

**Guidance for Laboratory Diagnosis of
Human Verotoxigenic *E. coli* Infection
produced by
The Laboratory Sub-Group
of the
VTEC Sub-Committee
of the
Health Protection Surveillance Centre
Scientific Advisory Committee, Ireland**

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Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive



Chapter 3: Laboratory Diagnosis of Human VTEC Infection

3.1 Introduction

There are a variety of issues that make the laboratory diagnosis of VTEC human infections challenging. These include the wide spectrum of presenting features of VTEC infection (ranging from mild diarrhoea to haemorrhagic colitis, to haemolytic ureamic syndrome-HUS), the large number of *E.coli* serogroups implicated in disease with varying virulence factors, and the lack of clear phenotypic distinguishing characteristics that separate VTEC from other pathotypes of *E. coli*. The diversity of laboratory diagnostic tests available for VTEC thus requires a rational selection of methodologies from the wide variety available. The choice of laboratory methods utilised will be influenced by the patients clinical symptoms and sample risk assessment/categorisation, together with the laboratory safety facilities and laboratory equipment available. The logistics of sample transport to the local, regional or national reference laboratory also requires consideration.

Risk assessments are required on all samples requiring VTEC analysis, and appropriate methods should be applied. All manipulations of samples and cultures should comply with relevant safety legislation.¹ Currently not all diagnostic clinical laboratories have the required containment level facilities to allow primary culture, enrichment and/or identification of VTEC organisms. At present, methods chosen are based on the facilities available locally and may not always be appropriate for the sample risk category. Rapid laboratory diagnosis of VTEC is essential to ensure early medical and public health intervention.

In addition to these safety requirements, there is a lack of standardisation in approach both nationally and internationally in the diagnosis of VTEC infections, in particular those caused by non-O157 VTEC. While *E. coli* O157 is still the commonest reported serogroup causing clinical disease internationally, there are nearly 200 serogroups of *E. coli* which are verotoxigenic. Despite the limited application of diagnostic methods for non-O157 infections in Ireland, Ireland has for many years the highest reported incidence of VTEC infection in Europe, with an increasing proportion of non-O157 VTEC infections. These factors pose a significant challenge to optimising laboratory diagnostics for VTEC in Ireland.

3.1.1 *Escherichia coli* (*E. coli*)

Escherichia coli (*E. coli*) normally live in the intestines of humans and animals. Most *E. coli* are non-pathogenic and are normal commensals of the human intestinal tract. However, many *E. coli* are pathogenic resulting in localized infections, e.g. UTI and gastroenteritis; bloodstream infection can result in distant spread. Enterocolitic strains resulting in diarrhoea can be transmitted through ingestion of contaminated water or food, or contact with animals. Person-to-person spread is a significant transmission route.

E. coli consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorised into various pathotypes. Six pathotypes are associated with diarrhoea and collectively are referred to as diarrhoeagenic *E. coli*.

- 1) Enterotoxigenic *E. coli* (ETEC),
- 2) Enteropathogenic *E. coli* (EPEC),
- 3) Enteroaggregative *E. coli* (EAEC),
- 4) Enteroinvasive *E. coli* (EIEC),
- 5) Diffusely adherent *E. coli* (DAEC),
- 6) Verotoxin-producing *E. coli* (VTEC).

Verotoxin-producing *E. coli* (VTEC) are also sometimes referred to as Shiga toxin-producing *E. coli* (STEC) or Enterohemorrhagic *E. coli* (EHEC). This pathotype is the one most commonly associated with severe clinical illness.

Each *E. coli* pathotype is characterised by the presence of specific genes that facilitate or regulate pathogenicity (Appendices 1 and 2). Detection of these genes can be used in the characterisation of *E. coli* strains. However strains are continuously evolving and variants have been identified that have genes of more than one pathotype, as was the case in the *E. coli* O104 outbreak in Germany in 2011.²

3.2 Epidemiology of VTEC

3.2.1 Reported VTEC epidemiology in Europe and the impact of laboratory practice

Surveillance data internationally shows considerable variation in the reported incidence and epidemiology of VTEC infection between countries. While some of the

difference is likely to be real, variation in data collection (e.g. case definitions, data sources, etc) and laboratory practice (e.g. specimen selection, sensitivity and specificity of methods, etc), and variation in public health contact screening practice, undoubtedly influence reported epidemiology.

The European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) annually publish data on the incidence of foodborne zoonoses in EU Member States (MSs) in the annual EU zoonoses report. The latest available report details data in EU MSs in 2012

(<http://www.efsa.europa.eu/en/efsajournal/pub/3547.htm>).³ Overall, the reported incidence rate for VTEC across the EU in 2012 was 1.15 per 100,000, considerably lower than the annual rate reported in Ireland over the last nine years (range 1.6 per 100,000 in 2004 to 12.1 per 100,000 in 2012). There was wide variation in the reported incidence rates between Member States ranging from 0/100,000 in Cyprus, Bulgaria, Greece and Latvia to 8.99 per 100,000 in Ireland. There was a particularly notable increase in the reported number of cases in the Netherlands between 2008 and 2012 (circa 10-fold). This was attributed to an increase in the number of laboratories implementing PCR for the diagnosis of VTEC infections.

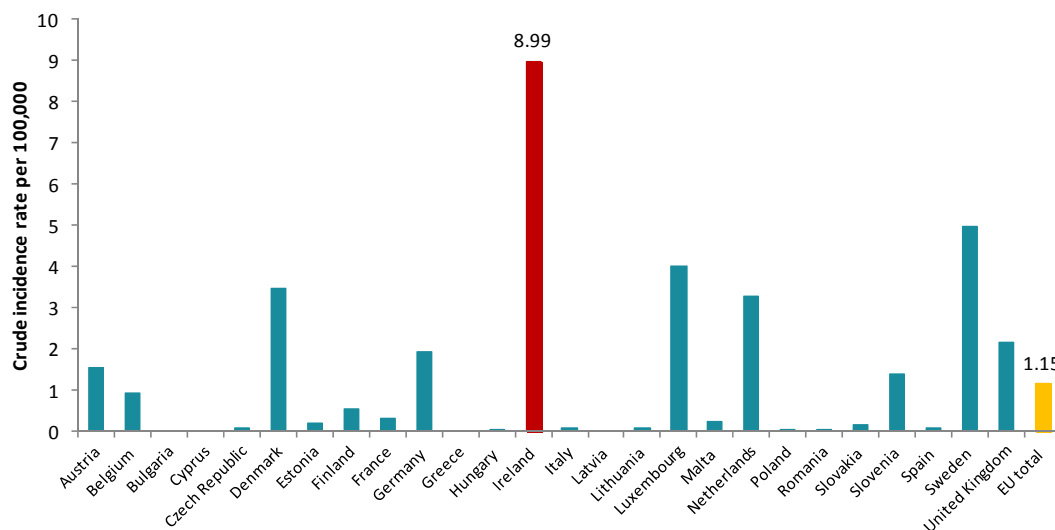


Figure 1. Crude incidence rate VTEC infection by reporting member state, EU 2012

(based on data reported in the EFSA and ECDC European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012 at <http://www.efsa.europa.eu/en/efsajournal/pub/3547.htm>)

There are also notable differences in the proportion of cases by MS for which serogroup data were available. For a high proportion of cases in the Netherlands and Germany, for example, no serogroup data are available; this might reflect more common use of culture-independent methods without subsequent strain isolation/characterisation.

In addition, there is variation in the serogroup distribution where this is available, with the United Kingdom, Spain, and Belgium reporting higher proportions of VTEC O157 cases (Figure 2). The report surmises that this can be due to a heavier reliance on culture methods focused towards VTEC O157 isolation. The top five non-O157 serogroups across Europe in 2012 were O26, O91, O103, O145 and O111. (For EU surveillance updates, see www.efsa.europa.eu and <http://www.ecdc.europa.eu/en/Pages/home.aspx>)

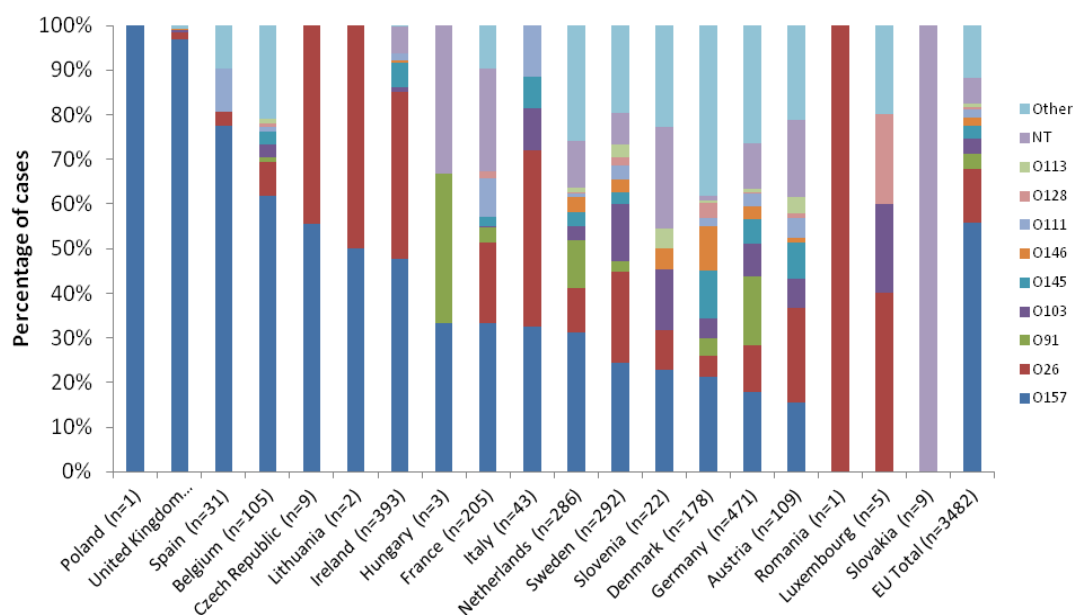


Figure 2. Reported serogroup distribution of VTEC cases by Member State, EU 2012

(based on data reported in the EFSA and ECDC European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012 at <http://www.efsa.europa.eu/en/efsajournal/pub/3547.htm>)

More recently, data from Ireland shows a progressively increasing number of VTEC notifications. In 2012, the great majority of this increase was accounted for by non-O157 VTEC infections (Figures 3 & 4). This development was accompanied by more widespread use of methods that detect both VTEC O157 and non-O157 VTEC, for example, PCR and chromogenic agars.

In addition, 2012 was notable for its high rainfall, particularly during the summer months, which may have contributed to the rise through a range of mechanisms, including contamination of drinking water.⁴

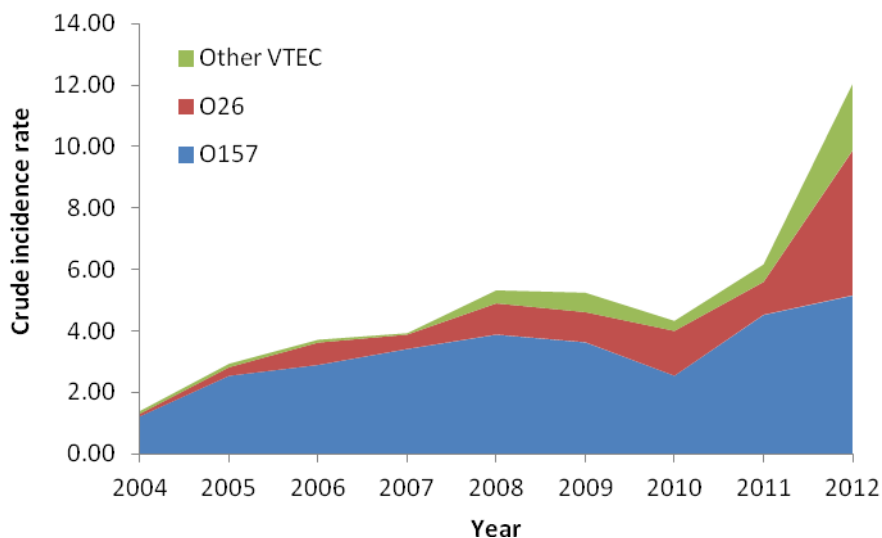


Figure 3. Annual crude incidence rate VTEC notifications by serogroup, Ireland 2004-2012

[Data source HPSC Annual Report 2012]

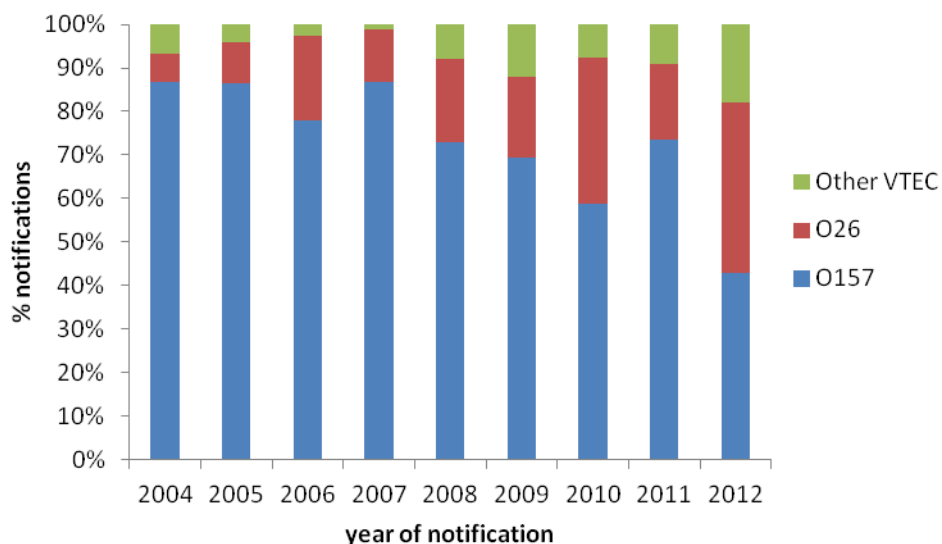


Figure 4. Annual percentage VTEC notifications by serogroup, Ireland 2004-2012

Data source HPSC Annual Report 2012

3.3 International Approaches to VTEC Diagnosis

It has been shown that increased use of laboratory methodology for non-O157 testing increases the proportion of non-O157 infections diagnosed.⁵ Health Protection Scotland (HPS), the Health Protection Agency (HPA) (now Public Health England) and the United States Centres for Disease Control and Prevention (CDC) have each provided guidance to their primary hospital laboratories:

- (i) on which stool samples received at the laboratory should be tested for VTEC;
- (ii) on approaches to testing both for O157 and non-O157 VTEC infection; and
- (iii) on the use of VTEC reference laboratory services.^{6, 7, 8}

In Scotland, the Health Protection Network [Guidance for the Public Health Management of Infection with Verotoxigenic *Escherichia coli*](#) recommends that:

- (1) All diarrhoeal specimens received at the primary hospital laboratory be tested for VTEC O157.
- (2) Faecal samples should be referred to the Scottish Enteric Reference Laboratory (SERL) for:
 - Cases of suspected haemolytic uremic syndrome (HUS)/ thrombotic microangiopathy (TMA), or bloody diarrhoea in whom conventional laboratory testing has failed to yield a pathogen;
 - All symptomatic contacts of cases of VTEC infection or any VTEC outbreak-associated case in whom conventional laboratory testing has failed to yield a pathogen;
 - All symptomatic contacts of cases of infection with sorbitol-fermenting (SF) VTEC O157, or non-O157 VTEC;
 - All asymptomatic contacts of cases of infection with SF VTEC O157.
- (3) The HPS also recommends submission of serum samples from likely cases of VTEC infection when culture and specialised techniques have failed to provide a diagnosis or where no stool sample is available for the patient.
- (4) In relation to typing services, it is recommended that diagnostic laboratories should immediately refer to SERL isolates of:
 - *E. coli* O157 for confirmation of identity, verotoxin gene detection and typing;

- Other strains of *E. coli* (non-O157) for confirmation of identity and verotoxin gene detection if there is high clinical suspicion of VTEC infection.⁶

In England and Wales, the HPA (now PHE) did not specify which stools samples received at the primary laboratory should be tested for VTEC, but state that when VTEC O157 is not isolated but the clinical symptoms are consistent with VTEC infection, the specimen may be sent to the Laboratory of Gastroenteric Pathogens (LGP) at Colindale where it will be tested for the presence of VTEC other than VTEC O157. The HPA also advises that it is possible to obtain evidence of infection by testing patient's serum for the presence of antibodies to the lipopolysaccharide of VTEC O157 and a limited number of other VTEC. The HPA recommends referral of all suspected VTEC to the Laboratory of Gastrointestinal Pathogens (LGP) for confirmation and typing.⁷

<http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/EscherichiaColiO157/Laboratory/>

In the US, the CDC [Recommendations for Diagnosis of Shiga Toxin--Producing *Escherichia coli* Infections by Clinical Laboratories](#) recommends that:

- (1) All stools submitted for testing from patients with acute community-acquired diarrhea should be cultured for VTEC O157 on selective and differential agar. These stools should be simultaneously assayed for non-O157 VTEC with a test that detects the verotoxins or the genes encoding these toxins.
- (2) Specimens or enrichment broths in which verotoxin VTEC are detected but from which VTEC O157 are not recovered should be forwarded as soon as possible to a state or local public health laboratory.
- (3) In relation to reference laboratory services, all VTEC O157 isolates should be forwarded as soon as possible to a state or local public health laboratory for confirmation and additional molecular characterisation (i.e. PFGE analysis and virulence gene characterisation).⁸

3.4 VTEC diagnosis in Ireland

3.4.1. VTEC case definition:

A clear case definition which incorporates clinical, epidemiological and laboratory criteria is essential for the effective identification and surveillance of VTEC cases, and the application of effective control measures. In 2004, VTEC became notifiable in Ireland under S.I. 707 -Infectious Disease Regulations 2003. Following an amendment in S.I 452 -Infectious Disease Regulations 2011, the case definition for VTEC and a number of other diseases were revised –see box for current VTEC case definition.

In common with other diseases, the VTEC case definition includes clinical, laboratory and epidemiological criteria (see below), which together results in a case classification of either possible, probable or confirmed status.

<p>Verotoxin-producing <i>E. coli</i> (VTEC)</p> <p>Clinical criteria Any person with at least one of the following three: (i)Diarrhoea (ii)Abdominal pain (iii)Haemolytic-uraemic syndrome (HUS)</p> <p>HUS Any person with acute renal failure AND at least one of the following two: Microangiopathic haemolytic anaemia Thrombocytopenia</p> <p>Laboratory criteria At least one of the following three: (i) Isolation of an <i>E. coli</i> strain that produces verotoxin or harbours <i>vt1</i> or <i>vt2</i> gene(s) (ii) Direct detection of <i>vt1</i> or <i>vt2</i> gene(s) nucleic acid (without strain isolation) (iii) Detection of free verotoxin in faeces</p> <p>Only for HUS the following can be used as laboratory criterion to confirm VTEC: <i>E. coli</i> serogroup-specific (LPS) antibody response</p> <p>Isolation of a VTEC strain and additional characterisation by serotype, phage type, <i>eae</i> genes, and subtypes of <i>vt1/vt2</i> should be performed if possible.</p> <p>Epidemiological criteria At least one of the following two: (i) Human to human transmission (ii) Exposure to a common source</p> <p>Case classification A. Possible case Any person meeting the clinical criteria for HUS (see note 1) B. Probable case Any person meeting the clinical criteria for VTEC and with an epidemiological link OR a laboratory confirmed case not meeting the clinical criteria, e.g. asymptomatic (see note 2) C. Confirmed case Any person meeting the clinical and the laboratory criteria</p> <p>Note 1: A person whose presentation meets the definition of HUS above, but whose condition is demonstrated to have been caused by another infectious agent, is not notifiable as a possible case of VTEC. Where that alternative infectious disease is notifiable, notification should be made according to that case definition. Note 2: Where no clinical information is available, laboratory confirmed cases should be notified as confirmed cases</p>

3.4.2 Laboratory methods for the diagnosis of VTEC infection

To make a laboratory confirmed diagnosis of VTEC, a number of methods can be used. These methods utilise either phenotypic or genotypic diagnostics.

Phenotypic:

These methods detect observable or biochemical characteristics of the organism. There are a number of methods that fit into this category and all involve culturing of the VTEC organism.

Enrichment

Although not a stand-alone method, enrichment is used to increase the number of bacteria present prior to the use of the detection method. Modified tryptone soya broth with novobiocin (MTSB) is the most commonly used enrichment broth for VTEC.

Advantages: Particularly useful for samples with very low numbers of VTEC present. Enrichment is also very useful in determining if VTEC organisms present are viable or non viable since only viable organisms will grow.

Disadvantages: Because it is a liquid culture stage all manipulations must be carried out in a CL3 laboratory.

CT-SMAC

Sorbitol MacConkey II Agar with Cefixime and Tellurite (CT-SMAC) is used as a selective and differential medium for the detection of *Escherichia coli* serogroup O157. This method relies on the fact that approximately 98% of all *E. coli* O157 are sorbitol negative. The non-sorbitol fermenting colonies (NSF) are visualised as colourless colonies on the media.

Advantages: The presence of antibiotics inhibits the growth of other gram negative bacteria (GNB) such as *Proteus mirabilis* that is also sorbitol negative. The quite distinct appearance of *E. coli* O157 on CT-SMAC makes visualisation/isolation easy.

Disadvantages: Inhibits the growth of many non-O157 VTEC. The small percentage of sorbitol fermenting (SF) *E. coli* O157 may be dismissed or

overlooked. Growth from stool samples with very low numbers of *E. coli* O157 or stressed *E. coli* O157 may not be sufficient for detection.

MacConkey

MacConkey agar is a differential culture media containing lactose, which allows differentiation of gram-negative bacteria based on their ability to ferment lactose. Organisms which ferment lactose (LF) produce acid end-products which react with the pH indicator neutral red, and produce a pink colour.

Advantages: Both O157 and non-O157 serogroups grow equally well.

Disadvantages: *E. coli* colonies appear the same colour as those of many other LF organisms, making it difficult to further identify or isolate the *E. coli*. Growth from stool samples with very low numbers of *E. coli* O157 or stressed *E. coli* O157 may not be sufficient for detection.

STEC-Chromagar

CHROMagar™ STEC is a chromogenic agar that detects a wide range of verotoxin-producing *E. coli*. VTEC will grow in mauve colony colour while other bacteria will grow in blue.

Advantages: Many other bacteria are inhibited. Visualisation of VTEC is very easy. Detection of O157 and non-O157 (especially O26) on a single media makes this media a good choice for VTEC screening.

Disadvantages: Sensitivity for some non-O157 serogroups is poor, e.g. the detection rate for *E. coli* O103 is only 70%. Growth from stool samples with very low numbers of *E. coli* O157 or stressed *E. coli* O157 may not be sufficient for detection.

Note: Further chromogenic agars are available; however these are for detection of O157 only. Other chromogenic media for O157 and non-O157 VTEC may become available and will need to be assessed by each laboratory.

Verotoxin ELISA

This Verotoxin assay is an enzyme-linked immunosorbent assay (ELISA) that may be used to screen for the presence of Verotoxins (VT1 and VT2).

Advantages: Detects the presence of verotoxins and is serogroup independent.

Disadvantages: Poor sensitivity has been widely reported.

IMS

Immunomagnetic separation utilises magnetic beads coated in serogroup specific antibodies, these bind to antigens present on the surface of cells thus capturing the cells and facilitating the concentration of these bead-attached cells. The concentration process is created by means of a magnet.

Advantages: An excellent method for recovering serogroup specific VTEC from samples with initial small numbers of VTEC present, this is due to a combination of enrichment and concentration using serogroup specific beads.

Disadvantages: Magnetic beads are serogroup specific and it is not feasible to test for all serogroups by this method. IMS requires strict CL3 conditions for sample manipulation. Beads are not routinely available for many non-O157 serogroups.

Serogrouping

When a bacterial culture is mixed with a specific antiserum directed against bacterial surface components, the cells are bound together through antigen-antibody bonds to form aggregates (agglutination). This is usually visible to the naked eye as clumps in the suspension. By mixing specific antisera with an *E. coli* culture, the O- and H antigens are determined.

Advantages: Rapid and no specific equipment needed. Polyvalent antisera can detect a number of serogroups simultaneously.

Disadvantages: Cross reactivity between serogroups is common; it is expensive to keep all 187 serogroup specific antisera and their associated controls.

Note: Manufacturers have different serogroups in their polyvalent antisera. Choice of polyvalent antisera should be based on the required serogroups (most common in Ireland are O157, O26, O145, O103, O111), and assessed by each laboratory.

Genotypic:

These methods examine the genetic makeup, rather than the physical appearance, of an organism. Detection of genes can be carried out directly from the sample or post enrichment, and as confirmation of toxin presence from pure cultures.

PCR

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA by several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. In the case of VTEC, verotoxin genes (as well as a large variety of other virulence and serogroup genes) may be targeted by gene-specific primers. PCR verotoxin assays can be in the form of ‘Laboratory developed assays’ or commercial kits. For either method to be regarded as positive, the characteristic amplification curves and a working crossing point (Cp) value of <36 cycles must be taken into account.⁹⁻¹²

Advantages: PCR is by far the most sensitive method for detecting VTEC. It is rapid and a high throughput is easily achieved.

Disadvantages: Non-viable as well as viable organisms are detected by PCR, making Public Health significance difficult to determine. ‘Laboratory developed PCR assays’ require a significant amount of validation particularly for accreditation, taking account of different matrices and potential inhibitors in clinical samples. However commercial PCR systems are very easy to implement. Some also have the advantage of not requiring the time consuming DNA extraction step.

3.4.3 Safety during transport of clinical specimens and isolates to and between laboratories

To ensure all samples are processed appropriately, samples and isolates will have to undergo transportation, whether from local to regional laboratories or regional to reference laboratory. The Regulations concerning the transport of infectious substances, including diagnostic specimens, by road, rail, sea, and air, state that

packaging must be United Nations (UN) licensed and packed in compliance with Packaging Instructions 602 (See appendix 3).

3.4.4 A strategy for the optimal use of clinical laboratory services for VTEC diagnosis and confirmation in Ireland

A co-ordinated national strategy for the diagnosis of VTEC infections is necessary in Ireland, which is based upon defined stratified standard methodologies that take into consideration the capacities of laboratory facilities and methodologies available regionally. The National VTEC Reference Laboratory in the PHL-HSE-DML would be available to support such a strategy.

A strategy for the effective screening of primary samples at local/regional level, with referral only of positives to the National VTEC Reference Laboratory would result in:

- (i) a more efficient and cost-effective National VTEC diagnostic service;
- (ii) a more comprehensive and accurate understanding of the true epidemiology of VTEC in Ireland, with lasting benefits for risk assessment and risk reduction programs for VTEC infection, particularly those relating to food and water safety, and
- (iii) a more uniform implementation of the safety legislation with respect to laboratory handling of VTEC organisms.

It is recommended that these new arrangements for VTEC diagnostic services will be divided into 3 categories:

- 1) *Local:* Local laboratories would carry out risk assessments. If the appropriate facilities/methodologies are available, samples can be processed locally using at a minimum an ‘acceptable’ culture based approach for low risk specimens (see Algorithm 2 later). If this is not possible, samples (higher and lower risk) should be referred to the appropriate regional laboratory.
- 2) *Regional:* Regional laboratories should at a minimum be able to detect VTEC via the ‘recommended method’ which utilises PCR for verotoxin gene detection from primary samples (see Algorithm 1 later). Further culture and serogrouping may also be available.
- 3) *National:* This is the reference service provided by the National VTEC Reference Laboratory at the Public Health Laboratory-HSE-DML in Cherry

3.5 Recommendations for Clinical VTEC Diagnosis and Confirmation in Ireland

3.5.1 Recommendations for the local/regional laboratory

1. All samples received at the laboratory should be categorised as ‘higher’ or ‘lower’ risk using Table 1
2. All presumptive positive VTEC samples/isolates should be referred to the National VTEC Reference Laboratory for confirmation and further molecular characterisation.

Table 1. Sample Categorisation

Category I (higher risk for VTEC)*	Category II (others, lower risk for VTEC)
i. Diarrhoeal stools ≤5 year olds. ii. Bloody diarrhoeal stools iii. Stool samples from confirmed VTEC patients or their contacts. iv. Presumptive Isolates for VTEC confirmation. v. VTEC outbreak samples vi. Stools from Public Health Risk Groups 1-4 , including where microbiological is clearance required (http://www.hpsc.ie/hpsc/A-Z/Gastroenteric/GastroenteritisorIID/RiskGroups/) vii. Samples from patients with HUS.	i. All non-Category I diarrhoeal stools from individuals >5yrs

*Samples have been included in Category I (i) if they have a higher probability of containing VTEC organisms, or (ii) if the occurrence of VTEC in the sample would have a higher public health risk

3. It is recommended that all stools in Category I be tested for VTEC O157 and non-O157 in accordance with Algorithm 1: ‘The Recommended VTEC Screen’ as outlined below.
4. It is recommended that all stools in Category II be tested for VTEC O157 and non-O157 VTEC in accordance ideally with Algorithm 1, but as a minimum, with Algorithm 2: ‘The Acceptable VTEC Screen’ as outlined below.

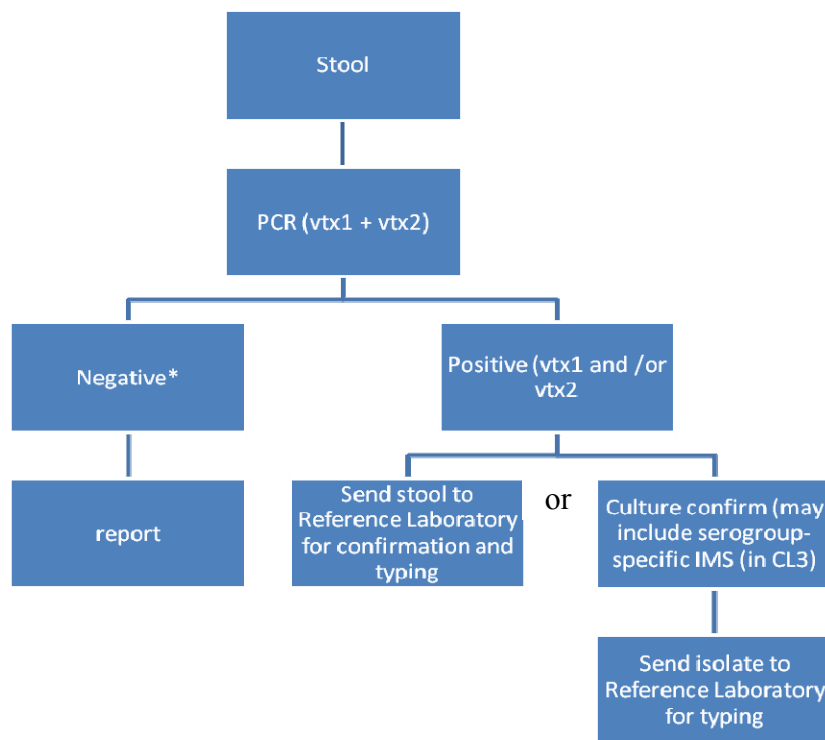
5. All stools positive by PCR for *vtx1* or *vtx2* at a primary hospital laboratory (and/or the associated presumptive VTEC isolates if culture was undertaken locally) AND all presumptive VTEC isolates identified at a primary hospital laboratory using the culture-based approach should be referred to the National VTEC Reference Laboratory for confirmation, culture and typing. However all subsequent serial stools from a known positive VTEC case, which are PCR positive for *vtx1* and/or *vtx2* at the primary hospital need not be referred to the reference laboratory. Intermittent confirmation at the reference laboratory can be agreed with the primary hospital.
6. All samples or suspect VTEC colonies referred to the National VTEC Reference Laboratory will be processed in accordance with Algorithm 3: 'VTEC Reference Service' as outlined below.
7. Where VTEC is not detected at the regional or local laboratory in a Category 1 stool sample from a patient with HUS (even when the molecular diagnostic approach has been used), the stool should then be referred to the National VTEC Reference Laboratory for further testing.
8. All specimens/isolates referred to the Reference Laboratory should be accompanied by (i) the name and contact details of the medical practitioner to whom the result is to be communicated, (ii) an outbreak code (preferably the CIDR outbreak code) if applicable, and (iii) it should be indicated on specimens from HUS cases that they have HUS.

Category I (higher risk for VTEC) stools

- a) All stool samples in Category I should at a minimum be tested for VTEC using the molecular diagnostic approach outlined in Algorithm 1.

Samples are considered higher risk if they fall into Category I. If a commercial PCR is used, there may be a heating step in the protocol. This may negate the need for the procedure to be carried out in a CL3, however, risk assessments need to be carried out locally before such a decision is made.

ALGORITHM 1
Category I – ‘Higher risk’ samples for VTEC screening at local/regional laboratories



*Samples from HUS cases that screen negative for verotoxin genes should be sent to the National VTEC Reference Laboratory

If the initial validated PCR screen for *vtx1* and *vtx2* is negative, then no further work is required with the exception of HUS cases (see section c). This ‘Recommended’ screening method is expected to detect the vast majority of VTEC cases. However, in cases where the bacterial load is very low, a pre PCR enrichment step is necessary; this is particularly true of HUS cases. This enrichment step is performed on all VTEC screens in the National VTEC Reference Laboratory (see Reference Laboratory section).

- b) Where testing of higher-risk (Category I) samples using the molecular diagnostic approach cannot be undertaken in the local laboratory for safety, expertise or equipment reasons, it is recommended that Category I stool samples be referred to another local or regional hospital laboratory that has the necessary VTEC Algorithm 1 capability. If this is not feasible,**

then the sample should be referred to the National VTEC Reference Laboratory directly where these stools can be tested appropriately.

- c) Where VTEC is not detected at the regional or local laboratory in a Category 1 stool sample from a patient with HUS (even when the molecular diagnostic approach has been used), the stool should then be referred to the National VTEC Reference Laboratory for further testing. Serodiagnosis may also be considered.**

Category II (lower risk for VTEC) stools

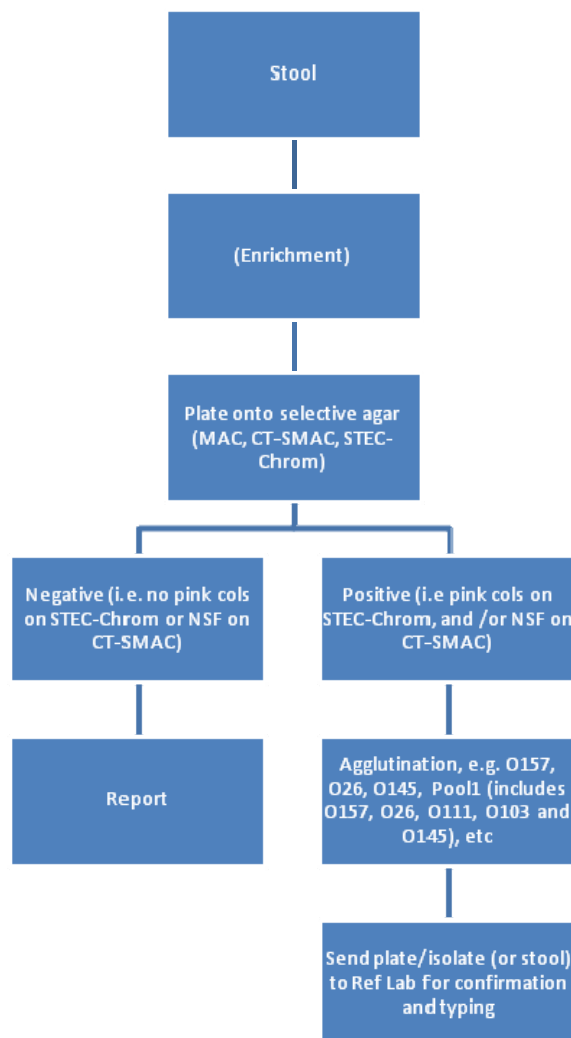
- a) Stool samples in Category II should be tested for both VTEC O157 and non-O157**

Where possible, the molecular diagnostic approach -Algorithm 1 should be used for all stool specimens that require VTEC testing. Unfortunately many hospital laboratories in Ireland do not have the capacity to carry out VTEC PCR. An 'acceptable' culture-based approach is outlined in 'Algorithm 2', which may be used for a VTEC screen on samples in Category II. This method is not optimal; therefore it is not recommended for higher risk (Category I) samples.

There are a number of Chromogenic/selective media available for O157 and non-O157 VTEC, and each laboratory may choose and evaluate the one that is most suitable to them, and combine this with sero agglutination of suspect colonies.

ALGORITHM 2

Category II – ‘Lower risk’ samples for VTEC screening at local/regional laboratories



3.5.2 Samples or isolates referred to the National VTEC Reference Laboratory (NRL-VTEC)

All stools and isolates referred for VTEC testing/confirmation are processed by a combination of culture and molecular methods (see Algorithm 3 below). In addition, all VTEC isolates are routinely typed using PFGE to the PulseNet protocol. Results are transmitted to referring laboratories, Departments of Public Health, outbreak control teams, HPSC, the Food Safety Authority of Ireland (FSAI), and ECDC as relevant. When indicated *vtx1* and *vtx2* subtyping by PCR is also undertaken. Full characterisation of VTEC multiple virulence genes detection by PCR is also available.

This is not offered as a routine service, but rather when public health risk assessment justifies its utilisation.

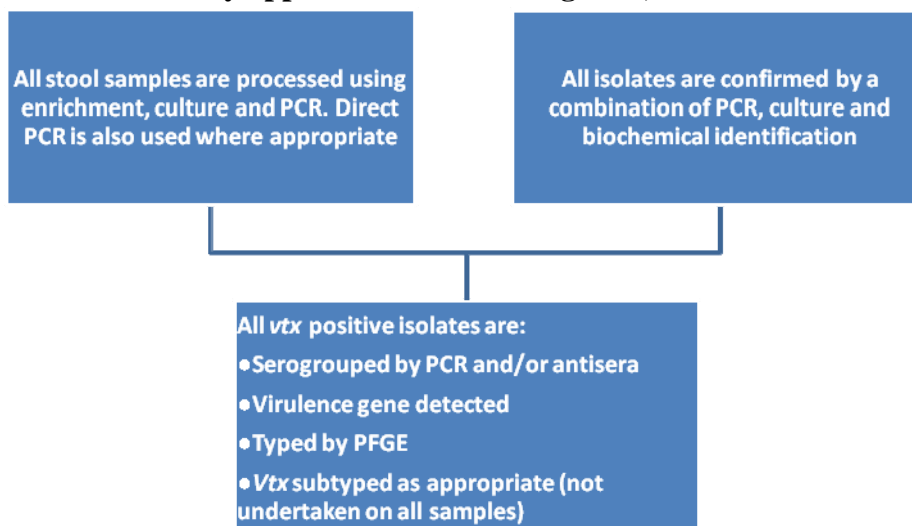
The range of *E. coli* serogroups for which there are antisera and PCR primers at the NRL-VTEC are listed in the PHL-HSE-DML Laboratory Manual available at hse.ie/eng/services/list/5/publichealth/publichealthlabs/Public_Health_Laboratory_Dublin/

Contact Details for National VTEC Reference Laboratory:

VTEC Reference Laboratory
Public Health Laboratory HSE, Dublin.
Cherry Orchard Hospital
Ballyfermot
Dublin 10
Tel 01 6206175

ALGORITHM 3

Reference Laboratory approach to VTEC diagnosis, confirmation and typing



3.5.3 Role and activities of the National VTEC Reference Laboratory

The National VTEC laboratory service was set up in the PHL-HSE-DML in 2001. It first provided diagnostic facilities with enhanced culture methodologies in its newly commissioned biosafety level 3 laboratory for this pathogen. Since then, it has

developed an international reputation for the pathogenesis, phenotypic and genotypic typing of VTEC and for its role in the detailed investigation of many national VTEC outbreaks. In close collaboration with Departments of Public Health, Environmental health service and the HPSC, it has documented the unique risks of VTEC associated with potable waters (private group water schemes and private wells) and childcare facilities in Ireland. The NRL-VTEC has undertaken important work supporting VTEC risk assessment in relation to food and water. Enhancement and implementation at PHL-HSE-DML of VTEC diagnostic methods now includes utilisation of culture, immunomagnetic separation, seroagglutination, automated DNA extraction, PCR for verotoxin genes, *vtx* subtyping and detection of virulence factors along with typing by PFGE to 'Pulsenet' protocol. This has resulted in a unique, highly specialised accredited national VTEC scope that supports our colleagues in Acute Health Care Facilities, Public Health and other national agencies to address the disease burden caused by these pathogens. The NRL-VTEC provides an important service to clinical microbiology, public health, the Environmental Health Service, and national and state agencies. The PHL-HSE-DML director is the national contact point for VTEC/EHEC to the ECDC-Food and waterborne disease (FWD) network and was the National Microbiology Focal Point contact to ECDC from 2006-2008. Recently the PHL-HSE-DML has been designated an ECDC EUPHEM (European Public Health Microbiology) Fellowship training site.

Crucially, the NRL-VTEC holds a bank of VTEC isolates which contains all confirmed clinical VTEC isolates in Ireland over the last 10 years together with food and water isolates. Ireland is one of the few countries that maintains such a comprehensive VTEC strain collection. It is therefore very important that all VTEC isolates (even if they are confirmed at a local/regional laboratory) continue to be forwarded to the VTEC reference laboratory.

3.5.4 Public Health response to VTEC laboratory results

Invariably there may be some discrepancies between the initial Primary/Regional VTEC screening laboratory results and the subsequent confirmatory reference laboratory results due to different sensitivity and specificity of the methods utilised.

The following table reflects various laboratory result scenarios and recommended Public Health actions.

Table 2: Public Health Action to be considered on the basis of laboratory results

Scenarios	PCR at Primary Lab	PCR at Reference Lab.	Isolate confirmation at NRL-VTEC.	Public health action
1. A case can be diagnosed by PCR in primary lab and have subsequent culture confirmation at NRL-VTEC	Positive	Positive	Positive	Should be notified by primary lab and should be retained on CIDR following confirmation by NRL-VTEC. Public health action as per National VTEC guidance for VTEC cases ¹²
2. A case can be diagnosed by PCR in primary lab and only have PCR confirmation at NRL-VTEC*	Positive	Positive	Negative	Should be notified by primary lab, and reported by NRL-VTEC as ‘PCR Positive, Culture negative – public health risk assessment required’. Public health risk assessment will primarily require consideration of symptoms / risk group: I. Symptomatic → follow up as a confirmed VTEC case II. Asymptomatic + risk group → follow up as probable VTEC case III. Asymptomatic + no risk group → assess if need to follow up as probable VTEC case Should be retained on CIDR as confirmed/probable VTEC case as appropriate if in symptom group I or II, and as probable case or not for symptom group III based on risk assessment. Public health action as per National VTEC guidance for all confirmed or probable cases. ¹²
3. A case can be diagnosed by PCR in primary lab and negative at NRL-VTEC by PCR	Positive	Negative	Negative	This should be considered a false positive. Should be notified by primary lab in first instance, but should be denotified if NRL-VTEC reports negative by PCR (unless independently of lab results, it is notifiable on the grounds that it meets the criteria for possible VTEC case or a probable epi-linked VTEC case). In this event, VTEC-specific public health action is not required
4. A specimen tested only at NRL-VTEC , e.g. contact of known case	Not done	Positive/ Negative	Positive/ Negative/ Not applicable	Public health action as per three scenarios above as appropriate ¹²
5. A case can be diagnosed by PCR in primary lab, and no sample be referred to NRL-VTEC as not sufficient leftover	Positive	Not applicable	Not applicable	Ideally a second sample should be requested. In the absence of a second sample being available for NRL-VTEC, these cases should default to having a public health risk assessment as in scenario 2 above.

* PCR positive but culture negative cases can arise for the following reasons:

- (1) The organism may be non viable, with PCR detecting the gene only: result may not be significant from a Public Health perspective or
- (2) Because PCR is a more sensitive methodology than culture, the number of organisms in the sample could have been below detection limit of the culture method: result may be significant from a Public Health perspective

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Appendix 1. *E. coli* pathotype-specific genes

Abbreviation	Pathotypes	Genes	Gene function
DAEC	Diffusely-adherent	<i>afa</i>	Afimbrial adhesion
EAEC	Enteraggregative	<i>aggR</i>	Regulator, plasmid encoded
EIEC	Enteroinvasive	<i>ipaH</i>	Invasive
ETEC	Enterotoxigenic	<i>estA eltB</i>	Heat stable/labile toxins
EPEC	Enteropathogenic	<i>eae</i> , EAF	Intimin attaching effacing, LEE (chr) Adherence factor (plasmid)
VTEC	Verotoxigenic	<i>vtx1, vtx2</i>	Verotoxins encoded on bacteriophages
EHEC	Enterohaemorrhagic	<i>vtx1, vtx2, eae</i>	Haemorrhagic colitis

Appendix 2: Virulence factors associated with VTEC

Verotoxins

Verotoxins are considered to be the major virulence factor of VTEC and comprise a family of structurally related cytotoxins with similar biological activity. The two main groups consist of Vtx1, which is nearly identical to the toxin of *S. dysenteriae* type 1, and Vtx2, which shares less than 60% amino acid sequence with Vtx1. The genetic information for the production of Vtx1 and Vtx2 is located in the genome of lambdoid prophages integrated in the VTEC chromosome. Whereas Vtx1 shows only minor sequence variations, several variants of Vtx2 with altered antigenic or biological characteristics have been described. Epidemiological studies have revealed that Vtx2 is more associated with severe human disease than Vtx1. To date, 7 subtypes of Vtx2 and 3 subtypes of Vtx1 have been described.

Attaching and effacing adhesion

Most VTEC included in the EHEC group colonise the intestinal mucosa with a mechanism that subverts the epithelial cell function and induce a characteristic histopathologic lesion, defined as "attaching and effacing" (A/E). The A/E lesion is due to marked cytoskeletal changes and is characterised by effacement of microvilli and intimate adherence between the bacteria and the epithelial cell membrane, with accumulation of polymerised actin directly beneath the adherent bacteria. The complex mechanism of A/E adhesion is genetically governed by a large pathogenicity island (PAI) defined as Locus of Enterocyte Effacement (LEE).

Other virulence factors

VTEC O157 possess a large virulence plasmid of approximately 90 Kb termed pO157. The nucleotide sequence of this plasmid showed that it encodes 35 proteins, some of which are presumably involved in the pathogenesis of EHEC infections. The enterohaemolysin (*hly*) operon is considered the best marker of the presence of pO157 and is also present in the large plasmids that can be detected in most non-O157 EHEC strains. Other putative virulence factors harboured by this plasmid comprise a katalase-peroxydase and a serine protease, encoded by *katP* and *espP* genes, respectively.

Other *E. coli* pathotypes have the ability to acquire verotoxin genes. The *E. coli* O104 outbreak in Germany in 2011 was caused by an enteraggregative *E. coli* that had acquired a verotoxin encoding bacteriophage. This strain also demonstrated extended-spectrum beta-lactamase (ESBL) resistance.

Appendix 3. Transport and packaging of VTEC samples:

References:

- **Transport of VTEC is covered by Carriage of dangerous goods by road, regulations 2001. S.I. No. 492 of 2001. This can be viewed at <http://www.irishstatutebook.ie/2001/en/si/0492.html>**
- **International air transport association (IATA). Dangerous goods regulations, Class 6.2, packing instruction 602, UN2814.**

Packing Instructions for UN602

1. Serum tubes, swabs, urines, tissues, etc. must be placed in a sealed primary container, sealed with parafilm.
2. Each sample must then be wrapped in cotton wool. The cotton wool should be sufficient to absorb the entire contents of the samples. Cotton wool is not required for solid substances.
3. Samples are then placed in watertight secondary container.
4. Request forms are placed in a separate biohazard bag with the relevant patient and referring Doctors details and urgent contact numbers. The request form is placed between the secondary and tertiary container.
5. Secondary container is then placed into the Outer Shipping Package. The outer packaging must comply and bear the compliance marks to satisfy UN 602 Packaging Requirements. Full performance testing includes pressure, water, drop and puncture testing, to required standards. Fully compliant packaging bears the marks UN 2814 and markings Class 6.2/year/differing marks according to country of production, together with relevant Control of Substances Hazardous to Health (COSHH) labelling. Packaging not bearing these marks must not be used.
6. The outer cardboard packaging must be labelled with:
 - a. Name and address of the consignee (destination of samples)
 - b. Name and address of sender.
 - c. A name and emergency contact telephone number.
 - d. All compliance testing markers and biohazard marking.

Appendix 4. Members of HPSC VTEC Sub-Committee Laboratory Subgroup

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Dr. Anne Carroll, Molecular Scientist PHL, HSE Dublin.

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Dr. Sarah Doyle, Specialist in Public Health Medicine, HSE-South-East

Dr. Patricia Garvey, Surveillance Scientist, HPSC

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Dr. Paul McKeown, Specialist in Public Health Medicine, HPSC

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Dr. Margaret O Sullivan, Specialist in Public Health Medicine, HSE-South