT	544	[13]
int-Ru	TTTATTTGCAGCCCCCAT		
int γ F	CGTTGAAGTCGAGTACGCCA	957	X60439
int γ R	TTCTACACAAACCGCATAGA		
int β F	CAACAGCGAAGGCAGATGGT	788	U62656
int β R	GAAAGCATTCTTATTGTTTACTCC		
eaeAF(mid)	TGCGGCACAACAGGCGA	2135	AF116899
int ϵ R	AGCTCACTCGTAGATGACGGCAAGCG		[20]
ehxC	GCTATGGGCTGTCTCTCCTCTG	1779	X86087
chxA	TGTCTTGCGTCATATCCATTCTCA		
etpDF	CGTCAGGAGGATGTTTACG	1061	Y09824
etpDR	CGACTGCACCTGTTCTTGATTA		
espPF	TTGCGAAAAATGGCGGAACTC	1146	X79542
espPR	GCTGACGGGGCATTGACTG		
katPF	CTTCCTGTTCTGATTCTTCTGG	2125	[21]
katPR	AACTTATTCTCGCATCATCC		
CNF1F	GGCGACAAATGCAGTATTGCTTGG	552	[22]
CNF1R	GACGTTGGTTGCGGTAATTTGGG		
CNF2F	GTGAGGCTCAACGAGATTATGCACTG	839	[22]
CNF2R	CCACGTTCTTCTTCAGTTGTTCCCTC		
astAF	CCATCAACACAGTATATCCGA	111	[23]
astAR	GGTCGCGAGTGACGGCTTTGT		
bfpAF	AAATCCTCCATGAAGCCAGT	1088	U27184
bfpAR	TTCTTCCGACTGGGCGCAA		

bacterial strains by phenol/chloroform extractions. Amplification was performed in a total volume of 50 μ l containing template DNA (500 ng), 25 pmol of each primer, 100 μ M of each dNTP, 5 μ l of 10 $\times$ PCR buffer, 20 mM MgCl₂ and 2 units of *Taq* DNA polymerase (Promega). After an initial denaturation step of 5 min at 94°C, 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension for 2 min at 72°C were performed. The reaction was completed with a final extension step of 5 min at 72°C. PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining.

Plasmid profile and Southern blot analysis

The plasmid profiles of the O115:H⁻ isolates were determined by standard methods as described previously [24]. DNA was transferred to nitrocellulose filters and probed with chemiluminescent-labelled probes (Amersham Pharmacia Biotech) derived from *ehxA*, CNF1 and CNF2 PCR amplicons as described in the manufacturer's instructions.

Bacterial adhesion to HEp-2 cells

Approximately 10⁷ cfu of bacteria were added to HEp-2 cells and incubated for 6 h by the method of Donnenberg and Nataro [25]. Non-adherent bacteria were removed by

washing with diluent. Bacterial microcolony formation was observed by scanning electron microscopy of HEp-2 cells that had been seeded on to 13-mm diameter glass coverslips and fixed in glutaraldehyde 3% v/v. The fluorescence actin staining (FAS) test was performed as described previously [26].

Results

Clinical and macroscopic findings

The affected lamb produced soft faeces from 5 days post inoculation (pi) until it was killed at 18 days pi. Macroscopic abnormalities were not seen in the tissues when sampled. The other three lambs in the group, in which lesions and O115 bacteria were not detected, also produced soft faeces from 5 days pi.

Bacterial recovery and identification

E. coli O157:H7 140065 Nal^r strain was recovered from the colon contents of the affected animal [19]. Two further isolates were recovered by direct plating from the colon and rectum, respectively, of the same lamb. These isolates had the lilac colony colour on CHROMagar O157 which is typical of *E. coli* O157:H7 but they failed to agglutinate in the O157 latex test. They were confirmed as serotype O115:H⁻ and toxin

typing by analysis of cytopathic effect on Vero cells indicated that they expressed cyto-necrotising factor (CNF) toxin but not Shiga-like toxin. These isolates were designated O115:H⁻ (CO) and O115:H⁻ (RC) for the colon and rectum isolates, respectively.

Light microscopy

Multiple foci of bacteria closely adherent to the mucosal surface, which appeared to be excavated in places (Fig. 1), were seen on the luminal epithelium in the caecum, the proximal loop of the ascending colon and the rectum. Bacteria associated with the lesions did not immunostain for the O157 antigen, but did stain positively with an O115 antiserum (Fig. 2), which was selected following recovery by culture of this serogroup.

Transmission electron microscopy

In the lesion identified in wax-embedded tissue, bacteria were observed to be closely adherent to the host cell membrane, and frequently on pedestals, in areas of microvillus effacement (Fig. 3).

PCR analysis

When PCR primers specific for *stx1*, *stx2* and the conserved region of *eaeA* were used in a multiplex PCR, the O115:H⁻ isolates gave positive results for the presence of the *eaeA* gene only. With additional oligonucleotide primers for the specific amplification of each of the five intimin (*eaeA*) subtypes thus far described, the O115:H⁻ isolates were determined to be of the ϵ subtype. The RC O115:H⁻ isolate gave negative results in the PCR analyses for the EHEC plasmid markers *ehxA* (enterohaemolysin), *katP* (catalase/peroxidase), *espP* (serine protease) and the type II secretion system (*etp*). However, the CO isolate gave

Fig. 1. Caecum, attaching-effacing lesion. Bacteria are closely adherent to enterocytes at the intestinal surface. Some bacteria-coated enterocytes are detaching (arrows). Haematoxylin and eosin, bar = 15 μ m.

Fig. 2. Caecum, attaching-effacing lesion. Immunolabelled mass of *E. coli* O115 organisms (arrows), adhering to the luminal aspect of an enterocyte and extending into a cleft. Immunoperoxidase, bar = 10 μ m.

positive PCR amplification with the primer pairs that amplify *ehxA* and *etpD*. PCR analyses of both isolates indicated the possession of both CNF1 and CNF2. Both isolates were also positive for EAST1 (*astA*), but were negative for the amplification of bundle-forming pili (*bfpA*). Table 1 summarises the characteristics of the *E. coli* O115:H⁻ isolates in comparison with other reference strains used as controls in the PCR analyses.

Plasmid profile and Southern blot analysis

Both the *E. coli* O115:H⁻ isolates had identical plasmid profiles, each harbouring a high and low mol. wt plasmid (Fig. 4). The low mol. wt plasmid appeared to be a high copy number plasmid, as indicated by the intensity of ethidium bromide staining. The predominant DNA species of the low mol. wt plasmid was visible as covalently closed circular (ccc) DNA with a secondary species visible as a slower migrating DNA band, most probably the open circular (oc) form. Southern hybridisation analysis of the plasmid profiles from each isolate with labelled CNF1, CNF2 and *ehxA* probes in separate experiments indicated that all three probes hybridised with the residual chromosomal DNA of O115:H⁻ (CO) but with neither of the two plasmids from this strain. Both the CNF1 and CNF2 probes hybridised with chromosomal DNA from strain RC, but the *ehxA* probe did not hybridise with any of the genetic material derived from the RC isolate (Fig. 5).

Bacterial adhesion to HEp-2 cells

The two O115:H⁻ isolates showed localised adherence to HEp-2 cells, forming small, sparse microcolonies after incubation for 6 h (Fig. 6) and were positive in the FAS test for the detection of AE lesion formation.

Fig. 3. Caecum, attaching-effacing lesion. Host enterocytes (H) have lost their microvillous surface and bacteria (B) are intimately attached to the plasma membrane. Some pedestals (P) have formed. Transmission electron micrograph, bar = 750 nm.

Fig. 4. Plasmid profiles of O115:H⁻ colon (1) and rectal (2) isolates showing high and low mol. wt plasmids in both open circular (oc) and covalently closed circular (ccc) forms, associated with each isolate.

Discussion

This report describes the recovery of two *E. coli* O115:H⁻ isolates that were associated with AE lesions [27] in a conventionally reared lamb. Although the lamb was experimentally inoculated with O157:H7, a known inducer of AE lesions in neonatal lambs [17], the lesions that were observed were unequivocally induced by O115 bacteria, with no evidence of associated O157 antigen. It is not known whether the presence of O157:H7 in the gastrointestinal tract was a predisposing factor for AE lesion formation by the O115:H⁻ strains.

The recovery of the O115:H⁻ organisms was serendipitous in that bacteriological analysis of samples from the animal was intended to select for the O157:H7 strain 140065 NaI^r by plating on CHROMagar O157 supplemented with nalidixic acid. Whether the strains were resistant to nalidixic acid before plating is unknown but, if not, sufficiently high numbers must have been plated in order to select and recover spontaneous resistant mutants. The fact that the O115 strains had the typical lilac appearance of O157:H7 organisms on CHROMagar O157 and grew despite two selective methods to eliminate non-O157 organisms (CHROMagar O157 and nalidixic acid supplementation), emphasises the importance of serological confirmation of isolates believed to be O157. The occurrence of *E. coli* strains that mimic the growth and appearance of *E. coli* O157:H7 on CHROMagar O157 has been established previously [28].

The induction of AE lesions by *E. coli* O115:H⁻ in sheep is a novel observation. The lamb was conventionally reared and therefore it suckled from its mother and was exposed to natural challenge by environmental bacteria before the O157:H7 experimental inoculation at 6 weeks of age. Therefore, it is most likely that the O115:H⁻ isolates were of natural origin.

To the best of our knowledge the combinations of virulence factors harboured by the two O115:H⁻ isolates have not been described previously. Several studies have demonstrated that *astA* may be present in human EPEC isolates in addition to *eaeA*. In one study, only 14 (22%) of 65 EPEC isolates examined were *astA*-positive [6], but in an outbreak of diarrhoeal disease, an O39:H⁻ isolate that was *astA*- and *eaeA*-positive was isolated from >100 cases [29]. An O111 isolate that was also *astA*- and *eaeA*-positive was implicated in an outbreak of diarrhoea in Finnish adults and schoolchildren [30]. Interestingly, EPEC strain 2348/69, the prototype EPEC strain used for volunteer studies, contains two copies of the *astA* gene, one in the chromosome and one in the EPEC adherence factor (EAF) plasmid, which also encodes bundle-forming pili on the *bfp* operon [6].

The two O115:H⁻ isolates described in this study are

Fig. 5. Southern blot analysis of O115:H⁻ colon (1) and rectal (2) isolates probed with CNF1 (A), CNF2 (B) and *ehxA* (C) PCR products showing the hybridisation of the residual chromosomal DNA but neither the large nor small (oc) plasmids.

Fig. 6. Microcolony of O115:H⁻ rectal isolate on HEp-2 cells after incubation for 6 h. Scanning electron micrograph, bar = 2 μ m.

eaeA-positive, and *bfpA*- and *stx*-negative and as such these isolates may be described as 'atypical' EPEC, as they lack the EAF plasmid [31]. Several case-control studies including *eaeA*-positive EPEC indicated that EAF plasmid-positive isolates were significantly associated with diarrhoea [32, 33]. However, atypical EPEC have also been reported as being the sole causative agent of acute diarrhoeal disease in several countries [32, 34–36].

In addition to *astA*, CNF toxin activity was demonstrated by the Vero cell assay and both isolates were confirmed as being positive for both CNF1 and CNF2. The possession of both CNF1 and CNF2 has not previously been noted in *E. coli*. Hybridisation analysis indicated that both the CNF1 and CNF2 genes were chromosomally encoded by both O115:H⁻ isolates. While CNF1 is normally chromosomally encoded [37],

CNF2 has hitherto only been reported to be encoded by a transferable F-like plasmid, Vir [38]. CNF1 and CNF2 toxins are expressed by *E. coli* strains isolated from intestinal infections and induce multinucleation of several eukaryotic cell types in culture [39]. CNF1 production has been demonstrated in enterotoxigenic *E. coli* (ETEC) strains from cases of diarrhoea, and from *E. coli* strains associated with urinary tract infections and bacteraemia in man, extra-intestinal infections in cats and dogs, enteritis in piglets, and diarrhoea or bacteraemia, or both, in calves [40]. CNF2-producing strains have been isolated from calves and lambs with diarrhoea or bacteraemia, or both [40]. Experimental inoculation of an *E. coli* strain with proven CNF2 toxin expression into colostrum-restricted newborn calves resulted in intestinal colonisation, causing long-lasting diarrhoea and bacteraemia with localisation in various internal organs [41].

To date, at least five different intimin subtypes have been described from EPEC and EHEC isolates [20] and their distribution may account for the ability of the intimin-producing strains to colonise different tissues and hosts. The ϵ intimin subtype is the most recent form of the *eaeA* gene to be described [20]. This intimin subtype has been identified among several STEC serotypes of humans and cattle, with the majority (19 of 25) being serogroup O103 [20]. Only 2 of the 25 ϵ intimin isolates (both O103:Hnd) were from non-human (cattle) sources [20]. In man, the EHEC O103:H2 serotype has been associated with HUS in Europe [42–44], the USA [45] and Canada [46], although it is only rarely associated with diarrhoeagenic disease in animals [47]. Clonal analysis of the pathogenic STEC serogroup O103 determined that it has a unique profile of virulence traits, including a distinct *eaeA* sequence [47], that differ from those of other STEC serotypes including O157:H7 [48].

AE lesions were formed on HEp-2 cells by the O115 isolates in the present study, but the lesions were not as developed as those observed *in vivo* in the lamb. This may reflect differences in the time periods over which the lesions developed, in the specificity of the bacteria–host interaction or features of the expression and regulation of ϵ intimin *in vivo* that may promote the formation of more extensive AE lesions. It is also possible that the isolates tested *in vitro* were attenuated by the selection on nalidixic acid.

O115:H⁻ (CO), but not O115:H⁻ (RC), was positive for the typically plasmid-borne elements *ehxA* and the *etp* locus by PCR, despite there being similar plasmid profiles for both strains. Southern blot analysis indicated that the *ehxA* gene was chromosomally located. The large plasmids of diarrhoeagenic *E. coli* are highly variable in their genetic composition [4]. EHEC O157:H7 possesses a large plasmid, pO157, that commonly encodes the *ehxA*-D genes necessary for the expression of enterohaemolysin [49], *katP* for expression of a catalase/oxidase system [21], the production of a serine protease encoded by *espP* [50], and a type II secretion system encoded by the *etp* locus [51]. The role of pO157-associated virulence determinants has not been fully established. The carriage of these plasmid-borne determinants is less common in non-O157 EHEC isolates [4] and they are rarely associated with *stx*-negative *E. coli* [48, 52–55]. Hybridisation experiments with a specific *etp* probe previously determined that the *etp* genes were found in 100% of the EHEC O157:H7 and 60% of the EHEC non-O157 isolates [51]. Of isolates from bovine faeces, only 10% of STEC carried the *etp* operon, and other *stx*-negative *E. coli* groups examined (EAggEC, EPEC, ETEC, EIEC) were all negative for the *etp* gene cluster [51]. Therefore, strain O115:H⁻ (CO) is unusual in being an *stx*-negative isolate carrying *ehxA* and the *etp* gene cluster and, to our knowledge, this is the first report of the presence of the latter in an *stx*-negative *E. coli* of animal origin. Furthermore, the location of these genes is unusual, as Southern blot analysis showed that neither of the O115:H⁻ plasmids hybridised with the *ehxA* probe, but only reacted with residual chromosomal material of the CO O115:H⁻ isolate. The *ehx* and *etp* loci lie directly adjacent to each other on pO157, and may have been acquired together by the CO isolate or were lost from the RC isolate upon selection [56, 57].

The O115:H⁻ isolates described in this report present a combination of virulence factors associated with EHEC, EPEC and ETEC isolates that may cause disease in man and animals. It is not known whether the mild clinical signs exhibited by the lamb infected with *E. coli* O115 were due to the O115:H⁻ isolate(s) or the inoculated O157:H7, or some other factor. Similar clinical signs were also seen with the three other O157:H7-inoculated lambs in the group, in which O115 was not detected. However, EPEC-like isolates

lacking the EAF locus, similar to these O115 strains, are associated with diarrhoea in calves and sheep. In one report a Shiga toxin-negative *E. coli* isolate was positively identified as being associated with AE lesion formation in the small and large intestines in a calf with severe enteritis [58]. This isolate was subsequently found to be *eaeA*-positive and *stx*- and *bfpA*-negative and of serotype O80:H⁻ [59]. *E. coli* O115 has been associated with neonatal septicaemia in sheep [60], but the virulence factors associated with the septicaemic strains were not reported. The potential of the O115:H⁻ isolates for causing human disease cannot be excluded, considering the combination of virulence factors they possess. The occurrence of novel, potentially pathogenic combinations of virulence factors in *E. coli* serovars that have hitherto not been associated with pathogenicity demonstrates the value of screening for specific virulence factors in routine surveillance.

We gratefully acknowledge Andy Skuse for the preparation of specimens for transmission electron microscopy, the VLA Electron Microscopy Unit for the preparation of scanning electron micrographs and Mr J. Conibear for the preparation of photographic prints. This work was supported by the Department of Environment, Food and Rural Affairs, UK, through the Food Safety and Zoonoses programme, project OZ0706, and by the VLA seedcorn funding programme, project SC0081.

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