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Serotypes and Virulence Profiles of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolates from Bovine Farms[▽]

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Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains are clinically significant food-borne pathogens. However, there is a dearth of information on serotype prevalence and virulence gene distribution, data essential for the development of public health protection monitoring and control activities for the meat and dairy industries. Thus, the objective of this study was to examine the prevalence of non-O157 STEC on beef and dairy farms and to characterize the isolates in terms of serotype and virulence markers. Bovine fecal samples (n = 1,200) and farm soil samples (n = 600) were collected from 20 farms throughout Ireland over a 12-month period. Shiga toxin-positive samples were cultured and colonies examined for the presence of stx_1 and/or stx_2 genes by PCR. Positive isolates were serotyped and examined for a range of virulence factors, including *eaeA*, *hlyA*, *tir*, *espA*, *espB*, *katP*, *espP*, *etpD*, *saa*, *sab*, *toxB*, *iha*, $lpfA_{O157/OI-141}$, $lpfA_{O113}$, and $lpfA_{O157/OI-154}$. Shiga toxin and intimin genes were further examined for known variants. Significant numbers of fecal (40%) and soil (27%) samples were *stx* positive, with a surge observed in late summer-early autumn. One hundred seven STEC isolates were recovered, representing 17 serotypes. O26:H11 and O145:H28 were the most clinically significant, with O113:H4 being the most frequently isolated. However, O2:H27, O13/O15:H2, and ONT:H27 also carried stx_1 and/or stx_2 and *eaeA* and may be emerging pathogens.

Shiga toxin-producing *Escherichia coli* (STEC), also referred to as verocytotoxogenic *Escherichia coli* (VTEC), has emerged as a group of highly pathogenic *Escherichia coli* strains characterized by the production of one or more Shiga toxins (stx_1, stx_2, or their variants). STEC is common in ruminants and related foods (4, 5, 15, 30, 47). Clinical manifestations in humans range from hemorrhagic colitis (HC) to hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (3) and are directly related to the virulence genes present in the causative agent. To date, STEC prevalence studies have focused primarily on *E. coli* O157:H7, because of its initial predominance in human clinical infection. Culture and molecular methods for the detection of STEC have thus been developed and optimized for that serotype, with little attention to and resultant underestimation of the risks posed by non-O157 serogroups. However, non-O157 infections are of primary concern in several countries (19), with half of all confirmed STEC infections in Europe caused by non-O157 STEC (3), and non-O157 infections outnumber O157 cases in the United States (10). Ireland has the highest per capita incidence (5.7 STEC cases per 100,000) in Europe (28).

E. coli O157:H7, the most extensively investigated serotype, has long been associated with ruminants, but more-recent work has suggested that these animals are also important reservoirs for other STEC serotypes (29). Six non-O157 O groups have been identified by the Centers for Disease Control and Prevention (CDC) as being responsible for over 70% of non-O157 STEC-associated illness (O26, O45, O103, O111, O121, and O145) (10), and both the European Food Safety Authority (EFSA) and the U.S. Department of Agriculture (USDA) have issued recommendations for laboratory testing for these pathogens (17, 19). However, serotype alone does not necessarily provide an accurate assessment of the ability to cause illness or the severity of disease, which requires virulence gene data. In addition to the *stx* genes, other virulence factors encoded by *eaeA* (intimin), *tir* (*espE*) (translocated intimin receptor), *espA* (EspA protein), and *espB* (EspB protein) are required for intimate attachment and the formation of the attaching and effacing (A/E) lesions characteristic of STEC infection (10, 13, 79). Other clinically significant virulence markers include *hlyA* (pO157 enterohemolysin releasing hemoglobin from red blood cells), *katP* (pO157 catalase peroxidase that defends the cell against oxidative damage), *etpD* (pO157, encodes part of a type II secretory pathway transporting proteins across the outer membrane) (73), *lpf* (chromosomal long polar fimbriae) (72), *espP* (pO157 extracellular serine protease autotransporter), *saa* (pO113 STEC agglutinating adhesion) (55), *sab* (pO113 STEC autotransporter) (33), *toxB* (pO157 adhesion), and *iha* (chromosomal iron-regulated gene A homolog adhesin) (68, 70).

Despite the increasing general recognition of the clinical significance of non-O157 STEC, there is a lack of data on (i)

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TABLE 1. Origin and serotype of isolates used as positive controls

Strain	Serotype	Origin	Target gene	Reference/source
3653/97	O78:H−	Patient with diarrhea	stx_{1c}	77
7139/96	O8:H−	Asymptomatic carrier	stx_{1d}	42
E32511	O157:H−	Patient with HC	stx_{2c}	34
EH250	ONT:H12	Patient with diarrhea	stx_{2d} (nonactivatable)	61
B2F1	O91:H21	Patient with HUS	stx_{2dact}	49
2771/97	ONT: H−	Patient with diarrhea	stx_{2e}	50
T4/97	O128:H2	Pigeon	stx_{2f}	67
EC132	O111:H−	Bovine feces	stx_1, stx_2, *eaeA*, and *hlyA*	Provided by University of Münster, Germany
98NK2	O113:H21	Patient with HUS	$lpfA_{O113}$, *saa*, *sab*	56
38094	O157:H7	Patient with HUS	*katP*, *etpD*, *tir*, *espP*, *espA*, *espB*, $lpfA_{O157/OI154}$, *toxB*, and *iha*	Provided by CDC, Atlanta, GA
C9490	O157:H7	Patient with HUS	*lpfA*	74

prevalence at the farm level, (ii) distribution of virulence factors, and (iii) emerging serotypes for inclusion in future monitoring programs. The objective of this study was to contribute to addressing these data gaps. Furthermore, as the interaction between different virulence gene products in pathogenesis is poorly understood, the presence of virulence genes in farm serotypes in this study that are not associated with human infection would also provide an important insight into the relative importance of virulence factors required for human illness.

MATERIALS AND METHODS

Sample collection. Twenty bovine (beef and dairy) farms located throughout the Republic of Ireland were selected on the basis of geographical location (covering the whole country) for inclusion in this study. Each farm was sampled bimonthly over a 1-year period (July 2007 to July 2008). Animals were largely on pasture for the duration of the study, although they were housed during the winter months. At each visit, 10 fresh fecal samples (from the ground) and 5 soil samples (taken from areas where the animals congregated, such as beside a water trough) were aseptically transferred into sterile containers (Sterilin Ltd., Medical Supply Co., Mulhuddard, Dublin, Ireland). A total of 1,200 fecal and 600 soil samples were taken throughout the study. Samples were stored at 4°C for no more than 24 h prior to analysis.

Bacterial cultures. The *E. coli* control strains used are described in Table 1. Control strains used in the typing of the Shiga toxin genes, STEC strain O111:H− (EC132) and STEC strain O113:H21 (98NK2), were kindly provided by Helge Karch (University of Münster, Germany) and Adrienne Patton (University of Adelaide, Australia), respectively.

Detection of *vt*-positive *E. coli*. Each sample (10 g) was homogenized with 90 ml of tryptone soya broth (TSB; CM0129; Oxoid, Basingstoke, United Kingdom) containing 4 μg ml^{-1} vancomycin hydrochloride (V2002; Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37°C. One-milliliter aliquots of the homogenized enrichment were harvested by centrifugation (7,426 $\times$ *g* for 10 min at 4°C). Genomic DNA was extracted from the resultant pellets (resuspended in maximum-recovery diluent [MRD] CM0733; Oxoid, Basingstoke, United Kingdom) by using the DNeasy tissue kit (69506; Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. The remainder of the enrichment samples were stored at 4°C. The sample DNA (2 μl) was screened for the presence of stx_1 and/or stx_2 genes by using PCR in a Peltier thermal cycler (PTC-200; MJ Research Inc., Watertown, MA) with primer sets and reaction conditions as described by Paton and Paton (54) (Table 2). The reaction mixture (reaction mix A) was modified by using 1× Green GoTaq reaction buffer (M891; Promega, Madison, WI) and made up to a final volume of 25 μl. PCR products (10 μl) were separated by electrophoresis on a 1.5% (wt/vol) agarose gel and visualized under UV light (GelDoc 2000 system; Bio-Rad Laboratories, Hercules, CA) by ethidium bromide staining (10 mg ml^{-1}). Product size was determined using a BenchTop 100-bp DNA ladder (G8291; Promega).

Isolation of *stx*-positive *E. coli*. Samples positive for the virulence genes stx_1 and/or stx_2 were serially diluted in MRD and plated onto Chromocult tryptone bile X-glucuronide agar (TBX; Merck, Germany) supplemented with sterile filtered streptomycin sulfate (10 μg ml^{-1}) (S6501; Sigma-Aldrich, Steinheim, Germany) and sulfamethazine (100 μg ml^{-1}) (S6256; Sigma-Aldrich, Steinheim, Germany). Preliminary studies in our laboratory (data not shown), designed to optimize non-O157 recovery, found that TBX agar gave maximum recovery of non-O157 STEC isolates. After overnight incubation at 37°C, 5 colonies of differing colony morphologies were taken from each sample and streaked onto nutrient agar (NA) (CM0003; Oxoid, Basingstoke, United Kingdom) and eosin methylene blue agar (EMBA) (CM0069; Oxoid, Basingstoke, United Kingdom). Agar plates were incubated overnight at 37°C. Genomic DNA was extracted from presumptive *E. coli* colonies (those showing a green sheen on EMB plates) by resuspending a single colony from the NA plate in PrepMan Ultra sample preparation reagent (Applied Biosciences, Foster City, CA) and extracting DNA as per the manufacturer's instructions. All isolates were assessed for the presence of stx_1, stx_2, *eaeA*, and enterohemorrhagic *E. coli* (EHEC) *hlyA* by using 2 μl of the DNA sample and the PCR protocol as described above (Table 2). All STEC isolates were preserved in a cryogenic bead storage system (Protect bacterial preservers; TSC Ltd., Heywood, United Kingdom) at −20°C and were routinely recultured.

Serotyping. Serotyping of the O (lipopolysaccharide) and H (flagellar) antigens was performed by the *E. coli* Reference Center at Pennsylvania State University, University Park, PA. The O antigen was determined using antisera produced against available serogroups designated O1 to O181, with the exceptions of O31, O47, O72, O93, O94, and O122, as these are not designated (52). The H antigen was established by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *fliC* gene, which is responsible for flagella (46).

Detection of putative virulence and adhesion genes. One cryogenic bead of each STEC isolate was individually cultured in 10 ml of TSB overnight at 37°C. Genomic DNA was extracted from a 1-ml aliquot of culture by using PrepMan Ultra reagent. In addition to stx_1, stx_2, *eaeA*, and *hlyA* genes, the template DNA of each STEC isolate was analyzed using PCR for the presence or absence of other putative virulence and adhesion genes. These include (i) genes associated with the locus of enterocyte effacement (LEE) (*tir*, *espA*, and *espB*), (ii) plasmid-encoding virulence genes associated with STEC O157:H7 (*katP*, *espP*, and *etpD*), and (iii) genes encoding other attachment mechanisms (*saa*, *sab*, *toxB*, *iha*, $lpfA_{O157/OI-141}$, $lpfA_{O113}$, and $lpfA_{O157/OI-154}$). The primer sets and target genes used in these analyses are also listed in Table 2. Reaction mixture A was used to amplify the *katP*, *etpD*, *tir*, *saa*, *toxB*, *iha*, $lpfA_{O157/OI-141}$, $lpfA_{O113}$, and $lpfA_{O157/OI-154}$ genes. Reaction mixture B was modified from the method of McNally et al. (48), in which 1× Green GoTaq reaction buffer was used to amplify the *espA*, *espB*, *espP*, and *sab* genes. Reaction mixtures, including 2 μl of DNA, were made up to a final volume of 25 μl, and PCR product (10 μl) was separated by electrophoresis on a 1.5% (wt/vol) agarose gel and visualized under UV light by ethidium bromide staining. Product size was determined using a BenchTop 100-bp DNA ladder.

Molecular characterization of *stx* variants. The Shiga toxin genes were subtyped using the primer sets, typing protocols, and PCR conditions given in Table 2. Briefly, isolates were initially examined by PCR using the primer pair KS7 and KS8 to detect stx_1 and the genetic variants (stx_{1c} and stx_{1d}) of stx_1 (66). Positive isolates were further assessed for the presence of vt_{1c} and vt_{1d} by using the primer pair stx1c-1 and stx1c-2 (77) and primer pair VT1AvarF and VT1varR (12). PCR and RFLP-PCR was used to examine each isolate for the presence of vt_2 and its genetic variants (stx_{2a}, stx_{2c}, activatable stx_{2d} [stx_{2dact}], stx_{2e}, stx_{2f}, and stx_{2g}) by

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TABLE 2. Target genes and primer sequences used for the detection of STEC virulence gene markers

Primer	Sequence (5′–3′)	Target (gene)	Reference
stx1-F	ATAAATCGCCATTCGTTGACTAC	*stxA*$_1$	54
stx1-R	AGAACGCCCACTGAGATCATC		
stx2-F	GGCACTGTCTGAAACTGCTCC	*stxA*$_2$	54
stx2-R	TCGCCAGTTATCTGACATTCTG		
eaeA-F	GACCCGGCACAAGCATAAGC	*eaeA*	54
eaeA-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	*hlyA*	54
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		
TIR-F	CATTACCTTCACAAACCGAC	*tir*	40
TIR-R	CCCCGTTAATCCTCCCAT		
EspAa	CACGTCTTGAGGAAGTTTGG	*espA*	48
EspAb	CCGTTGTTAATGTGAGTGCG		
EspBa	CGATGGTTAATTCCGCTTCG	*espB*	48
EspBb	CGATGGTTAATTCCGCTTCG		
ESPPa	AAACAGCAGGCACTTGAACG	*espP*	48
ESPPb	AGACAGTTCCAGCGACAACC		
D1	CGTCAGGAGGATGTTCAG	*etpD*	65
D13R	CGACTGCACCTGTTCCTGATTA		
wkat-B	CTTCCTGTTCTGATTCTTCTGG	*katP*	11
wkat-F	AACTTATTTCTCGCATCATCC		
lpfO141-F	CTGCGCATTGCCGTAAC	*lpfA*$_{O157/OI\text{-}141}$	69
lpfO141-R	ATTTACAGGCGAGATCGTG		
lpfA-F	ATGAAGCGTAATATTATAG	*lpfA*$_{O113}$	16
lpfA-R	TTATTTCTTATATTCGAC		
O154-FCT	GCAGGTCACCTACAGGCGGC	*lpfA*$_{O157/OI\text{-}154}$	71
O154-RCT	CTGCGAGTCGGCGTTAGCTG		
SAADF	CGTGATGAACAGGCTATTGC	*saa*	55
SAADR	ATGGACATGCCTGTGGCAAC		
toxB.911F	ATACCTACCTGCTCTGGATTGA	*toxB*	70
toxB.1468R	TTCTTACCTGATCTGATGCAGC		
iha-I	CAGTTCAGTTTCGCATTCACC	*iha*	68
iha-II	GTATGGCTCTGATGCGATG		
LH0147-BamHI	CCCGGATCCGGAAACTCCAAGAGTATTGC	*sab*	33
LH0147-EcoRI	CCCGAATTCCCTTGCTTTTCCCTGTTACC		
KS7	CCCGGATCCATGAAAAAAACATTATTAATAGC	*stx*$_{1b}$	66
KS8	CCCGAATTCAGCTATTCTGAGTCAACG		
Stx1c-1	TTTTCACATGTTACCTTTCCT	*stxA*$_{1c}$	77
Stx1c-2	CATAGAAGGAAACTCATTAGG		
VT1varF	CTTTTCAGTTAATGCGATTGCT	*stxA*$_{1d}$	12
VT1varR	AACCCCATGATATCGACTGC		
LP43	ATCCTATTCCCGGGAGTTTACG	*stxA*$_2$ variants	13
LP44	GCGTCATCGTATACACAGGAGC		
GK3	ATGAAGAAGATGTTTATG	*stxB*$_2$ and *stxB*$_{2c}$	32
GK4	TCAGTCATTATTAAACTG		
SLT-II-vc	ACCACTCTGCAACGTGTCGC	*stx*$_{2c}$ and *stx*$_{2dact}$	36
CKS2	ACTGAATTGTGACACAGATTA		
VT2-cm	AAGAAGATATTTGTAGCGG	*stxB*$_{2d}$	61
VT2-f	TAAACTGCACTTCAGCAAAT		
FK1	CCCGGATCCAAGAAGATGTTTATAG	*stxB*$_{2e}$	21
FK2	CCCGAATTCTCAGTTAAACTTCACC		
128-1	AGATTGGGCGTCATTCACTGGTTG	*stxA*$_{2f}$	67
128-2	TACTTTAATGGCCGCCCTGTCTCC		
209F	GTTATATTTCTGTGGATATC	*stx*$_{2g}$	44
781R	GAATAACCGCTACAGTA		
VSAAF	ACTCGCATAATTGGTGGTG	*saa* variants	45
VSAAR	ATCATTGGTATTGCTGTCAT		
EaeVF	AGYATTACTGAGATTAAG	*eae* variants	64
EaeVR	AAATTATTYTACACARAY		
EaeZetaVR	AGTTTATTTTACGCAAGT		
EaeIotaVR	TTAAATTATTTTATGCAAAC		

using previously published methods (Table 2). In brief, all STEC isolates were examined by PCR with the primer pair LP43 and LP44 for the detection of *stx*$_2$, *stx*$_{2c}$, *stx*$_{2dact}$, and *stx*$_{2e}$ (13); primer pair 128-1 and 128-2 for the detection of *vt*$_{2f}$ (67); and primer pair 209F and 781R for the detection of *vt*$_{2g}$ (44). Isolates positive for *stx*$_2$ were further differentiated by PCR screening using the primer pair GK3 and GK4 (32) to detect *stx*$_2$, *stx*$_{2c}$, and *stx*$_{2dact}$. The resulting PCR products were incubated with the HaeIII restriction enzyme (R0107; New England BioLabs, Hertfordshire, United Kingdom) according to the manufacturer's instructions. Bands at 137 bp and 151 bp (32) indicated the presence of the variant *stx*$_{2c}$ or *stx*$_{2dact}$. PCR-RFLP methods were also used to differentiate between *stx*$_{2c}$ and *stx*$_{2dact}$. PCR was preformed using the primer pair SLT-II-vc and CKS2. The resulting PCR product (890 bp) was incubated in the PstI

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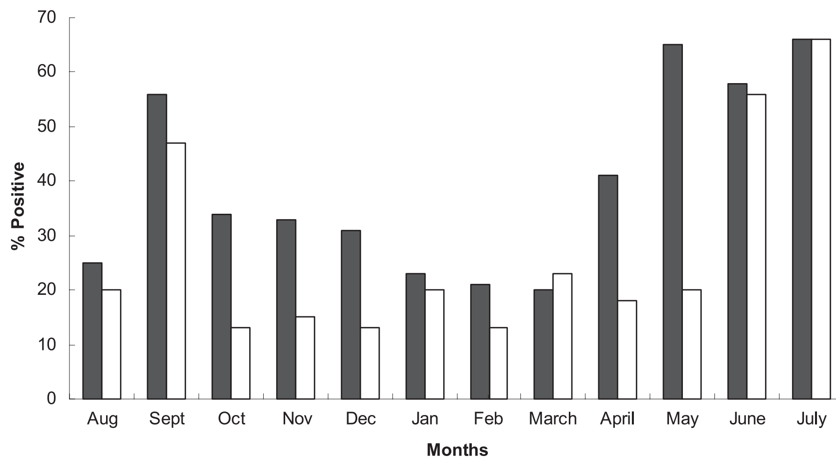

FIG. 1. The seasonal distribution of STEC-positive enrichment samples in feces (■) and soil (□).

restriction enzyme (R0140; New England BioLabs) according to the manufacturer's instructions. The absence of any band in the amplicon was indicative of stx_{2dact}, with 504-bp and 386-bp bands indicative of stx_2 and stx_{2c}, respectively (36).

Molecular characterization of intimin variants. All intimin-positive STEC isolates were further characterized using intimin typing as described by Ramachandran and colleagues (64). Briefly, a single forward primer (EaeVF) and 3 reverse primers (EaeVR, EaeZetaVR, and EaeIotaVR) (Eurofins MWG Operon, Ebersberg, Germany) (Table 2) were used to amplify an 834- to 876-bp fragment (size varied depending on the variant) representing the 3′-variable regions of all reported intimin variants. The resulting PCR products (10 μl) were incubated along with 3 restriction endonucleases, AluI, RsaI, and CfoI (New England Biosciences, Ipswich, MA), following the manufacturer's instructions. The restriction fragments were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. To determine which intimin type was present in each isolate, the resulting RFLP patterns were compared to published RFLP profiles (64).

Sequencing analysis. All PCR products for the intimin variants and the vt_{2g} gene were purified using the QIAquick PCR purification kit (28106; Qiagen, Crawley, West Sussex, United Kingdom) by following the manufacturer's guidelines and commercially sequenced (Eurofins MWG Operon, Ebersberg, Germany). Sequences were analyzed using the BLASTN program to search nucleotide databases (2), and sequences were aligned with the sequence of the vt_{2g} gene and intimin variants.

RESULTS

Of the 1,800 samples analyzed, 40% (480/1,200) of fecal and 27% (162/600) of soil samples were stx_1 and/or stx_2 positive. STEC were cultured from 1.9% (23/1,200) of fecal and 0.7% (4/600) of soil samples, with the majority of the 107 isolates obtained from samples taken from May to September (Fig. 1). The serotypes obtained included O2:H27, O6:H8, O13/O150:H2, O20:H19, O26:H11, O86:H21, O109:H5, O113:H4, O116:H28, O119:H5, O136:H2, O136:H16, O145:H28, O168:H8, O168:H27, O171:H2, and O174:H21 (Table 3). There were 6 isolates which could not be assigned to O-antigen serogroups but had H4, H17, H18, or H27 flagellar antigens. The most frequently occurring serotypes were O113:H4 (29%), O26:H11 (13%), and O2:H27 (12%), and the most widely distributed were O26:H11 (5 farms) and O113:H4 (4 farms).

stx_1, stx_2, and a combination of both were present in 22%, 42%, and 36% of isolates, respectively. The stx_{1c} and stx_{1d} gene variants were not detected, but stx_{2d}, stx_{2dact}, and stx_{2g} were present in 77%, 11.2%, and 8.4% of isolates, respectively. The distribution of target virulence genes in the farm isolates is shown in Table 3. The intimin gene (*eaeA*) was present in 18% of isolates: serotypes O2:H27 (variant untypeable), O13/O150:H2 (ζ), O26:H11 (β1), O145:H28 (γ), and ONT:H27 (θ). Other virulence genes detected included *hlyA* (26%; O2:H27, O13/O150:H2, O20:H19, and O26:H11), *tir* (14%; O2:H27, O26:H11, and O145:H28), *espA* (0.9%; O145:H28), *espB* (0.9%; O145:H28), $lpf_{O157/OI-141}$ (10.3%; O109:H5, O119:H5, O145:H28, and ONT:H4), $lpfA_{O113}$ (48.5%; O2:H27, O6:H8, O13/O150:H2, O20:H19, O26:H11, O86:H21, O109:H5, O116:H28, O136:H2, O136:H16, O168:H8, O168:H27, O171:H2, O174:H21, ONT:H4, and ONT:H18), *espP* (23%; O2:H27, O13/O150:H2, O20:H19, O26:H11, O86:H21, O116:H28, O136:H2, O136:H16, O168:H8, O168:H27, O171:H2, O174:H21, ONT:H4, and ONT:H18), *saa* (5.6%; O20:H19, O86:H21, ONT:H4, and ONT:H18), *toxB* (15.9%; O2:H27, O26:H11, and O145:H28), and *iha* (73.8%; O2:H27, O6:H8, O13/O150:H2, O20:H19, O26:H11, O86:H21, O109:H5, O113:H4, O145:H28, O168:H8, O168:H27, O171:H2, O174:H21, ONT:H4, ONT:H17, and ONT:H18). Virulence genes *katP*, *etpD*, $lpfA_{O157/OI-154}$, and *sab* were not detected in any of the isolates.

Interestingly, different virulence gene profiles were detected within strains from the same serotype; for example, O26:H11 (10 isolates) displayed 3 different virulence profiles: stx_1 stx_2 stx_{2d} *eaeA hlyA tir* $lpfA_{O113}$ *espP toxB iha* (7 isolates), stx_1 *eaeA hlyA tir* $lpfA_{O113}$ *espP toxB iha* (2 isolates), and stx_1 *eaeA hlyA* $lpfA_{O113}$ *espP toxB iha* (1 isolate).

DISCUSSION

Forty percent of bovine fecal and 27% of farm soil samples were positive for Shiga toxin genes (stx_1 and/or stx_2), with culture-based prevalences of 1.9% and 0.7%, respectively. The difference in the molecular and culture-based analyses was attributed to the relative sensitivities of the two methods and the presence of phage DNA. The increased prevalence in late summer-early autumn has been widely reported (38, 60, 62) and corresponds to an increase in human cases for both O157 and non-O157 STEC (31).

Seventeen different serotypes were isolated from bovine feces and beef farm soil samples, including O2:H27, O6:H8,

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TABLE 3. Serotypes, sources (fecal or soil), number of isolates, virulence factors, and variants[a]

Serotype (source)	No. of isolates	stx_1	stx_{1c}	stx_{1d}	stx_2	stx_{2c}	stx_{2dact}	stx_{2d}	stx_{2e}	stx_{2f}	stx_{2g}	*eaeA*	*hlyA*	*tir*	*espA*	*espB*	$lpfA_{O157}$	$lpfA_{O113}$	*espP*	*saa*	*toxB*	*iha*
O2:H27 (1F)	1	+	−	−	−	−	−	−	−	−	−	+ (NT)	+	+	−	−	−	+	+			
O2:H27 (2F)	2	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	+
O2:H27 (2F)	2	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	+
O2:H27 (2F, 1S)	3	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O2:H27 (4F, 4S)	5	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
O6:H8 (1S)	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
O13/O150:H2 (2F)	2	+	−	−	+	−	−	+	−	−	−	+ (ζ)	+	−	−	−	−	+	+	−	−	+
O20:H19 (1F)	1	+	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	+	+	+	−	+
O26:H11 (6F)	6	+	−	−	−	−	−	−	−	−	−	+ (β1)	+	+	−	−	−	+	+	−	+	+
O26:H11 (7F)	7	+	−	−	+	−	−	+	−	−	−	+ (β1)	+	+	−	−	−	+	+	−	+	+
O26:H11 (1S)	1	+	−	−	−	−	−	−	−	−	−	+ (β1)	+	−	−	−	−	+	+	−	+	+
O86:H21 (1F)	1	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−	+
O109:H5 (1F)	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
O113:H4 (3S, 23F)	26	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
O113:H4 (3F)	3	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
O113:H4 (1S, 1F)	2	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O116:H28 (4F, 2S)	6	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−
O119:H5 (3F, 3S)	6	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
O136:H2 (1F)	1	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−
O136:H16 (2F)	2	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−
O145:H28 (1F)	1	+	−	−	−	−	−	−	−	−	−	+ (γ)	−	+	+	+	+	−	+	−	+	+
O168:H8 (7F)	7	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	+
O168:H8 (2F)	2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
O168:H27 (F)	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
O171:H2 (4F)	4	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	+
O174:H21 (7F)	7	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	+
ONT:H4 (3F, 1S)	3	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	+
ONT:H17 (1S)	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ONT:H18 (1F)	1	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	+	+	+	−	+
ONT:H27 (1F)	1	+	−	−	−	−	−	−	−	−	−	+ (θ)	−	−	−	−	−	−	+	+	−	−

[a] F, fecal sample; S, soil sample. Numbers of samples from each source are also indicated. NT, nontypeable.

O13/O150:H2, O20:H19, O26:H11, O86:H21, O109:H5, O113:H4, O116:H28, O119:H5, O136:H2, O136:H16, O145:H28, O168:H8, O168:H27, O171:H2, and O174:H21. Of these, O20:H19, O26:H11 O113:H4, and O171:H2 are common in cattle (7). Indeed the most prevalent serogroup in our study, O113, is the predominant serogroup reported in the majority of European bovine surveys (79). However, our study also discovered a range of new serotypes (O6:H8, O13/O150:H2, O86:H21, O109:H5, O116, H28, O119:H5, and ONT:H17) not previously reported in the lists of non-O157 strains previously published (8, 35, 79). Furthermore, 6 of the 107 STEC strains were O nontypeable, highlighting the need for new antisera for the detection of emerging STEC serogroups. In total, 55% of the strains belonged to 5 serotypes (O2:H27, O26:H11, O113:H4, O136:H2, and O174:H21) previously associated with disease in humans. Moreover, the second most prevalent serotype (O26:H11) has been widely associated with HUS.

STEC strains from patients suffering severe disease such as HC or HUS are frequently stx_2 and *eaeA* positive and many also carry the *hlyA* gene (23). The majority of the strains obtained in this study were stx_2 positive, with 77% of isolates carrying the stx_2 and stx_{2d} variants, 13% stx_{2dact}, and 8% stx_{2g}. A high prevalence of stx_2 genes in bovine STEC has been previously reported (6, 10), in particular stx_2, stx_{2d}, and stx_{2g} (78). In more general terms, the high incidence of stx_2 genes observed in this and other studies is a matter of some concern, as the carriage of stx_2 genes and in particular stx_{2c} and stx_{2dact} has been linked to more-severe *E. coli* infection (25), and the presence of stx_{2c} and stx_{2dact} in O168:H8, O171:H2, and ONT:H18 is of particular concern, as all three serotypes, while *eaeA* negative, possessed an alternative attaching mechanism ($lpfA_{O113}$).

stx_1 was present in 11% of isolates, but neither stx_{1c} nor stx_{1d} was detected. STEC strains harboring stx_{1c} or stx_{1d} are more commonly associated with ovine sources (41) and are rarely recovered from patients suffering from HC or HUS (23, 24).

The *eaeA* gene was detected in less than one-fifth of isolates (18/107), a finding consistent with previous studies which reported a lower frequency in bovine than in human strains (8). Intimin-positive serotypes included O2:H27, O13/O150:H2, O26:H11, and O145:H28 as well as ONT:H27, with the O26:H11 strains carrying type β1. This subtype has been previously associated with O26:H11 and O157:H7 (8, 64), and its high specificity for both bovine and human cells may underlie its association with clinically significant STEC (9). O145:H2 and ONT:H27 strains carried intimin γ and θ, respectively, which along with β1 are commonly recovered from HC and HUS outbreak patients (53, 64). Different intimin types may have different host cell tropisms (73), and differentiation of different intimin alleles is an important diagnostic and epidemiological tool. The O2:H27 intimin variant was untypeable, suggesting there are more subtypes yet to be identified and characterized.

With the exception of those mentioned above, most STEC serotypes were *eaeA* negative and therefore unlikely to be associated with large outbreaks and HUS (39), a situation previously reported for the majority of bovine STEC (75, 76). However, while there is a strong association between the carriage of *eaeA* and the capacity to cause severe human disease (14), non-*eaeA* strains have been responsible for sporadic cases and small outbreaks in both the United States and Australia (20, 59) as well as the recent outbreak associated with intimin-negative O104:H4 in Germany (18). Attachment of *eaeA*-negative human pathogenic strains is therefore mediated by alternative adherence factors such as the STEC autoagglutinating

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adhesion (Saa), found in 5.6% of the strains in this study. Furthermore, to the best of our knowledge, this is the first study reporting the presence of *saa* in serotypes O20:H19, O86:H21, ONT:H4, and ONT:H18. All of the *saa*-positive strains were *eaeA* negative, supporting the hypothesis that these virulence factors are mutually exclusive (1, 37, 43, 58, 71, 78, 79). The *eaeA* gene is located in the LEE region of the chromosome, while the *saa* gene is found on pO113; thus there is no known reason as to why both could not be present in the same STEC strain. Further research is required.

The hemolysin gene, *hlyA*, was present in 30% of our strains, while the *etpD* and *katP* genes were absent in all isolates, despite all 3 genes being carried on pO157. Several studies have shown that these 3 genes are almost always present in O157:H7 but occur sporadically in non-O157 strains (22). Indeed, the *etpD* gene is rarely detected in bovine STEC (22). All of the O113:H4 (the most prevalent serotype) isolates were *eaeA* negative, a characteristic particularly associated with the O113 serogroup (57). In recent years, intimin-negative STEC O113:H4 has been associated with clinical cases in Ireland (26, 27). Intimin-negative O113:H4 has also been implicated in sporadic cases in other countries, where patients displayed an array of symptoms, including colitis and watery diarrhea (63).

Conclusion. Despite the low prevalence of STEC using culture-based methods, the diversity of serotypes provides further evidence that cattle are an important reservoir of STEC. The isolation of *eaeA*-positive O2:H27, O13/O150:H2 O26:H11, O145:H28, and ONT:H27 represents a risk to public health, as does the presence of phage-mediated HUS-associated stx_{2dact} genes in 6% of our isolates. Moreover, cattle are also an important source of atypical STEC, with this study reporting several previously unknown *saa*-positive STEC serotypes. Our work also highlights several knowledge gaps, including the limitation of current intimin typing and O-serotyping methods as well as in our understanding of virulence factors, specifically why *eaeA* and *saa* are rarely if ever found in the same strains.

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