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1 **Running Title:** Numerous antibiotic resistant VTEC isolated in faeces and soil
2 **Detection of numerous verotoxigenic *E. coli* serotypes, with multiple antibiotic**
3 **resistance from cattle faeces and soil**

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17 **Abstract**

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

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

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1 **1. Introduction**

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

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

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

23

1 **2. Materials and Methods**

2 **2.1 Isolation and Identification of Isolates**

3 *2.1.1 Collection of samples*

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

11

12 *2.1.2 Enrichment of samples*

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

21

1 2.1.3 Phenotypic characterisation

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

4

5 2.1.4 Virulence determination

6 After resuscitation from storage beads, template DNA was prepared from a single colony of
7 each isolate. DNA was purified from the culture with a DNeasy extraction kit (Qiagen,
8 Crawley, UK). All isolates were screened for virulence factors commonly associated with
9 VTEC, *eae*, *hly*, *vt1* and *vt2* genes using a multiplex PCR method (Paton and Paton, 1998).
10 The presence of virulence genes was also confirmed by independent PCR analysis by the
11 Laboratorio de Referencia de *E. coli* (LREC, Lugo, Spain) (Blanco *et al.*, 2004b). Isolates
12 positive for one or more of these genes were also screened for the presence of the *rfb*_{O157}
13 gene (Paton and Paton, 1998) and *fliC*_{h7} gene (Fratamico *et al.*, 2000). PCR products were
14 separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and
15 visualised under UV illumination. The PCR products of *eaeA*, *hly*, *vt1* and *vt2* genes were
16 purified using a PCR purification kit (Nucleospin Extract 11, Machery-Nagel, Germany) and
17 sequenced commercially in duplicate (MWG Biotech, Ebersberg, Germany). Sequences were
18 initially compared with the current GenBank sequence databases using the BLAST suite of
19 programs (Altschul *et al.*, 1997). ClustalW amino acid sequence alignments were produced
20 for comparison online at <http://www.ebi.ac.uk/clustalw>.

21

1 2.1.5 Toxin production

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

5

6 2.1.6 Serotyping

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

13 2.2 Antibiotic susceptibility testing

14

15 2.2.1 Phenotypic characterisation

16 Isolates positive for virulence genes, were examined for susceptibility to 12 antibiotics using
17 the Bauer – Kirby disc diffusion method (Bauer *et al.*, 1966). The following discs (Oxoid, U.
18 K.) were used: ampicillin 10µg, kanamycin 30µg, cefixime 5µg, cefaclor 30µg, streptomycin
19 10µg, trimethoprim 5µg, nalidixic acid 30µg, compound sulphonamides 300µg,
20 chloramphenicol 30µg, tetracycline 30µg, ciprofloxacin 5µg and moxalactam 30µg. *E. coli*
21 strain ATCC 25922 and *S. aureus* strain ATCC 25923 were used for quality control. (Oxoid ,
22 UK). After incubation, the diameter (in millimeters) of the zone of inhibition of each
23 antibiotic was measured. Isolates were classed as sensitive or resistant to each antibiotic
24 according to Committee for Laboratory Standards Institute (CLSI) guidelines (NCCLS,
25 2003).

1 2.2.2 *β-lactamase and Class 1 Integron associated genes*

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

8

9 2.2.3 *DNA sequence analysis of Gene Cassettes*

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

17

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19 **3. Results**

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

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

6 *3.1 Virulence determination*

7 From the 103 *E. coli* isolates tested, 7 were positive for the presence of *vt1* or *vt2* genes as
8 presented in Table 1. Of these isolates, 4 were detected from cattle in Farm A, one from soil
9 in Farm A and 2 isolates were detected from cattle faeces taken in Farms B and D. All 7
10 isolates were negative for the presence of the *rfb*_{O157} and *fliC*_{h7} genes. Two isolates of were
11 also identified positive for the presence of *eae* and *hly* genes. Both isolates were serotyped
12 as O123:H2. However as these isolate did not contain *vt* gene they are not included in the
13 data presented in this paper.

14 *3.2 PCR product sequencing*

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

1 3.3 Verotoxin production

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

4 3.4 Serotyping

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

8 9 3.5 Antibiotic susceptibility

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

14 15 3.5.1 β -lactamase and Class 1 Integron associated genes

16 The *bla*-TEM gene was present in all 7 of the VTEC isolates with the exception of isolate 50
17 and 84. None of the other *bla* genes tested were found to be present. *E. coli* O63:H- was the
18 only strain found to contain complete class 1 integron structures. The integrase1 (*int1*),
19 quaternary ammonium compound resistance (*qacDE1*), and sulphonamide resistance (*sull*)
20 genes in addition to 4 gene cassettes (of sizes including 1.0, 1.2, 1.6, kb) were detected after
21 PCR.

22

23 3.5.2 DNA sequence analysis of gene cassettes

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

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1 4. Discussion

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

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

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

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

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

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

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

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

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

10 The identification of rare non-O157, multiply antibiotic resistant VTEC in this
11 study is of concern. Findings from the current study suggest that targeted sampling and
12 method development utilizing the antibiotic resistant characteristics may reveal many
13 more of these serotypes in the bovine population and possibly elsewhere. Antibiotic
14 resistant *E. coli* are known to be disseminated through the food chain, (Johnson *et al.*,
15 2005, DeFrancesco *et al.*, 2004) therefore the bacteria from this study may represent a
16 new group of clinically significant food-borne pathogens. This data highlights the need
17 for increased monitoring for the presence of non-O157 VTEC in cattle and humans in
18 addition to monitoring the level of antimicrobial resistance in order to ascertain the
19 potential public health risk of these emerging strains.

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**Table 1 Phenotypic and genotypic characteristics of VTEC isolates from cattle
faeces and soil**

Farm	Animal ID	Isolate origin	Serotype	<i>vt</i>	Verotoxin produced	<i>hly</i>	<i>eae</i>	Antibiotic resistance pattern¹
A	12	dairy calf	O2:H27	<i>vt2</i>	VT2	+	-	A, S, S3, C, T,
A	50	soil,	O149:H1	<i>vt1</i>	VT1	-	-	S
A	58	beef cow	O26:H11	<i>vt1</i>	VT1	+	+	A, S, S3, C, T,
A	80	beef calf	O63:H-	<i>vt1</i>	VT1	+	+	A, K, S, W, N, S3, C, T, CIP,
A	84	beef calf	O148:H8	<i>vt2</i>	VT2	+	-	S
B	92	steer	ONT:H25	<i>vt1</i>	NT	-	-	A, K, S, W, S3, T,
D	101	calf	O174:H21	<i>vt2</i>	VT2	-	-	A, S, S3, C, T,

¹: Anti-microbial agents tested: A = ampicillin ; K = kanamycin ; S = streptomycin ; W= trimethoprim ; S3= compound sulphonamides ; CIP = ciprofloxacin; C = chloramphenicol ; T = tetracycline and N = nalidixic acid
 ONT = untypeable
 NT = not tested