

VEROCYTOTOXIGENIC ESCHERICHIA COLI

SUMMARY

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans. Some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. Since 1977, it has been recognised that some diarrhoeagenic strains of *E. coli* produce toxins that have an irreversible cytopathic effect on cultured Vero cells. Such verocytotoxigenic *E. coli* (VTEC) belong to over 100 different serotypes. *Escherichia coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic *E. coli* (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce verocytotoxins, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid. In the past two decades, VTEC O157:H7 has risen in importance world-wide as a public health problem. Other non-O157 serogroups, including O26, O91, O103, O104, O111, O113, O117, O118, O121, O128 and O145, have been associated with occasional outbreaks of human disease, and others may be associated with sporadic cases. Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds. Cattle are considered to be the main reservoir of *E. coli* O157:H7 infection for humans. Despite its pathogenicity for humans, infection in animals with *E. coli* O157:H7 is invariably asymptomatic. By contrast, the EHEC serogroups, O26, O111 and O103 may be pathogenic for both humans and animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain by faecal contamination of milk products, contamination of meat with intestinal contents during the slaughter process or contamination of fruit and vegetables by contact with infected manure. VTEC are also transmitted through contaminated water and by direct contact with infected people or animals.

Identification of the agent: Diagnostic procedures for VTEC have been developed, primarily for *E. coli* O157:H7, and seek to overcome the problems of isolating low numbers of target organisms from complex matrices such as animal faeces, food and clinical specimens. Identification of *E. coli* O157:H7 in subclinical animal carriers depends on enrichment of faeces samples in liquid media, usually buffered peptone water with or without the addition of vancomycin, cefsulodin and cefixime, for 6 hours at 37°C followed by immunomagnetic separation using commercially available paramagnetic particles or beads coated with anti-O157 lipopolysaccharide antibody. Beads with bound bacteria are plated on to selective agar, commonly 1% sorbitol MacConkey agar containing cefixime and potassium tellurite, and incubated for 18 hours at 37°C. Non-sorbitol-fermenting colonies are confirmed biochemically as *E. coli* and by serum or latex agglutination as possessing the O157 somatic antigen and/or H7 flagellar antigen. Potential virulence for humans is confirmed by the demonstration of verocytotoxin production by Vero cell assay, enzyme-linked immunosorbent assay (ELISA) or agglutination tests or the demonstration of genes encoding verocytotoxin by polymerase chain reaction. Detection of non-O157 VTEC relies on direct analysis of colonies on nonselective plates by, for example, immunoblotting or DNA probing for verocytotoxin production. Numerous immunological and nucleic acid-based recognition tests have been described to provide a more rapid presumptive diagnosis of VTEC and many are available commercially. Phage typing and pulsed field gel electrophoresis are widely used by reference laboratories for subtyping VTEC O157 for epidemiological purposes.

Serological tests: Serological tests are not used routinely in animals to diagnose VTEC infection, but it has been shown that cattle infected with VTEC produce serum antibodies to the O157 lipopolysaccharide that can be detected by ELISA.

Requirements for vaccines and diagnostic biologicals: No vaccines are currently available for controlling VTEC infections in animals or humans, but a variety of experimental vaccines are being developed.

A. INTRODUCTION

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans of which only some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. *Escherichia coli* are routinely characterised by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Since 1977, it has been recognised that some diarrhoeagenic strains of *E. coli* produce toxins that have an irreversible cytopathic effect on cultured Vero cells (19). Such verocytotoxigenic *E. coli* (VTEC) have been shown to belong to over 100 different serotypes (16, 33). They are also described as Shiga toxin-producing *E. coli* (STEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of *Shigella dysenteriae* (28). In the past two decades, VTEC O157:H7 has increased in importance world-wide as a public health problem. *Escherichia coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic *E. coli* (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce VT, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid (27). Other non-O157 serotypes, including O26:H11, O104:H21, O111:H- and O145:H-, have been associated with occasional outbreaks of human disease, and others still with sporadic cases (16).

Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds; these species can be transiently colonised by the organisms (4, 16). Surveys have shown that O157 strains normally represent a minority of the VTECs that colonise the intestinal tract of animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain via faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contamination of fruit and vegetables by contact with contaminated manure. VTEC are also transmitted through water and by direct contact with infected people, animals or animal waste. Contaminated water, used for irrigating or for washing vegetables, can also be source of infection for humans or animals. Cattle are considered to be the main reservoir of *E. coli* O157:H7 infection for humans, although the organism has been isolated from a variety of farmed animals, horses, dogs, rabbits, birds and flies. Despite its ability to cause severe disease in humans (29), infection in animals with *E. coli* O157:H7 is invariably subclinical. Some non-O157 serotypes, however, are pathogenic for animals and humans and include O26:H11; O103:H2; O111:H- (3, 16).

VTEC are also associated with oedema disease in piglets with four serotypes responsible for the majority of outbreaks world-wide, namely O45:K+, O138:K81, O139:K82 and O141:K-. The main virulence factors are a fimbrial adhesin, F18, involved in colonisation and the VT2e toxin, which is responsible for clinical signs. A high degree of genetic relatedness between O101 strains harbouring stx2e genes of human and porcine origin has been demonstrated. The role of pigs as subclinical carriers of STEC in the epidemiology of human disease needs further research.

Because *E. coli* O157:H7 has become the predominant zoonotic VTEC, diagnostic methods have been developed to detect selectively this serotype in human clinical cases (33) and in food sources (34). For the latter, a validated International Standard detection method is available (EN ISO 16654:2001). In this chapter, however, emphasis will be given to the isolation and identification of O157 and other VTEC from carrier animals (11).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

o Samples

In most cases, samples taken from animals for VTEC isolation will be faeces collected for surveillance purposes or as part of an epidemiological trace-back exercise following an outbreak of disease in humans. Samples may be taken from the rectum or from freshly voided faeces on the farm or from intestinal contents after slaughter. A variety of VTEC are present in healthy animals and not all are thought to be pathogenic for humans. *Escherichia coli* O157:H7, which is the most significant VTEC in human disease, is carried subclinically in animals. Cattle are thought to be the most important reservoir of this serotype. In an infected herd, only a proportion of the animals will be detectably infected, the organism is usually present in carriers in low numbers and is shed intermittently in faeces. Shedding is influenced by the age of the animals, diet, stress, population density, geographical location

and season (25). Some animals are thought to contribute disproportionately to transmission of infection and have been termed "super-shedders" (24). Isolation rates may be improved by taking faeces samples in preference to rectal swabs, by increasing the sample size, by increasing the number of individuals sampled and by repeat sampling. Use of recto-anal mucosal swabs is reported to improve detection of colonised as distinct from transiently infected cattle (30). Precautions should be taken to avoid cross-contamination of samples in transit and at the laboratory. Samples should be kept cool and cultured as soon as possible after collection.

o Safety

Care should be exercised when handling VTEC-positive samples as the infective dose capable of causing severe human infection may be low (possibly 100 organisms for VTEC O157:H7) and laboratory-acquired infections have been reported (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

o Isolation

a) Liquid enrichment media

Clinical samples are routinely plated directly on to solid media for isolation of *E. coli*, but the number of target VTEC organisms in faeces from healthy carriers is usually low and enrichment in liquid media improves recovery. Commonly used enrichment media are buffered peptone water either unsupplemented (which gives good recovery) or supplemented with 8 mg/litre vancomycin, 10 mg/litre cefsulodin and 0.05 mg/litre cefixime (BPW-VCC) to suppress the growth of Gram-positive organisms, *Aeromonas* spp. and *Proteus* spp.; modified trypticase–soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10 mg/litre acriflavin to reduce the growth of Gram-positive organisms; or modified *E. coli* broth with 20 mg/litre novobiocin (mEC+n). EHEC *E. coli* grow poorly at 44°C. The optimal incubation for bovine faeces to minimise overgrowth by other organisms is 6 hours at 37°C. For meat samples, enrichment for 6 hours at 41–42°C is used and for water and dairy products, 24 hours at 41–42°C. Nonselective pre-enrichment is necessary for the effective recovery of low levels of stressed *E. coli* O157. Enrichment broths should be pre-warmed to prevent cold-shocking the organisms and slowing their initial growth; 24 hours' incubation may increase recovery if the organisms are stressed.

b) Immunomagnetic separation

Immunomagnetic separation (IMS) has been used as a selective concentration technique to improve isolation of *E. coli* O157:H7 where numbers of the organism are low (10). Commercially available paramagnetic particles or beads coated with anti-lipopolysaccharide (LPS) antibody are mixed with an aliquot of incubated broth. Beads with bound bacteria are separated from the supernatant by a magnetic field and after washing are plated on to selective agar and incubated for 18 hours at 37°C to isolate suspect colonies. The technique is serogroup specific. Commercial systems are available for manual or automated separation (9). Recovery may be affected by the bead-to-organism ratio (optimum is 3:1), the enrichment broth used and the problem of nonspecific adsorption of *E. coli* to the magnetic beads (which can be reduced by the use of a low ionic strength solution in the IMS procedure and careful washing). These factors should be taken into account when trying to maximise the sensitivity of the technique for detecting target *E. coli*.

c) Selective culture for *Escherichia coli* O157

There are no biochemical characteristics that distinguish the majority of VTEC from other *E. coli*, however, the inability of most strains of *E. coli* O157:H7 to ferment D-sorbitol rapidly and their lack of beta-glucuronidase activity can be exploited in the isolation and identification of these organisms. However, the less common sorbitol fermenting and beta-glucuronidase positive *E. coli* O157:H– variants (nonmotile due to lack of expression of the H7 antigen), will not be identified by isolation in such selective media chosen for these biochemical characteristics (18). MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC) is a useful and inexpensive medium on which non-sorbitol-fermenting *E. coli* grow as small, round greyish-white colonies. Selectivity is improved by the addition of 0.5% rhamnose, and addition of 0.05 mg/litre cefixime (CR-SMAC) inhibits overgrowth by *Proteus* spp. While fewer presumptive colonies require testing on this medium, rhamnose is an expensive supplement. An alternative modification is the addition of 2.5 mg/litre potassium tellurite in addition to cefixime (CT-SMAC), which has a greater inhibitory effect against non-O157 *E. coli* and other non-sorbitol fermenters, such as *Aeromonas*, *Plesiomonas*, *Morganella* and *Providencia*, than against *E. coli* O157 (28). This is currently the most commonly used medium for isolating *E. coli* O157.

Media containing fluorogenic or chromogenic glucuronides are used to distinguish non-beta-glucuronidase-producing *E. coli* O157:H7 from beta-glucuronidase-producing *E. coli*. Hydrolysis of 4-methylumbelliferyl-beta-D-glucuronide (MUG) by beta-glucuronidase activity produces a fluorescent compound visible under UV light. The addition of 0.1 g/litre 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (BCIG) to SMAC differentiates white colonies of *E. coli* O157:H7 from green-blue colonies of sorbitol negative, beta-glucuronidase positive organisms. Commercially available chromogenic and fluorogenic media may be found

by reference to media catalogues. While advances have been made in improving the selectivity of media for *E. coli* O157:H7, isolation rates, particularly of stressed organisms, may be adversely affected by the additives used. To mitigate against these effects, addition of recovery agents such as 1% sodium pyruvate to tryptone–soy agar or delaying exposure of stressed cells to selective agents can aid recovery of the organism (8).

Sorbitol-fermenting (SF) *E. coli* O157:H– have been isolated from patients with diarrhoea and HUS but the epidemiology of this infection is poorly understood and only rarely has the organism been isolated from animals, including cattle (22). The majority of SF *E. coli* O157:H– isolates are susceptible to tellurite and cannot be identified on CT-SMAC. Microbiological analysis for this organism is laborious and entails plating IMS-separated organisms onto SMAC and testing individual SF colonies by latex agglutination for the O157 antigen. Alternatively, colony sweeps are tested by polymerase chain reaction (PCR) for the presence of *vt₂*, *eae*, *rfb_{O157}* and *sfpA* (see below). Well-spaced colonies from growth positive by PCR are then tested by colony hybridisation with probes for *vt₂*, *eae* and *sfpA* or colony immunoblot using specific antibody (18, 22).

d) Isolation of other VTEC

Non-O157 VTEC grow well on media that permit the growth of *E. coli*, such as blood agar or MacConkey agar, and the majority can only be differentiated from other *E. coli* by their ability to produce VT. The large number of different VTEC serotypes precludes the use of O-antisera for the routine screening and presumptive identification of colonies on these media. IMS can be used for selective concentration of serogroups O26, O103, O111 and O145 from a pre-enriched sample, as for the O157 strains. These serogroups are the non-O157 VTECs most commonly associated with human disease, and commercially produced beads are currently available.

The inability of O26 strains to ferment rhamnose has led to the recent development of media that may prove to be useful in differentiating O26 *E. coli* from other enteric organisms. The first is rhamnose-MacConkey agar (RMAC) in which the lactose in the MacConkey medium is replaced by 10 g/litre rhamnose. Addition of 2.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime (CT-RMAC) is said to improve specificity. The second is a chromogenic rhamnose agar incorporating 10 g/litre rhamnose and 0.02 g/litre phenol red in ES coliform agar (an indicator medium for beta-galactosidase activity) to which is added 0.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime. On this medium, O26 colonies are reported to be dark blue to black, other *E. coli* serotypes are green, and enterobacteria other than *E. coli* are green, yellow or colourless.

One potentially useful virulence marker for VTEC is enterohaemolysin production, which causes haemolysis of washed sheep erythrocytes after overnight incubation on blood agar supplemented with calcium. This characteristic is shared by 90% of VT-producing *E. coli* isolated from human infections. However, the finding that a proportion of disease-producing VTEC can be negative for enterohaemolysin production reduces the value of enterohaemolysin agar as a screen.

In most cases, therefore, isolation of VTEC relies on direct analysis of colonies on plates by immunoblotting or DNA probing for VT production to identify colonies for further characterisation. Colonies are first replicated so that positive colonies can be isolated after replicates have been tested. Colonies may be blotted on to suitable membranes (nitrocellulose or nylon) from which replicates are made or picked off into 96-well microtitre plates containing broth for replication before transferring aliquots to appropriate filters. Colonies are then analysed using nucleic acid probes or antibodies to identify any VTEC (33). Hull *et al.* (14) developed a mitomycin immunoblot assay for detecting VTEC in faeces that was simple enough to use in routine diagnostic laboratories. Serial dilutions of faeces in broth are inoculated on to MacConkey agar plates and incubated overnight at 37°C. Using standard replica plating techniques, growth from the plate yielding approximately 200 colonies is transferred to two 0.45-µm pore-size nitrocellulose filters laid on Syncase agar with 25 ng mitomycin/ml. This medium induces vegetative growth of bacteriophages carrying the genes for VT and enhances toxin expression. (Alternatively, bacterial or faecal suspensions may be plated directly on to the filters.) The plates are incubated overnight at 37°C. After overnight growth, filters are removed from the plates, immersed in a chloroform bath for 15 minutes, then blocked for 1 hour with 5% non-fat milk in 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 8) (TNT). The filters are incubated for 1 hour in antisera raised against VT1 or VT2, given three 5-minute washes in TNT, then incubated for 1 hour with alkaline phosphatase-conjugated anti-immunoglobulin G followed by three further 5-minute washes in TNT. Any reaction is visualised by colour development with nitroblue and 5-bromo-4-chloro-3-indolyl-phosphate. VT1, VT2 and VT-negative control *E. coli* are tested in parallel. The use of polyclonal antibodies results in some false positives that are eliminated by using monoclonal antibodies. When the use of DNA probes was compared with the use of the mitomycin immunoblot colony assay, it was shown that the results were comparable. The immunoblot assay has the advantage of being simpler to perform than DNA probing. Mitomycin plates have a long shelf life when stored in the dark at 4°C.

Colony immunoblotting or probing are labour intensive techniques and may be better applied to samples that have been screened and shown to be positive for the presence of VT or VT genes by, for example, enzyme-linked immunosorbent assay (ELISA) or PCR.

o Identification and characterisation of suspect colonies

Colonies growing on solid media that are suspected to be VTEC must be confirmed biochemically or genotypically (e.g. by *GadA* PCR) to be *E. coli*. Somatic 'O' and flagellar 'H' antigens are identified serologically. Not all VTEC isolated from animals are thought to be pathogenic for people. Some isolates of *E. coli* O157, particularly from pigs, are non-verocytotoxigenic and nonpathogenic for humans. Diagnosis, therefore, must include the demonstration of known virulence factors in the isolates. These include the verocytotoxins VT1 (Stx1) and VT2 (Stx2) and their genes and an outer membrane adhesion protein associated with attaching and effacing lesions, intimin, which is encoded by the *eae* gene (21). For VTEC O157 strains, subtyping methods are available in reference laboratories for epidemiological investigations.

a) Biochemical tests

VTEC are biochemically similar to other *E. coli*. VTEC O157:H7 strains differ in failing to ferment sorbitol, failing to produce beta-glucuronidase and fermenting raffinose and dulcitol. *Escherichia coli* can be distinguished from *E. hermannii* by lack of growth in the presence of potassium cyanide and failure to ferment cellobiose. *Escherichia hermannii* is positive for both tests. Ninety-eight per cent of *E. hermannii* strains have a characteristic yellow pigment on nutrient agar that is not seen in VTEC. *Escherichia coli* may be confirmed by demonstration of the use of tryptophan and beta-galactosidase activity (see below) or by commercially available biochemical test strips.

b) Serological tests

Commercial latex kits are available for O157, O26, O91, O103, O111, O128, O145 and H7. Tests should be carried out according to the manufacturer's instructions and should incorporate positive and negative control organisms and control latex. Presumptive diagnosis may also be made using slide or tube agglutination tests with anti-O LPS antiserum (antisera to 181 O- antigens are available). O157 antiserum has been shown to cross-react with other organisms including *E. hermannii* (frequently found in foods), *Salmonella* O group N, *Yersinia enterocolitica* serotype O9 and *Citrobacter freundii*, indicating the need to confirm putative VTEC colonies as *E. coli*. Isolates can be tested for the presence of flagellar antigen (antisera have been raised to 56 H antigens), but this may require passage through motility medium. Some pathogens are nonmotile.

c) Verocytotoxin production in Vero cell assay (16)

The Vero cell assay remains a standard method for the confirmation of VT production (see below). Vero cells have a high concentration of globotriaosylceramide (Gb₃) and globotetraosylceramide (Gb₄) toxin-binding receptors in their plasma membranes and will normally detect all variants of VT. The test can be used on faecal suspensions, culture filtrates or live cultures. In mixed faecal cultures, the sensitivity of the assay is increased by treating the suspension with polymyxin B or mitomycin to release cell-associated toxin. While the test is sensitive, it is not available in most routine diagnostic laboratories. It is labour intensive and results can take 3–4 days after the cell culture is inoculated. Where tissue culture facilities are not available, other methods may be used for detecting VT production, including ELISA or agglutination and PCR can detect the *vt* genes. All of these methods are now available as commercial kits.

d) Subtyping of *Escherichia coli* O157 for epidemiological studies

A variety of methods is available in reference laboratories to help discriminate between strains of *E. coli* O157:H7 to aid epidemiological investigations of outbreaks of human disease (13, 33). These methods vary in technical complexity and more than one technique is required to provide useful differentiation. Techniques include phage typing, biotyping and antimicrobial sensitivity testing (resistance being uncommon in strains from most countries), plasmid profiling, restriction fragment length polymorphism analysis, ribotyping, pulsed field gel electrophoresis (PFGE) and various PCR-based analyses (random amplification of polymorphic DNA; repetitive DNA element PCR; amplified fragment length polymorphism analysis). Of these, only phage typing and PFGE are widely used. Despite some difficulties with interpretation of profiles, PFGE has emerged as the standard method used by public health reference laboratories for subtyping VTEC O157 due to its high level of discrimination and accuracy and reproducibility. It is used in 'Pulsenet', a network of public health laboratories performing a standardised PFGE method that allows comparison of fingerprints held on an electronic database by the Centres for Disease Control and Prevention in the USA (www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm). The European Union's 'Enter-net' system for the surveillance of *Salmonella* and VTEC relies largely on phage typing to subtype *E. coli* O157:H7 strains. Use of subtyping of genes for intimin and VT has proved valuable for epidemiological studies and source attribution (5, 6). Subtyping methods for non-O157 serotypes have been less well explored, however, similar molecular approaches to those used for VTEC O157 can be taken.

o Non-culture techniques for detecting VTEC

Although definitive diagnosis of VTEC relies on the isolation and characterisation of pure cultures, cultural methods for VTEC are time-consuming and labour intensive. This has led to the development of a range of

immunological and nucleic acid hybridisation tests for rapid identification of O and H antigens, VT or genes associated with VT production in the sample. As the tests have a detection level above the numbers at which the target organism is normally present in the faeces, an enrichment step (preferably nonselective for isolation of injured or stressed bacteria) is required to increase the numbers prior to testing.

a) Immunological methods

Immunoassays to identify O and H antigens and VT may be used to confirm the identity of the organisms once isolated from clinical, food or environmental samples, while others, including dipstick and membrane technologies, microplate assays, colony immunoblotting, immunofluorescence and ELISA, are used as rapid methods for detecting the presence of potential pathogens in samples prior to isolation, thus shortening the time for a presumptive diagnosis. Most assays for somatic and flagellar antigens are designed to detect the O157 LPS and H7 flagellar antigen. Toxin assays have the advantage of detecting all VTEC. Enzyme immunoassays for O157 and VT, visual immunoassays for O157 and agglutination tests for O157, H7 and VT are available commercially as kits (7, 11, 27, 33). Not all have been validated for use with faeces. Specialised reagents in which anti-O157 LPS antibodies are conjugated to fluorescein, peroxidase or phosphatase are also available. Of the enzyme immunoassays, the most commonly used format is a sandwich assay. Antibody is bound to a carrier surface to capture a specific VTEC antigen; following the addition of an appropriate substrate, a second antibody with an enzyme label binds to this antigen and produces a colour reaction. The kits have been validated with specific pre-enrichment protocols and reagents to ensure reproducible results. Some use heat-treated samples thus improving the safety of the test, and some incorporate an automated processing system to screen large numbers of samples. Others are blot ELISAs developed to screen colonies for O157 antigen. The commercial kits have the advantage of being easy to perform in routine laboratories, and tests should be carried out according to the manufacturers' instructions. Kits validated for food and carcase samples or for human clinical samples may lack sensitivity for animal faeces samples. Immunological assays only give a presumptive result, which must be confirmed by isolation and characterisation of the organisms producing the O157 antigen or the toxin. The availability of kits is changing and the OIE Reference Laboratories should be able to provide the latest information on validated diagnostic kits.

b) Nucleic acid recognition methods

i) *Colony hybridisation assays*

Colony hybridisation is a useful means of detecting VTEC in mixed culture for further characterisation. DNA probes and synthetic oligonucleotide probes are available labelled with digoxigenin or biotin and therefore suitable for use in routine diagnostic laboratories. Assays have been described to detect VT genes, the 60 MDa plasmid in *E. coli* O157 and the *eae* gene individually and in combination (27, 29, 33). Hybridisation assays are less sensitive for detecting VTEC in broth cultures or faecal extracts.

ii) *PCR for VT genes and other virulence markers*

Many PCRs are described in the literature for detection of VT1, VT2 and VT2 variant genes (27, 29, 33), and a number of these toxin-typing PCR methods has recently been compared (36). Demonstration of the genes associated with VT-production does not confirm gene expression and hence production of toxin. PCR can be used on pure or mixed plate or broth cultures, and extracts from food or faeces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for VTEC in epidemiological studies. Amplification of target genes in bacterial DNA extracts from faeces is less successful than from pure cultures, and careful preparation of the sample is required to improve sensitivity. Faeces contain nonspecific PCR inhibitors and no single method of removing these is ideal. Sensitivity is improved by nonselective enrichment prior to testing, but remains lower than using IMS or the Vero cell cytotoxicity assay. Commercial assays are available.

DNA probes, PCR assays and microarrays have also been developed to detect other genes in VTEC shown to be associated with virulence in humans, including *eae* (encoding for intimin), *ehx* (encoding for enterohaemolysin production), *fliC* (encoding the H7 antigen), O157 *rfb* (encoding O157 LPS), *uidA* (the mutant glucuronidase gene in beta-glucuronidase-negative *E. coli* O157:H7) and *katP* (a gene carried on the large plasmid of *E. coli* O157:H7 encoding a novel catalase peroxidase) (2, 27, 29, 33). A variety of multiplex assays has been developed to detect simultaneously several diagnostic genes. These assays are of value in the characterisation of pure cultures. On mixed populations of bacteria in food or faeces samples, they may have a use in identifying samples to which isolation procedures should be targeted.

o Screening faeces for *Escherichia coli* O157:H7

Escherichia coli O157:H7 is the VTEC of greatest public health concern in most countries. Its carriage in the intestinal tract of healthy animals, particularly cattle, represents a source of direct and indirect infection to humans. Screening relies on cultural techniques designed to overcome the problems of isolating low numbers of organisms, possibly in a stressed state, from a competing background flora followed by identification of suspect

colonies and demonstration of known virulence characteristics. These methods are still evolving and the following is a description of the methods routinely employed in one national veterinary laboratory. Suitable precautions should be taken to avoid human infection (see Chapter 1.1.2).

a) Pre-enrichment

- i) Transport faeces in sterile, leak proof, closed containers at 4°C and culture as soon as possible, preferably within 2 hours of collection. Faeces intended for long-term storage should be frozen at -70°C.
- ii) Mix faeces at a dilution of 1/10 in warmed buffered peptone water (BPW) in a labelled container.
- iii) Incubate at 37°C±2°C for 6 hours.
- iv) Include positive and negative control cultures.

b) Immunomagnetic separation

- i) Use of Dynabeads® anti-*E. coli* O157 product 710.04 (DynaL Biotech, ASA, Oslo, Norway) meets the requirements of AFNOR (DYN 16/02-0696 and DIN 10167); it is cited in the USA Food and Drug Administration's Bacteriological Analytical Manual (www.cfsan.fda.gov/~ebam/bam-4a.html) and the Health Canada Compendium of Analytical Methods (www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_3/e_mflp9001.html) and is the official method of the Japanese Health Ministry.
- ii) Following the instructions of the manufacturers, carry out immunomagnetic separation (IMS) on the pre-enriched samples using the manual (MIMS) or automated (AIMS) method. Care should be taken to mix the beads well before use and to avoid cross-contamination between prepared tubes. If using the manual method, adherence to the instructions for careful washing of the bead-bacteria complexes is essential.
- iii) After the final wash, use a micropipette to transfer 50 µl of each bead-bacteria suspension to a labelled sorbitol MacConkey agar plate containing cefixime and potassium tellurite (CT-SMAC) (35) taking care to avoid cross-contamination.
- iv) Using a sterile swab, spread the drop over one-third to one-half of the plate to break up the complexes. Using a sterile 10 µl loop, dilute the bead-bacteria complexes further over one quadrant by streaking out at right angles from the previously streaked area. Using a second sterile loop, streak out at right angles from this quadrant into the final unstreaked area of the plate to obtain single colonies. Incubate at 37°C±2°C for 16–18 hours (sorbitol-fermenting colonies lose colour after this time and may be confused with non-sorbitol fermenting *E. coli* O157). An alternative method for isolating sorbitol-negative colonies is to spread the entire inoculum over the surface of a dry CT-SMAC plate with a sterile bent rod.

c) Colony identification

- i) Pick off up to 10 white, sorbitol-negative colonies per plate and test by O157 latex agglutination following the manufacturer's instructions (include appropriate positive and negative control organisms and latex control).
- ii) Subculture agglutination-positive colonies on to solid medium without antibiotics (e.g. 5% sheep blood agar). Streak to obtain single colonies. Incubate at 37°C±2°C overnight.

d) Confirmation of *Escherichia coli*

- i) Inoculate o-nitrophenyl beta-D-galactopyranoside (ONPG) broth. Set up positive and negative controls. Incubate overnight, aerobically at 37°C. *Escherichia coli* produce a positive result indicated by a change to yellow colouration confirming beta-galactosidase activity.
- ii) Place a circle of 0.45 µm cellulose nitrate membrane filter paper on to a plate of tryptone bile agar (TBA) using sterile forceps. Use a 1 µl loop to remove a loopful of growth to be tested and inoculate a pea-sized area on the surface of the Millipore filter. Set up positive and negative controls. Incubate at 44°C for at least 17 hours. Transfer the membrane to filter paper soaked with indole reagent for the detection of the use of tryptophan. *Escherichia coli* show a positive reaction indicated by a purple/pink colouration.
- iii) A commercial reagent for detection of indole is available. The reagent is placed on to filter paper and a portion of the colony rubbed into the reagent spot. This requires less than 5 minutes and can be backed up by the described test if suspicious colonies appear negative. Indole medium is also available commercially.
- iv) Alternatively, use commercially available biochemical test kits to confirm *E. coli*.

e) Somatic determination (24)

- i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Schlecht broth (31). Incubate at 37°C±2°C overnight.
- ii) Boil the Schlecht broth for a minimum of 1 hour at 100°C.
- iii) Dispense 25 µl of 0.85% saline into wells 2 to 12 of a U-well microtitre plate. Dispense 50 µl of O157 antiserum into well 1. Make a doubling-dilution series of the antiserum to 1/1024, discarding 25 µl after mixing well. Add 50 µl of boiled broth suspension to wells 1 to 12. Cover the plate to prevent evaporation and incubate at 37°C for 6 hours. Use a black background to identify agglutination in the wells.

f) Vero cell assay

- i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Mundell (26) broth. Incubate at 37°C±2°C overnight.
- ii) Set up broths with control strains of organisms producing no toxin, thermolabile enterotoxin (LT), cytotoxic necrotising factor (CNF) and verocytotoxin (VT). Incubate at 37°C±2°C overnight.
- iii) Dispense Vero cells (African green monkey kidney cells, reference ATCC CCL81, seeding rate 2 ×10⁵/ml) into flat-well microtest plates, 200 µl to each well, 24 hours before inoculation. Incubate at 37°C±2°C in 5% CO₂ for 24 hours.
- iv) Add 100 µl of a 400,000 units/ml solution of polymyxin B sulphate in sterile distilled water to each overnight broth culture. Incubate at 37°C±2°C for 5 hours.
- vi) Centrifuge the broths at 3000 rpm for 30 minutes.
- vii) Remove supernatants into labelled sterile containers (approximately 1.5 ml required).
- viii) Place the Vero cell plate on a numbered worksheet to identify each well. Inoculate 10 µl of prepared supernatant into the relevant well of Vero cells. Return Vero cells to the CO₂ incubator and incubate for 3 days.
- ix) Examine cells after 24 hours, 48 hours and 72 hours to observe any cytopathic effect. Compare with positive and negative test controls. With VT-positive samples, the cell sheet becomes disintegrated with blackened, shrivelled cells observed between 24 and 72 hours.

g) Multiplex PCR for VT1, VT2 and eae (1, 15, 32)

Multiplex PCR is used to confirm the presence of virulence determinants using primers as shown below:

Target gene	Accession no.	Primer sequence	Nucleotide position	Amplicon size (bp)
VT1	M19437	F (5'-CGC-TCT-GCA-ATA-GGT-ACT-CC-3')	287–306	256
		R (5'-CGC-TGT-TGT-ACC-TGG-AAA-GG-3')	522–541	
VT2	X07865	F (5'-TCC-ATG-ACA-ACG-GAC-AGC-AG-3')	623–642	185
		R (5'-GC-TTC-TGC-TGT-GAC-AGT-GAC-3')	788–807	
eaeA	X60439	F (5'-GC-TTA-GTG-CTG-GTT-TAG-GAT-TG-3')	271–293	618
		R (5'-CCA-GTG-AAC-TAC-CGT-CAA-AG-3')	871–890	

- i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 1 ml of Luria-Bertani broth. Set up three appropriate control broths. Incubate at 37°C±2°C overnight.
- ii) Boil the broths for 15 minutes at 100°C. Remove from waterbath and allow to cool.
- iii) Prepare master mix for 48 µl per sample containing:
1 × Saiki buffer (50 mM KCl; 10 mM Tris, pH 8.5; 100 µg/ml gelatin); 3 mM MgCl₂; 0.5 U Taq polymerase; 25 pmoles of each primer (forward and reverse primers for VT1, VT2 and eaeA); 0.2 mM each of dATP, dCTP, dGTP and dTTP.
- iv) Mix by inverting tubes and dispense 48 µl into each PCR reaction tube.

- v) Add 2 µl of boiled culture (crude DNA extract) to the bottom of each reaction tube (include three control extracts and a media blank).
- vi) Run the PCR using cycling parameters of initial denaturation at 94°C for 2 minutes; 25 cycles of 94°C for 1 minute, 62°C for 1.5 minutes and 72°C for 2 minutes; with a final extension of 72°C for 5 minutes. The reaction is held at 4°C until required for electrophoresis.
- vii) Electrophorese 15 µl of each PCR sample on a 1.5% agarose gel in E buffer (10× strength solution made by adding to distilled water in the following order: 109 g/litre Tris, 55.6 g/litre ortho-boric acid, 9.3 g EDTA, made up to 1 litre with distilled water and adjusted to pH 8.0 with 10 ml concentrated hydrochloric acid diluted in distilled water before use). Run 100 bp step ladder molecular weight marker for comparison.
- ix) Stain in ethidium bromide and view by transillumination.
- x) Inspect control lanes to identify positions of VT1, VT2 and eae amplicons. Compare with bands present in test sample lanes. Record the results.

2. Serological tests

In humans, serodiagnosis of VTEC can be valuable, particularly later in the course of the disease when the causative organism becomes increasingly difficult to isolate from faeces. LPS has emerged as the antigen of choice, and production of serum antibodies to the LPS of a wide range of prevalent serotypes of VTEC has been demonstrated. Serological tests are not used for diagnosis of animal infection with VTEC. However, it has been shown that exposure of cattle to *E. coli* O157:H7 infection results in the production of antibodies against the O157 LPS, which persist for months, demonstrable by the indirect ELISA (17). Cross-reactions have been demonstrated between O157-LPS and the LPS antigens of other bacteria including *E. coli* O55, *Salmonella* spp., *Yersinia enterocolitica*, *Brucella abortus* and *V. cholerae* non-O1 strains. To reduce cross-reactivity, a blocking ELISA using a monoclonal antibody specific for *E. coli* O157 as the competing antibody has been developed for detection of serum antibodies to O157 antigen in cattle (20). Serum antibodies to VT1, but not to VT2, have been demonstrated in cattle by toxin neutralisation tests in Vero cell assays (17). Other studies have shown greater prevalence of VT1 neutralising antibodies in cattle sera than VT2 which may be explained by the greater prevalence of VT1-producing VTEC in cattle and/or the lesser immunogenicity of VT2.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are currently no vaccines available to control zoonotic VTEC. Various approaches to the immunological control of EHEC infections in humans are being explored (23). These are aimed at preventing colonisation, intestinal disease or the serious sequelae of haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. They include the use of conjugate vaccines (e.g. O157 polysaccharide linked to the B-subunit of VT1 and VT2 as carrier proteins), live-vector vaccines, toxoid vaccine or passive immunisation with hyperimmune globulin or monoclonal antibodies against VT. However, were an effective vaccine to become available, there is debate about the social, political and economic consequences of widespread vaccination of people against pathogens in their food. As animals, mainly cattle, are thought to be the reservoirs of infection for the human population, a novel strategy being explored is to vaccinate cattle in order to reduce colonisation with pathogenic VTEC and thereby reduce contamination of food and the environment (i.e. to make food safer as opposed to protecting people against their food). One approach is to use a live, toxin-negative colonising strain as an oral vaccine to induce antibodies against surface components, and another is to deliver colonisation factors, such as intimin, as an edible vaccine in transgenic plants (12).

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NB: There is an OIE Reference Laboratory for *Escherichia coli* (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).