Laboratory-based surveillance of human verocytotoxigenic *Escherichia coli* infection in the Republic of Ireland, 2002–2004

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The aim of this study was to examine the frequency and distribution of human verocytotoxigenic *Escherichia coli* (VTEC) O157 and non-O157 in the Republic of Ireland, and also to examine the presence of virulence genes in these isolates. This genetic information combined with phenotypic tests was used to produce a complete laboratory-based surveillance of human clinical VTEC infection in the Republic of Ireland between 2002 and 2004. Between January 2002 and December 2004 a total of 207 VTEC isolates were studied (one isolate per patient), 185 (89 %) of these were *E. coli* O157. The remaining 22 (11 %) were non-O157 *E. coli*, made up of 15 (7.2%) *E. coli* O26, one (0.5%) *E. coli* O103, one (0.5%) *E. coli* O146, one (0.5%) *E. coli* O145, two (1%) *E. coli* O111 and two (1%) ungroupable VTEC. These isolates originated from the eight health boards in the Republic of Ireland and represented over 90 % of the clinical cases of VTEC in the Republic of Ireland during this period. The results showed that VTEC O157 was the predominant serogroup and had a predominant toxin genotype of VT2 alone. Phage type 32 was the most common phage type of *E. coli* O157 identified. Non-O157 VTEC was a small proportion of all VTEC (10 % in 2002, 8 % in 2003, 15.5 % in 2004). In 2004 it was noted that there was an increase in the number and variety of non-O157 VTEC strains; however, this requires further monitoring in the future to see if this trend is sustained. It was also noted throughout the study period that the incidence of VTEC was higher in rural areas. Implementation of real-time PCR for the detection and subtyping of VTEC has aided outbreak investigations and is important for enhanced surveillance of VTEC in the Republic of Ireland.

**INTRODUCTION**

Since its identification as a pathogen in 1982 (Riley *et al.*, 1983), verocytotoxigenic *Escherichia coli* (VTEC) has emerged as an important cause of gastrointestinal disease in humans. VTEC infections may result in life-threatening conditions such as haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (Karmali, 1989). There are at least 100 *E. coli* serogroups that are capable of producing verocytotoxins (Nataro & Kaper, 1998), of which *E. coli* O157 is the most common. *E. coli* O157 can cause clinical infection with a very low infectious dose; this is attributed to the presence of verocytotoxins (Gyles, 1992) and other accessory virulence factors such as intimin and enterohaemolysin (Frankel *et al.*, 1998). Intimin is carried on the chromosomally located locus of enterocyte effacement (LEE) pathogenicity island (Levine *et al.*, 1987). The eae gene carried on this island allows the bacteria to produce intimin, resulting in attaching and effacing lesions in the host intestinal mucosa cells. This has the effect of increasing the virulence of the bacteria to the host (Kaper, 1998). Schmidt *et al.* (1995) suggested that enterohaemolysin (encoded by the hlyA gene) acts synergistically with Shiga toxins to disrupt important cell functions.

Verocytotoxins can be divided into two major subclasses, VT1 and VT2. VT2 is further subdivided into five subtypes VT2, VT2c, VT2d, VT2e and VT2f (Wang *et al.*, 2002). Variants of VT1 have also been described (Paton *et al.*, 1995); however, VT1 is more homogeneous than VT2. VTEC strains can carry one or both verocytotoxins. Strains associated with human disease and in particular haemolytic uraemic syndrome more commonly carry VT2 rather than VT1 alone (Kleanthous *et al.*, 1990; Ostroff *et al.*, 1989).

*E. coli* O157 is part of the normal gut flora of cattle; however, it is also carried by sheep and other animals (Chapman *et al.*, 1997). Contact with animals is an important route of transmission for human infection (Parry *et al.*, 1995; Milne *et al.*, 1999). However, the consumption of contaminated food has been the source of well-documented outbreaks of
VTEC infection (Duffell et al., 2003; Pennington, 1997). The risk foods include meat and dairy products and in particular undercooked beef, unpasteurised milk and ready-to-eat products contaminated with animal waste, e.g. salad, vegetables and fruit (Griffin & Tauxe, 1991). Person-to-person transmission is also frequently observed in families, nurseries and nursing homes (McMaster et al., 2001; Carter et al., 1987).

To date the general epidemiology of human VTEC in the Republic of Ireland has been documented by the National Disease Surveillance Centre (NDSC) (NDSC Annual Report, 2002, 2003). Outbreaks have been associated with person-to-person spread (McMaster et al., 2001), probable food transmission and also with waterborne transmission (Finnegan, 2004). The incidence of VTEC in the Republic of Ireland was first reported in 1996 at 0·22 per 100 000 (NDSC Annual Report, 1999) and has increased since, with the incidence in 2003 documented as 2·1 per 100 000 (NDSC Annual Report, 2003). However, VTEC only became a statutory notifiable disease in the Republic of Ireland in January 2004, so the accuracy of voluntary surveillance data prior to this date may be an underestimate of the true incidence. To date there are no reports on the prevalence of E. coli verocytotoxin, eae or hly genes from human clinical strains in the Republic of Ireland; however, data from Northern Ireland have been published (Crothers et al., 2004). The aim of this study was to examine the incidence of these virulence loci in E. coli O157 and non-O157 VTEC and combine this data with serology, phage types and antimicrobial resistance to produce a complete laboratory-based surveillance of human VTEC infection in the Republic of Ireland between 2002 and 2004.

METHODS

Bacterial strains. Two hundred and seven patients were identified with VTEC infection between 2002 and 2004. One hundred and eighty-five E. coli O157, 15 E. coli O26, one E. coli O103, one E. coli O146, one E. coli O145, two E. coli O111 and two ungroupable human isolates were obtained, with only one isolate per patient being documented. The organisms were isolated from human faeces submitted to the Public Health Laboratory - Health Service Executive - South Western Area (PHL-HSE-SWA). Other VTEC isolates were referred to the PHL-HSE-SWA for confirmatory VTEC studies and typing.

Culture

E. coli O157. Facies was plated directly onto cefixime tellurite sorbitol MacConkey (CT-SMAC) agar (LIP) and simultaneously inoculated into modified Tryptone Soya broth (mTSB, LIP), both of which were incubated aerobically overnight at 37°C. The inoculated mTSB broth was then utilized for immunomagnetic separation (IMS, IMS beads DYNAL Biotech), post-IMS beads were plated onto CT-SMAC and MacConkey agar (LIP) and incubated aerobically overnight at 37°C. Suspect colonies (non-sorbitol-fermenters) were identified as putative E. coli O157 by latex agglutination with E. coli O157 antisera (wellcolex) and confirmed biochemically as E. coli by API20E (Biomerieux).

Non-O157 E. coli. Facies was plated directly onto MacConkey, CT-SMAC and Columbia Blood agar (LIP), and incubated aerobically overnight at 37°C. Suspect colonies (lactose fermenters on MacConkey or predominant sorbitol fermenters on CT-SMAC) were identified by slide agglutination with a range of E. coli antisera (Murex). If agglutination occurred with single E. coli antisera it was then further biochemically confirmed as E. coli by API20E, and PCR for verocytotoxin detection was performed (see below). Ungroupable VTEC isolates were sent to the Laboratory for Enteric Pathogens, Health Protection Agency, Colindale, UK, for further serogrouping.

PCR for verocytotoxin, virulence and rfbE: O157 genes

Primers. PCR for vt1, vt2, O157, eae and hlyA genes was carried out using the primer pairs detailed in Table 1.

Method. Using a 1 μl loop suspect colonies (a single colony if there was a pure growth or a sweep from a mixed plate, ensuring that there was at least one colony from each colony type on the plate) were picked from primary and/or post-IMS plates. These were emulsified in 100 μl 1× TAE buffer [0·04 mol Tris acetate l−1 (pH 8·3), 0·001 mol EDTA l−1] in a 1·5 ml microcentrifuge tube and incubated at 100°C for 10 min in a heating block. The cell suspension was centrifuged at 12 500 g for 5 min and 5 μl of the supernatant was added to the following PCR mix: 25 μl reaction containing 1× Sybr green PCR master mix (Bio-Rad Laboratories) and 50 ng of each primer (Sigma Genosys). Samples were amplified using a Bio-Rad I-Cycler at a cycle of 95°C for 2 min, 30 cycles of 45 s at 95°C, 45 s at 58°C and 1 min at 72°C, and a final extension step of 72°C for 10 min. This amplification reaction was followed by a melting temperature analysis of PCR amplimers.

Table 1. Primer sequences used for PCR amplification of VTEC genes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene targeted</th>
<th>Primer sequence (5′–3′)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT1F</td>
<td>stx1</td>
<td>ACACCTGGATGATCTCAGTGG</td>
<td>601</td>
<td>Gannon et al. (1992)</td>
</tr>
<tr>
<td>VT1R</td>
<td>stx2</td>
<td>CTAATCCCCCCTCCATTATG</td>
<td>780</td>
<td>Gannon et al. (1992)</td>
</tr>
<tr>
<td>VT2F</td>
<td>stx2</td>
<td>CCATGACAAGGGACAGCAGTT</td>
<td>780</td>
<td>Gannon et al. (1992)</td>
</tr>
<tr>
<td>VT2R</td>
<td>stx2</td>
<td>CCTGCAACTGACGACTTGT</td>
<td>780</td>
<td>Gannon et al. (1992)</td>
</tr>
<tr>
<td>EaeF</td>
<td>eae</td>
<td>TCAGTCAGGTCAGGGCTGTC</td>
<td>1109</td>
<td>Beebakhee et al. (1992)</td>
</tr>
<tr>
<td>EaeR</td>
<td>eae</td>
<td>CGAGGCTTATGACCGCTGAAAGT</td>
<td>1109</td>
<td>Beebakhee et al. (1992)</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Serogroups

Of the 4185 human clinical stool samples and 1152 isolates examined between January 2002 and December 2004 at PHL-HSE-SWA, 207 E. coli isolates were confirmed as VTEC by detection of one or both of the genes that encode the verocytotoxins VT1 and VT2. These confirmed VTEC isolates were divided into six serogroups, O157 (n = 185), O26 (n = 15), O111 (n = 2), O145 (n = 1), O146 (n = 1) and O103 (n = 1) and ungroupable VTEC (n = 2). Isolates detailed in this paper represent over 90% of all VTEC cases reported to the NDSC from 2002 to 2004.

E. coli O157 is the predominant serogroup, accounting for 90% of all VTEC isolates throughout the 3 years (Fig. 1). In 2002 one VTEC O103 (2%) and five VTEC O26 (9%) isolates were detected. In 2003 O26 was the only non-O157 serogroup detected and represented 8% of VTEC isolates (Fig. 1). However, in 2004 there were four different non-O157 VTEC serogroups (O26, 3; O111, 1; O145, 1; O146, 1) plus two ungroupable VTEC isolates. The non-O157 isolates accounted for 15.5% of the total 2004 VTEC isolates. This increase in the variety of non-O157 isolates and the complexity of serogroups has many implications for laboratory methodology and related public health interventions. Currently in the Republic of Ireland there are no standard methods utilized for the detection of non-O157 VTEC. Many diagnostic laboratories rely on culture methods alone and few utilize molecular methods to detect verocytotoxin genes; therefore there is probably an under-detection of non-O157 VTEC infections. This may hamper the implementation of relevant public health interventions. The increase in non-O157 VTEC has also been documented in other countries, for example in Italy there has been an increase in the incidence of E. coli O26 (Tozzi et al., 2003). The predominance of the E. coli O157 serogroup in the Republic of Ireland correlates with the UK, the Czech Republic, Finland, Spain and the Netherlands (Fisher et al., 2003); however, other European countries such as Denmark, Italy, Germany and France have a higher incidence of non-O157 VTEC compared to O157 VTEC (Tozzi et al., 2003; Caprioli et al., 1997; Fisher et al., 2003).

Apart from producing verocytotoxins, non-O157 VTEC do not differ significantly from each other in their biochemical characteristics. This makes it difficult to diagnose non-O157 VTEC via culture methods alone, as no specific selective media are available. This heightens the need for molecular detection and typing of VTEC by PCR and PFGE. PCR is rapid, sensitive and specific, yielding information on the presence of specific virulence genes, whereas PFGE yields valuable information on the genetic similarity of strains. The increased variety of non-O157 serogroups may reflect an increased awareness and pursuit in the diagnosis of non-O157 VTEC. However, to optimize detection, there needs to be standardization of diagnostic procedures for VTEC (O157 and non-O157) in primary laboratories.

Origins of isolates

VTEC isolates originated from all eight health boards in the Republic of Ireland. These health board regions are Eastern Region Health Authority (ERHA), North Western Health Board (NWHB), Mid Western Health Board (MWHB), Midlands Health Board (MHB), South Eastern Health Board (SEHB), Western Health Board (WHB), North Eastern Health Board (NEHB) and the Southern Health Board (SHB) (Fig. 2). The incidence in the various health boards fluctuated from year to year due to the location of outbreaks. Predominantly urban areas and densely populated regions, e.g. ERHA, had a consistently low incidence of VTEC, while health boards with a mainly rural population, e.g. WHB, and a high level of agriculture had consistently higher levels of VTEC. These findings are consistent with those of Locking et al. (2001), who found that contact with animal faeces is a very important risk factor for sporadic E. coli O157 infection.

Fig. 1. Serogroups of VTEC isolates in the Republic of Ireland from 2002 to 2004. Colours: black, ungroupable; purple, O145; green, O146; yellow, O26; blue, O111; pink, O103; red, O157.
Antimicrobial resistance

Although the role of antimicrobials in the treatment of VTEC infection is debatable (Safdar et al., 2002), susceptibility to antimicrobials may be a useful and easy primary epidemiological marker of VTEC infection. All VTEC O157 isolates were tested for susceptibility to a panel of 13 antibiotics (Table 2). In general there was a low level of antimicrobial resistance in VTEC O157; the percentages of isolates that showed resistance to one or more antibiotics were 2.8% in 2002, 13.9% in 2003 and 12.2% in 2004. Most of the resistant isolates were resistant to two or more antibiotics.

Seasonality

In the period 2002–2004 the peak incidence (26–35 cases) of positive VTEC in the PHL-HSE-SWA was seen in late summer to early autumn (quarter 3), with numbers falling with the onset of winter (Fig. 3). These trends for VTEC have been observed in other countries (Willshaw et al., 2001). Contributing factors to the seasonality of VTEC may be related to open grazing of cattle (or other domestic animals) coupled with rainfall, leading to heavy environmental contamination.

Verocytotoxins

The presence of VT1 and VT2 genes varied greatly between the different serogroups of the VTEC isolates. In all study years VTEC O157 were predominantly VT2 alone (>70%), with smaller percentages (2002, 25%; 2003, 17.8%; and 2004, 24.5%) carrying both VT1 and VT2 genes (Table 3, Fig. 4). No VTEC O157 carried the VT1 gene alone. In addition to VTEC O157 there were a small number of toxin-negative E. coli O157 isolates (2002, n = 1; 2003, n = 2). The Republic of Ireland VTEC O157 results are consistent with those seen in Northern Ireland, where in 2000 80.5% of VTEC O157 isolates examined possessed the VT2 gene alone and 16.7% possessed both VT1 and VT2 genes (Crothers et al., 2004). Similar results have also been observed in England and Wales (Chalmers et al., 1999), and Scotland (Locking et al., 2003).

Over the three study years 22 non-O157 VTEC were detected, of which 59% possessed VT1 alone, 32% possessed both VT1 and VT2 genes and just 9% had VT2 alone (Table 3, Fig. 4). Three of the four E. coli O26 isolates in 2004 were verocytotoxin positive, whereas in 2002 and 2003 only 50% of E. coli O26 isolates were verocytotoxin positive. This reiterates the clinical significance of non-O157 VTEC, and therefore the importance and the need for standardized laboratory diagnosis of these organisms.

Phage types

All O157 VTEC were phage typed and a number of trends were observed (Fig. 5). In all study years the predominant

Table 2. Percentage of VTEC O157 isolates that are resistant to each antibiotic in the Republic of Ireland, 2002–2004

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Percentage of isolates resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (10 μg)</td>
<td>11.8</td>
</tr>
<tr>
<td>Ampicillin (10 μg)</td>
<td>3.8</td>
</tr>
<tr>
<td>Trimethoprim (5 μg)</td>
<td>3.2</td>
</tr>
<tr>
<td>Minocycline (30 μg)</td>
<td>3.2</td>
</tr>
<tr>
<td>Tetracycline (5 μg)</td>
<td>3.2</td>
</tr>
<tr>
<td>Sulphonamide (300 μg)</td>
<td>4.8</td>
</tr>
<tr>
<td>Kanamycin (30 μg)</td>
<td>1.0</td>
</tr>
<tr>
<td>Gentamicin (10 μg)</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Phage type in the Republic of Ireland was phage type 32. It accounted for 60% of VTEC O157 isolates in 2002, 39% in 2003 and rose to 63.5% in 2004. Although at much lower levels, phage type 8 was the second most common phage type. It accounted for 17% of isolates in 2002, 25% in 2003 and 10.5% in 2004.

Phage type 21/28 is the commonest phage type in Scotland (Locking et al., 2003), but its incidence is very different in the Republic of Ireland. Phage type 21/28 was first documented in the Republic of Ireland in 1999; however, its presence has fluctuated greatly. In 2001 no phage type 21/28s were observed, and the frequency was 11% in 2002 and 19% in 2003 but decreased in 2004 to just 4%. However, with such small numbers it is hard to determine the significance of these trends. The trends observed in Northern Ireland (Crothers et al., 2004) have shown that phage type 32 was the predominant type in the 1990s but was eroded in 2000, when phage type 21/28 emerged as the predominant type. It is interesting that although our countries are geographically close the VTEC phage types can differ.

Phage type 14 is present in the Republic of Ireland in low numbers and accounted for 8% of isolates in 2002, 9.6% in 2003 and 10.5% in 2004. In the Republic of Ireland in 2003 three new non-travel-related phage types emerged, these were phage type 1 (one in 2003 and one 2004), phage type 2 (three in 2003) and phage type 31 (one in 2003 and one in 2004). Phage type 51 ($n = 1$) emerged in 2004.

Phage typing has proven to be a useful epidemiological tool. Potentially strains can be differentiated into more than 80 phage types. However, many countries, like we have seen in the Republic of Ireland, have a predominance of a few major phage types (Smith et al., 2002). When phage typing is used in conjunction with other molecular typing methods such as PCR and PFGE, better epidemiological discrimination of VTEC O157 is achieved (Smith et al., 1998).

Virulence genes

VTEC O157 were tested for the presence of other virulence genes, i.e. $eae$ and $hlyA$. The $eae$ gene was present in 100% of VTEC O157 isolates and $hlyA$ was present in 96% of verocytotoxin-producing isolates. Crothers et al. (2004) also reported that all VTEC O157 isolates tested in Northern Ireland carried the $eae$ gene. The significance of the Republic of Ireland virulence gene data may become more apparent when linked to the enhanced surveillance data on individual clinical symptomatology and risk exposures collected by the NDSC on VTEC infections. It may be possible to link certain symptoms or exposures to the presence of particular virulence genes and subsequent illness.

### Table 3. Verocytotoxin genotypes of human VTEC O157 and non-O157 strains in the Republic of Ireland, 2002–2004

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Year</th>
<th>No. of isolates</th>
<th>VT1 alone</th>
<th>VT2 alone</th>
<th>VT1+VT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>2002</td>
<td>0</td>
<td>39</td>
<td>13</td>
<td>52</td>
</tr>
<tr>
<td>O157</td>
<td>2003</td>
<td>0</td>
<td>69</td>
<td>15</td>
<td>84</td>
</tr>
<tr>
<td>O157</td>
<td>2004</td>
<td>0</td>
<td>37</td>
<td>12</td>
<td>49</td>
</tr>
<tr>
<td>Non-O157</td>
<td>2002</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Non-O157</td>
<td>2003</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Non-O157</td>
<td>2004</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>All</td>
<td>All</td>
<td>All</td>
<td></td>
<td></td>
<td>207</td>
</tr>
</tbody>
</table>

Fig. 4. Toxin genotypes of VTEC isolates received in the PHL-HSE-SWA in 2002–2004. Bars: hatched, VT1+VT2; black, VT2 alone; white, VT1 alone.

Fig. 5. Major phage types of VTEC O157 isolates in the PHL-HSE-SWA in 2002–2004. Phage types are expressed as a percentage of the total number of VTEC O157 isolates. The combined totals for each of the phage types were as follows: PT 32, 93; PT8, 34; PT 21/28, 23; PT 14, 16. Symbols: $\bullet$, PT32; $\blacksquare$, PT8; $\blacktriangle$, PT21/28; $\blacktriangleleft$, PT 14; $\times$, other.
Conclusions

This 3 year study has provided detailed laboratory surveillance data on human VTEC infections. When these data are linked with public health enhanced VTEC surveillance data, they will aid the determination of the true burden of illness related to VTEC infection in humans in the Republic of Ireland.

ACKNOWLEDGEMENTS

We would like to thank all of the consultant microbiologists from the Republic of Ireland for sending us their VTEC isolates. We would also like to thank the Laboratory for Enteric Pathogens, HP A, Colindale, and in particular Dr Geraldine Smith for providing us with an excellent phage typing service, and finally a thank you to the dedicated staff of PHL-HSA-SWA for all their excellent work.

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