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Microarray-based detection of virulence genes in verotoxigenic *Escherichia coli* O157:H7 strains from Swedish cattle

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SUMMARY

Verotoxigenic *Escherichia coli* (VTEC) serotype O157:H7 strains from a Swedish cattle prevalence study ($n=32$), and livestock-derived strains linked to human disease ($n=13$), were characterized by microarray and PCR detection of virulence genes. The overall aim of the study was to investigate the distribution of known virulence determinants and determine which genes are linked to increased pathogenicity in humans. A core set of 18 genes or gene variants were found in all strains, while seven genes were variably present. This suggests that the majority of VTEC O157:H7 found in Swedish cattle carry a broad repertoire of virulence genes and should be considered potentially harmful to humans. A single virulence gene type was significantly associated with strains linked to human disease cases ($P=0{\cdot}012$), but no genetic trait to explain the increased virulence of this genotype could be found.

Key words: Molecular biology, molecular epidemiology, Shiga-like toxin-producing *E. coli*, zoonotic foodborne diseases.

INTRODUCTION

Verotoxigenic *Escherichia coli* (VTEC) is a major zoonotic pathogen worldwide, with the serotype O157:H7 the most frequently isolated from outbreaks and severe human disease [1]. The first cases of infection by VTEC in humans in Sweden were reported in the mid-1990s and infection with VTEC O157:H7 has been a notifiable disease since 1996. In 2004, all serotypes of VTEC were included in the official notification system and the number of reported cases of human VTEC infection during 2004–2009 has varied between 196 and 385 cases per year (www.smittskyddsinstitutet.se). Of these cases, about half have been reported as serotype O157:H7. In 50–80% of cases, infection is contracted within Sweden, with the highest number occurring during the summer months. Geographically the number of reported cases varies significantly, with the majority of cases reported from the west coast and particularly from the county of Halland (www.smittskyddsinstitutet.se).

Ruminants, and especially cattle, are asymptomatic carriers of VTEC O157:H7 (reviewed by Gyles [2]), and there is evidence of association between cattle and farm density and human infection in a region [3]. Studies in Sweden have shown that 3·4% of cattle carry VTEC O157:H7 in faeces at slaughter [4]. Of these, most are younger animals [4, 5]. It has also

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been shown that about 12% of cattle presented for slaughter carry VTEC O157:H7 on the hide [4]. In a prevalence study performed on dairy cattle farms between 1998 and 2002, VTEC O157:H7 was detected in 8·9% of all sampled herds. The bacterium was more commonly found in cattle sampled in the south and central Sweden, but there were no findings from the north of Sweden. The highest prevalence was found in the county of Halland, where 23% of the dairy herds were positive [6].

Sources of human infection include direct contact with animals, contaminated food, e.g. unpasteurized milk and meat, contaminated water, and person-to-person contact [1]. The infectious dose for VTEC O157:H7 is very low and it may suffice to ingest <100 bacteria for disease to develop (reviewed by Karch *et al*. [1]). Clinical manifestations vary from no symptoms at all to mild watery diarrhoea that may develop to severe bloody diarrhoea [7]. The diarrhoea may be followed by complications such as haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP) [7]. Serious illness is more frequent in children [8].

The pathogenicity of VTEC O157:H7 derives from virulence-associated genes acquired by the bacteria through horizontal gene transfer [9]. A principal virulence factor of VTEC O157:H7 is the ability to produce verotoxins (VTs) [10]. Several other virulence factors are known, including genes conferring the ability to cause attaching-effacing lesions, other factors encoded in the locus of enterocyte effacement (LEE) and genes on the large virulence plasmid pO157 [10]. To date there is no comprehensive definition of which virulence determinants are necessary for VTEC to cause disease in humans [11].

Retrospective typing studies performed on Swedish cattle strains of VTEC O157:H7 ($n=200$) isolated during 1996–2002 have revealed that the strains, isolated from randomly sampled cattle in prevalence studies, demonstrated great diversity regarding phage types, verotoxin subtypes and pulsed field electrophoresis (PFGE) patterns [12]. Conversely, strains that were isolated from cattle farms that had been linked to human cases of illness during 1996–2002 presented a more homogenous pattern. Among these strains a specific predominating group of strains was identified, which were of phage type (PT)4, carried two verotoxin genes (vtx_2 and vtx_{2c}) and had similar PFGE profiles. This group of strains, hereafter called VTEC O157:H7 (PT4;vtx_2,vtx_{2c}), was isolated on 16/18 farms linked to human cases of VTEC O157 infection [12]. Moreover, VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains isolated during 2001–2008 accounted for more than two thirds of human VTEC O157 cases in Sweden [S. Löfdahl, Swedish Institute of Infectious Disease Control (SMI), personal communication].

In the present study we investigated the repertoire of virulence factors in a subset of isolates from the above-mentioned retrospective studies performed by Aspán & Eriksson [12], to investigate the distribution of known and suspected virulence determinants in Swedish VTEC O157:H7 cattle strains. Of particular interest was whether the virulence gene profile of the isolates from the prevalence studies would differ from the virulence profile of the more homogenous group of VTEC O157:H7 strains isolated from farms associated with human VTEC cases, which were assayed using the same methods. Most of these isolates were VTEC O157:H7 (PT4;vtx_2;vtx_{2c}). Such information is of great importance to characterize the findings of VTEC O157:H7 in primary production systems and in food products, specifically in order to predict whether a strain that has been isolated is likely to cause serious human illness.

DNA microarray technology enables the simultaneous detection of a large number of target sequences. For pathogenic *E. coli* as well as many other bacteria, different low-density arrays with comparatively few but relevant target genes have been successfully used to profile the presence of virulence factors and other genetic markers [13–17]. This type of assay requires comparatively simple equipment and data analysis, and is thereby well suited for high throughput use and routine diagnostics. In the studies described here we employed a commercially available microtube array system containing 124 *E. coli* gene probes in duplicate, including controls, designed to detect virulence genes in *E. coli* of various pathotypes.

METHODS

Bacterial strains and DNA extraction

Bacterial strains were stored in glycerol stocks at -70 °C at the National Veterinary Institute (Uppsala, Sweden) since isolation. Thirty-two isolates were included from a Swedish dairy cattle prevalence study of VTEC O15:H7 performed during 1998–2000 [6], with one strain representing each farm on which VTEC O157:H7 was detected in the study, excluding one farm for which the representing isolate was lost in

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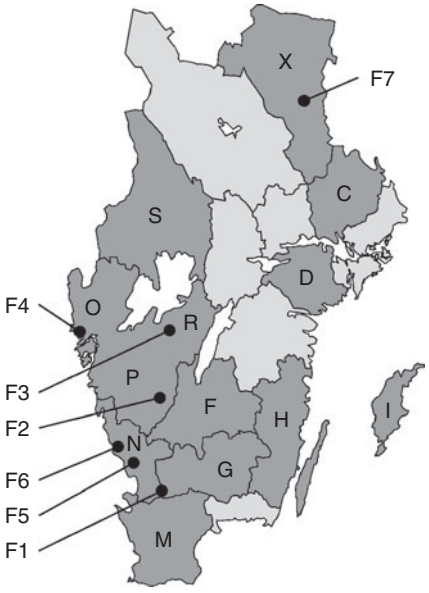

Fig. 1. Map of the southern part of Sweden detailing the geographic origin of isolates. The VTEC O157:H7 isolates analysed were from the counties of Gävleborg (X), Uppsala (C), Södermanland (D), Värmland (S), Gotland (I), Kalmar (H), Skåne (M), Kronoberg (G), Jönköping (F), Halland (N) and what is currently Västra Götaland county, with the historical subregions of Bohuslän (O), Skaraborg (R) and Älvsborg (P). The location of the seven farms (F1–F7) where livestock VTEC O157:H7 caused cases of disease in humans during the same period are indicated by black dots (●).

storage. Thirteen isolates were available from the seven different farms in Sweden where human VTEC infection had been confirmed, arising either from direct or indirect contact with livestock on those farms during the same time period (1998–2000), and where human and livestock strains had been linked by comparison of verotoxin gene profiles and PFGE [12]. The geographical origin of the isolates is presented in Figures 1 and 2. Finally, five different well characterized *E. coli* strains with different virulence gene profiles were analysed. These included two strains of VTEC O157, CCUG (Culture Collection, University of Gothenburg) 42744 (*E. coli* O157 vtx_1^+, vtx_2^+, $eaeA^+$, EHEC-$hlyA^+$, $fliC^+$) and CCUG 42901 (*E. coli* O157 vtx_1^-, vtx_2^-, $eaeA^-$, EHEC-$hlyA^-$, and $fliC^-$), and three ETEC strains; one O101 carrying genes for STa, F41 and F5/K99, one O141 carrying genes for STa, STb, VT2e and F18, and one O149 carrying genes for EAST1, STa, STb, LT, F6/987P, and F4/K88, as determined by conventional PCR analysis. DNA extraction was performed by boiling colonies in PCR grade water and using the lysates for BioRobot EZ1 DNA extraction (Qiagen, Austria).

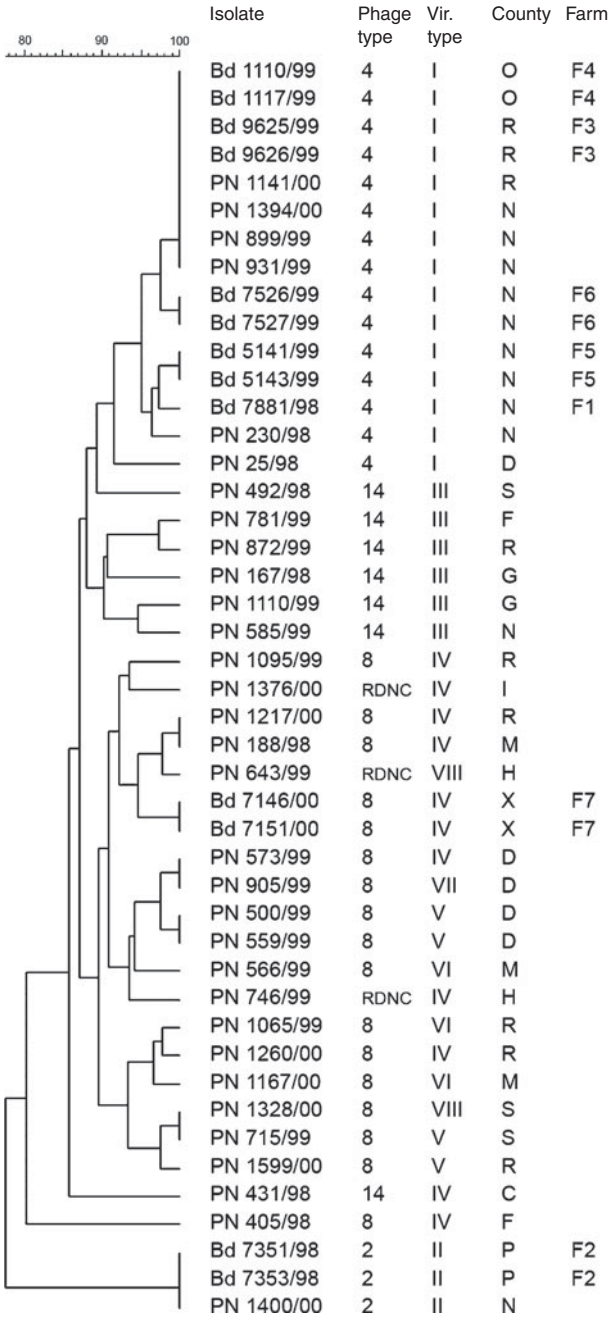

Fig. 2. PFGE patterns, phage types, virulence (vir.) types and geographic origin of studied isolates. UPGMA dendrogram based on *Xba*I restriction patterns using the Dice algorithm. RDNC = Reacts, but does not conform. Farm of origin is presented for isolates associated with human cases of illness.

Characterization of strains

Virulence typing was performed by PCR to identify genes encoding VT1 and VT2 (vtx_1 and vtx_2), intimin (*eaeA*) and EHEC-haemolysin (EHEC-*hlyA*) according to Paton & Paton [18] and H7 (*fliC*) according to

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Table 1. *Primer sequences*

Gene	Primers	Primer sequences (5′–3′)	Product size
efa-1 5′	Efa1 upper/	TATGAGACTGCCAGAGAAAGT	1205 bp
	Efa1 lower	GATGGGTTGTTGTTGTATTTCTTC	
efa-1 3′	Efa1 3′ fwd/	TGCGCACAATTGACTACAGAGGAA	713 bp
	Efa1 3′ rev	ATACGACCATCAGGGGAATCAC	
icf	O157 icf up/	TGGTGGCCCGCATACAGC	501 bp
	O157 icf lo	TGCCCAGTCAGCCCAGGTTA	
sodC	O157-2For/	GCAGAACAGGAAGTCCCAA	467 bp
	O157-2Rev	TTATTGAATGATGCCGCAGG	
tccP	TccP-F1/	ATGATTAACAATGTTTCTTCACTT	1154 bp*
	TccP-R1	TCACGAGCGCTTAGATGTATTAATGCC	
ureA	ureA F/	GACTCCAAGAGAAAAAGACAAACTA	271 bp
	ureA R	CAGATTATCGGATTATGGACGGTA	
terB	terB1/	AGGCCGTGACGAACTGACC	286 bp
	terB2	TCGCAACGGCAATACCAACACG	

* Variable.

Gannon *et al*. [19]. VT2-positive isolates were further investigated by PCR–RFLP to determine the VT2 gene subtype, as described by Pierard *et al*. [20].

Seven E. coli virulence determinants were analysed by PCR according to harmonized protocols developed in Work Package (WP)26 – 'virulotyping of *Salmonella* and *E. coli*' of the MedVetNet project (www.medvetnet.org), with primers developed in WP26 or adopted from the literature: *efa-1* 5′-region, *efa-1* 3′-region [21], *icf, sodC, tccP* [22], *ureA* [23] and *terB* [24]. The primer sequences are presented in Table 1. Each PCR reaction consisted of 0·5 U of AmpliTaq Gold polymerase, 1 × GeneAmpPCR buffer II, 0·1 M of each dNTP, 2·25 mM $MgCl_2$, (all from Applied Biosystems, USA), each primer at 0·27 μM, 5 μl DNA template, and PCR grade water up to a final volume of 25 μl. The PCR programmes were for *efa-1* 5′, *sodC* and *terB*: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s (annealing) and 72 °C for 1 min, and finally 72 °C for 7 min. For *efa-1* 3′ and *tccP* the same programme was used but with 58 °C annealing temperature, *icf* annealing temperature 56 °C, *ureA* annealing temperature 63 °C. The presence and size of PCR products were analysed by submarine electrophoresis on 1·5% agarose gels supplemented with ethidium bromide.

Phage-typing using published methods [25, 26] was performed at the Laboratory of Enteric Pathogens (Central Public Health Laboratory, London, England). PFGE was performed as described previously [5].

Microarrays (Identibac Ec v. 3) containing 124 *E. coli* virulence gene probes including controls were purchased from Identibac (New Haw, UK) and used according to the manufacturer's instructions. Array images were processed in IconoClust 3.0 (Clondiag, Germany), and signals were analysed using the *gapA*-positive control gene for normalization and with cut-offs as recommended by the manufacturer (>0·4 = present, 0·4 to 0·3 = ambiguous, <0·3 = absent, relative to the *gapA* signal). Probes which produced ambiguous signals, mostly representing duplicates targeting known variants of the same gene, were excluded from further analysis, excluded probes are indicated in the Supplementary Appendix (available online). Genes were considered present in the isolate if at least one well-performing probe gave a positive signal.

Statistical association between belonging to a given virulence gene type and belonging to the group of isolates with known links to human disease was tested using two-sided Fisher's exact test implemented in R 2.7.1 (r-project.org). Association between possession of a virulence gene and belonging to a given phage type/virulence gene type was tested as above. For statistical testing, identical duplicate isolates from the same farm were treated as single. A P value of $\leqslant$0·05 was considered significant.

RESULTS

PCR detection of virulence genes

For a summary of results, see Figures 2 and 3. All cattle isolates (n = 32) were positive for *fliC* (H7), vtx_2, *eaeA*, *hlyA, sodC*, *terB*, *ureA*, and the 5′-region

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Table 2. *Virulence genes in control strains*

Control strain	Serotype	Virulence genes detected by WP26 PCR	Virulence genes detected by microarray
853/67	O149	*ureA, terB, icf*	*K88* (F4/K88), *cba, fasA* (F6/987P), *lthA* (LT), *sta, stb, iha, sepA*
Bd 3437/83	O101	*icf*	*fanA* (F5/K99), *fim41a* (F41), *mchB, mchC, mchF, mcmA, sta*
60/84	O141	*ureA, terB*	*cba, cma, fedA* (F18), *fedF* (F18), *sta, stb,* vtx_2, *iha*
CCUG 42901	O157	None	*stb, lpfA*
CCUG 42744	O157	*tccP, sodC, efa-1* 5′, *ureA, terB*	vtx_1, vtx_2, *eaeA, hlyA, tccP, tir, katP, etpD, espP, espF, espJ, toxB, iha, nleA, nleB, nleC, cba*

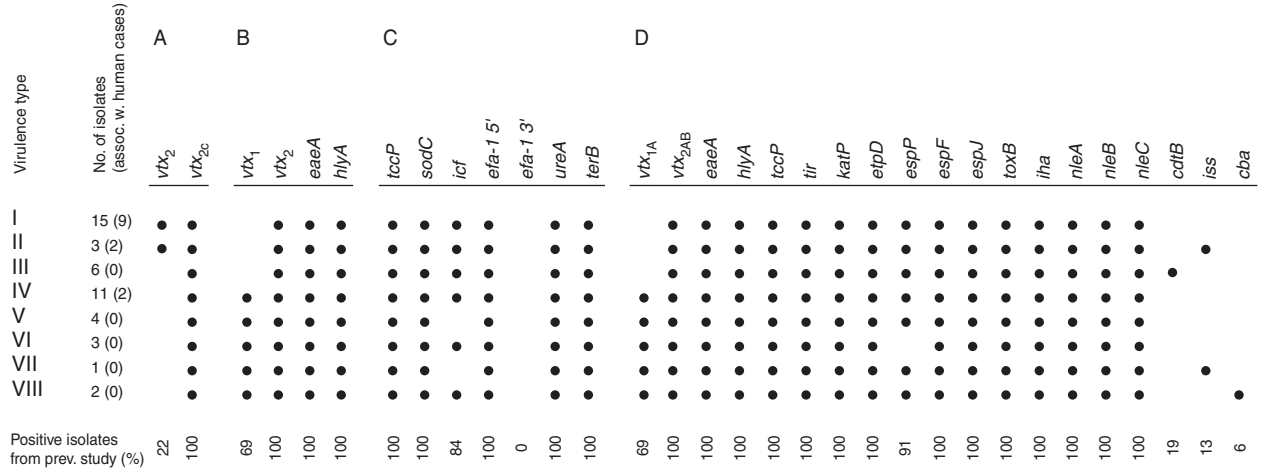

Fig. 3. Virulence genes in Swedish VTEC O157:H7. Definition of virulence types based on present genes/gene variants (●) as determined by (A) PCR–RFLP according to Pierard *et al.* [20]. (B) Multiplex PCR according to Paton & Paton [18]. (C) PCR according to the MedVetNet WP26 PCR protocol. (D) Identibac Ec v. 3 microarray assay. Only genes positive for at least one VTEC O157:H7 strain are shown. Percentage of isolates carrying each gene was calculated based on the prevalence study isolates ($n = 32$).

of *efa-1*. All cattle isolates were also positive for *tccP*, with PCR product sizes exceeding the expected 1154 bp for the isolates Bd7146/00, Bd7151/00 and PN188/98, and PCR products of <1154 bp for PN643/99, PN1095/99, PN1217/00, PN1376/00, PN431/98 and PN746/99. All isolates were negative for the 3′-region of *efa-1*. *icf* was present in the majority of the cattle isolates, while absent in PN1599/00, PN500/99, PN559/99, PN715/99 and PN905/99. vtx_1 was present in 21 of the cattle isolates. PCR results for control strains are presented in Table 2.

Microarray analysis

For two genes, *astA* (EAST1) and *espA*, no well-performing probe was found, and these genes were therefore excluded from further analysis. All VTEC O157:H7 isolates tested positive for vtx_2AB, *eaeA*, *hlyA*, *tccP*, *tir*, *katP*, *etpD*, *espF*, *espJ*, *toxB*, *iha*, *nleA*, *nleB* and *nleC*, while vtx_1A, *cdtB*, *espP*, *iss* and *cba* were variably absent or present. For detailed results, see Figures 2 and 3. Full probe-level results are available in the Supplementary Appendix. Genes known from previous characterization to be present in positive control strains and which were represented on the array (*eaeA*, *hlyA*, vtx_1, vtx_2, *sta*, *stb*, *fanA*, *fim41a*, *lthA, fasA, fedA, fedF*, *K88*) were successfully detected, while not detected in control strains known not to carry the genes. All *E. coli* isolates were positive for the control genes *gad* and *ihfA*. Microarray results for the control strains are presented in Table 2.

Virulence types

Based on the absence or presence of virulence genes and gene variants, the cattle isolates were assigned to eight different virulence types coded by roman numerals (see Fig. 3 for details). All pairs of isolates from the same farm had the same virulence type.

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Virulence type I was highly significantly associated with the group of isolates linked to human disease cases compared to all other virulence types grouped ($P=0{\cdot}012$). Virulence type I/PT4 was significantly associated with the presence of two *vtx* gene variants (vtx_2 and vtx_{2c}) compared to all other types ($P<0{\cdot}001$). Apart from the previously noted association between the county of Halland and the (PT4;vtx_2,vtx_{2c}) variant [12] shown here to belong to virulence type I, no obvious connection between geographic origin and virulence type was observed. As evident in Figure 2, all isolates of PT2 and PT4 and most isolates of PT14 had identical virulence gene types and similar PFGE patterns within each group, while PT8 isolates and isolates not corresponding to known phage types (RDNC, 'reacts but does not conform') were more variable. The association between virulence type III, with the majority of the PT14 isolates included in the study, and *cdtB* was statistically significant ($P<0{\cdot}001$), as was the association between PT2/virulence type II and *iss* ($P=0{\cdot}004$). However, in the latter case this was based on only two independent observations.

DISCUSSION

A highly topical question concerning VTEC O157:H7 is highlighted in this study, namely: which virulence factors are found in human pathogenic strains of VTEC O157:H7, and to what extent are these factors found in the average strain found in the cattle reservoir? Improved knowledge in this area can, at an early stage such as in primary production or later in the food chain, predict whether strains of VTEC O157:H7 are more or less likely to cause disease in humans. In this way, targeted intervention measures can be deployed at an early stage and thereby reduce the risk of spread of highly pathogenic VTEC O157:H7.

Using combined data from PCR and microarray analysis we have found a well-conserved core set of virulence genes in all VTEC O157:H7 isolates investigated. Apart from the verotoxins, all isolates carried the LEE-associated genes for intimin, the translocated intimin receptor (*tir*) and the effector protein gene *espF*. From the large virulence plasmid pO157, in addition to haemolysin (*hlyA*) the genes for catalase/peroxidase P (*katP*), the type II secretion pathway related protein gene *etpD*, and toxin B (*toxB*) were detected in all isolates. One further pO157 virulence gene, the serine protease gene *espP*, was indicated by microarray analysis to be present in all isolates except three, all of which were PT8 and none of which were associated with human disease cases. This suggests that these isolates do carry the large virulence plasmid, but with sequence divergence or a deletion. Brunder *et al*. [27] found *espP* in all of 63 non-sorbitol-fermenting human VTEC O157:H7/H$^-$ isolates by colony blot hybridization, two of which were PCR negative due to a partially deleted *espP* gene in the first and an insertion sequence in the second, showing that substantial alterations to the *espP* gene does not render the bacteria incapable of causing human infections.

All isolates tested positive for the genes *tccP*, by both microarray and PCR, as well as *espJ* by microarray only, both of which are described as located on the prophage CP-933U. This is in agreement with the results of Garmendia and co-workers [22], who investigated 365 O157:H7 isolates from several countries on different continents and found all to be $tccP^+/espJ^+$. Furthermore, we noted variant *tccP* gene sizes for nine isolates, all PT8 and two of which (Bd7146/00 and Bd7151/00) have been implicated in causing human illness. Again this is in agreement with the findings of others that strains with gene size variations due to different numbers of proline-rich repeat elements encoded in this gene can infect humans [22]. Similarly co-located on O157:H7 genomic islands are the tellurite resistance gene *terB*, the urease *ureA* and the adhesin *iha* [24], all of which were consistently positive in the assayed cattle isolates. Interestingly, these three genes were either all present or all absent in the non-O157 control strains.

The *efa-1* gene appears to generally be truncated in non-sorbitol-fermenting O157:H7 strains [28], and in the present study PCR analysis could not detect the 3′-region of the gene in any isolate, while the 5′-region was present in all O157:H7 cattle strains. The *efa-1* gene is also represented on the microarray, targeting a part of the 3′-region absent in EDL933. As expected, this probe also produced negative results for all isolates. The adhesion factor *lpfA* was found in the control strain CCUG 42901, but not in the O157:H7 cattle isolates. This was not surprising as the *lpfA* probe of this microarray system is designed against AY057066 which corresponds to $LpfA_{O113}$, while O157:H7 isolates generally carry $LpfA_{O157/OI\text{-}141}$ and $LpfA_{O157/OI\text{-}154}$ [29].

A few additional genes were variably absent or present: the *icf* (*paa*) gene, known to participate in O157:H7 cell adhesion in model systems [30], was

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absent in four isolates, all of PT8 and none related to human cases of disease. Additionally, in a few strains that were tested using the arrays we detected genes not traditionally associated with VTEC O157:H7 pathogenicity. Cytolethal distending toxin has been described as highly prevalent in sorbitol-fermenting VTEC O157:NM strains [31], and has been found in atypical *eae*-negative O157 [28]. In O157:H7, *cdt* has previously been described as most common in phage types 34 and 14, while rare in phage types 4, 8 and 2, and was shown to be significantly more common in clinical isolates from patients with diarrhoea than in patients with HUS or asymptomatic carriers [31]. In the present study, six PT14 isolates out of seven, all from different, geographically dispersed farms, but with similar PFGE patterns, tested positive for *cdtB*. We found the gene coding for increased serum survival (*iss*) in a single PT8 isolate and all three PT2 isolates assayed, two of which were linked to human illness. Increased serum survival exists in several plasmid-encoded and chromosomal variants, and mediates complement resistance. It has been implicated as a key virulence determinant for avian extraintestinal *E. coli* infection and neonatal meningitis-associated *E. coli* [32], but has to our knowledge no known association with enteric disease. The plasmid-carried colicin B (*cba*) was present in a few otherwise unrelated strains, one PT8 and one RDNC, as well as in the control strains CCUG 42744, 853/67 and 60/84.

The majority of the isolates related to human cases of disease investigated in this study were of the (PT4,vtx_2^+,vtx_{2c}^+) variant which is known to frequently cause severe illness in Sweden. All these isolates had identical virulence gene profiles, and the same were present in all PT4 isolates assayed. This further strengthens previous conclusions based on PFGE that these hyper-virulent strains form a distinct genetic group. However, although the isolates belonging to the (PT4,vtx_2^+,vtx_{2c}^+) group shared a unique virulence type, these isolates did not carry any distinguishing genes to explain the high virulence of this variant. Neither could we identify any distinguishing feature separating all isolates with connection to human cases of illness from the prevalence study isolates. The particular virulence of certain VTEC O157:H7 might well depend on some key virulence trait not covered by the comparatively limited set of genes investigated in this study, there is also evidence that certain groups of VTEC O157:H7 strains have higher gene expression of key virulence factors and that these differences are correlated to different prevalence in human and cattle hosts [33, 34]. Screening the cattle population for strains with potentially higher virulence might thereby have to rely on detection of regulatory elements or clonal markers identifying known high-virulence genogroups rather than detection of the presence of the virulence genes themselves.

The opportunity to compare data with PCR systems for a number of key VTEC virulence determinants also allowed us to evaluate the employed microarray system for routine characterization of VTEC isolates. Where comparison was possible the microarray performed well, with full and consistent agreement between PCR and the microarray for vtx_1, vtx_2, *hlyA*, *eaeA* and *tccP*. We additionally detected a substantial number of expected virulence genes in non-O157 control strains.

In conclusion, we have investigated the virulence gene profile of 32 VTEC O157:H7 cattle isolates from a prevalence study and 13 cattle isolates with strong epidemiological links to cases of human disease using DNA microarray analysis complemented by PCR assays. We have found a remarkably well-conserved core set of 18 virulence factors in all assayed isolates, while only a small number of genes were variably absent or present. This suggests that a majority of VTEC O157:H7 bacteria found in Swedish cattle carry a broad virulence gene repertoire and should be considered a potential threat to human health. Most isolates associated with known human disease cases had identical virulence gene profiles, but we found no particular genetic trait to explain the increased virulence of this genotype. The identification of such markers will be the objective of further study.

NOTE

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/hyg).

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DECLARATION OF INTEREST

None.

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