Comparison of Human and Cattle *E. coli* O26 Isolates by Pulsed Field Gel Electrophoresis (PFGE)

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Executive Summary

In recent years, *Escherichia coli* (*E. coli*) O157, a verotoxin-producing *E. coli* (VTEC), has emerged as a pathogen of increasing importance, responsible for numerous outbreaks of gastro-intestinal infection worldwide. It is now apparent, however, that *E. coli* isolates of serogroups other than O157 (termed non-O157 VTEC) also make a significant contribution to human diarrhoeal disease and in many European countries, non-O157 VTEC are isolated more frequently than O157 VTEC. To date, in excess of 200 non-O157 VTEC serotypes have been identified worldwide with over 100 types associated with human illness, including Haemolytic Uraemic Syndrome (HUS). Although non-O157 VTEC are not thought to not contribute significantly to the development of HUS in Scotland at present (surveillance of childhood HUS in Scotland between1997 and 2001 identified that 89% of reported cases had VTEC O157 infection), their contribution to diarrhoeal disease remains to be established.

In 2003, the Scottish *E. coli* O157 Reference Laboratory (SERL) received or isolated 31 non-O157 *E. coli* from 30 individuals, of which 16 possessed virulence factors (*vtx*$_1$, *vtx*$_2$, *eae* or *hly*). These included four apparently unrelated strains of *E. coli* O26 isolated over a six-week period. Although it was not possible to trace the source of any of the non-O157 VTEC isolated in Scotland, some of the serogroups isolated from Scottish human cases have recently been isolated from Scottish cattle. Preliminary results of a Food Standard Agency funded project (Project SO1014) determining the prevalence of specific non-O157 VTEC in Scottish beef cattle farms has shown that VT positive *E. coli* O26 isolates are common in Scottish beef cattle.

Over the past few years, *E. coli* O26 strains have become the most frequently isolated non-O157 VTEC associated with human disease, having been reported in Europe, Japan, Asia, USA, Australia, New Zealand, Argentina and Brazil. The organism has been isolated from cattle and humans and is considered as pathogenic for both. *E. coli* O26 has recently been associated with outbreaks in the Republic of Ireland and Japan and is known to survive well in bovine faeces and withstand substantial periods of stress. Typing of *E. coli* O26, by various methods has revealed great genetic heterogeneity within the group.

It is well documented that Scottish *E. coli* O157 isolates responsible for clinical disease are also circulating in the cattle population, sharing common virulence profiles, the same phage types and indistinguishable pulsed-field gel electrophoresis profiles. However, although cattle are thought to be a reservoir of infection of *E. coli* O26 isolates, there has been no direct evidence of bovine VTEC O26 causing human infection to date.

A total of 185 *E. coli* O26 isolates from human (33) and cattle (152) sources were analysed in this study to determine whether common PFGE profiles of *E. coli* O26 are circulating in both the cattle and clinical populations of Scotland. All bovine isolates were obtained from finishing cattle aged 12 to 30 months of age collected during the Food Standards Agency funded project (SO1014). The clinical collection consisted of all human O26 strains, isolated from Scottish cases between January 2002 and October 2003 and a selection of English and European *E. coli* O26 isolates.

All isolates were examined for virulence determinants (*vtx*$_1$, *vtx*$_2$, *eae* & *hly*) and were analysed by pulsed field gel electrophoresis using *Xba* I. Digital images of PFGE profiles were analysed visually and with BioNumerics (v 3.0) software. BioNumerics data was further analysed using SAS (v 8.2) and differences between human and cattle isolates were evaluated by assigning isolates to statistically significant groups.

Results demonstrated that the most common virulence profile of *E. coli* O26 was the possession of *vtx*$_1$ in conjunction with the *eae* gene and the *hly* gene (*vtx*$_1$+, *eae*+, *hly*+). This was also the most common virulence profile in Scottish bovine isolates analysed in this study. This concurs with recently published data on the virulence factors of non-O157 VTEC in a cohort of Scottish beef calves and their dams and investigators have previously reported the preponderance of this profile in human isolates of *E. coli* O26. None of the Scottish *E. coli* O26 isolates investigated possessed the *vtx*$_2$ gene. Although VTEC, including isolates of *E. coli* O26, which produce VT1, have been shown to cause HUS, there is a more established association between the production of VT2 and severe clinical disease. However, *vtx* genes in non-O157 VTEC are known to be unstable and stressful conditions, such as the exposure of VTEC to a number of antibiotics and growth-promoting compounds in animals may influence the horizontal transfer of *vtx* genes between bacteria. Surveillance of *E. coli* O26 in Germany and the Czech Republic has established that they can acquire *vtx*$_2$ genes over a short timeframe. It is possible that the acquisition of the *vtx*$_2$ gene may then confer on *E. coli* O26 a greater pathogenic potential for humans.

Following PFGE analysis, the human isolates were assigned to 30 unique PFGE profile groups and the animal isolates were allocated to 88 unique PFGE profile groups, 83 of which were unique to individual farms. The human strains did not share 100 % similarity with any of the animal strains. Statistical analysis of PFGE profiles obtained in this study established that clinical isolates were widely distributed among cattle isolates. The assignment of isolates to four groups confirmed that human and cattle isolates were intermingled, with each group including *E. coli* O26 of human and cattle origin.

E. coli O26 occurs commonly on Scottish farms and isolates also share common virulence profiles with the clinical isolates. Although an exact interpretation of relatedness of human and animal isolates is not possible, comparison of *E. coli* O26 PFGE profiles has shown that, within the limits of technology used, human and cattle isolates cannot be regarded as being distinct populations, and the possibility therefore remains that Scottish cattle are a credible source of human infection of *E. coli* O26. Despite this, the epidemiological association between cattle and human infection is lacking and may not be readily established. The link may be elucidated by information gained from enhanced screening for all non-O157 VTEC, establishment of their reservoirs of infection and epidemiological studies to follow up clinical cases.

It is clear that further work is required to monitor the incidence of non-O157 VTEC, in humans, animals and the environment, to determine their contribution to human diarrhoeal disease and to identify their reservoirs and routes of transmission to humans.

Nomenclature for Cytotoxins Produced by Escherichia coli

There have been varying systems of nomenclature for the cytotoxins produced by *Escherichia coli*, with some authors referring to Shiga-Like Toxins (SLT) and others using the term Verotoxin or Verocytotoxin (VT). The authors of this report have used the term "verotoxin" (VT1, VT2) which are encoded for by the "verotoxin genes" (*vtx*$_1$ and *vtx*$_2$). *Escherichia coli* that produce verotoxins are referred to as verotoxin-producing *E. coli* (VTEC).

Serogroups of *E. coli* other than O157 that also possess verotoxin genes are termed non-O157 VTEC. These include serogroups such as *E. coli* O26 and *E. coli* O111.

Glossary

HUS - Haemolytic Uraemic Syndrome
IMS - ImmunoMagnetic Separation technique
PCR - Polymerase Chain Reaction
VCA - Vero Cell Assay
SERL - the Scottish *E. coli* O157 Reference Laboratory
SAC - Scottish Agricultural College
PFGE - Pulsed Field Gel Electrophoresis
LEP – the Laboratory of Enteric Pathogens, Health Protection Agency, Colindale
UPGMA - Unweighted Pair Group Mean Average method
SCIEH – the Scottish Centre for Infection and Environmental Health
CGI - Comparative Genomic Indexing

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1. Aims and Objectives of the Investigation

In recent years, *Escherichia coli* (*E. coli*) O157, a verotoxin-producing *E. coli* (VTEC), has emerged as a pathogen of increasing importance[1, 2], responsible for numerous outbreaks of gastro-intestinal infection worldwide[3, 4]. In humans, *E. coli* O157 can cause a broad spectrum of disease ranging from non-bloody and bloody diarrhoea to haemorrhagic colitis, haemolytic uraemic syndrome and ultimately death [5, 6]. The pathogenic potential of *E. coli* O157 has traditionally been associated with the production of verotoxins, encoded for by the verotoxin genes (vtx_1 and vtx_2), and the ability to form attaching and effacing lesions in the intestine (*eae* gene) [6,7]. However, pathogenicity is also associated with other virulence factors, some of which are still being discovered [8, 9].

It is now apparent, however, that *E. coli* isolates of serogroups other than O157 (termed non-O157 VTEC) also possess verotoxin genes and also make a significant contribution to human diarrhoeal disease[10]. To date, in excess of 200 non-O157 VTEC serotypes have been identified worldwide with over 100 types associated with human illness, including Haemolytic Uraemic Syndrome (HUS)[11].

In many European countries, non-O157 VTEC are isolated more frequently than O157 VTEC[5] and it is thought that non-O157 VTEC may play a more important role in disease than O157 VTEC in Argentina, Australia, Chile and South Africa[12]. In Japan, the incidence of VTEC infection caused by non-O157 isolates has risen steadily since 1995[4] and, non-O157 isolates have also been documented in cases of human infection in the USA[13]. In Continental Europe, the most common non-O157 serogroups are *E. coli* O111, O26, O103 and O145[10], which have been reported in 11, 11, 7 and 5 countries respectively[14, 15]. In Australia, non-O157 serotypes such as O111 predominate and *E. coli* O157 is classed as uncommon[16] and in Italy, *E. coli* O26 is now more prevalent than *E. coli* O157[17]. In a recent study of non-O157 VTEC associated with disease in the UK, *E. coli* O145 and *E. coli* O26 were the most frequently isolated serogroups causing clinical disease[18]. Serotype O26, in particular, was the most common type found among human non-O157 VTEC in Spain, and the first or second most common type in human non-O157 infection in Germany, Belgium, Denmark, Finland, Canada, US and Japan[12].

Many laboratories do not routinely screen for non-O157 VTEC either because they do not recognise their role in disease or because resources are limited. Non-O157 VTEC lack a unique biochemical identifier that will visually distinguish them from a background flora of commensal *E. coli* on standard isolation media for O157 strains and therefore, specific additional techniques must be employed. Immunomagnetic separation (IMS) techniques are available to isolate specific serogroups (O26, O111, O103 and O145), but these will fail to detect other non-O157 VTEC strains. Techniques for the identification of non-O157 VTEC focussing on identifying the presence of verotoxin (VT) genes using techniques such as polymerase chain reaction (PCR) or dot blot hybridisation have been developed [19, 20, 21, 22]. Alternatively, the presence of free verotoxin in the stool can be detected using a verocell assay (VCA) or one of the many commercial kits now available. Most routine diagnostic laboratories lack the capacity to carry out such screening and consequently, the prevalence of infection with non-O157 VTEC strains is currently underestimated.

Non-O157 VTEC do not contribute significantly to the development of HUS in Scotland at present with the first Scottish case of non-O157 associated HUS only being identified in 2003. Surveillance of childhood HUS in Scotland between1997 and 2001 identified that 89% of reported cases had VTEC O157 infection[23]. The contribution non-O157 isolates make to diarrhoeal disease, however, remains to be established. The Scottish *E. coli* O157 Reference Laboratory (SERL) is receiving an increasing number of faecal samples for screening for these organisms, largely due to recommendations made in the *E. coli* O157 Task Force Report[24]. In 2003, SERL received or isolated 31 non-O157 *E. coli* from 30 individuals of which 16 (51.6 %) possessed virulence factors (*vtx*$_1$, *vtx*$_2$, *eae* or *hly*). The serogroups isolated from Scottish cases have all been isolated previously from human and bovine sources worldwide[11] and some of those serogroups have recently been isolated from Scottish cattle[25].

Ruminants, principally cattle, are now accepted to be the primary reservoir of *E. coli* O157[26, 27]. Transmission to humans occurs through direct contact with farm animals or their faeces or through consumption of undercooked meat, unpasteurized dairy products or contamination of products or water with animal faeces[28, 29, 30]. The extent to which Scottish cattle carry and excrete non-O157 VTEC in their faeces is currently being determined but their routes of transmission to humans remain undetermined and may differ from those of *E. coli* O157[10].

The Scottish Agricultural College (SAC) have recently completed the field sampling for a Food Standard Agency funded project (Project SO1014) to determine the prevalence of specific non-O157 VTEC in Scottish beef cattle farms. The preliminary results show that VT positive O26 isolates occur commonly in faeces from Scottish cattle. The weighted farm level prevalence was 19.8% (68/338) for *E. coli* serogroup O26. In comparison, 50 farms (15.3%) were positive for serogroup O157 strains. The proportion of individual faecal pats positive was 4.6% (249/6086) for serogroup O26 and 262 samples (4.1%) were positive for serogroup O157 strains. As a selective enrichment medium for O26 strains is not available and as the sensitivity of the *E. coli* O157 testing is probably higher than for serogroup O26 it is likely that estimates of sensitivity for individual serogroup prevalence are underestimates of the proportion of cattle carrying the organism and suggests a widespread distribution of VTEC O26 isolates in Scottish cattle (C. Low, personal communication). Whilst this project was underway, SERL and primary diagnostic laboratories identified four Scottish clinical cases of *E. coli* O26, during a six-week period in June/July 2003,[31]. All cases involved children less than 3 years old who presented with diarrhoea. Apart from two siblings, the cases were confirmed by molecular typing methods to be unrelated and the source of infection was undetermined in each case.

Escherichia coli strains of serogroup O26 were first recognized as causes of infantile diarrhoea in 1951[32]. In 1977, five years before the identification of *E. coli* O157 as a pathogen, production of verotoxin (VT) was identified in *E. coli* O26 strains isolated from infants with diarrhoea[33] and an isolate of *E. coli* O26 isolated from a human case in the Czech Republic in 1965 has recently been characterised[34]. *E. coli* O26 was thought to be the cause of a nosocomial outbreak in Hungary during the same time period[34]. Over the last few years, *E. coli* O26 strains have been the most frequently isolated non-O157 VTEC associated with human disease, having been reported in Europe, Japan, Asia, USA, Australia, New Zealand, Argentina and Brazil[11]. The

organism has been isolated from animals and humans and is considered as pathogenic for both cattle and humans[35]. They have recently been associated with outbreaks in the Republic of Ireland[36] and Japan[37] and are known to survive well in bovine faeces and withstand substantial periods of stress[38]. Typing of *E. coli* O26, by various methods has revealed great genetic heterogeneity within the group[39, 40, 41, 42].

It is well documented that Scottish *E. coli* O157 isolates responsible for clinical disease are also circulating in the cattle population[43, 44, 45]. They share common virulence profiles and have the same phage types. Previous pulsed field gel electrophoresis (PFGE) work carried out on *E. coli* O157 has shown that human and cattle populations share many indistinguishable PFGE profiles. Indeed, one of the most common PFGE profiles observed in clinical *E. coli* O157 isolates is also observed in cattle isolates, reaffirming the view that cattle are a reservoir of clinical infection of *E. coli* O157 (L. Allison personal observations). Although there has been no direct evidence of bovine VTEC O26 causing human infection to date[18], VTEC O26 isolated from cattle have been shown to share the same virulence profiles as human strains of the same serotype[39, 46].

The aim of this study is to determine whether common PFGE profiles of *E. coli* O26 are circulating in both the cattle and clinical populations of Scotland. This work will help the Food Standards Agency, Scotland identify whether Scottish cattle are a reservoir for clinical infection of *E. coli* O26 and thus have the potential to contribute to human diarrhoeal disease as observed in many other European countries.

2. Materials and Methods

2.1 *Bacterial Isolates*

A total of 185 *E. coli* O26 isolates from human (33) and cattle (152) sources were analysed in this study. Scottish, human *E. coli* O26 isolates (12) were provided by the Scottish *E. coli* O157 Reference Laboratory, Edinburgh. The clinical cases resided at various locations throughout Scotland (Figure 1 – originating laboratories shown in red) between January 2002 and October 2003. All other human isolates (21) were kindly gifted by Dr. Henry Smith, (LEP, Colindale, London).

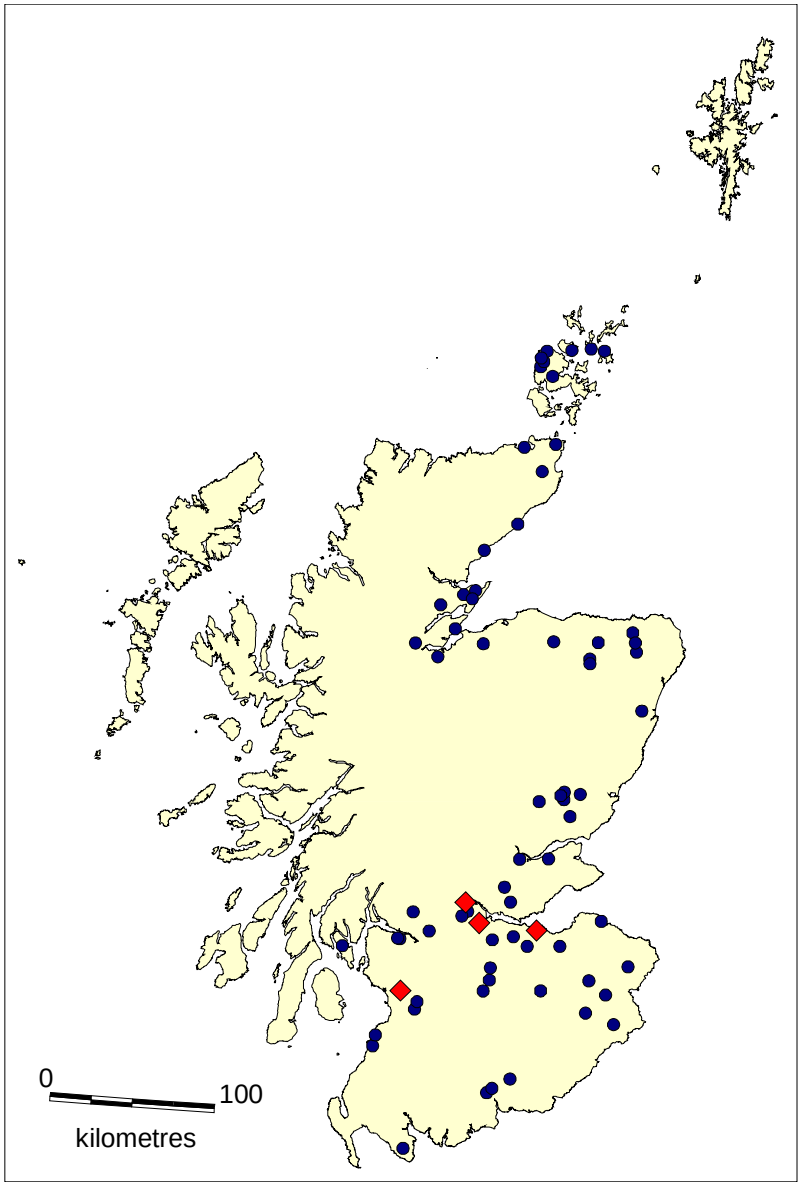

Figure 1: Location of Scottish *E. coli* O26 Isolates. Source of bovine isolates marked in blue and human isolates marked in red.

All bovine isolates of *E. coli* O26 (152) were obtained from finishing cattle aged 12 to 30 months of age collected during a Food Standards Agency funded project (SO1014) to determine the prevalence of certain non-O157 VTEC in Scottish beef cattle farms. Study SO1014 included the examination of all isolates for virulence determinants and all VT positive *E. coli* O26 and a representative selection of *E. coli* O26 isolates containing the *eae* and/or *hly* gene from this study were kindly donated by Dr. Chris Low (SAC, Edinburgh). The bovine isolates analysed originated from 57 farms (allocated numerical codes prefixed by "E"), located throughout Scotland (Figure 1 – shown in blue). All farms were visited and sampled on one occasion only, on different dates between June 2002 and January 2004 (with the exception of Farms E228 and E229 which were sampled on the same day). One *E. coli* O26 from a rabbit was also included in this study as were two isolates that subsequently serotyped as O? serogroup. All isolates were serotyped using the LEP serotyping scheme[47].

A list of all strains analysed in this study is contained in Appendix 1. The dates of sampling, source of strains and virulence profiles are also listed.

2.2 *PCR for Virulence Genes*

The presence of various virulence genes was determined at each sending laboratory, using the primers of Paton & Paton (1998)[48]. The genes detected were vtx_1, vtx_2, *eae* and *hly*. At the SERL, PCR amplifications were performed in 25ul volumes using 1.0 ul of boiled cell lysate. The PCR reaction was performed in a GeneAmp PCR system 9700 using BioLine Taq polymerase. Products of PCR amplification were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining.

2.3 *Pulsed-Field Gel Electrophoresis (PFGE)*

The *E. coli* O26 isolates from human and animal sources were compared by PFGE of *Xba I* digests. Isolates were grown overnight in 3 ml Difco broth at 37^{o}C, washed and resuspended in EET buffer (10mM Tris-HCl, 100mM EDTA, 10mM EGTA, pH 8.0) to give an OD_{450} of approximately 0.4 (20 % transmittance using the BioMerieux Vitek colorimeter). The plugs were prepared by mixing an equal volume of cell suspension with 1 % molten agarose (MastGel BB; Mast) prepared in 0.5 x TBE buffer (50mM Tris-HCl, 50mM boric acid, 0.5 mM EDTA, pH 8) dispensed into wells of plug moulds. When set, the plugs were placed in lysis buffer (50 mM Tris, 50 mM EDTA, 1 % N-laurylsarcosine, 1 mg.ml^{-1} proteinase K, pH 8.0) and incubated overnight at 50^{o}C. Following a 1 hr wash in 1mM phenylmethylsulfonylfluoride (PMSF), the plugs were washed three times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The DNA in the plugs was subsequently digested with *Xba I* (30U) following manufacturer's instructions (Promega).

Restriction fragments were separated by electrophoresis on a 1 % agarose gel in 2.2 l of 0.5 x TBE buffer in a CHEF-DR® III electrophoresis system (BioRad). A linearly ramped switch time of 5 to 50 s was applied for 38 h at a voltage of 5.4 V.cm^{-1}, an included angle of 120 degrees and a temperature of 14^{o}C.

After electrophoresis, the gels were stained with ethidium bromide (1mg.ml^{-1}) and the image visualised under UV light on a Gel Doc 2000 system (BioRad).

One isolate was untypeable by PFGE using the above method. A PFGE profile was subsequently obtained by adding thiourea (at a final concentration of 200μM) to the TBE buffer in the CHEF tank.

2.4 *PFGE Analysis*

Digital images of PFGE profiles were stored electronically as TIFF files and analysed visually and with BioNumerics (v 3.0) software (Applied Maths, Belgium). Bands smaller than 48.5 kb (the smallest band of the Lambda ladder) were not used in this analysis, as resolution at this level tends to be poor. The degree of similarity of DNA profiles was determined using the Jaccard similarity coefficient with 1.3 % tolerance and 1 % optimisation settings and dendrograms constructed by the unweighted pair group mean average (UPGMA) method[49]. The Jaccard similarity coefficient is the ratio of the number of DNA fragments of matching lengths to the number of DNA fragments between each pair of isolates, and was used in preference to the Dice coefficient because it provides an unbiased estimate of the proportion of shared bands. Each unique PFGE profile was allocated a profile identifying code. Isolates sharing 100 % similarity, shared the same code.

2.5 *Statistical Analysis*

Data from the BioNumerics Jaccard similarity matrix were further analysed using SAS (v 8.2). Similarities were converted into a data-based distance matrix from which a dendrogram was constructed using the UPGMA method.

Differences between human and cattle isolates were evaluated by assigning isolates to statistically significant groups. Group membership was initially determined by estimating an optimum cut-off value using dendrogram-based distances, and isolates separated by less than this value (0.393) assigned to the same group. The optimum cut-off value was calculated by:

1. randomly choosing a value between 0.00 and 1.00;
2. treating isolates within groups identified by the cut-off value as identical (with a distance of 0) and isolates between groups as being entirely different (with a distance of 1). A group-based distance matrix consisting of zeros and ones was constructed;
3. calculating the correlation coefficient between the comparable elements of the group and data-based distance matrices;
4. repeating the procedure 1000 times and selecting the cut-off value associated with the highest correlation coefficient.

Statistical support for each group was evaluated using the co-phenetic correlation. This is the correlation coefficient between comparable elements of the data and dendrogram-based distance matrices. The statistical significance of the co-phenetic correlation was evaluated using a Mantel test[50] in which the probability of the estimate occurring by chance is estimated by permutating or (for large groups) repeatedly randomising the elements in one of the matrices and enumerating how often the observed co-phenetic correlation is observed by chance. Programme zt[51] was used for this purpose using 1000 simulations, and groups regarded as statistically significant when the probability of the co-phenetic correlation occurring by chance was 5% or less. Where a putative group was not statistically significant, a node forming a group with a higher distance was used, providing this did not subsume pre-existing statistically significant groups.

3. Results & Discussion

3.1 *PCR Characterisation of E. coli O26 isolates*

The *E. coli* O26 isolates were categorised based on their virulence determinants (Table 1).

Source	Location	No. of Isolates	PCR Characteristics [a]			
			vtx$_1$	*vtx*$_2$	*eae*	*hly*
Human O26	Scotland	5	+	-	+	+
	Scotland	3	-	-	+	+
	Scotland	1	-	-	+	-
	Scotland	3	-	-	-	-
	England	2	-	+	+	+
	England	2	+	-	+	+
	Eire	5	+	+	+	+
	Eire	5	+	-	+	+
	Eire	1	-	+	+	+
	Belgium	2	+	-	+	+
	Italy	1	-	+	+	+
	Sweden	2	+	-	+	+
	Sweden	1	-	+	+	+
Cattle O26	Scotland	19	+	+	+	+
	Scotland	53	+	-	+	+
	Scotland	20	+	-	+	-
	Scotland	16	+	-	-	-
	Scotland	7	+	+	-	-
	Scotland	5	+	+	+	-
	Scotland	2	+	-	-	+
	Scotland	15	-	-	+	+
	Scotland	14	-	-	+	-
Rabbit O26	Scotland	1	-	-	+	-
Bovine O?	Scotland	1	+	+	-	+
Bovine O?	Scotland	1	+	+	+	+

Table 1: Source and PCR Characteristics of *E. coli* O26 Isolates

[a] +, gene present; -, gene absent

Of the 33 human isolates analysed, 26 possessed verotoxin genes; 16 possessed *vtx*$_1$ only, 5 possessed *vtx*$_2$ only and 5 possessed both *vtx*$_1$ and *vtx*$_2$. Of 152 animal *E. coli* O26 analysed, 122 were verotoxigenic, with 91 isolates possessing *vtx*$_1$ only, and 31 possessing both *vtx*$_1$ and *vtx*$_2$ genes. No cattle *E. coli* O26 isolate possessed the *vtx*$_2$ gene only.

In 26/26 human verotoxigenic isolates, the *eae* and *hly* genes were invariably present. However, this was not the case in the cattle isolates where only 97/122 (79.5 %) of verotoxigenic isolates also possessed the *eae* gene and only 74/122 (60.7 %) possessed the *hly* gene. Of 33 clinical O26 isolates studied, 7/33 did not possess verotoxin genes. Of these, 4 did possess the *eae* and/or *hly* gene(s), but 3 did not

possess any of the virulence factors looked for in this study. The most common clinical virulence profile (*vtx$_1$*+, *eae*+, *hly*+) observed in 16/26 verotoxigenic isolates was also the most common virulence profile in the cattle isolates, being observed in 53/122 verotoxigenic isolates.

Previous investigators have found *E. coli* O26 to be a remarkably heterogeneous group possessing either the *vtx$_1$* gene or the *vtx$_2$* gene, *vtx$_1$* and *vtx$_2$* genes in combination or neither of these genes[14,39]. In this study, human isolates of O26 possessed verotoxin genes in all combinations but the most common virulence profile was the possession of *vtx$_1$* in conjunction with the *eae* gene and the *hly* gene (*vtx$_1$*+, *eae*+, *hly*+). Other workers have reported the preponderance of this profile in human isolates. In a study conducted by Schmit *et al*, 10/11 clinical isolates of *E. coli* O26 possessed this particular virulence profile[46]. This was also the most common virulence profile in Scottish cattle isolates analysed in this study. This concurs with recently published data on the virulence factors of non-O157 VTEC in a cohort of Scottish beef calves and their dams[25]. Pearce *et al* demonstrated that in excess of 90 % of *E. coli* O26 isolates carried the *vtx$_1$*, *eae* and *hly* genes (*vtx$_1$*+, *eae*+, *hly*+).

E. coli O26 isolates from clinical cases lacking both *vtx* and *eae* genes were also identified in this study and have been observed previously[40]. In addition, four human isolates were identified which were *vtx* negative but *eae* positive and strains with these virulence profiles were also present in Scottish cattle. *vtx* negative isolates are likely to have lost their *vtx* genes or may be progenitors of VTEC O26 strains[52]. The spontaneous loss of verotoxin genes appears to occur frequently in non-O157 VTEC[53]. In a study of 57 *E. coli* O26 isolates from various countries, *vtx* negative isolates accounted for 78 % (45/57) although 93 % of these isolates did possess the *eae* gene[40]. In contrast, in *E. coli* O157, the *vtx* genes appear to be relatively stable but *vtx* negative *E. coli* O157 have still been reported[54] and can still cause severe clinical disease[55]. Although *vtx* negative *E. coli* O26 were detected in Scottish patients with clinical illness, their exact role in the disease process remains uncertain but may involve the possession of as yet undetermined virulence factors.

The majority of Scottish human isolates possessed the *hly* gene. Other workers[14,56] have noted a relationship between the possession of the *eae* gene and the possession of the *hly* gene in VTEC in general, where *hly* carriage was found to be associated with the carriage of the *eae* gene. In this study, of 30 isolates possessing the *eae* genes, 29 also possessed the *hly* gene. However, other investigators have shown that it may be dispensable as a virulence factor as O26 isolates lacking the *hly* gene have been associated with human infection[39].

3.2 *PFGE Analysis - Human E. coli O26 Isolates*

Following PFGE analysis, the 33 human isolates of *E. coli* O26 were assigned to 30 unique profile groups. The Scottish strains were designated PFGE profile groups A – I and all other strains were designated groups LEP A – T. PFGE revealed three sets of two isolates (designated Profile Groups I, II and III) which shared 100 % similarity (Figure 2).

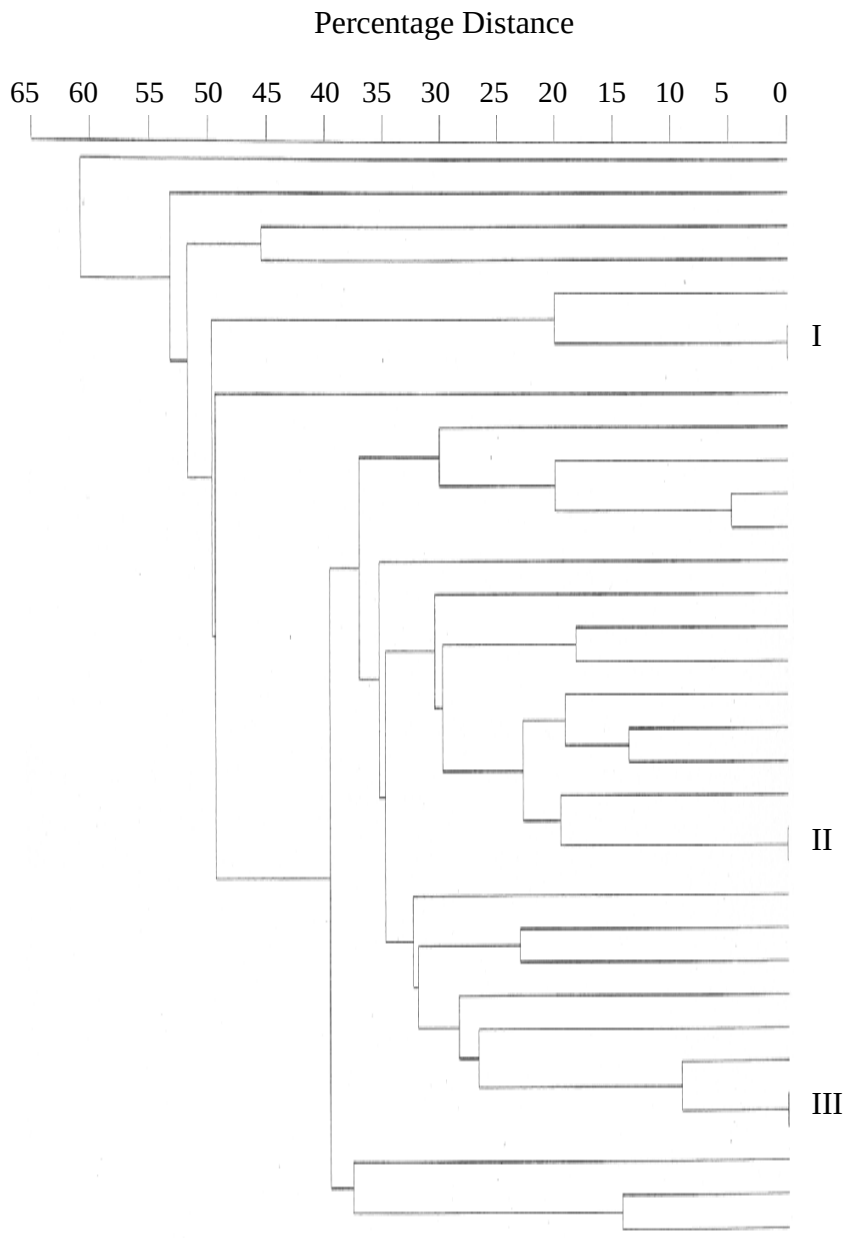

Figure 2: An UPGMA dendrogram of all human *E. coli* O26 isolates based on Jaccards distance coefficient.

The Profile Group I and Group II isolates were isolated in Scotland in 2003. Profile Group I isolates were isolated from two brothers in June 2003: one age 2 years and the other aged 10 months. Profile Group II isolates isolated in Sept/Oct 2003 were from one brother, aged 2 months and a sister age 2 years. All children originally presented with diarrhoea.

The isolates in Profile Group III were isolated in Eire where they were both involved in an outbreak of infection (G. Willshaw, personal communication).

3.3 *PFGE Analysis - Cattle E. coli O26 Isolates*

Following PFGE analysis, it was possible to assign the cattle *E. coli* O26 strains to 88 PFGE profile groups on the basis of their uniqueness or the fact they shared 100 % similarity. Each profile group was allocated a PFGE profile code 1 – 88 (Figure 3). Digitised images of each isolate with each profile code allocation are listed in Appendix 2.

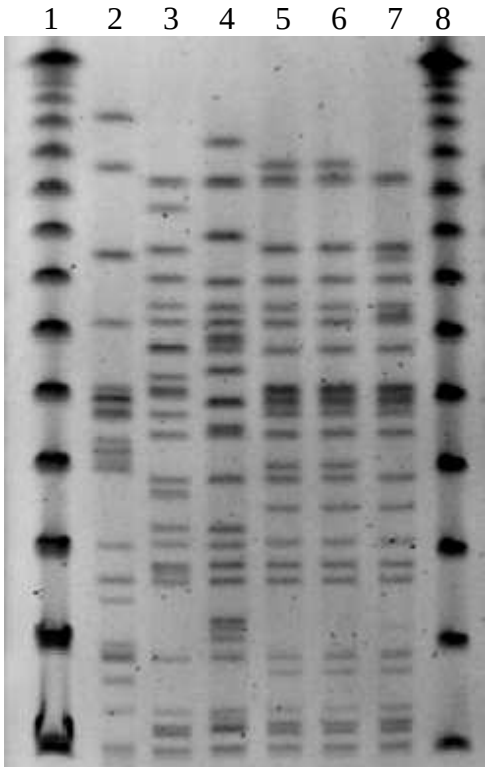

Figure 3: PFGE Profiles of six representative cattle *E. coli* O26 isolates. Lanes 1 & 8 - molecular weight standard (Lambda ladder). Lane 2 – allocated PFGE profile code 76; lane 3 – allocated PFGE profile code 43; lane 4 – PFGE profile code 36; Lanes 5 & 6 – PFGE profile code 24; Lane 7 – PFGE profile code 58.

A single isolate of *E. coli* O26 was received from 26 farms (Table 2) with more than one *E.coli* O26 isolate received from the remaining 31 farms. Nine of these farms had only one PFGE profile present (Table 3) but multiple PFGE profiles were identified on the remaining 22 farms (Table 4).

Farm	Profile Identifier	Farm	Profile Identifier
E047	1	E237	4
E077	11	E267	71
E124	78	E328	74
E139	66	E332	63
E154	9	E338	10
E161	24	E339	13
E176	49	E348	36
E182	26	E368	68
E221	73	E421	75
E225	7	E429	6
E228	35	E431	28
E234	17	E438	82
E236	12	E443	59

Table 2: Farms where a single isolate of *E. coli* O26 was recovered

Farm	Profile Code	No Isolates
E003	45	3
E076	56	4
E138	3	2
E185	34	3
E242	15	3
E296	80	2
E432	64	2
E437	65	4
E445	30	4

Table 3: Farms with multiple *E. coli* O26 isolates
but only one PFGE *Xba* I profile identified

Farm	No Profiles	No Isolates	Farm	No Profiles	No Isolates
E065	3	3	E229	2	2
E067	2	2	E238	2	2
E071	3	3	E273	3	3
E075	5	5	E279	4	9
E085	2	2	E299	2	6
E093	2	2	E314	2	2
E095	2	2	E334	2	2
E112	3	5	E341	2	2
E127	2	4	E363	4	14
E180	3	5	E409	6	7
E224	2	13	E465	2	5

Table 4: Farms with Multiple PFGE *Xba* I Profiles

Eighty-three PFGE profiles were unique to individual farms but five different PFGE profiles were observed on more than one farm (Table 5), with PFGE profile 36 being isolated from three different farms. Four of the profiles were isolated from farms in Orkney and Highland and interestingly, PFGE profiles 43 and 58 were isolated from the same farm in Orkney (E065) and the same farm in Highland Region (E 279).

Profile Identifier	Farm	No. Isolates	Location	Sample Date
17	E234	1	Grampian	18/08/2003
	E363	5	Lothian	18/11/2002
24	E065	1	Orkney	22/07/2002
	E161	1*	Highland	28/01/2003
36	E229	1	Orkney	11/08/2003
	E348	1	Orkney	23/09/2002
	E279	1	Highland	10/12/2003
43	E065	1	Orkney	22/07/2002
	E279	1	Highland	10/12/2003
58	E065	1	Orkney	22/07/2002
	E279	6	Highland	10/12/2003

Table 5: PFGE *Xba* I Profiles Common to Multiple Farms

In addition, PFGE profile 24 from Farm E161 (denoted by *) that shared 100 % similarity with the PFGE profile of an *E. coli* O26 isolate from Farm E065 was actually a serotype "O"? isolate. Table 6, however, demonstrates that, with the exception of two isolates of Profile 17 (denoted by *), the isolates from different farms sharing a common PFGE profile actually have different PCR virulence profiles.

Profile Identifier	Farm	No. Isolates	VT1	VT2	eae	hly
17	E234	1 *	+	-	-	-
	E363	1 *	+	-	-	-
		4	+	-	+	-
24	E065	1	+	-	+	+
	E161	1	+	+	-	+
36	E229	1	+	-	-	-
	E348	1	+	-	+	-
	E279	1	+	+	-	-
43	E065	1	+	-	+	+
	E279	1	+	+	-	-
58	E065	1	+	-	+	+
	E279	4	+	+	-	-
		2	+	+	+	+

Table 6: PCR Virulence Profiles of Common PFGE Isolates

Further typing work would have to be carried on the Profile 17 isolates to establish whether they belonged to one clonal group, present on both farms.

3.4 *Comparison of Human and Animal PFGE Profiles*

Following PFGE analysis, the human isolates were assigned to 30 unique PFGE profile groups and the animal isolates were allocated to 88 unique PFGE profile groups. The human strains did not share 100 % similarity with any of the animal strains. It is perhaps not surprising that no common *E. coli* O26 PFGE profile was detected since there was no epidemiological link between the Scottish clinical cases of *E. coli* O26 and cattle.

Four groups A, B, C and D comprising 5, 16, 19 and 142 isolates were identified from the UPGMA dendrogram of isolates (Figure 4).

Inspection of the dendrogram suggests that although there is some clustering of human isolates, the overall impression is of human isolates interspersed amongst cattle isolates. The closest neighbour of a human isolate is almost as often a cattle isolate (for 13 profiles) as a human isolate (on 16 occasions). All four groups identified included *E. coli* O26 isolates of human and bovine origin (Table 7). Full membership of each dendrogram grouping is given in Appendix 1 and the complete statistical report is appended (Appendix 3).

Groups	No. Human Isolates	No. Cattle Isolates
A	2	3
B	3	16
C	25	117
D	2	14

Table 7: No. human and cattle isolates allocated to each group

Five isolates were not assigned to a cluster. These comprised three cattle strains, one Scottish human strain and one rabbit strain. It is probable that the rabbit acquired the *E. coli* O26 from cattle during communal grazing. This has been reported previously for *E. coli* O157[57].

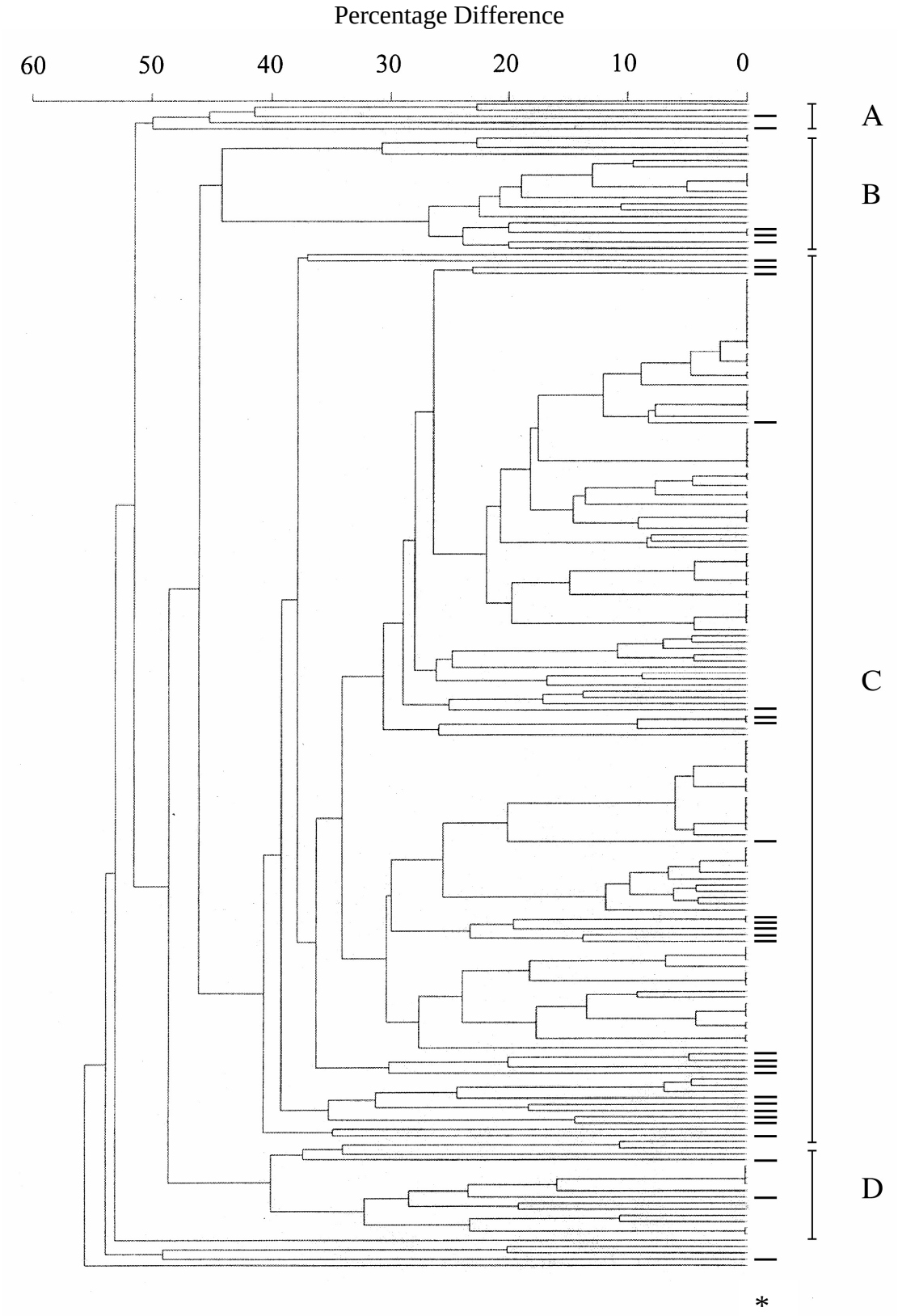

Figure 4: UPGMA Dendrogram of Isolates based on Jaccards Distance Coefficient showing the grouping of cattle and human isolates.

* Horizontal line denotes position of human strains within the dendrogram

4. Final Discussion

In this study, the source of the human *E. coli* O26 strains was not determined and indeed, it has not been possible to trace the source of any of the non-O157 VTEC isolated in Scotland. Little is known about the reservoir of *E. coli* O26, or the routes of transmission although cattle have been reported as a potential reservoir[58] and other animals such as pigs have also been implicated [59]. *E. coli* O26 has also been isolated from sheep[60], diarrheic lambs[61] and mastitic cattle[62]. The source of infection in an *E. coli* O26 crèche outbreak was unknown[36] and the source of the Japanese O26 outbreak was also undetermined although contaminated food did subsequently contribute to the spread of the organism[37]. Despite *E. coli* O26 being isolated from humans, cattle and their products, the only evidence directly linking bovine VTEC O26 to human infection occurred in 2001 when two Austrian cases of HUS were indirectly linked to consumption of unpasteurized cow's milk[63]. The *E. coli* O26 isolates from the human cases and from cattle from the farm producing the incriminated milk shared the same virulence profile and the same pulsed-field gel electrophoresis (PFGE) profile.

PFGE is a molecular typing technique that has been successfully applied to the subtyping of many pathogenic organisms, including VTEC[4, 64]. The technique is of particular value during outbreaks of infection and the submission of PFGE profiles to a national database (as demonstrated in the United States by PulseNet) has made possible the rapid detection of outbreaks, not only on a local scale, but also nationally[65].

Analysis of pulsed-field profiles has also been used to provide evidence of transmission between cattle and humans[66]. The first link between a human case and animal case of VTEC O157 was demonstrated in 1993[67, 68]. In a subsequent study of 19 human/animal incidents occurring in Scotland, an indistinguishable PFGE profile was found in strains from animals and clinical cases on 10 occasions[44]. These isolates also shared the same phage type and had the same plasmid profiles providing irrefutable evidence of animal to human transmission. PFGE has also been used to infer relatedness within different populations of cattle[69, 70] or human[71] isolates or between cattle and human populations[72]. At SERL, relationships between isolates are inferred following PFGE analysis using up to two restriction enzymes and consideration of epidemiological information from enhanced surveillance carried out by the Scottish Centre for Infection and Environmental Health (SCIEH). In addition, typing methods such as PCR and phage typing are used in conjunction with PFGE.

The aim of study S11001 was to establish whether specific PFGE profiles of human *E. coli* O26 isolates were also circulating in the cattle population. Statistical analysis of PFGE profiles obtained in this study established that human isolates did not group together but were widely distributed among cattle isolates. The assignment of isolates to groups confirmed that human and cattle isolates were intermingled, with each of the four groups including *E. coli* O26 of human and cattle origin and the dendrogram provided evidence that human and cattle isolates were often more closely related to each other than themselves.

One should, however, be cautious in interpreting these results, as there are several possible explanations for this pattern of interspersion. The construction of a similarity coefficient from PFGE data assumes that fragments of DNA of the same size are homologous but this is not necessarily the case. There are also difficulties in resolving fragments of near identical sizes; what is assumed to be one band may actually be multiple bands that have co-migrated. It is only recently that PFGE has been evaluated as a tool for establishing genetic relationships[73]. Davis *et al* concluded that if relationships between isolates must be inferred from PFGE data alone, then PFGE analysis by six or more restriction enzymes would be required to provide an estimate of genetic relatedness[73]; the results of this study are based on a single restriction enzyme. A further technical reason for the dendrogram pattern is that UPGMA assumes that the rate of molecular evolution of isolates is equal and differences in the rate of molecular evolution between isolates will distort the resulting dendrogram. Although there are powerful statistical methods, which do not require this assumption, PFGE data is not suited to their use.

E. coli O26 occurs commonly on Scottish farms and isolates also share common virulence profiles with the clinical isolates. Although a definitive interpretation of relatedness of human and animal isolates is not possible, comparison of *E. coli* O26 PFGE profiles suggests that, within the limits of technology used, human and cattle isolates cannot be regarded as being distinct populations, and cattle are therefore a credible source of human infection. Thus Scottish cattle may not only be a potential reservoir but a source for human infection of *E. coli* O26. However, only five of 88 PFGE profiles were common to more than one farm and only in one case was an *E. coli* O26 isolate sharing indistinguishable PCR and PFGE profiles, identified on more than one farm. This may suggest either that *E. coli* O26 strains are genetically stable and diverse or that they are genetically unstable and undergoing considerable evolution. Recently, Anjum *et al* used a technique called comparative genomic indexing (CGI), to assess the clonal diversity of a small number of clinical and animal isolates of *E. coli* O26[52]. This technique used a microarray approach to establish core genes and regions of difference between isolates. Their results indicated a greater genetic homogeneity for this serogroup than had been previously proposed and the technique may prove to be a valuable tool in increasing our understanding of the diversity within this serogroup.

Investigators have proposed that the presence of isolates with indistinguishable PFGE profiles (termed recurrent clonal subgroups) may be an indication of the organisms' ability to establish a successful relationship with its host, allowing it to survive and propagate[74]. This situation has been commonly observed with *E. coli* O157, which is generally accepted to have recently emerged and to be of a limited clonal type[70, 75]. Conversely, it may be postulated that unique PFGE profiles, which are observed on only one occasion, are unstable and may fail to persist[76]. However, other investigators have demonstrated the presence of recurrent clonal subgroups in *E. coli* O26, one of which had persisted in the clinical population for 30 years[39]. Another VT 2-producing clonal group was identified which was widely distributed over Germany and the Czech Republic and had a propensity to cause outbreaks and HUS[39]. Since little longitudinal work has been conducted on cattle farms our results do not give an indication of strain stability.

It is possible that the *E. coli* O26 isolates circulating in the human and bovine populations have a low level of virulence in humans. Although VTEC, including isolates of *E. coli* O26, which produce VT1, have been shown to cause HUS[46], there is a more established association between the production of VT2 and severe clinical disease[46, 48]. In outbreaks of *E. coli* O26 occurring in Japan and Ireland, the organism produced VT1 only and none of the patients developed HUS[35, 36]. In 1999, a cluster of HUS cases associated with VT2 producing *E. coli* O26 was reported in Germany[77] and two HUS cases caused by VT2-producing *E. coli* O26 were recently reported in Austria[63]. This study has shown that, although human and cattle isolates share common virulence profiles, the most common virulence profile observed in this study involved the production of VT1 and, of the Scottish *E. coli* O26 isolates investigated, none possessed the vtx_2 gene. However, in a study of 55 clinical VTEC O26 isolates from Germany and the Czech Republic, Zhang observed a shift in *vtx* genotype from vtx_1 (which was identified exclusively up to 1994) to vtx_2 where 71 % of *E. coli* O26 isolated after 1997 possessed this gene only[39]. The trigger for this shift was not established but may have resulted by infection of *E. coli* O26 isolates with vtx_2-encoding bacteriophages. The investigators believed that this switch in *vtx* genotype might have contributed to an increased incidence of *E. coli* O26 cases associated with HUS. In non-O157 VTEC, *vtx* genes are known to be unstable and it is apparent that *E. coli* O26 can acquire vtx_2 genes over a very short period of time (1-2 years). Stressful conditions, such as the exposure of VTEC to a number of antibiotics[78] and growth-promoting compounds[79] in animals can increase the amount of free *vtx*-encoding bacteriophages in the intestinal environment thus influencing the horizontal transfer of virulence genes between bacteria. The acquisition of vtx_2-encoding bacteriophages may then confer on *E. coli* O26 a greater pathogenic potential for humans.

Despite showing Scottish cattle to be a potential source of human infection by *E. coli* O26, the epidemiological association between cattle and human infection is lacking and may not be readily established. The link may be elucidated by information gained from enhanced screening for all non-O157 VTEC, establishment of their reservoirs of infection and epidemiological studies to follow up clinical cases.

In 2001, the Task Force report on *E. coli* O157 made a number of recommendations designed to improve diagnosis of VTEC infection and influence the clinical outcome and the public health management of infection[24]. Clinical laboratories are beginning to formalise these recommendations in their laboratory protocols and an increasing number of laboratories are forwarding human faecal samples for more comprehensive VTEC screening carried out at SERL. However, because not all diagnostic laboratories are able to participate, insufficient information is available to monitor any change in incidence of non-O157 VTEC infection in the Scottish population and it is not possible to determine the prevalence of individual serogroups.

In 2004, the SCIEH instituted enhanced surveillance of clinical non-O157 VTEC infection. This involves the follow-up of all VTEC cases with public health and environmental health teams in an attempt to gain information, including possible sources of infection. Microbiological investigations of human, animal, food and environmental samples occurring during the investigation of a case or a cluster of infection usually concentrates on detection of *E. coli* O157. However, referral of

samples for more comprehensive VTEC screening is more appropriate in investigation of non-O157 cases.

It is clear that further work is required to monitor the incidence of non-O157 VTEC, in humans, animals and the environment, to determine their contribution to human diarrhoeal disease and to identify their reservoirs and routes of transmission to humans.

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