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Detection of Numerous Verotoxigenic E. Coli Serotypes, with Multiple Antibiotic Resistance from Cattle Faeces and Soil

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Running Title: Numerous antibiotic resistant VTEC isolated in faeces and soil

Detection of numerous verotoxigenic *E. coli* serotypes, with multiple antibiotic resistance from cattle faeces and soil

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Abstract

Verotoxigenic *E. coli* (VTEC) belong to a diverse range of serotypes. Serotypes O157 and O26 are predominately identified in VTEC-associated disease in Europe, however due to difficulty in detection little is known about the epidemiology of non-O157 serotypes. This study reports the identification of 7 VTEC serotypes from cattle faeces and soil. Cattle faeces samples (n=128) were taken from animals in 6 different farms, with soil samples (n=20) obtained from one farm. After sample incubation in modified tryptone soy broth (mTSB) supplemented with streptomycin sulphate samples were plated onto sorbitol MacConkey (SMAC) also supplemented with streptomycin sulphate. Bacteria detected on the plates were subjected to biochemical testing, antibiotic resistance profiling, and PCR to detect typical virulence genes, ß lactamase and Class 1 Integron associated genes. Serotyping was performed on isolates positive for virulence genes. *E. coli* was identified from 103 samples,

with verotoxin genes present in 7 *E. coli* isolates. Of these 7 isolates, 5 were resistant to five or more antibiotics. The isolate resistant to 9 antimicrobials contained a Class 1 Integron structure. Serotyping identified 7 separate VTEC, O2:H27, O26:H11, O63:H-, O148:H8, O149:H1, O174:H21 and ONT:H25. Six of these VTEC have been previously associated with human disease, however with the exception of O26:H11, these serotypes have been rarely reported worldwide. Increased surveillance is required to determine the prevalence of these and other non-O157 VTEC. The presence of multi-antibiotic resistance in these isolates is of concern, and the overall implications for public health must be ascertained.

1. Introduction

Since the emergence of verotoxigenic *E. coli* (VTEC) as human pathogens, contamination of foods of animal origin has been a major public health concern. Cattle are considered the main reservoir of VTEC, with infection associated with the consumption of contaminated beef and beef products (Griffin and Tauxe, 1991, Elder *et al.*, 2000) or direct contact with animals and animal faeces on the farm (Howie *et al.*, 2003, Crump *et al.*, 2003). VTEC can produce severe illness in humans, leading to this infection being listed as a notifable disease in all European countries (EU-Report, 2001, Sanco 2001**).** The virulence of VTEC can be characterized by the expression of genes for potent verotoxins (VT1 and VT2), genes for intimin production (*eae),* and *hly* gene. (Frankel *et al.*, 1998, Donnenberg *et al.*, 2001).

In the US, Canada, United Kingdom and Japan, serotype O157 is predominately identified in VTEC-associated disease. Unlike other *E. coli* serotypes, VTEC O157:H7 do not ferment sorbitol and are β -glucuronidase negative. These unique properties make the identification of this strain on selective media, such as sorbitol MacConkey (SMAC), straightforward (Mora *et al*., 2004). Infections with strains from serogroups such as O26, O111, O103 and O145 are the most common non-O157 VTEC associated with illness in humans (Blanco *et al*., 2004a). However, non-O157 VTEC infections may be frequently overlooked as they are indistinguishable from normal intestinal coliforms on the routinely used SMAC. Information on the source of non-O157 VTEC strains and their potential role in human disease is thus limited and needs further examination.

The present study compares the phenotypic and genotypic characteristics of unusual non-O157 VTEC strains, which were isolated from healthy cattle and soil.

2. Materials and Methods

2.1 Isolation and Identification of Isolates

2.1.1 Collection of samples

Faeces and soil samples were collected from 6 unrelated farms in the months of July to November. One faeces sample (10 g) was taken from the rectum of each animal (n=108) while soil samples (20 g), were obtained once from 5 paddocks on the same farm (n=20). Each animal was sampled once. In accordance with EU regulations, none of the animals was given food that contained anti-microbial agents. The soil samples were obtained from paddocks, which were empty of cattle at the time of sampling, although they had been grazed previously. There was no known contact of personnel or animals between the farms.

2.1.2 *Enrichment of samples*

Faeces samples were enriched in mTSB broth, (Oxoid, UK) containing streptomycin sulphate (1000 μg/ml) stomached in a Colworth Stomacher (Model BA 6024, A. J. Steward & Co.Ltd. London, UK), and incubated at 37°C for 24 h. Aliquots were plated onto sorbitol MacConkey agar (Oxoid, UK) containing streptomycin sulphate (1000 μg/ml), (SMAC-strep), and incubated at 37°C for 24 h. Following incubation, a single colony was taken from each sample and stored on cryoprotective beads at –20°C until required for biochemical and molecular testing (Protect, Technical Services Consultants, Lancashire, UK). The presence of any bacterial growth on the plates after enrichment was considered a positive sample.

2.1.3 Phenotypic characterisation

Each isolate was Gram stained and characterized using API 20E and API 50CH commercial kits according to manufacturers instructions (Biomerieux).

2.1.4 Virulence determination

After resuscitation from storage beads, template DNA was prepared from a single colony of each isolate. DNA was purified from the culture with a DNeasy extraction kit (Qiagen, Crawley, UK). All isolates were screened for virulence factors commonly associated with VTEC, *eae*, *hly*, *vt*1 and *vt*2 genes using a multiplex PCR method (Paton and Paton, 1998). The presence of virulence genes was also confirmed by independent PCR analysis by the Laboratorio de Referencia de *E. coli* (LREC, Lugo**,** Spain) (Blanco *et al*., 2004b). Isolates positive for one or more of these genes were also screened for the presence of the rfb_{O157} gene (Paton and Paton, 1998) and $fliC_{h7}$ gene (Fratamico *et al.*, 2000). PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised under UV illumination. The PCR products of *eaeA*, *hly*, *vt*1 and *vt*2 genes were purified using a PCR purification kit (Nucleospin Extract 11, Machery-Nagel, Germany) and sequenced commercially in duplicate (MWG Biotech, Ebersberg, Germany). Sequences were initially compared with the current GenBank sequence databases using the BLAST suite of programs (Altschul *et al.*, 1997). ClustalW amino acid sequence alignments were produced for comparison online at http://www.ebi.ac.uk/clustalw.

2.1.5 Toxin production

Isolates positive for *vt1* or *vt2* genes following PCR were tested for verotoxin production, using the commercial ELISA Premier-VTEC kit according to the manufacturers instructions (Meridian, Bioscience).

2.1.6 Serotyping

Serotyping was performed on isolates positive for virulence genes. The determination of O and H antigens was carried out by the LREC, (Lugo, Spain) as previously described, (Guinée *et al.*, 1981) employing all available O (O1-O185) and H (H1-H56) antisera. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O antisera were produced in the LERC and the H antisera were obtained from the Statens Serum Institute (Copenhagen, Denmark).

2.2 Antibiotic susceptibility testing

2.2.1 Phenotypic characterisation

Isolates positive for virulence genes, were examined for susceptibility to 12 antibiotics using the Bauer – Kirby disc diffusion method (Bauer *et al.*, 1966). The following discs (Oxoid, U. K.) were used: ampicillin 10μg, kanamycin 30μg, cefixime 5μg, cefaclor 30μg, streptomycin 10μg, trimethoprim 5μg, nalidixic acid 30μg, compound sulphonamides 300μg, chloramphenicol 30μg, tetracycline 30μg, ciprofloxacin 5μg and moxalactam 30μg .*E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923 were used for quality control. (Oxoid , UK). After incubation, the diameter (in millimeters) of the zone of inhibition of each antibiotic was measured. Isolates were classed as sensitive or resistant to each antibiotic according to Committee for Laboratory Standards Institute (CLSI) guidelines (NCCLS, 2003).

2.2.2 β-lactamase and Class 1 Integron associated genes

Template DNA from seven serotyped isolates was examined by PCR for several β-lactamase (*bla*) encoding genes including; *tem, carb, shv, oxa* and *ctx*, as described previously (Wichard, 2005; Henriques *et al.* 2006; Batchelor *et al.* 2005, Pomba *et al*. 2006). Variable regions containing the gene cassette(s) associated with class 1 integron structures were amplified, as described previously (O'Mahony *et al.* 2005). The genes mapping to the 5'- and 3'-conserved structures of class 1 integrons, were also identified by PCR.

2.2.3 DNA sequence analysis of Gene Cassettes

Amplicons of interest were extracted directly from agarose gels using a QIAGEN gel extraction kit (QIAGEN, West Sussex, UK). The recovered DNA fragment of interest was purified and quantified (as described above) prior to being sequenced commercially (Qiagen, Hilden, Germany). Sequence text files were subsequently obtained and used to search the current GenBank databases using the BLAST suite of programs (Altschul *et al.*, 1997). CLUSTALW amino acid sequence alignments were produced for comparison (Thompson *et al.*, 1994).

3. Results

From 128 samples collected, 103 isolates were obtained which grew on SMAC-strep, covering the plate with a sorbitol fermenting (pink), mucoid growth. Each sample was individually sub-cultured onto SMAC-strep from which a single colony was taken for subsequent testing. Upon subculturing, large (2 mm diameter), round, mucoid colonies were observed and Gram-negative rod-shaped bacteria were identified following staining. All

isolates were negative for Voges-Proskaur, hydrogen sulfide, citrate, oxidase and urease production and were positive for indole and fermented glucose, mannitol and sorbitol. The isolates were confirmed as *E. coli* following analysis of API 20E and API 50CH strip results. The API results were confirmed by PCR amplification of the 16S rRNA gene (data not shown).

3.1 Virulence determination

From the 103 *E. coli* isolates tested, 7 were positive for the presence of *vt*1 or *vt*2 genes as presented in Table 1. Of these isolates, 4 were detected from cattle in Farm A, one from soil in Farm A and 2 isolates were detected from cattle faeces taken in Farms B and D. All 7 isolates were negative for the presence of the rfb_{O157} and $fliC_{h7}$ genes. Two isolates of were also identified positive for the presence of *eae* and *hly* genes. Both isolates were serotyped as O123:H2. However as these isolate did not contain *vt* gene they are not included in the data presented in this paper.

3.2 PCR product sequencing

Isolates obtained from animals 92 and 80 were not included in the PCR product sequencing analysis as either the *vt*1 gene was lost following storage (isolate 92) or the isolate could not be resuscitated (isolate 80). BLAST searches showed that PCR products from positive *vt*1, *vt*2, *hly* and *eaeA* reactions were homologus to similar sequences in known bacteria carrying these genes. Deduced amino acid sequences from each PCR product were compared with selected known bacteria using ClustalW. The alignment demonstrated a similar amino acid homology across the regions examined. In summary it can be stated that the genes for *vt1*, *vt2*, *eae* and *hly* were present in the isolates examined.

3.3 Verotoxin production

Verotoxin production was confirmed for each isolate positive for the presence of *vt1* or *vt2* gene.

3.4 Serotyping

Serotyping was performed and 7 different VTEC serotypes were identified; O2:H27, O149:H1, O26:H11, O63:H-, O148:H8 and O174:H25 with one untypeable serotype ONT:H25 (Table 1).

3.5 Antibiotic susceptibility

Multi drug resistance (the resistance to three or more different antibiotic classes) was demonstrated in 5 of the 7 isolates. The AR profiles (Table 1) show that some of the isolates share a common resistance pattern. Serotype O174:H21 obtained from Farm D was resistant to the same 5 antibiotics as serotypes O2:H27 and O26:H11 isolated from Farm A.

3.5.1 β-lactamase and Class 1 Integron associated genes

The *bla*-$_{\text{TEM}}$ gene was present in all 7 of the VTEC isolates with the exception of isolate 50 and 84. None of the other *bla* genes tested were found to be present. *E. coli* O63:H- was the only strain found to contain complete class 1 integron structures. The integrase1 (*int1*), quaternary ammonium compound resistance (*qacDE1*), and sulphonamide resistance (*sull*) genes in addition to 4 gene cassettes (of sizes including1.0, 1.2, 1.6 kb) were detected after PCR.

3.5. DNA sequence analysis of gene cassettes

Sequence analysis of two gene cassettes 1.0 kb and 1.2 kb (Accession numbers EU 938126 and EU938125) amplified from the *E. coli* O63:H-, gave 100% sequence identity to the *aadA1* gene in *E. coli.* The remaining gene 1.6 kb gene cassette (Accession No. EU938127) contained two ORF's in the classical 'head-to-tail' orientation, with complete identity to a *dfr1* gene and *aadA1* gene in *E. coli.* The *aadA1* (adenyltransferase) gene confers aminoglycoside resistance and the *dfr* (dihydrofolate reductase) gene confers trimethoprim resistance.

4. Discussion

This study reports on the isolation of multiple VTEC serotypes from cattle faeces and soil. Of 103 antibiotic AR *E. coli* isolates in this study, 7 verotoxigenic serotypes, were identified. To detect whether these VTEC had been previously reported, a reference list was used which summarises published data on the serotypes and origin of non-O157 VTEC up to 2003 (www.microbionet.com.au/vtectable.htm), in addition to a list of VTEC reported from the *E. coli* reference lab in Lugo, Spain (http://www.lugo.usc.es/ecoli/). Comparison with these, suggest that the serotypes found in the current study have been rarely reported previously, with the exception of O26:H11. Serotype O63:H- has never been reported from any source.

In contrast to *E. coli* O157:H7, the identification of non–O157 VTEC is complex, with no obvious characteristics to consistently distinguish them from other *E. coli* serotypes.. In the present study, two of the seven serotypes detected, were obtained from farms remote from each other, where just six and four animals were sampled. This is in contrast to previous reports where extensive sampling and screening of multiple coliform colonies were obtained in order to isolate different serotypes. Beutin *et al.* (1997), examined 114 faeces samples from 19 cattle, and isolated 11 VTEC serotypes. In a VTEC prevalence study in Japan, faecal samples were taken from 358 animals and 20 coliform colonies per sample were tested for *vt* encoding genes, revealing 25 different serotypes (Kobayashi *et al.*, 2001).

In the present study all of the isolates fermented sorbitol and therefore would be overlooked if non-sorbitol fermenting colonies only were selected, as is usual with routine methods. The fact that these isolates are resistant to a number of anti-microbial

agents may aid their detection in future studies by supplementing selective media with the appropriate anti-microbials. It should be noted, however, that as the methodology in the current study selected for streptomycin resistant VTEC only, a different protocol may have detected additional, non AR VTEC isolates. The results from the current study suggest that the prevalence and variety of non-O157 VTEC in food animals may be grossly underestimated, with the lack of standardised methods leading to difficulty in ascertaining the prevalence of these and other unidentified VTEC serotypes present in cattle and the food chain.

The acquisition of verotoxin genes by enteric bacteria is well documented and the horizontal transmission of virulence factors has been crucial to the emergence of VTEC as important pathogens (Moxon *et al.*, 1994). The isolates identified in this study contained many virulence factors that are associated with human disease and serotypes O2:H27, O149:H1, ONT:H25, O148:H8 and O174:H21 have been each been identified as infrequent human pathogens from several countries in Europe and North America. Verotoxin (VT) production was confirmed in just six of seven isolates as a *vt* gene was lost from serotype ONT:H25 following longterm storage (9 months). The loss of *vt* gene-carrying phages has been previously reported following storage and cultivation of *E. coli* O157:H7 and non O157 isolates (Iguchi *et al.*, 2002, Murase *et al.*, 1999).

The production of VT2 alone is associated with more serious clinical disease (Ostroff *et al.*, 1989, Boerlin *et al.*, 1999) and this toxin was produced by three strains in the current study, including serotype O174:H21, which has been previously associated with HUS. Additional virulence factors associated with increased human pathogenicity were determined in five of the nine isolates. The *eae* gene was identified in two isolates

and serotypes carrying this gene have been linked with severe illness, such as haemorrhagic colitis and HUS (Oswald *et al.,* 2000). The newly identified serotype O63:H- produced VT1 and contained *hly* and *eae* genes indicating that this serotype has the potential to be pathogenic to humans. The VTEC strains identified in the present study contain the necessary virulence genes required to cause human disease, and must be considered as potential pathogens that could be involved in future outbreaks of haemorrhagic colitis and HUS. Screening for these and other serotypes should be carried out in suspect clinical cases from which *E. coli* O157 is not isolated.

E. coli are a rapidly evolving species capable of developing new pathogenic variants (Donnenberg *et al*., 2001). LeClerc *et al.,* (1996) reported that 1% of *E. coli* O157:H7 serotypes had spontaneous rates of mutation that were 1,000 fold higher than those of typical *E. coli*. This ability to hyper-mutate may lead to the acquisition of virulence genes and development of other properties, such as antibiotic resistance, that increase bacterial diversity thus conferring a competitive advantage. Recently increasing numbers of multi-resistant VTEC have been isolated from humans, cattle and food (vonMüffling *et al,* 2007, Mora *et al.*, 2005.) The findings of the current study indicate that the VTEC serotypes were simultaneously resistant to several anti-microbial classes, including penicillins, aminoglycosides, tetracyclines, sulphonamides and fluroquinolones with strain O63:H- resistant to 9 antibiotics. Multiple antibiotic resistance may be acquired through mobile genetic elements such as plasmids, transposons and Class 1 integrons, (Mora *et al.*, 2005, Singh *et al.*, 2005), or due to the presence of overexpression from chromosomally encoded multi-drug efflux pumps (Poole, 2004). A complete class I integron structure was detected in strain O63:H and the *bla*-$_{\text{TEM}}$ gene,

which is generally plasmid encoded, was present in all isolates, suggesting the potential horizontal transfer of antibiotic resistance to other bacteria. Although anti-microbial treatment of VTEC infection is not recommended due to the potential for verotoxin release (Wong *et al.*, 2000) the development of multiple resistant VTEC is nevertheless a development that public health professionals must note. It is unknown whether these AR serotypes have always been present, with a recent increase in prevalance in response to an unknown environmental stimulus. Future work characterizing the potential for resistance transfer from these isolates is critical to ascertain the significance of multiple antibiotic resistance in these pathogens.

The identification of rare non-O157, multiply antibiotic resistant VTEC in this study is of concern. Findings from the current study suggest that targeted sampling and method development utilizing the antibiotic resistant characteristics may reveal many more of these serotypes in the bovine population and possibly elsewhere. Antibiotic resistant *E. coli* are known to be disseminated through the food chain, (Johnson *et al.*, 2005, DeFrancesco *et al.*, 2004) therefore the bacteria from this study may represent a new group of clinically significant food-borne pathogens. This data highlights the need for increased monitoring for the presence of non–O157 VTEC in cattle and humans in addition to monitoring the level of antimicrobial resistance in order to ascertain the potential public health risk of these emerging strains.

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