Methods for Detection and Molecular Characterisation of Pathogenic *Escherichia coli*

CO-ORDINATION ACTION FOOD-CT-2006-036256
Pathogenic *Escherichia coli* Network

PEN

Methods for Detection and Molecular Characterisation of Pathogenic *Escherichia coli*

CO-ORDINATION ACTION
FOOD-CT-2006-036256

Pathogenic *Escherichia coli* Network

Editors
J. O'Sullivan, D. J. Bolton, G. Duffy, C. Baylis, R. Tozzoli,
Y. Wasteson and S. Lofdahl

Editorial Team

John O'Sullivan   Ashtown Food Research Centre
Teagasc, Ashtown,
Dublin 15, Ireland

Declan Bolton   Ashtown Food Research Centre
Teagasc, Ashtown,
Dublin 15, Ireland

Geraldine Duffy   Ashtown Food Research Centre
Teagasc, Ashtown,
Dublin 15, Ireland

Chris Baylis   Campden & Chorleywood Food
Research Association
Chipping Campden,
loucestershire,
GL55 6LD, UK

Rosangela Tozzoli   Community Reference Laboratory
for *E. coli*
Istituto Superiore di Sanità,
Viale Regina Elena 299,
00161 Rome, Italy

Yngvild Wasteson   Norwegian School of Veterinary
Science
PO Box 8146, Oslo 0033, Norway

Sven Lofdahl   Swedish Institute of Infectious
Disease Control
(Smittskyddsinstitutet)
171 82, Nobels väg 18,
Solna, Sweden

Contents

1. Introduction

Escherichia coli is the predominant facultative organism in the human gastrointestinal tract. Pathogenic forms of *E. coli* can cause a variety of diarrhoeal diseases in hosts due to the presence of specific colonisation factors, virulence factors and pathogenicity associated genes which are generally not present in other *E. coli*. Of the strains that cause diarrhoeal diseases, six pathotypes are now recognised. Figure 1 shows the complex relationships between these pathotypes. These pathotypes are:

- − Verocytotoxigenic *E. coli* (VTEC)
- − Enterotoxigenic *E. coli* (ETEC)
- − Enteroinvasive *E. coli* (EIEC)
- − Enteropathogenic *E. coli* (EPEC)
- − Enteroaggregative *E. coli* (EAggEC)
- − Diffusely adherent *E. coli* (DAEC)
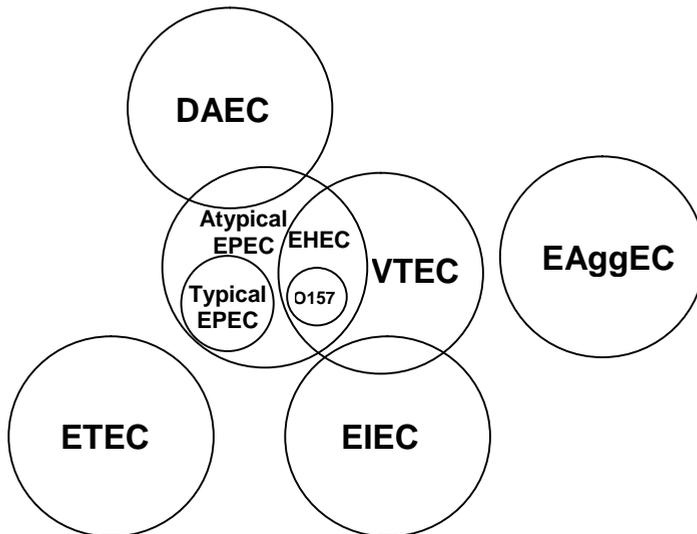
Figure 1: Venn diagram illustrating the relationships between *E. coli* pathotypes causing diarrhoeal disease (modified from Donnenberg, 2002)

Figure 1 shows ETEC and EAggEC to be distinct, separate groups. There are varying degrees of overlap between DAEC and EPEC, EPEC and VTEC, and VTEC and EIEC.

1.1 Verocytotoxigenic *E. coli* (*VTEC*)

E. coli that produce verocytotoxin/Shiga toxin (VT/Stx) (VTEC/ Shiga toxin producing *E. coli*, STEC) are characterised by the production of cytotoxins that disrupt protein synthesis within host cells. These toxins are synonymously either called verocytotoxins (VT), because of their activity on Vero cells, or Shiga toxins (Stx) because of their similarity with the toxin produced by *Shigella dysenteriae*. The terms VTEC and VT will be used throughout this document.

Enterohaemorrhagic *E. coli* (EHEC) are a subset of VTEC that are considered to be human pathogens. Figure 1 shows EHEC as a subset of VTEC and EPEC as, like VTEC, they produce VT and have the ability to form attaching and effacing lesions (A/E lesions) on epithelial cells, a characteristic of EPEC. EHEC also possess an approximately 60-MDa "EHEC plasmid". The most important EHEC (and VTEC) serotype in public health terms is *E. coli* O157.

VTEC infection occurs via the faecal-oral route and results in symptoms ranging from mild uncomplicated diarrhoea to severe bloody diarrhoea. Complications including haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) can occur in some cases, both of which can result in death. The infectious dose of VTEC has been calculated to be as low as 10 – 100 cells.

The disease causing ability of VTEC in humans is associated with its ability to express VT. The types of VT produced are classed as VT1 and VT2, along with VT2 subtypes. The capacity of EHEC to form A/E lesions in the intestine after ingestion is also linked to disease in humans. These A/E lesions are characterized by localized destruction (effacement) of brush border microvilli, attachment of the bacteria to the enterocyte membrane, and formation of a cup

like pedestal at the site of bacterial contact. Proteins required for the formation of A/E lesions are encoded for by the locus of enterocyte effacement (LEE), located on the chromosome. This contains genes such as *eae* which codes for intimin. In human pathogenic VTEC, the main virulence factors are *vtx1*, *vtx2*, *vtx2c* and *eae*.

Ruminant animals, in particular cattle are the main reservoir for VTEC. Beef has historically been most linked to VTEC infections however a wide variety of other sources have been implicated in infection, including unpasteurised milk and fruit juice, sprouts, lettuce, spinach, cantaloupe, cheese, mushrooms, sprouts and salami. Waterborne transmission occurs through swimming in contaminated lakes, pools, or drinking untreated water. Direct contact with animal faecal material through recreational activities and person to person contact are also sources of infection.

1.2 Enteropathogenic *E. coli* (EPEC)
Following the ingestion of EPEC, the organisms adhere to the epithelial cells of the intestine, causing either watery or bloody diarrhoea. The former is associated with the attachment to, and physical alteration of, the integrity of the intestine. Bloody diarrhoea is associated with attachment and an acute tissue-destructive process. Low grade fever and vomiting are also associated with infection.

EPEC, unlike VTEC, do not produce any classic toxins. Their virulence mechanism involves the formation of A/E lesions followed by interference with host cell signal transduction. Virulence factors located on the LEE (such as *eae* which codes for intimin and *esp* which codes for secreted proteins) and on the EPEC adherence factor (EAF) (*bfp* which codes for bundle-forming pili, BFP) are responsible for this mechanism. Not all EPEC strains form BFP. Those that do are referred to as typical EPEC, and those that do not are termed atypical EPEC (Figure 1). EPEC virulence genes are encoded on plasmids (*bfp*) and the chromosome (*eae*).

Water and food contaminated with EPEC have been linked to EPEC infection, with the most common foods implicated in outbreaks being raw beef and chicken.

1.3 Enterotoxigenic *E. coli* (ETEC)
ETEC are a major cause of traveller's diarrhoea worldwide. Infection with ETEC leads to watery diarrhoea which lasts up to a week, but can be protracted. Abdominal cramps, sometimes with nausea and headache, occur and fever is usually absent.

On infection, ETEC first establishes itself by adhering to the epithelium of the small intestine via one or more colonization factor antigens (CFA). This is followed by the expression of one or more heat-stable (ST) or heat labile (LT) enterotoxins. These enterotoxins cause inhibition of sodium absorption and stimulation of chloride secretion, which leads to watery diarrhoea. Distinct groups of the enterotoxins exist: for the heat stable, STa (STI) and STb (STII) – encoded for on plasmids – and for the heat labile, LTI and LTII – encoded for on the chromosome.

Infection occurs when a person ingests food or water contaminated with ETEC bacteria.

1.4 Enteroinvasive *E. coli* (EIEC)
EIEC are transmitted through the faecal-oral route. Even minimal contact is adequate for transmission. Following the ingestion of EIEC the organisms invade the epithelial cells of the intestine resulting in a mild form of dysentery often mistaken for dysentery caused by *Shigella* species. The illness is characterized by the appearance of blood and mucus in the stools of infected individuals.

Characteristic features of EIEC strains are their ability to induce their entry into epithelial cells and disseminate from cell to cell. The genes required for entry into host cells are clustered on a 220 kb virulence-associated invasion plasmid in EIEC strains. This plasmid is also present in *Shigella* spp. Expression of several plasmid-encoded proteins is required for the complete virulence

phenotype of EIEC. These invasion plasmid antigens (Ipa) proteins are encoded in the *ipa* operon.

EIEC infection can occur through contaminated food or water, or through mechanical vectors such as flies. Outbreaks have been associated with hamburger meat and unpasteurised milk.

1.5 Enteroaggregative *E. coli (EAggEC)*
EAggEC (also known as EAEC) are associated with acute or persistent diarrhoea, especially in developing countries. Infection is typically followed by a watery, mucoid, diarrhoeal illness with little to no fever and an absence of vomiting.

The precise mechanisms by which EAggEC cause diarrhoea and the role of the various pathogenicity factors are poorly understood. EAggEC strains are characterised by their ability to aggregatively adhere to tissue culture cells in a distinctive "stacked, brick-like" manner. Aggregative adherence in EAggEC is mediated by either aggregative adherence fimbrae I (AAF/I) or AAF/II, which are encoded for by *aggR* genes. EAggEC also produce an enteroaggregative heat-stable toxin (EAST1). EAST1 is similar to ST, and may be responsible for the symptoms of infection. EAST1 is encoded on a plasmid by *astA* genes.

Infant foodstuffs and formulae, milk and water have all been implicated in EAggEC outbreaks.

1.6 Diffusely Adherent *E. coli (DAEC)*
DAEC are a major cause of urinary tract infections worldwide, but its role as a causative agent of diarrhoea is controversial.

DAEC are comprised of heterogeneous groups of organisms with variable virulence. They are identified by their adherence to Hep-2 cells in a diffuse pattern. DAEC are divided into two classes, those which harbour afimbrial adhesins (Afa)/Drori antigen (Dr) adhesins and those that express an adhesin involved in diffuse adherence, which is a potential cause of infantile diarrhoea. DAEC infection is

characterised by the growth of long finger-like cellular projections that wrap around the adherent bacteria. 75% of DAEC strains produce the F1845 fimbrial adhesin (encoded for by the *daaC* gene) or a related adhesin.

Sources implicated in outbreaks of DAEC include contaminated food, especially undercooked ground beef, contaminated water and contact with livestock and other animals.

1.7 Isolation, detection and characterisation of pathogenic *E. coli*

Pathogenic *E. coli* represent a phenotypically diverse group of pathogens and no single method or approach can be used to detect or isolate all of the pathotypes of concern. Consequently methods have been developed to specifically detect or isolate certain pathotypes.

Pathogenic *E. coli* strains that ferment lactose and are not adversely affected by elevated temperatures (e.g. 44°C) can be isolated using standard procedures for *E. coli*. Most pathogen detection in foods is performed on a 25g sample which is enriched in 225ml of a suitable enrichment broth. In the Food and Drug Administration Bacteriological Analytical Manual (FDA BAM) method (available online at www.cfsan.fda.gov/~ebam/bam-24.html) the recommended procedure for pathogenic *E. coli* is to pre-enrich the sample in 225ml brain heart infusion (BHI) broth at 35°C for 3 hours to facilitate resuscitation of sub-lethally injured cells. The entire pre-enrichment is transferred to 225ml of tryptone phosphate (TP) broth and incubated at 44°C for 20 hours, after which time an aliquot of enriched broth is plated onto eosin-methylene blue (EMB) agar and MacConkey agar plates. These are incubated at 37°C for 24 hours. Some pathogenic *E. coli* strains may exhibit atypical colony morphology on these media. Therefore 10 typical (green metallic sheen on EMB or red colony on MacConkey agar) and 10 atypical colonies should be selected for further identification.

Identification and confirmatory steps include biochemical tests, serotyping and examination for key virulence associated genes. Typing of pathogenic *E. coli* may involve the use of a variety of typing techniques, e.g. pulsed field gel electrophoresis, multiple-locus variable-number tandem repeat analysis, amplified fragment length polymorphism and ribotyping, but these are often applied to specific pathotypes during epidemiological investigation. DNA probes may also be developed based on the sequences of genes specific to a particular pathotype. Examples of probes and PCR targets suitable for the detection of various pathotypes of *E. coli* are provided in the FDA BAM. This document aims to give specific methods for isolation, detection and characterisation of the six pathotypes of diarrhoeagenic *E. coli*.

2. Verocytotoxigenic *E. coli*
2. 1 Isolation
There are many challenges associated with the isolation of verocytotoxigenic *E. coli* (VTEC) from complex sample matrices. While clinical specimens may have high numbers of the pathogen present, animal faeces, environmental and food samples may contain very low numbers of VTEC. In all matrices there may be very high levels of background micro-flora and natural inhibitors which interfere with isolation and subsequent detection of the pathogen. In many samples, VTEC may be present in an injured or stressed condition as a result of food processing stresses (pH, temperature, preservatives, etc.) or the presence of antibiotics in clinical specimens. Unless an enrichment step is built into the protocol these cells may not be recovered, resulting in false negative results. These problems result in a necessary trade off between the need to incorporate antibiotics and other inhibitory agents into the enrichment broth and agar to enhance selectivity and the potential inhibition of stressed cells by these selective agents.

2.1.1 Enrichment
A liquid enrichment step may be included in methods for the isolation of VTEC from all sample matrices in order to increase numbers of the pathogen to a detectable level and to allow recovery of injured cells. While growth of VTEC is usually carried out at 37°C, in samples where there is a very high level of background micro-flora incubation at 42°C is favoured. It should however be noted that incubation at 42°C may hinder the recovery of injured VTEC cells.

Two of the most successful enrichment media used for *E. coli* O157 and other VTEC serogroups are tryptone soy broth (TSB) and *E. coli* broth (EC) with or without modifications to their original formulation. Modification to TSB media may include addition of bile salts and dipotassium phosphate while modified EC broth contains less bile salts. These basal media may also be supplemented with various selective agents (Table 1).

For food and animal feeding stuffs there is an International Organisation for Standardisation (ISO) reference method (ISO 16654) for isolation of *E. coli* O157. This method advocates enrichment in modified TSB with novobiocin (mTSBn) at 41.5℃ for an initial period of 6 hours and then for a further period of 12 to 18 hours. At present there is no standardised enrichment protocol for non-O157 VTEC though a number of enrichment protocols have been reported which will allow for the isolation of a wide range of VTEC or which have been optimised for recovery of selected serogroups (Table 1).

Table 1. Enrichment media for VTEC

Enrichment Media	Group/ Serogroup
Non selective enrichment media	
Tryptone Soya Broth (TSB)	VTEC
Modified TSB	VTEC
Buffered Peptone Water	VTEC
E. coli Broth	VTEC
Selective enrichment media	
TSB+ Vancomycin + Potassium Tellurite	O111
TSB + Vancomycin + Cexifime + Potassium Tellurite	O26
Modified TSB + Novobiocin	O157 & other VTEC
Modified TSB + Acriflavin	O157
Modified TSB + Cefixime + Cefsulodin + Vancomycin	O157
Modified *E. coli* Broth + Novobiocin	O157, O26
Modified *E. coli* Broth + Novobiocin	O26
Enterobacteriaceae Enrichment Broth + Novobiocin	O157
Buffered Peptone Water + Vancomycin + Cefsulodin + Cefixime	O157
Buffered Peptone Water + Vancomycin	O26, O111

2.1.2 Immunomagnetic Separation

A number of isolation methods using antibodies specific to particular VTEC serogroups are available. Immunomagnetic separation (IMS) recovers target cells from the enrichment broth using paramagnetic beads. These beads are coated with polyclonal antibodies specific for a particular VTEC serogroup. To date beads coated with antibodies against serogroups O157, O26, O111, O103 and O145 are commercially available. The cell bead complex is recovered from the medium by the application of a magnetic field that causes the complex to be concentrated in a tube. The bulk of the medium is decanted off leaving the cell bead complex in the tube. The concentrated cell bead complex can then be examined by culturing onto solid media or by a rapid method such as PCR. IMS can be fully automated (e.g. BeadRetriever™; Dynal Biotech Ltd, Wirral, UK).

In the gold standard cultural O157 method (ISO 16654) the inclusion of IMS is a prerequisite step before cultural isolation onto plating media. While there is no standardised protocol for other VTEC, IMS has been shown to be useful in recovery of specific serogroups from food and faecal samples.

2. 2 Detection
2.2.1 Culture plating media

The majority of commercial agars for VTEC still focus predominantly on the identification of *E. coli* O157. The inability of most *E. coli* O157 to ferment sorbitol is exploited in sorbitol McConkey agar (SMAC). Cexifime and potassium tellurite may be added to the SMAC (CT-SMAC) to increase selectivity in heavily contaminated samples. *E. coli* O157 generally produces colourless colonies when cultured on this media, thus distinguishing it from other VTEC serogroups and other micro-flora. This is the media of choice in the ISO standard protocol (ISO 16654) for *E. coli* O157, together with a second appropriate selective agar.

Although non-sorbitol fermenting *E. coli* O157 predominate, sorbitol fermenting *E. coli* O157 (non motile) have emerged as causes of HUS in Europe and Australia. These particular variants will not be readily identified on SMAC as they will produce coloured colonies which will appear similar to the other micro-flora present. Additionally the presence of potassium tellurite in CT-SMAC may actively inhibit the growth of sorbitol fermenting *E. coli* O157. Thus alternative strategies are required for cultural detection of these strains, an example of which exploits the fact that in general *E. coli* O157 (including the sorbitol-fermenting strains) are β-glucuronidase (GUD) negative. Commercially developed examples of media which utilise this characteristic include 4-methylumbelliferyl-β-D-glucoronide (MUG) agar and 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (BCIG) agar. Other agars in use for recovery and identification of *E. coli* O157 are listed in Table 2.

Non-O157 VTEC strains display a heterogeneous range of phenotypic properties making it difficult to find a common agar to selectively and differentially recover these pathogens. There are however some commercial chromogenic agars including Chromocult and Rainbow agar which differentiate between O157 and other selected VTEC serogroups (O111, O26, O103 and O145) on a colour basis.

Recent research has focused on the development of a differential media based on a chromogenic compound and a mixture of selected carbohydrates which can identify and discriminate between serogroups O26, O103, O111, O145 and O157 on a colour basis. The specificity of this new isolation method is currently being evaluated.

Other agars that have been optimised for the identification of specific VTEC serogroups and are listed in Table 2.

16

Table 2. Solid Media used for detection of VTEC

Selective Agar	Serogroup(s)
Sorbitol McConkey Agar (SMAC)	O157
SMAC + Potassium Tellurite + Cefixime (CT-SMAC)	O157
5-bromo-4-chloro-3-indolyl-β-D-glucoronide (BCIG) Agar (BCIG)	O157
SMAC-BCIG	O157
SMAC-BCIG + Potassium Tellurite + Cefixime (SMAC-BCIG-CT)	O157
4-methylumbelliferyl-β-D-glucoronide (MUG)	O157
Lactose Monensin Glucoronate Agar (LMG)	O157
Enterohaemolysin Agar	VTEC
Enterohaemolysin Agar with Vancomycin + Cefixime + Cefsulodin	VTEC
Phenol Red Sorbitol Agar + MUG (PRS-MUG)	O157
Fluorocult™ *E. Coli* O157 Agar	O157
CHROMagar O157	O157
BCM O157	O157
O157-ID Agar	O157
Chromocult	O157, O111, O26, O1O3, O145
Chromocult Agar + Cefixime + Cefsulodin + Vancomycin	O157, O111, O26, O1O3, O145
Rainbow Agar© O157	O157, O26, O111, O48
Rhamnose McConkey Agar (RMAC)	O26, O157
RMAC + Cefixime	O157
RMAC + Potassium Tellurite + Cefixime (CT-RMAC)	O26

2.2.2 Immunological methods

Immunological methods are widely used for the detection of VTEC. The methods utilize specific poly- or monoclonal antibodies targeting surface antigens and thus detect specific VTEC serogroups while others detect the toxins produced by VTEC.

Most commercial kits target the O157 antigen expressed by bacteria in a mixed culture with few, if any, targeting other VTEC serogroups. These are available in various formats including enzyme-linked immunosorbent assays (ELISA), reversed passive latex agglutination (RPLA) and automated systems combining IMS and ELISA. All commercial kits require prior enrichment of the target cells to reach detectable levels. These assays are generally less sensitive than traditional cultural techniques; however they offer advantages in terms of rapidity, reduced labour costs and high volume throughput. All immunological techniques should be adequately standardised and controlled to reduce the possibility and impact of false positive and false negative results. This is more prevalent in samples with high levels of diverse background microflora. In all cases suspect positive samples should be subject to further confirmatory tests as the coating antibody may cross-react with other organisms. False positive results occur when the immunological material cross-reacts with a non-VTEC organism (i.e. antibody is insufficiently specific). False negative results occur when the immunological material does not detect VTEC cells when they are present in the sample under examination (i.e. antibody can find a target binding site on the VTEC organism).

2.2.3 Serodiagnosis

Alternative approaches may be considered in clinical diagnosis of VTEC infection where the pathogen cannot be cultured, or where a rapid answer is needed, aiming at the detection of antibodies against VTEC serogroups in the patients' sera.

Specifically these antibodies target the lipopolysaccharide (LPS) component of the VTEC cell. ELISA kits and immunoblotting assays have been commercially developed to detect the antibodies

to VTEC LPS. Clinical diagnosis can be made from blood, faecal or saliva samples as they all contain the VTEC LPS antibodies.

2.3 Verocytotoxin assay
The ability of VTEC to produce VT can be assessed by exploiting the ability of these cytotoxins to induce a cytopathic effect on Vero cell monolayers. The supernatant of suspected VTEC cultures or the sonicated bacteria are inoculated onto Vero cell monolayers and the cytopathic effect (CPE) appearance can be scored after 24 hours by microscopic observation. The VT can also be detected as free faecal toxin directly in stool extracts thus speeding up the clinical diagnosis. Commercial kits are available for this purpose based on the use of latex agglutination assay (VTEC Reversed Passive Latex Agglutination).

2.4 Molecular methods
2.4.1 Polymerase Chain Reaction
A number of nucleic acid based methods have been reported for the detection and characterisation of VTEC. The most commonly reported methods are based on the use of the polymerase chain reaction (PCR) to amplify a specific gene target in VTEC.

The primers used in the PCR may detect a characteristic virulence factor in VTEC, i.e. *vt* genes (*vtx1*, *vtx2* or *vtx2* subtypes) as well as others such as *eae* (intimin) gene sequences. Closely related strains of *E. coli* have *rfb* genes encoding different O antigens and this can be exploited to differentiate between different serogroups of VTEC. Serogroup target genes include rfb_{O157} (O157 antigen), *flicH7* (H7 antigen), *wzx* (O-antigen flippase in O26); *wzy* (O-antigen polymerase in O103), *galE* (galactose operon in O103), *Wbdl* (transferase gene in O111) and *Sil* (silver resistance in O145).

While *vtx1* has a relatively homogeneous nucleotide sequence, several variants of *vtx2* have been described. There are a number of primer pairs available which will detect most *vtx2* variants in a single assay and others which discriminate between the variants. This can give additional characterisation of the strains and

information regarding their virulence potential with some variants more associated with severe clinical illness.

Conventional PCR relies on amplification of the target gene(s) in a thermocycler, separation of PCR products by gel electrophoresis, followed by visualisation and analysis of the resultant electrophoretic patterns, a process that can take a number of hours. The development of real-time PCR which uses fluorescence to detect the presence/absence of a particular gene in real time has greatly increased the sensitivity and speed of PCR-based detection methods. Real-time PCR assays have been developed for the detection of VTEC carrying the major associated virulence genes i.e. *eae*, *vtx1* and *vtx2* and also for serogroup specific gene targets for O157, O26, O111, O103 and O145. Some of these targets have been combined into multi-plex assays.

2.5 Quantification of VTEC
There is no standard protocol for enumeration of *E. coli* O157 or other VTEC serogroups from food or environmental samples although quantitative data is essential for the development of quantitative risk assessment models.

Cultural approaches to the enumeration of *E. coli* O157 from food samples have involved plating the diluted sample directly onto SMAC, SMAC-CT or alternative selective agar. The sensitivity of this approach is generally low ($\sim 10^2$ CFU g^{-1}) and bacteria which are stressed or injured may not be detected. To overcome this issue, samples likely to contain stressed cells may be initially plated onto a non selective agar such as trytone soy agar, incubated for a number of hours ($\sim$ 3 to 4 hours) to allow stressed cells to recover and then overpoured with a selective agar and re-incubated.

An alternative approach is to use a most probable number (MPN) method.

The enumeration of non-O157 serogroups (O111, O26, O145, O103) is more difficult due to the lack of an agar which clearly morphologically differentiates colonies of different serogroups. This means that many colonies must be identified using serological or molecular methods which is very labour and resource intensive.

An alternative approach in the future may be the application of quantitative real time PCR. This approach has been applied to directly quantify VTEC in food and faecal samples but the detection limit is reportedly very high at 10^5 CFU g^{-1} for food samples and 10^2 to 10^7 CFU g^{-1} for faecal samples.

2.6 Typing of VTEC
Typing of confirmed VTEC isolates is essential in surveillance and epidemiology of infection, particularly in outbreak investigations. Also, typing to determine the pathogenicity and the virulence capacity of a VTEC isolate is essential for control measurements and risk-based legislation. The following methods can be used to demonstrate whether individual isolates may, or may not, be linked.

Isolates can be typed by either phenotypic or DNA-based methods.

2.6.1 Phenotypic methods
Biochemical profiling
Biochemical profiling involves the reaction of a particular isolate to a range of biochemical tests, for example fermentation of carbon sources. The result is a metabolic fingerprint of the isolate. Fingerprinting can be done using commercially available phenotypic arrays (e.g. Biolog Inc., Hayward, California, USA).

VTEC serogroups lack common biochemical characteristics. *E. coli* O157 has some biochemical characteristics that have been used in its typing such as its lack of ability to ferment D-sorbitol, in contrast to about 75 to 94% of other *E. coli* strains. *E. coli* O157 is also unable to produce β-glucuronidase, which hydrolyzes MUG and related substrates, unlike most other *E. coli* serogroups.

Serotyping
Serotyping is based on the use of specific antisera and the detection of somatic O- and H- antigens expressed by these bacteria. Currently, a total number of 181 O-antigens and 53 H-antigens are available. Different approaches can be applied for agglutination of these specific antisera, including screening of all types of antigens

simultaneously in a microplate format, or using several pools of antisera (e.g. 10 by 10) which are tested separately. Kits for O serotyping and H serotyping for VTEC are commercially available (see table 3 for examples of VTEC O serotyping kits). Certain isolates may be non-motile, requiring supplementary motility tests in specific motility media. However, a minority of strains do not serotype satisfactorily. Serotyping can also be done by DNA-based methods (see below). Full serotyping is usually carried out in national reference laboratories.

Table 3. Examples of commercial VTEC O serotyping kits (source LREC, Lugo, Spain)

Kit Available	O antisera
Human and Animal VTEC	O1, O2, O4, O5, O6, O8, O9, O15, O16, O17, O20, O22, O25, O26, O27, O32, O39, O41, O45, O46, O48, O55, O64, O74, O75, O77, O81, O82, O84, O86, O88, O91, O98, O101, O103, O104, O105, O109, O110, O111, O112, O113, O116, O117, O118, O119, O121, O123, O126, O128, O132, O136, O139, O141, O145, O146, O150, O153, O157, O162, O163, O165, O166, O168, O171, O172, OX3=O174, O176, O177, O178
Human VTEC (only the most prevalent)	O26, O48, O91, O103, O111, O113, O118, O128, O145, O146, O157
Bovine VTEC (only the most prevalent)	O2, O4, O5, O6, O8, O16, O20, O22, O26, O39, O41, O45, O46, O64, O74, O77, O82, O91, O103, O105, O109, O113, O116, O126, O128, O136, O141, O145, O146, O153, O157, O162, O163, O168, O171, O172, O174=OX3, O177

Bacteriophage typing

In Europe, the most widely used conventional sub-typing method for *E. coli* O157 is bacteriophage typing. However, this technique is

not applicable to non-O157 VTEC. The susceptibility of each isolate to lysis by a panel of 16 bacteriophages is determined and the lytic patterns obtained usually allow typing into one of 82 possible types. Bacteriophage typing does not usually provide the level of discrimination required for epidemiological and outbreak investigations, as the number of different types identified routinely may not be sufficient for confident interpretation of results.

2.6.2 DNA-fingerprinting methods

These methods are based on the analysis of total DNA from an isolate. There are a large number of methods available mainly based on two basic approaches: either the randomised analysis of whole genome DNA or the analysis of probed or amplified targeted DNA.

RFLP-based methods are commonly used in routine typing. The general principle of operation of these techniques is detection of restriction enzyme sites on genomic DNA. RFLP-based methods are based on pattern recognition of DNA fragments which can be visualized by gel electrophoresis. The ability to standardise and digitalise data generated for interlaboratory comparisons has its limitations.

The search for new tools for molecular epidemiology which facilitates web based communication and exchange of data is ongoing. The variable-number tandem repeats (VNTR) method targets the polymorphism in the number of tandemly repeated short DNA sequences. Results obtained using this method are more easily interpreted and communicated.

Single nucleotide polymorphism (SNP) is another approach using different kind of genes as targets for analysis. SNP detects nucleotide substitutions, by using sequencing, PCR or oligonucletide hybridizations (e.g. microarrays).

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) is an example of a randomised approach of RFLP and is currently considered as the

"gold standard" method for typing of *E. coli* O157. Using restriction enzymes which cleave the bacterial genome infrequently, it is possible to generate small numbers of large DNA fragments to form macrorestriction profiles characteristic for one or more isolates. Satisfactory separation of large DNA fragments can only be achieved in an alternating electrical field. Comparison of macrorestriction profiles for different isolates will indicate whether they are indistinguishable and thus potentially linked. PFGE has proved to be invaluable in investigation of outbreaks of food-borne infection caused by VTEC. The technique requires highly technically skilled personnel but is very reproducible and has the advantage of generating genetic data which can be statistically analysed. PulseNet, administrated by Centers for Disease Control and Prevention (CDC) in the USA, utilises highly standardised molecular subtyping methodologies, sophisticated data analysis software and an open communication platform, via the internet, that allows for rapid dissemination of information to all participating laboratories.

Targeted RFLP
Another approach of RFLP is the detection of regions of DNA complementary to molecular probe(s) targeting particular genomic characteristics either by DNA probing or PCR (PCR-RFLP). Several genomic targets have been exploited as epidemiological markers including ribosomal RNA genes, insertion sequence elements, bacteriophage lambda and VT-encoding genes integrated into the bacterial chromosome. Depending on the target, closely related groups of bacteria may not exhibit sufficient variation in these traits to allow effective discrimination except in defined circumstances. Generally, PFGE has more discriminatory power than targeted RFLP.

Multilocus variable-number tandem repeat analysis
Multilocus variable-number tandem repeat analysis (MLVA) determines the number of repeats at multiple variable-number tandem repeat (VNTR) loci, i.e. areas that evolve quickly in the bacterial genome. It is a fast and simple method and has

successfully been used for the rapid detection of outbreaks of *E. coli* O157. It has been shown to have excellent discriminatory ability, its sensitivity being equal or superior to that of PFGE. The output is highly objective, and the method is easily standardised for comparisons among different laboratories. In order to establish MLVA on a new pathotype or serotype of *E. coli*, the fully sequenced genome has to be available.

An MLVA assay has been published based on the *E. coli* reference collection (ECOR), consisting of 72 strains that are representative of the genomic diversity of the species.

Typing of virulence factor encoding genes
Genes encoding variants of verotoxins, A/E adhesion factors, enterohemolysins and other potential pathogenicity factors can be used as targets for characterisation and typing. The content of combinations of pathogenicity factor encoding genes together with serotypes has been used to classify a particular VTEC isolate into different seropathotypes (A – E).

DNA based serotyping
Serotyping can also be done by molecular methods including analysis of genes/DNA sequences within and just outside the O-antigens cluster and *fli*C (H-antigens) sequence analysis. This molecular serotyping is based on either DNA sequencing, DNA hybridization (microarray), PCR or PCR-RFLP.

3. Enteropathogenic *E. coli*
3.1 Detection
There are several published target sequences used for PCR detection of EPEC. These include the EAF plasmid and the gene encoding BFP (*bfpA*). Some of the genes found in EHEC (e.g. *eae*) are also present in EPEC strains. In order to distinguish between these pathotypes it may be necessary to confirm the presence of *eae* and the absence of *vtx* genes.

3.2 Typing and sub-typing approaches

Typing of confirmed EPEC isolates is essential in surveillance and epidemiology of infection, particularly in outbreak investigations. EPEC were traditionally defined by their serotype based on somatic O and flagellar H antigens and to a lesser extent on K antigens as, in general, serotype correlates with specific pathotypes of *E. coli*. While this practice continues, it is now more common to define *E. coli* pathotypes based on their pathogenic characteristics. This is necessary because of the existence of strains with the same serogroup which belong to different pathotypes, e.g. *E. coli* O111:H- may belong to the EPEC and EHEC groups depending on the presence or absence of certain virulence genes (e.g. *vtx*, *eae*). The pathogenic characteristics are determined using histopathology and molecular tools such as PCR and probes to detect the presence of specific virulence genes.

Histopathology

Histopathology, specifically the pattern of *E. coli* cell adherence in tissue/tissue culture is a very useful phenotypic test to define specific pathotypes of diarrhoea causing *E. coli*. EPEC strains exhibit a typical A/E histopathology which can be observed in intestinal biopsy specimens. This is characterised by effacement of micovilli and intimate adherence between the bacteria and the epithelial cell membrane and the formation of distinct pedestal like structures. Typical EPEC strains possess the *eae* gene and the EAF probe of *bfp* sequences which indicate the presence of the EAF plasmid. In contrast, atypical EPEC strains possess the *eae* gene without the EAF plasmid.

PFGE

PFGE has successfully been used for typing of EPEC in epidemiological studies and in outbreak investigations, although no international database for comparisons of patterns has been established.

26

Targeted RFLP
As for VTEC, several genomic targets have been exploited as epidemiological markers, including ribosomal RNA genes on the bacterial chromosome which makes targeted RFLP possible for EPEC typing.

MLVA
MLVA can be used for typing of EPEC as long as the required sequence information is available and has been used in several applications. Some EPEC strains are fully sequenced, and the same principle for setting up a MLVA assay will apply for EPEC as for VTEC.

Typing of virulence factor encoding genes
The intimin encoding genes (*eae*) are the most usual targeted virulence factor genes that are used in this method for typing of EPEC. This can be done by using PCR or hybridizations (arrays).

DNA-based serotyping
Serotyping can also be done by molecular methods including analysis of genes/DNA sequences within and just outside the O-antigens cluster and *fliC* (H-antigens) sequence analysis. This molecular serotyping is based on either DNA sequencing, DNA hybridization (microarray), PCR or PCR-RFLP.

4. Enterotoxigenic *E. coli*
4.1 Detection
Traditionally ETEC detection was reliant on detection of the enterotoxins LT and/or ST. Detection of ST was originally done using the rabbit ligated ileal loop assay which was later replaced by the suckling mouse assay. LT is detected using cell culture assays, e.g. the Y1 adrenal cell assay and Chinese hamster ovary cell assay. Detection of both ST and LT by bioassays has been replaced by ELISA tests and more recently by PCR based techniques. PCR targets include the genes encoding LT and ST. The FDA BAM also provides information on the application of DNA probes for the detection of LT and ST.

4.2 Typing and sub-typing approaches

Characterization of ETEC has been traditionally based on the detection of phenotypic traits (serotypes) and virulence-associated factors such as LT and ST as well as the colonization factors involved in the adherence of the bacterium to intestinal epithelial cells.

5. Enteroinvasive *E. coli*
5.1 Detection

EIEC strains are biochemically, genetically and pathogenically closely related to *Shigella* species. Most strains are lysine decarboxylase negative, non-motile and 70% are unable to ferment lactose. Strains of EIEC may therefore be difficult to distinguish from *Shigella* species.

The invasive potential of EIEC may be tested using the Sereny or guinea pig keratoconjunctivitis assay or using HeLa cell tissue culture methods where the ability of EIEC to form plaques correlates with virulence characteristics of these bacteria.

Both EIEC and *Shigella* species strains share a 140-MDa invasion plasmid (pINV) that encodes several outer-membrane proteins involved in cellular invasion. The genes encoding these invasion plasmid antigens (*ipa* genes) are conserved and used to distinguish EIEC from other pathogenic *E. coli*. The similarity between the *ipa* genes (*ipaA*, *ipaB*, *ipaC* and *ipaD*) using RFLP analysis with *Sal*I and *Hind*II digests has been demonstrated.

The FDA BAM also provides information on the application of DNA probes for the detection of pINV. However, as strains of EIEC can lose all or part of the pINV plasmid on *in vitro* passage or storage, these should be hybridised with probes as soon after excretion or initial isolation as possible.

5.2 Typing and sub-typing approaches

In principle, all typing methods mentioned in the previous sections can also be applied to other pathogenic *E. coli*. However, as less

28

knowledge and less data are available for EIEC there is less experience in the development and use of typing approaches for this group. Serotyping is used and strains of EIEC may belong to a limited number of serotypes including O159:H2, O28:H-, O124:H30, O164:H- and O143:H-.

6. Enteroaggregative *E. coli*
6.1 Detection

EAggEC strains are diverse and not all of them have AAF/I or AAF/II (encoded for by *aggR*) and EAST1 (encoded for by *astA*). While the *astA* gene is not specific for EAggEC, it may be used in combination with other specific EAggEC genes to facilitate detection. The *aggR* genes have also been targeted for EAggEC detection. The antiaggregation protein (*aap*) genes, which are related to the virulence factors of EAggEC and the chromosomally encoded genes *aaiA* (involved in aggregative adherence) have also been shown to be suitable target genes for the identification of typical and atypical EAggEC strains.

6.2 Typing and sub-typing approaches

Although serotyping is used, EAggEC represent a heterogeneous group which makes their classification based on serology of limited use. EAggEC are currently defined as *E. coli* strains that adhere to Hep-2 cells in an aggregative adherence pattern but which do not secrete enterotoxins ST or LT. The Hep-2 adherence assay is therefore the gold standard for the diagnosis of EAggEC. The distinct pattern of aggregative adherence is characterised by the layering of bacteria in a "stacked, brick-like" configuration on the surface of the host cells.

7. Diffusely Adherent *E. coli*
7.1 Detection

DNA probes have been developed for the detection of DAEC. Targets for probes include the *daaC* gene which is associated with the expression of the F 1845 fimbriae. False positive reactions can occur. Reports for PCR detection of DAEC in the scientific

literature are currently limited although this work is ongoing. DAEC phenotype is characterised by the ability to adhere to Hep-2 cells in a diffuse pattern.

7.2 Typing and sub-typing approaches
As with the EAggEC, DAEC represent a heterogeneous group characterised by plasmid borne virulence genes. Consequently, serotyping is of limited value. There are no other typing methods currently available.

8. Further Reading

Baylis, C., Heuvelink, A.E., Hofstra, H. and de Boer, E. (2001). Practical considerations and difficulties associated with the detection of verocytotoxin producing *Escherichia coli* (VTEC) in foods. *In* Verocytotoxigenic *E. coli*. (2001). Editors, Duffy, G., Garvey, P., and McDowell, D.A. Publishers: Blackwell Publishing Inc., Malden, Massachusetts. ISBN: 9780917678523

Baylis, C. L., Penn, C. W. Thielman, N. M., Guerrant, R. L. Jenkins, C., and Gillespie, S. H. (2006). *Escherichia coli* and *Shigella* spp. pp 347-365. In Hawkey, P M., and Gillespie, S. H. (Eds), Principles and Practice of Clinical Bacteriology, John Wiley & Sons Ltd, London.

Catarame, T.M.G., O'Hanlon, K.A., Duffy, G., Sheridan, J.J., Blair , I.S. and McDowell, D.A. (2003). Optimisation of Enrichment and Plating Procedures for the recovery of *E. coli* O111 and O26 from Minced Beef. *J. Applied Microbiology* 95: 949-957.

Donnenberg, M. S. (2002). Introduction pp. I *In* Donnenberg, M. S. (Ed.) *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen. Elsevier Ltd, UK. ISBN 0122207513.

Donnenberg, M. S. (2002). Enteropathogenic *Escherichia coli*. Chapter 40, p. 595-612. *In*: Infections of the Gastrointestinal Tract, 2nd Edition. Editors, Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL. Publishers: Lippincott Williams & Wilkins, Philadelphia. ISBN 0-781702267.

Feng, P., Weagant, S. D. and Grant, M. A. (1998) Enumeration of *Escherichia coli* and the Coliform Bacteria. *In* United States Food and Drug Administration Bacteriological Analytical Manual (online) www.cfsan.fda.gov/~ebam/bam-4.html.

Feng, P. and Weagant, S. D. (1998) Diarrheagenic *Escherichia coli*. *In* United States Food and Drug Administration

Bacteriological Analytical Manual (online) www.cfsan.fda.gov/~ebam/bam-4a.html.

International Standards Organisation (16654). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Escherichia coli* O157.

Kaper, J. B., Nataro J. P., and Mobley H. L. (2004) Pathogenic *E. coli*. *Nature Review of Microbiology.* Feb; 2(2):123-40.

Nataro, J. P. and Kaper. J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, vol. 11, no. 1. (142-201).

Perelle, S., Dilasser, F., Grout, J. and Fach P. (2006). Screening food raw materials for the presence of the world's most frequent clinical cases of Shiga toxin-encoding *Escherichia coli* O26, O103, O111, O145 and O157. *Int J Food Microbiol.* 15; 113(3):284-288.

Posse, B., De Zutter, L., Herman, L. and Heynrickx, M. (2007). A novel selective isolation procedure for shigatoxin producing *E. coli* serotypes O26, O103, O111, O145 and both sorbitol positive and negative O157. *In* Proceedings of Conference on "Methods of detection and molecular characterisation of pathogenic *Escherichia coli*", EU Pathogenic *E. coli* Network, www.pen-project.eu

Sekse, C., Solberg, A., Petersen, A., Rudi, K. and Wasteson, Y. (2005). Detection and quantification of shiga toxin encoding genes in sheep faeces by real time PCR. *Molecular and Cellular Probes* 19: (5) 363-370**.**

Smith, J. L., Fratamico, P. M. and Gunther, N. W. (2007) Review: Extraintestinal pathogenic *Escherichia coli. Foodborne Pathogens and Disease.* 4: 134-163.