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Verocytotoxin *Escherichia coli*-Associated Haemolytic Uraemic Syndrome

V. Murphy[1,3], A.M. Carroll[1], K. Forde[1], R. Broni[2], E.B. McNamara[1,3]

1. Public Health Laboratory, Health Service Executive Dublin Mid-Leinster, Cherry Orchard Hospital, Ballyfermot, Dublin 10, Ireland
2. Dublin Institute of Technology, Kevin Street, Dublin 2, Ireland
3. Department of Clinical Microbiology, Trinity College Dublin, Ireland

Abstract

Aims
To describe laboratory data on clinical human Verotoxigenic *E. coli* (VTEC) strains causing haemolytic uraemic syndrome (HUS) and to characterise the VTEC strains, thus contributing to risk mitigation to decrease HUS incidence in Ireland.

Methods
Laboratory characterisation was performed on isolates from 52 VTEC-associated HUS cases identified in the National clinical VTEC Reference Laboratory (NRL-VTEC) for the years 2012-2014. Data were analysed with respect to age, gender, serogroup and verotoxin type and subtype.

Results
52/83 (62.6%) culture positive HUS cases were identified from laboratory data; 30 (57.7%) cases occurred in females. Seven HUS-associated serogroups and eleven patterns of verotoxin subtypes are described.

Conclusion
Ireland has the highest incidence of VTEC infection in Europe and a variety of VTEC serogroups causing clinical infection, suggesting any viable VTEC may potentially cause HUS. A broad diagnostic approach, to detect uncommon serotypes, should be considered when analy**s**ing clinical and food samples for VTEC.

Introduction

Verocytoxin- producing *E. coli* (VTEC) is an important cause of gastrointestinal infection, predominantly in children[1]. Clinical infection presentation ranges from asymptomatic infection to mild, watery, short-lived diarrhoea to severe, bloody diarrh**o**ea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) or even death (3-5% in HUS cases, less in non-HUS cases)[2]. VTEC infection became a statutory notifiable disease in Ireland in 2004.

VTEC pathogenesis derives from production of shiga-like toxins or verocytotoxins (VT1, VT2 or both); in addition the *E. coli eae* gene encodes intimin production to create an attaching and effacing lesion and other virulence factors (such as haemolysin (*hlyA*) gene) may produce a range of gastrointestinal disease. It is one of a number of enteropathotypes. At least 100 VTEC serotypes are implicated in human infection[3]; serotypes O157, O26, O111, O145, O103 and O104 are of particular importance and considered the 'big 6' globally. In addition to these, other non-O157 serotypes, such as serotypes O91, O80 and O121 may frequently occur[4]. A rare hybrid VTEC strain caused a large outbreak in Germany in 2011. This outbreak was associated epidemiologically with the consumption of fenugreek sprouts. It was notable for its unusually high HUS, and mortality, rate[5]. The unique causative VTEC-producing strain of serotype O104:H4 carried verotoxin genes, but was also ESBL-positive (an extended beta-

lactamase producer). The strain was *eae* negative but carried virulence factor genes characteristic of human-associated Enteroaggregative *E. coli*.

Ireland has the highest incidence of VTEC infection in Europe[6]. Since 2013, over 700 cases of human VTEC infection are reported each year to the Health Protection Surveillance Centre (HPSC), Ireland's national specialist agency for the surveillance of communicable disease. In the period 2012-2014, the median incidence of VTEC in Ireland was 13.1/100,000 (IQR 3.3)[7, 8, 9].

VTEC cases in Ireland occur sporadically or are associated with outbreaks. Cattle and other animals such as sheep are the main reservoirs for VTEC[10]. Transmission occurs through consumption of contaminated food or water or from contact with infected animals or their environment or via person-to-person (P-P) spread. Ireland has a particular problem with contaminated water from non-public supplies implicated in VTEC outbreaks along with high secondary (P-P) spread within childcare facilities[11].

HUS is characterised by thrombocytopenia, acute renal failure and non-immune microangiopathic haemolytic anaemia. Typical and atypical forms exist; they differ in terms of causes, epidemiology, incidence, treatment and prognosis. Typical HUS commonly causes paediatric acute renal failure but up to 70% of children recover from the acute phase of infection[12]. Atypical HUS is associated with poorer outcomes than typical HUS. The association between administration of antibiotics and typical HUS is a cause of debate[13]. In the period 2012-2014, 90 VTEC-associated cases of HUS were notified in Ireland[7,8, 9].

Ireland's NRL-VTEC receives VTEC isolates and faecal specimens for diagnosis, confirmation and typing from all around the country. Samples are referred from patients linked to outbreaks, both symptomatic and asymptomatic, and from sporadic cases of infection. NRL-VTEC performs a combination of polymerase chain reaction (PCR) and culture methods to detect verocytotoxin and virulence genes[14]. A laboratory database containing the details of all VTEC isolates is maintained for each year.

Methods

Laboratory data were analysed in this study.

Between 2012 and 2014, NRL-VTEC diagnosed 1986 VTEC cases according to its ISO 15189 accredited VTEC scope. 52 isolates were from known HUS cases: these were further assessed by PCR for verotoxin subtype, *eae* and *hlyA* genes.

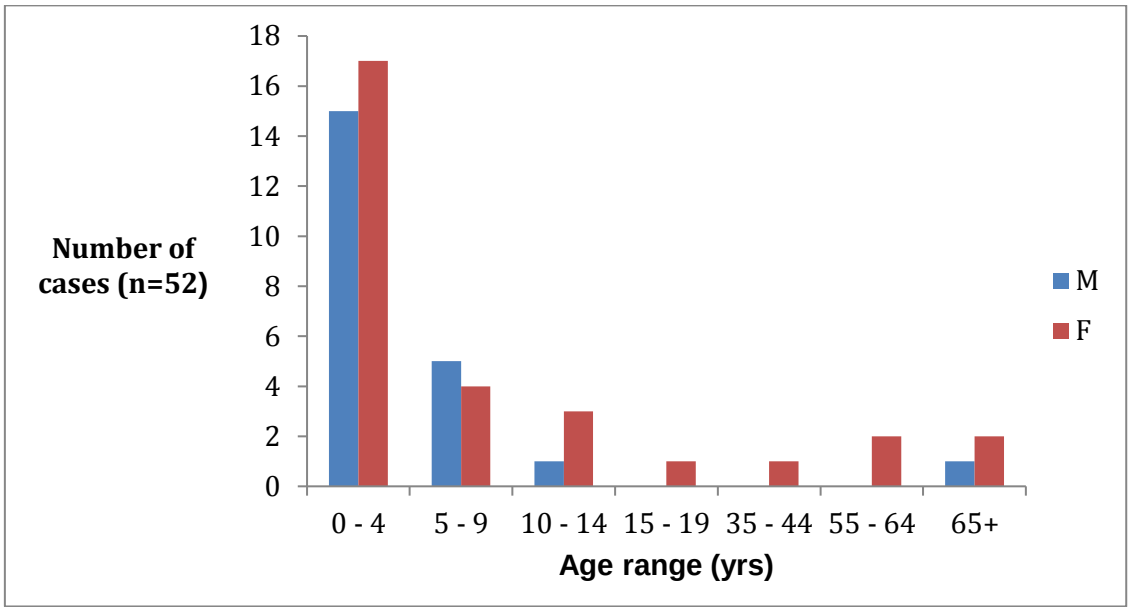
NRL-VTEC has a comprehensive laboratory VTEC database of VTEC isolates. This includes associated clinical symptoms (where provided on the original request form) and VTEC strain characteristics. Data for this 3-year study were derived from this NRL-VTEC database. Each database entry has a unique specimen number, with only one VTEC isolate listed per patient. The database was examined looking for specific reference to a clinical diagnosis of HUS, age and gender. However, clinical information on case diagnosis e.g. 'HUS' was missing or not recorded in some cases.

Results

From HPSC published data, it was known that there were 90 VTEC-associated HUS cases notified between 2012-2014, 83 of which were culture positive for VTEC [7,8,9]. NRL-VTEC laboratory database had HUS provided as the clinical diagnosis in 52/83 (62.6%) of these HUS cases. These 52 HUS-associated VTEC isolates formed the study cohort and were compared to the 1934 non HUS-associated VTEC strains.

Of the 52 cases of HUS thus identified, 30 (57.7%) occurred in female patients and 22 (42.3%) in male patients. Gender was not of statistical significance in HUS (p=0.68). Both genders were almost equally affected at the extremes of age. A preponderance of females occurred among cases >10 years of age (Fig.1). 46/52 (88.5%) cases of HUS were diagnosed in patients ≤ 15 years of age. The single greatest number of cases 35 (67.3%) occurred in young children up to the age of 5 years (OR 2.2, CI 1.2-3.7, p=0.005).

Fig. 1 Age range (years) and gender of VTEC associated HUS cases between 2012 – 2014 in Ireland

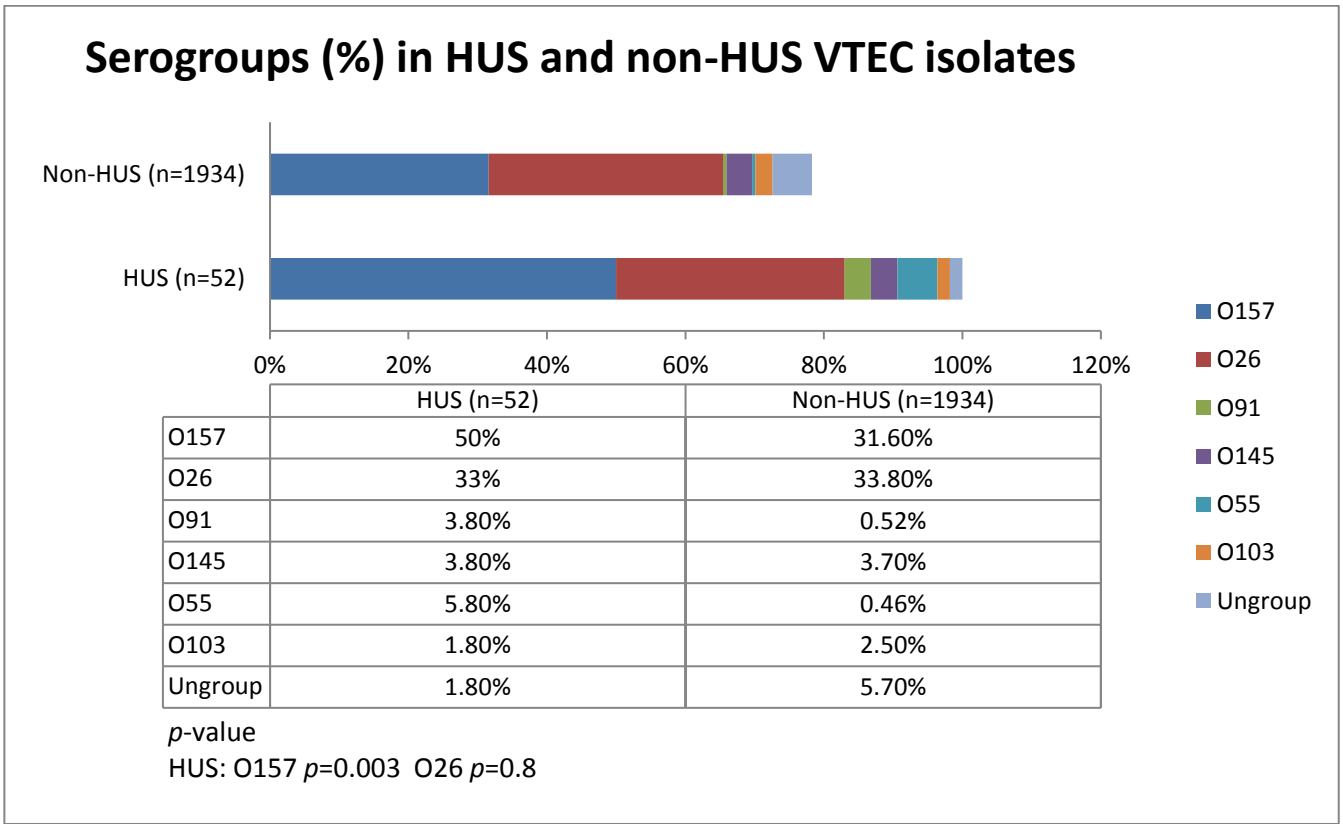


VTEC serogroups of HUS cases:

There were 7 different HUS-associated serogroups identified: serogroup O157 - 26 cases; (50%), O26 -17 cases; (32.8%), O91- 2 cases; (3.8%), O145 - 2 cases; (3.8%), O55 - 3 cases; (5.8%), O103 - 1 case; (1.9%) and ungroupable -1 case; (1.9%) (Fig. 2).

Serogroup O157 was associated with a higher risk of developing HUS (OR 2.2, CI 1.3-3.9, *p*=0.003)

Fig.2 Serogroups (%) of VTEC isolates in HUS & non-HUS cases in Ireland 2012 - 2014



	HUS (n=52)	Non-HUS (n=1934)
O157	50%	31.60%
O26	33%	33.80%
O91	3.80%	0.52%
O145	3.80%	3.70%
O55	5.80%	0.46%
O103	1.80%	2.50%
Ungroup	1.80%	5.70%

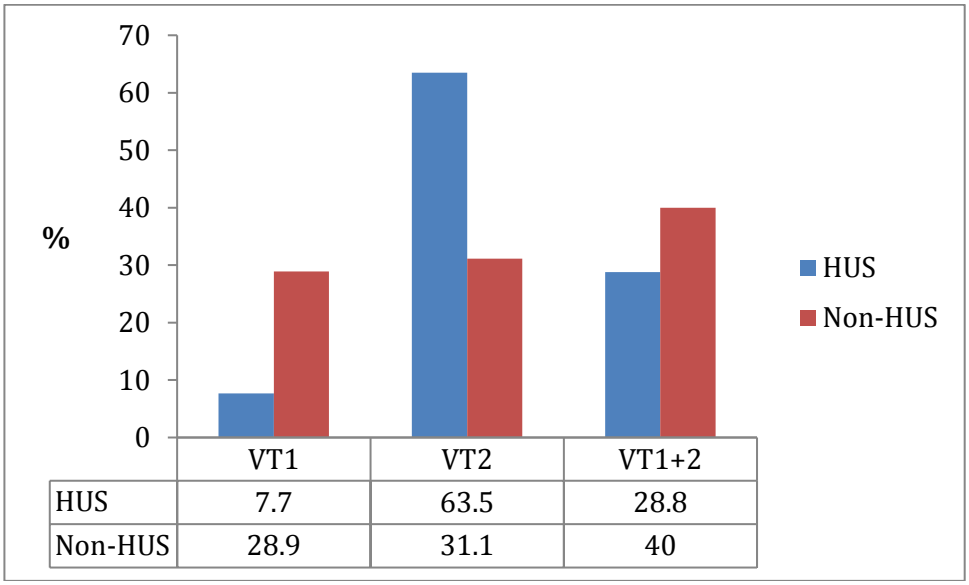
p-value

HUS: O157 *p*=0.003 O26 *p*=0.8

Toxin genotype of VTEC HUS

In HUS-associated VTEC isolates (n=52): there were 4 verocytotoxin-1 (VT1) producing organisms, 33 verocytotoxin-2 (VT2) producers and 15 VTEC isolates that produced both VT1 and VT2. VT2 producers were associated with a higher risk of developing HUS (OR 3.85, CI 2.17-6.8, $p<0.001$). The VT1 producers occurred in 2 male and 2 female patients; all were *eae*-positive, 3 were of serotype O26 while 1 was of serotype O91. The patients' ages ranged from 1 to 56 years. In contrast, the pattern of verocytotoxin genes in isolates not associated with HUS (n=1934) was more evenly distributed (Fig. 3).

Fig. 3 Verotoxin genotype (%) of HUS- and non HUS-associated VTEC cases



	VT1	VT2	VT1+2
HUS	7.7	63.5	28.8
Non-HUS	28.9	31.1	40

Eleven patterns of verotoxin subtypes were found among 51/52 HUS VTEC isolates. (1 isolate was not available for verotoxin subtyping*) (Table 1). Verotoxin subtyping was not performed on all non-HUS related isolates, thus not available for comparison.

Table 1. Verotoxin subtyping patterns of HUS-associated VTEC isolates (n=51)

Patterns of verotoxin subtypes in VTEC HUS- associated strains (n=51*)		Serogroup (no. of isolates)
Subtypes	n	
vtx1a	4	O26 (3), O91 (1)
vtx2a	13	O26 (1), O55 (2), O157 (8), unknown (2)
vtx2a, 2c	13	O157 (12), O- (1)
vtx2a, 2d	5	O26 (2), O145 (2), O55 (1)
vtx2a,2b, 2c	1	O91
vtx2a,2c, 2d	1	O157
vtx1a,2a	6	O26 (5), O157 (1)
vtx1a,1c, 2a	2	O26 (1), unknown (1)
vtx1a,1c, 2c, 2d	1	Unknown
vtx1a,2a,2c	2	O157 (1), O103 (1)
vtx1a,2a, 2d	3	O26 (3)

Other virulence genes

45/52 (86.5%) HUS isolates were positive for the *eae* gene. Serogroup O157 predominated (22 isolates, 52.4%) in this group of isolates. The remainder of the *eae*-positive isolates were of serogroups O26 (13 isolates, 30.9%), O145 (2 isolates, 4.8%), O55 (3 isolates, 7.1%), O91 (1 isolate, 2.4%) and O103 (1 isolate, 2.4%). These HUS isolates that were positive for the *eae* gene were predominantly VT2 producers (27 isolates, 64.3%). 17/52 (32.7%) of HUS-associated VTEC isolates were positive for the *hlyA* gene.

Discussion

Irish VTEC-associated HUS cases from 2012-14 predominated in children less than 4 years old. This trend is replicated in the United Kingdom[15], Europe[16] and also globally[17] and likely reflects the immaturity of the developing immune system which is unable to cope with the infectious insult of this virulent pathogen. We note equal gender distribution of HUS cases under 4 years old, but increasingly there is evidence of Irish adult females getting HUS which is unusual yet reminiscent of the 2011 German O104 VTEC outbreak. Perhaps this is a diet-related phenomenon attributable to higher consumption of fruit and vegetables by females[18]. Our data revealed 3 HUS cases in those aged 65 years or older. This is a patient cohort in which the associated mortality is high[19].

The serogroups of Irish HUS-associated VTEC isolates included five of the 'big six' and seem broadly similar to those found globally but they also included the rarer serogroup O91 which was previously reported as the most common VTEC serogroup isolated from adult patients in Germany[20]. The same serogroups were encountered in HUS and non-HUS cases although in different proportions, with serogroup O157 accounting for half of HUS cases and almost a third of non-HUS cases. Almost identical percentages of serogroup O26 and O145 occurred in both HUS and non-HUS cases. Half of HUS-associated isolates were of serogroup O157, 33% serogroup O26 and 17% were comprised of other serogroups, primarily O103, O145 and O111. Non-HUS causing isolates reveal that a third were serogroup O157, a third were serogroup O26 and a third were labelled as 'other' (Fig. 2)

Verotoxin genotyping of HUS-associated isolates demonstrated, as expected, a predominance of VT2 producers. In contrast, verotoxin types (VT1, VT2 and VT1+2) were more evenly distributed among non-HUS associated isolates. VT1 producers occurred much more frequently in non-HUS cases. A surprisingly large percentage of HUS-associated isolates were *eae* negative (13.5%) while 67% were *hlyA* negative. Thus, these genes should not be deemed exclusive genes for virulence. Data on *eae* positivity in non-HUS related isolates are not available. This may suggest that host susceptibility is a major factor in progression of infection to HUS disease despite the presence or absence of virulence genes. Taking into account these unique virulence findings and that additional serogroups outside the recognised 'big six' also caused HUS, demonstrates that all VTEC strains have the potential to cause HUS in Ireland. These results also have implication on the appropriateness of implementing ISO 13136 rigorously in Ireland. This is the method for detection of VTEC in food by primary PCR of VT and *eae* genes and any of the 'big six' serogroup-specific genes. Only those genes positive proceed to culture. This, as demonstrated by our data, would imply this method is too restrictive in its scope of VTEC detected and the need for *eae* positivity to be an appropriate method to determine food safety from VTEC in Ireland, as some of our HUS-associated VTEC strains would not be detected.

Eleven patterns of verotoxin subtypes were obtained in our study indicating a broad diversity in pathotype. The predominance of *vtx1a* or *vtx2a* in all of our HUS-associated isolates is notable. Carriage of *vtx2* gene subtypes *vtx2a* and *vtx2c* is commonly associated with HUS[21]. It is widely reported that *vtx2a* and *eae* occur frequently in HUS cases[22] and that together they result in greater enhancement of the virulence potential of VTEC[23]. However, it is difficult to predict full pathogenicity to cause HUS from our HUS data.

The identification of four HUS-associated *vtx1a eae*-positive isolates (3-O26, 1-O91 serogroup) in our study is a rare finding. In Europe, VTEC without *vtx2* and *eae* is infrequently associated with HUS[24]. *Vtx2a* is antigenically different from *vtx1a* and is more commonly associated with HUS than is *vtx1a*[25]. These four Irish isolates of non-O157 serotypes raise the question whether non-O157 associated HUS cases are more complex than previously thought. Perhaps this may represent a country-specific difference thus making it difficult to compile European-wide VTEC mitigation guidance advice.

A limitation of our study is that only 63% of HUS cases could be examined in the laboratory database due to inadequate clinical diagnostic information being provided on the laboratory request form and, in 7 culture negative cases, an organism was never available to us. Characterisation of 52/83 available HUS isolates raises the possibility of data on up to 31 HUS isolates being inadvertently included in the non-HUS data. The submission of some samples to

the laboratory from patients not known to have HUS could have preceded a subsequent clinical diagnosis of HUS being made.

The trends seen among VTEC strains causing HUS in Ireland generally mimic those described globally. However, we detected small but important varieties of VTEC causing HUS in this country that would substantiate our current approach that any viable VTEC has potential to cause HUS. Therefore, we cannot limit our investigations in either clinical or food samples to merely the common 'big six' serogroups with *eae* positivity to define a virulent VTEC strain.

Declaration of conflicts of interest:
The authors have no conflicts of interest to declare.

Corresponding Author:
Dr. Vivien Murphy,
Department of Clinical Microbiology,
St. James's Hospital,
James's Street,
Dublin 8.
Ireland
Email: murphyv3@tcd.ie

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