Influence of geographical origin, host animal and *stx* gene on the virulence characteristics of *Escherichia coli* O26 strains

Ilknur Aktan,^{1,2} Ben Carter,² Hendrik Wilking,³ Roberto M. La Ragione,² Lothar Wieler,³ Martin J. Woodward² and Muna F. Anjum²

Correspondence

Muna F. Anjum

m.anjum@vla.defra.gsi.gov.uk

¹Department of Veterinary Clinical Science and Animal Husbandry, University of Liverpool, Neston, South Wirral CH64 7TE, UK

²Department of Food and Environmental Safety, Veterinary Laboratories Agency (VLA), Weybridge, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK

³Institute of Microbiology and Epizootics, Free University Berlin, D-10115 Berlin, Germany

The influence of geographical origin, host animal and presence of the *stx* gene on the virulence of *Escherichia coli* O26 strains from ruminants was determined in this study. A clear association was found between the virulence profile and geographical origin of Shiga-toxigenic *E. coli* (STEC) O26 strains, with UK STEC O26 strains harbouring virtually identical profiles, whilst central European strains showed considerable heterogeneity in plasmid-encoded genes. The former group were also more likely to be non-motile and *katP* gene positive. Comparison of UK STEC and atypical enteropathogenic *E. coli* (aEPEC) O26 strains showed that the presence of the *stx1* gene was positively correlated with the presence of *espP* and *katP* genes and negatively associated with the presence of the *yagP*–*yagT* region and with rhamnose fermentation. In contrast to the uniform profiles of STEC O26 strains from ruminants in the UK, aEPEC O26 strains of bovine and ovine origin showed diverse profiles both within and between groups, and could not be separated into discrete groups. These results indicate that the characteristics of UK O26 strains from ruminants are distinct from those of O26 strains from ruminants and humans in other regions in central Europe. Such differences are expected to influence the zoonotic potential of this pathogen and the subsequent incidence of O26-associated human disease.

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INTRODUCTION

Escherichia coli O26 strains are zoonotic pathogens that have been isolated from ruminants, where they are the putative cause of diarrhoeal disease and oedema (Aktan *et al.*, 2004; Cid *et al.*, 2001; Fremaux *et al.*, 2006; Paiba *et al.*, 2002; Pearce *et al.*, 2004; Wells *et al.*, 1991; Wieler *et al.*, 1996b). Human infection is via the faecal–oral route, and similarity in genotypes and virulence attributes of O26 strains of human and animal origin provide evidence that animals serve as a reservoir for this human pathogen (Leomil *et al.*, 2005).

Enterohaemorrhagic *E. coli* (EHEC) O26 is a subset of Shiga toxin (Stx)-producing *E. coli* (STEC), which can cause a broad spectrum of disease from diarrhoea to haemolytic uraemic syndrome (HUS; Nataro & Kaper, 1998). STEC O26 is important as a cause of human

infection in central and southern Europe (Tozzi *et al.*, 2003; Zhang *et al.*, 2000a) and is the major cause of HUS and haemorrhagic colitis in Italy, surpassing STEC O157 (Tozzi *et al.*, 2003). In contrast, the number of O26 infections reported in the UK, North America and Japan is much smaller than for *E. coli* O157 (Banatvala *et al.*, 2001; Brooks *et al.*, 2005; Hiramatsu *et al.*, 2002; McMaster *et al.*, 2001; Pearce *et al.*, 2006; Smith *et al.*, 1998). However, it is possible that the incidence of non-O157:H7 STECs such as STEC O26 has been under-reported and thus overlooked as a human pathogen as there is no convenient medium, such as sorbitol MacConkey's agar used for O157:H7, that will reliably screen for STEC O26 in a cost-effective manner.

E. coli O26 strains that lack the *stx* genes have been classified as atypical enteropathogenic *E. coli* (aEPEC), as they have the ability to form attaching and effacing lesions on the intestinal epithelium but lack the EPEC adherence factor plasmid (Nataro & Kaper, 1998). Investigation of aEPEC O26 strains from humans and animals has concluded that two major groups exist, one group comprising EHEC/STEC and aEPEC strains with very

Abbreviations: aEPEC, atypical enteropathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; HPI, high-pathogenicity island; HUS, haemolytic uraemic syndrome; STEC, Shiga toxin-producing *E. coli*; Stx, Shiga toxin.

similar genotypes and virulence markers, differentiated by the *stx* gene, and the other comprising only aEPEC O26 strains with characteristics unlike those of the former group (Leomil *et al.*, 2005).

These data, taken collectively, indicate that O26 strains isolated from different geographical regions and host animals may harbour diverse virulence characteristics. Therefore, the aim of this study was to compare the virulence profiles of STEC O26 strains from ruminants, the primary reservoir for these pathogens, from the UK with those of strains from other regions, mainly in central Europe, to determine the influence of geographical origin on virulence characteristics. In addition, the virulence profiles of aEPEC and STEC O26 ruminant strains mainly from the UK were compared to determine whether an association of risk factors such as host species and virulence could be linked to distinguish STEC-like O26 strains from other aEPEC O26 strains.

METHODS

Bacterial strains and biochemical characteristics. A total of 50 *E. coli* O26 strains were investigated in this study comprising 21 bovine and 12 ovine strains from the UK and nine German, six Belgian, one Swiss and one USA bovine strain. The UK O26 strains were from the Veterinary Laboratories Agency (VLA, Weybridge, UK) Enteric Bacteriology Reference Unit strain collection, collected between 1999 and 2002. The geographical distribution of the UK strains is given in Tables 2 and 3. The remaining O26 strains were collected between 1989 and 1995 at the Institute of Microbiology and Epizootics (Berlin, Germany). Biochemical characterization was established using the API 20E strip (bioMérieux) on all strains following the manufacturer's protocol.

PCR amplification of virulence genes. PCR amplification was performed in a total volume of 50 µl containing 100 ng template DNA, which had been purified using a DNeasy kit (Qiagen), 0.5 µM each primer (MWG Biotech), 100 µM each dNTP, 5 µl 10 × PCR

buffer, 2 mM MgCl₂ and 2 U *Taq* DNA polymerase (Promega). Multiplex PCR was performed on all strains to detect specific genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*) and intimin (*eae*) (La Ragione *et al.*, 2002). The primers and conditions used to detect intimin subtypes (*eaeβ*, $-\alpha$, $-\gamma$, $-\epsilon$ and $-\kappa$), enterohaemolysin (E-hlyA), serine protease (*espP*), type III secreted proteins (*espB* and *espBβ*), periplasmic catalase–peroxidase (*katP*), bundle-forming pili (*bfpA*), high-pathogenicity island (HPI) genes (*irp2* and *fyuA*) and flagella (*fliC*) genes were as described previously (Cookson *et al.*, 2002; La Ragione *et al.*, 2002; Sandhu *et al.*, 1997; Schubert *et al.*, 1998). The somatic O-type was also confirmed for all O26 strains using O26-specific PCR amplification of the *wzx* and *wzy* genes as described previously (DebRoy *et al.*, 2004).

The remaining PCR primers used in this study (*espA*, *fimB*, *fimE* and *yagP*) were designed within the conserved region identified from multiple sequence alignments within each gene family, using sequences available in GenBank. For *espA*, which encompasses a large and diverse gene family, degenerate primers were designed to account for allelic variation. The GC content, presence of hairpin loops and the annealing temperature of primer pairs were checked using CLONE 3 software (Peterson & Ward, 1990). BLASTN searches were performed to ensure high specificity of the primers. The PCR amplification conditions and fragment sizes are given in Table 1. Control strains EDL933, MG1655, LT2 and PT4 were used to determine the reliability and specificity of the primers. For *espA*, the primer concentration in the PCR mix was varied between 0.25 and 2 µM and PCR was performed at three annealing temperatures for the control strains. For test strains, the *espA* PCR was performed at 55 °C, whilst the remaining PCR conditions were as outlined in Table 1.

Haemagglutination and motility assays. Mannose-sensitive haemagglutination to determine the expression of type 1 fimbriae and motility tests were performed as described previously (La Ragione *et al.*, 2001). The presence of the *fimB* and *fimE* regulator genes, determined by PCR, and expression of type 1 fimbriae were combined as one marker for this study.

Data analysis. A 2 × 2 contingency table was used and a two-sided Fisher's exact test was carried out to test the association between the risk factors (host, country and *stx1* status) and virulence markers (Fleiss, 1981).

Table 1. PCR primers designed in this study

Target gene*	Sequence (5'→3')†	GenBank accession no.	PCR conditions/annealing temperature‡	Amplicon size
<i>espA</i> (F)	TARTGYGARGCGHGAGTWCTT	AJ277443, AY223511, AF071034	59, 55 and 54 °C, 45 s	371 bp
<i>espA</i> (R)	ATCACCAGCGCYTAAWTCAG			
<i>fimB</i> (F)	ATGAAGAATAAGGCTGATAAC	NC_000913	60 °C, 45 s	603 bp
<i>fimB</i> (R)	CTATAAAACAGCGTGACGCT			
<i>fimE</i> (F)	AGAAGTTCAGGCCATGATGC	AE005174	60 °C, 45 s	417 bp
<i>fimE</i> (R)	CACGCTCCGCCAGTTCATAA			
<i>yagP</i> – <i>yagT</i> (F)	CAATTGCTGCCGCCATTCTT	U00096	§	5.2 kb
<i>yagP</i> – <i>yagT</i> (R)	ACTCTCGCAACGCACTCTTA			

*F, Forward primer; R, reverse primer.

†R=A or G; Y=C or T; H=A, C or T; W=A or T; V=A, C or G.

‡All PCRs, unless specifically stated, were denatured at 94 °C for 30 s and amplified at 72 °C for 1 min.

§Long-range PCR was used to amplify a 5.2 kb fragment spanning the *yagP*–*yagT* region. Samples were subjected to 10 PCR cycles consisting of 30 s at 94 °C, 45 s at 51 °C and 8 min at 68 °C, followed by 20 cycles of 45 s at 50 °C and 7 min at 68 °C.

Table 2. Virulence profiles of the STEC O26 strains from different geographical regions

Virulence profiles are given for O26 STEC strains from bovine hosts with the *stx1* marker present, grouped into UK and non-UK strains. Genotypic or phenotypic characteristics that were present (*stx1*, *fimBE* and haemagglutination, *espA* and *espBβ*) or absent (rhamnose fermentation) from all strains have been omitted for clarity (the *fimB*, *fimE* and haemagglutination profiles were grouped together and scored positive if either gene was present in a strain showing mannose-sensitive haemagglutination). Boxes highlight those strains that have the most frequently occurring virulence marker profile. Numbers in bold show the deviation from the most frequently occurring virulence profile.

Strain ID	Serotype	<i>yagP–yagT</i>	<i>stx2</i>	Motility	<i>espP</i>	<i>espBβ</i>	<i>katP</i>	<i>E-hlyA</i>
UK bovine STEC O26 strains*								
EC1840/00 (L)	O26:K60	0	0	0	1	1	1	1
EC172/00 (L)	O26:K60	0	0	0	1	1	1	1
EC1722/00 (L)	O26:K60	0	0	0	1	1	1	1
EC183/99 (Pr)	O26:K60	0	0	0	1	1	1	1
EC1537/99 (P)	O26:K60	0	0	0	1	1	1	1
EC1044/01 (T)	O26:K60	0	0	0	1	1	1	1
EC157/00 (T)	O26:K60	0	0	0	1	1	1	1
EC1721/00 (L)	O26:K60	0	0	0	1	1	1	1
EC306/01 (Pr)	O26:K60	0	0	0	1	1	1	1
EC1702/00 (L)	O26:K60	0	0	1	1	1	1	1
EC690/02 (P)	O26:K60	0	0	1	1	1	1	1
EC537/01 (W)	O26:K60	0	1	1	1	1	1	1
Non-UK bovine STEC O26 strains†								
IMT3748 (B)	O26:H1	0	0	1	1	1	1	1
IMT3750 (G)	O26:H11	0	0	1	1	1	1	1
IMT3857 (B)	O26:H11	0	0	1	1	1	1	1
IMT3856 (B)	O26:H1	0	0	1	1	1	1	1
IMT3909 (G)	O26:H11	0	0	1	1	1	1	1
IMT3836 (B)	O26:H1	0	0	1	1	1	1	1
IMT3846 (B)	O26:H1	0	0	1	1	1	1	1
IMT3861 (G)	O26:H11	0	0	1	1	1	1	1
IMT4400 (B)	O26:H1	0	0	1	1	1	0	1
IMT3837 (S)	O26:H11	0	0	1	1	1	0	1
IMT3887 (G)	O26:H11	0	0	1	1	1	0	1
IMT3853 (G)	O26:H11	0	0	1	0	1	0	0
IMT477 (G)	O26:H [−]	0	0	0	0	1	0	0
IMT3870 (G)	O26:H [−]	0	0	0	0	1	0	0
IMT4040 (G)	O26:H [−]	0	0	0	0	1	0	0
IMT3753 (U)	O26:H11	1	0	1	0	1	0	1

*Geographical origin of UK strains: L, Langford; Pr, Preston; P, Penrith; T, Thirsk; W, Winswick.

†Geographical origin of non-UK strains: B, Belgium; G, Germany; S, Switzerland; U, USA.

RESULTS

Performance and reliability of PCR primers designed in this study

To assess the specificity and reliability of the degenerate *espA* PCR primer set used in this study, DNA from the sequenced *E. coli* O157 strain EDL933 was used as the positive control, whilst *E. coli* K12 strain MG1655 was used as the negative control. At all primer concentrations (0.25–2.0 μM), a single band of the appropriate size for *espA* was detected in EDL933 samples but not in MG1655 at

annealing temperatures of 59 °C (Fig. 1a) and 55 °C (data not shown). However, at a lower annealing temperature (54 °C), a faint non-specific band was detected in the negative control samples (Fig. 1b). For amplification of the *fimE* and *yagP* genes, MG1655 was used as the positive control and the sequenced *Salmonella* Typhimurium LT2 strain as the negative control. In both instances, bands of the appropriate size were detected only in MG1655 (data not shown). For *fimB* amplification, MG1655 was used as the positive control and the sequenced *Salmonella* Enteritidis strain PT4 strain as the negative control; as expected, a band of approximately 600 bp was detected

Table 3. Virulence profiles of the aEPEC O26 strains

Virulence profiles are shown for the O26 aEPEC strains from bovine and ovine hosts with the *stx1* and *stx2* markers absent, grouped according to host. Boxes highlight those strains with the most frequently occurring virulence marker profile. Numbers in bold show the deviation from the most frequently occurring virulence profile.

Strain ID*	Serotype	<i>fimBE</i> and haemagglutination†	<i>espA</i>	<i>yagP-yagT</i>	Rhamnose fermentation	Motility	<i>espP</i>	<i>espBβ</i>	<i>katP</i>	<i>E-hlyA</i>
UK bovine aEPEC O26 strains										
EC38/99 (P)	O26:K60	0	1	1	1	0	0	1	0	0
EC459/01 (C)	O26:K60	0	1	1	1	0	0	1	0	0
EC1001/01 (T)	O26:K60	0	1	1	1	1	0	1	0	0
EC1002/01 (T)	O26:K60	0	1	1	1	1	0	1	0	0
EC196/02 (L)	O26:K60	1	1	1	0	0	1	1	1	1
EC1021/01 (P)	O26:K60	0	1	1	0	1	1	1	1	1
EC225/00 (P)	O26:K60	1	1	1	1	0	0	1	0	1
EC1725/00 (T)	O26:K60	0	0	1	1	0	1	0	0	0
EC80/02 (T)	O26:K ⁺	1	0	1	1	1	0	0	0	0
German bovine aEPEC O26 strain										
IMT3862 (G)	O26:H11	1	1	0	0	1	1	1	1	1
UK ovine aEPEC O26 strains										
EC740/01‡	O26:K60(H ⁻)	1	1	1	1	0	0	1	0	1
EC746/01‡	O26:K60(H ⁻)	1	1	1	1	0	0	1	0	1
EC738/01‡	O26:K60	1	1	1	1	0	0	1	0	1
EC744/01‡	O26:K60	1	1	1	1	0	0	1	0	1
EC747/01‡	O26:K60	1	1	1	1	0	0	1	0	1
EC1388/00 (W)	O26:K60	1	1	1	1	1	0	1	0	0
EC1389/00 (W)	O26:K60	0	1	1	1	0	0	1	0	0
EC335/98‡	O26:K60	1	1	1	0	1	0	1	0	1
EC513/02‡	O26:K60	0	1	1	1	1	0	0	0	0
EC460/02‡	O26:H11:K ⁻	0	1	1	1	1	0	1	0	0
EC417/02‡	O26:H11:K60	0	1	1	1	1	0	1	0	0
EC401/02‡	O26:K ⁻	0	0	1	1	0	0	0	0	0

*Geographical origin of bovine strains: C, Carmarthen; L, Langford; Pr, Preston; P, Penrith; T, Thirsk; W, Wales; G, Germany.

†The *fimB*, *fimE* and haemagglutination profiles were grouped together and scored positive if either gene was present in a strain showing mannose-sensitive haemagglutination.

‡These samples were from previously published work (Cassar *et al.*, 2004).

only in MG1655 (data not shown). These results were reproduced in two further experiments.

Common characteristics of O26 strains

The presence of the somatic O26 antigen in all 50 O26 strains in the collection, established by classical serotyping prior to this study, was confirmed by PCR amplification of the O26-specific *wzx* and *wzy* genes. All strains were found by PCR to harbour *eaeβ* and *fliC* genes, irrespective of motility (see below). The *fyuA* and *irp2* genes encoded within the HPI region were also present in all O26 strains tested. However, all strains lacked the *bfp* gene, which is

harboured by the EPEC adherence factor plasmid (Kaper, 1998).

Characteristics of STEC O26 strains from different regions

Genotypic and phenotypic profiles were compared for 28 UK and non-UK STEC strains of bovine origin (Table 2). All strains were negative for rhamnose fermentation, were type 1 fimbriated and harboured the *stx1*, *espA* and *espBβ* genes (data not shown). With the exception of strain IMT3753, all of the STEC O26 strains also lacked the 5.2 kb chromosomal *yagP-yagT* region.

Fig. 1. Determination of the specificity of the *espA* primers designed in this study. (a) The specificity of the degenerate *espA* primers was determined from PCR amplifications at an annealing temperature of 59 °C, using the sequenced strains EDL933 (lanes 2, 4, 6 and 8) and MG1655 (lanes 3, 5, 7 and 9) at the following primer concentrations: 0.25 µM (lanes 2 and 3), 0.5 µM (lanes 4 and 5), 1 µM (lanes 6 and 7) and 2 µM (lanes 8 and 9). (b) The annealing temperature was reduced to 54 °C to see the effect on primer specificity using MG1655 (lanes 2, 4 and 6) and EDL933 (lanes 3, 5 and 7) at primer concentrations of 0.25 µM (lanes 2 and 3), 0.5 µM (lanes 4 and 5) and 1 µM (lanes 6 and 7). The amplified PCR products were analysed by 0.8% agarose gel electrophoresis. Lanes 1 and 8, 1 kb DNA ladder (BenchTop).

In addition, the STEC strains from the UK showed an identical profile for all genotypes and phenotypes tested except for three strains, EC1702/00, EC690/02 and EC537/01. These three strains were motile, whilst the remaining O26 UK strains were non-motile. Also, EC537/01 harboured the *stx1* and *stx2* genes. In contrast, only eight of the 16 *stx1*-positive non-UK strains showed identical profiles. This profile was identical to that of the UK STEC strains in all characteristics except motility. The greatest variability in non-UK STEC O26 strains was seen in the plasmid-encoded *E-hlyA*, *espP* and *katP* genes. The strains could be divided into three groups according to their plasmid gene-related profile. One group was positive for both *E-hlyA* and *espP* genes but negative for the *katP* gene (IMT4400, IMT3837 and IMT3887); strains in this group were from Belgium, Switzerland and Germany. The second group (IMT3853, IMT477, IMT3870 and IMT4040) showed

absence of all three plasmid-encoded genes and comprised only strains from Germany. The third group, containing the only strain from the USA (IMT3753), was negative for *espP* and *katP* and positive for the *E-hlyA* gene. The majority of non-UK strains were motile.

When an association between the region of isolation (UK or non-UK) and the presence of virulence markers in STEC strains was tested using a two-sided Fisher's exact test, motility ($P=0.0035$) and the presence or absence of the *katP* gene ($P=0.0081$) presented strong evidence of an association with the region of isolation, with UK strains being less likely to be motile but more likely to possess the *katP* gene. The *espP* ($P=0.1060$) and *E-hlyA* ($P=0.2308$) genes presented weak evidence of an association with the region of isolation.

Characteristics of aEPEC O26 strains

The aEPEC strains in our study comprised strains of both ovine and bovine origin of which 21 were from the UK and one from Germany. All aEPEC O26 strains harboured the *yagP-yagT* region with the exception of the German strain, IMT3862, and the majority (18/22) fermented rhamnose (Table 3). The strains showed mixed profiles for the remaining genotypic and phenotypic characteristics, which were generally very different from the consensus profiles for STEC O26 strains (Table 2). Within the UK bovine aEPEC strains, the profile was varied, with three of the nine strains harbouring O26 plasmid-encoded genes, of which two were unable to ferment rhamnose. Four strains within this group (EC38/99, EC459/01, EC1001/01 and EC1002/01) showed profiles identical to each other apart from motility. Within the ovine strains, five (EC740/01, EC746/01, EC738/01, EC744/01 and EC747/01) showed identical profiles to each other. These strains were non-motile and fermented rhamnose but had the *E-hlyA* gene. Two other ovine aEPEC strains, EC460/02 and EC417/02, showed identical profiles; they were both motile but did not possess any O26 plasmid-associated genes. The only *stx*-negative non-UK strain present in our study, IMT3862, was identical in genotypic and phenotypic profile to the consensus profile for STEC O26 German strains (Table 2), except for the absence of the *stx1* gene. Several aEPEC strains showed absence of the locus of enterocyte effacement (LEE)-encoded *espA* and *espB* genes, possibly indicating the presence of a truncated LEE region within these strains that needs to be confirmed in future studies.

Using a two-sided Fisher's exact test to compare traits with the host species, the combined *fimBE* and haemagglutination marker exhibited an association ($P=0.0498$) with the host animal (ovine).

Comparison of UK O26 strains and predicted profiles for each group

Genotypic and phenotypic profiles from STEC and aEPEC O26 strains from the UK were compared. The results

Table 4. Correlation matrix for virulence markers of the UK O26 strains

Numbers in bold relate to those markers that exhibited a negative correlation, whilst boxed cells relate to those with a positive association.

	<i>stx1</i>	<i>fimBE</i> and haemagglutination	Motility	<i>espABβ</i>	<i>espP</i>	<i>yagP–yagT</i>	Rhamnose fermentation	<i>katP</i>
<i>stx1</i>								
<i>fimBE</i> and haemagglutination	0.41							
Motility	−0.10	−0.13						
<i>espAB</i>	0.25	0.09	−0.25					
<i>espP</i>	0.82	0.19	−0.09	0.09				
<i>yagP–yagT</i>	−1.00	−0.41	0.10	−0.25	−0.82			
Rhamnose fermentation	−0.73	−0.41	0.04	−0.34	−0.76	0.73		
<i>katP</i>	0.88	0.31	−0.05	0.28	0.94	−0.88	−0.83	
<i>E-hlyA</i>	0.48	0.68	−0.18	0.28	0.45	−0.48	−0.53	0.55

showed that, whilst the profiles for UK STEC strains were nearly identical, the profiles of the aEPEC strains were diverse (Tables 2 and 3). A correlation matrix was constructed to determine whether the presence or absence of the *stx1* gene was associated with any other virulence determinant present in this study (Table 4). It was found that the *stx1*, *espP* and *katP* genes were positively associated, whereas *yagP–yagT* and rhamnose fermentation were both negatively associated with the presence of the *stx1* gene. A weak association was also seen between the presence of the *E-hlyA* gene and the presence of the combined markers *fimBE* and haemagglutination (Table 4).

A predicted profile was constructed for each of the four groups (country of origin, bovine or ovine host and *stx1* status) based on the majority consensus (Table 5). All four groups harboured unique profiles, although features such as absence of the *yagP–yagT* region and inability to ferment rhamnose generally distinguished STEC O26 from aEPEC strains. Comparison of the predicted consensus profiles for the STEC non-UK and UK strains showed motility to be the only variable within the two predicted consensus

profiles. Expression of type 1 fimbriae and possession of the *E-hlyA* gene were variable features between bovine and ovine aEPEC from the predicted consensus profiles. However, the reliability of the predicted consensus profile for the aEPEC group is questionable due to the variability shown by this group, especially within bovine strains.

DISCUSSION

E. coli serogroup O26 comprises STEC and aEPEC pathogens that cause human and animal infections worldwide and are commonly shed by ruminants, which act as a reservoir for these pathogens. Differences in the zoonotic potential of these pathogens were investigated by studying the effect of geographical location on virulence in STEC strains from ruminants in the UK and in three countries from central Europe (Germany, Belgium and Switzerland), using a combination of genotypic and phenotypic markers. The majority of markers used in this study were from previously published work, and previously described conditions were used to amplify and detect these genes.

Table 5. Predicted profiles for each O26 pathogroup

The number of strains found to be consistent is shown, with the total number of strains in that group shown in parentheses. 1, Marker present; 0, marker absent.

Pathogroup	Region	Serotype	Host	No. of strains	<i>stx1</i>	<i>fimBE</i> and haemagglutination	<i>espABβ</i>	<i>yagP– yagT</i>	Rhamnose fermentation	<i>stx2</i>	Motility	<i>espP</i>	<i>katP</i>	<i>E- hlyA</i>
STEC	Non-UK*	O26	Bovine	6 (16)	1	1	1	0	0	0	1	1	1	1
	UK	O26	Bovine	9 (12)	1	1	1	0	0	0	0	1	1	1
aEPEC	UK	O26	Bovine	2 (9)	0	0	1	1	1	0	0	0	0	0
			Ovine	5 (12)	0	1	1	1	1	0	0	0	0	1

*The atypical German strain IMT3862 was excluded from this table.

However, primers were designed for the four genes (*espA*, *fimB*, *fimE* and *yagP*) specific to this study. The specificity and reliability of these primers were validated using sequenced positive- and negative-control strains under the conditions specified. In the case of *espA*, due to degeneracy of the primers, a range of primer concentrations was tested at different annealing temperatures. At the higher temperatures (59 and 55 °C), at all primer concentrations tested, a single band of the appropriate size was detected only in EDL933, indicating the primers to be specific and reliable. However, at the lower annealing temperature (54 °C), some faint non-specific bands were detected in the negative-control MG1655, indicating a decrease in the specificity of the primers, possibly due to its degeneracy.

The major differences in characteristics between the UK and non-UK STEC O26 strains were motility status and the presence or absence of plasmid-encoded genes, with the remaining chromosomally encoded genes and phenotypes being indistinguishable between the two groups. For UK STEC O26 strains, the profile was essentially identical for all determinants except for motility, where the majority of strains were non-motile. However, the non-UK strains were highly likely to be motile and to show variation in plasmid-encoded genes (*katP*, *espP* and *E-hlyA*). Motility has previously been attributed to many strains of the O26:H11 complex, especially those forming part of the group 1 complex (i.e. O26 STEC and aEPEC strains; Durso *et al.*, 2005; Leomil *et al.*, 2005; Zhang *et al.*, 2000a). It should be noted that UK strains were collected between 1999 and 2002, whilst non-UK strains were collected between 1989 and 1995. The difference in time of collection of the UK and non-UK strains may have influenced the genotypic and phenotypic differences seen between the two groups, and further studies of O26 isolates collected during the same time span from these regions are required to verify categorically the differences reported here.

Variations in the sizes and combinations of plasmid-encoded genes have been noted previously in STEC O26 human strains from central Europe (Zhang *et al.*, 2000b) and complement our data from ruminant strains gathered from this region. These studies also noted differences in PFGE patterns in STEC O26 human strains, indicating the presence of diverse clonal groups. This is in contrast to studies of Australian and North American STEC O26 strains, which have shown limited clonal diversity (Cobbold & Desmarchelier, 2001; Murinda *et al.*, 2004; Whittam *et al.*, 1993). These inconsistencies are probably due to differences in the genomic composition of STEC O26 strains from different regions and may be key to differences seen in the incidence of O26-associated human disease between the UK/USA and central and southern Europe. However, the similarities seen in this study between UK O26 STEC isolates from ruminants need to be confirmed with similar studies using strains from human infections in the UK. Data gathered from such studies will also establish whether ruminants are the main

source of O26 STEC infections in humans, or whether human-to-human contact plays a greater role than anticipated.

This and previous studies have shown that strains within the O26 serogroup share many common characteristics. These include a core genome (Anjum *et al.*, 2003) and possession of common virulence determinants such as the LEE-encoded β -intimin gene (*eae β* ; Kaper, 1998) and the HPI *fyuA* and *irp2* genes (Bielaszewska *et al.*, 2005; Karch *et al.*, 1999). In contrast, characteristics that consistently distinguish STEC and aEPEC O26 strains are difficult to find. In our study, STEC O26 strains were easily differentiated from aEPEC O26 strains due to the absence of the chromosomally encoded *yagP*–*yagT* region in all but one STEC strain present in our panel.

Absence of the 5.2 kb *yagP*–*yagT* region, encoding open reading frames of unknown function in STEC O26 strains, was first identified by comparative genomic microarray studies that showed this region to be present in other pathogenic and non-pathogenic *E. coli* strains including STEC O157, EPEC O111, aEPEC O26 and MG1655 (K12), but absent in STEC O26 strains (Anjum *et al.*, 2003). Although the significance of the absence of this region is not yet known, it may nevertheless be a useful marker for identifying STEC O26 strains. However, the only STEC O26 strain included in this study from North America, IMT3753, harboured this region. Further studies are required to determine whether strain IMT3753 is an exception or whether it is a variant group representing North American STEC O26 strains.

Similarly, although all STEC O26 strains included in our study were unable to ferment rhamnose, as expected (Hiramatsu *et al.*, 2002; Wieler *et al.*, 1995), approximately 18 % of the aEPEC O26 strains were also unable to ferment rhamnose. Previous studies have found such exceptions in 40–82 % of aEPEC O26 strains tested (Bielaszewska *et al.*, 2005; Murinda *et al.*, 2004). In fact, the inability to ferment rhamnose has previously been associated with the presence of the *E-hlyA* gene in both STEC and aEPEC O26 strains (group 1), whereas the ability to ferment rhamnose was associated with aEPEC O26 strains carrying the plasmid encoding the α -haemolysin gene (Leomil *et al.*, 2005; Wieler *et al.*, 1995). However, no significant association was found between the rhamnose and *E-hlyA* markers in this study, as several aEPEC strains that fermented rhamnose also harboured the *E-hlyA* gene, but lacked the *hlyD* gene encoding α -haemolysin (data not shown).

All STEC O26 strains in our panel were of bovine origin and harboured the *stx1* gene; only one STEC strain also harboured the *stx2* gene. Indeed, STEC strains of bovine origin, including STEC O26, are more likely to harbour *stx1* than *stx2* or *stx1* and *stx2* together (Pearce *et al.*, 2004, 2006; Wieler *et al.*, 1996a; Zschock *et al.*, 2000). In contrast, STEC strains, especially O26, harbour either the *stx1* or the *stx2* gene, although a recent shift from *stx1* to *stx2* has been detected in human STEC O26 strains (Bielaszewska *et al.*,

2005). It has been hypothesized that the emergence of *stx2*-harbouring strains may be due to better adaptation to the host or to changes in food consumption (Zhang *et al.*, 2000b). Therefore, the question arises as to the origin of the *stx2*-harbouring O26 strains found in human infection and whether cattle products and manure are the source of infection.

Comparison of genotypic and phenotypic profiles of UK O26 *stx*⁺ and *stx*⁻ strains showed that aEPEC strains harboured diverse profiles with limited consensus among strains, and that the host animal had no influence on any marker except expression of type 1 fimbriae. The latter was more likely to be present and expressed in strains of ovine origin. A weak association was seen between the presence of the *E-hlyA* gene and expression of type 1 fimbriae, although several strains expressed type 1 fimbriae but lacked the *E-hlyA* gene and vice versa. Curiously, in a previous study (Anjum *et al.*, 2003), the *fimB* gene, which regulates expression of type 1 fimbriae (Sohanpal *et al.*, 2004), was found to be absent in all O26 strains that lacked the *E-hlyA* gene.

In contrast to the aEPEC strains, the UK STEC O26 strains showed extremely similar profiles, with the presence of the *stx1* gene being highly correlated with possession of the *espP*, *katP* and *E-hlyA* genes and expression of type 1 fimbriae. Only one aEPEC strain shared the profile of its STEC counterparts. This strain, IMT3862, was unusual in that it was also the only aEPEC O26 strain that lacked the *yagP*–*yagT* region. It is possible that this strain was a STEC that had lost the *stx*-encoding phage. Loss of the lambdoid *stx*-encoding prophage from STEC strains is a common phenomenon, especially upon subcultivation (Karch *et al.*, 1992; Mellmann *et al.*, 2005), and is the probable cause of aEPEC-associated HUS cases (Sayers *et al.*, 2006). Overall, the results of our aEPEC study are in contrast to previous work, which has shown aEPEC strains of both human and animal origin (including ruminants) to be divided into two clear groups, with one aEPEC group showing identical characteristics to STEC O26 strains with exception of the *stx* gene (Leomil *et al.*, 2005). As ruminants are a likely source of human infection, it is expected that the diversity seen within UK aEPEC strains in this study will be reflected in human O26 isolates from the UK, which remain to be studied for these determinants. Such studies will also provide information on the proportion of O26 isolates passing through the food chain and causing human infection that are indeed from ruminants.

Currently, phylogenetic studies are being performed on the panel of UK and non-UK O26 strains to determine whether aEPEC and STEC O26 strains have evolved through different clonal lineages, and to see how closely related different aEPEC O26 strains from this study are to each other. Such studies will also determine whether a recent clonal spread of O26 isolates has occurred in the UK, especially within the STEC pathotype.

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