Isolation and identification methods for *Escherichia coli* 0157 and other Vero cytotoxin producing strains

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Introduction

Strains of *Escherichia coli* that produce a cytotoxin active on Vero cells, termed Vero cytotoxin (VT) or Shiga-like toxin, are now recognised as important aetiological agents of diarrhoeal diseases in man and animals. Two main types of VT, VT1 and VT2, have been defined. The properties of Vero cytotoxin producing *E coli* (VTEC) and infection by these organisms have been reviewed recently.^{1,2}

Clinical features

In man VTEC are associated with diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS). Haemorrhagic colitis is characterised by grossly bloody diarrhoea, usually without pyrexia. It is frequently preceded by abdominal cramps and watery diarrhoea. HUS is defined by three clinical features—acute renal failure, microangiopathic haemolytic anaemia, and thrombocytopenia. HUS can occur in all age groups but is more common in infants and young children and is a major cause of renal failure in childhood. There are two subgroups of HUS—typical HUS associated with a prodromal bloody diarrhoea and an atypical form without a diarrhoeal phase. It is only the typical form of HUS that is associated with infection by VTEC. Some patients have features resembling thrombotic thrombocytopenic purpura (TTP), such as neurological disorders and fever.

Epidemiology

Disease associated with VTEC has been documented in many countries, with most reports coming from North America and Britain. Most human infections are associated with strains belonging to serogroup 0157, usually possessing the flagellar antigen H7, although some non-motile 0157 strains have been isolated. These cases are found over a wide geographical area with the infections peaking in summer and autumn. Outbreaks of 0157 VTEC have occurred in the community, in nursing homes for the elderly, hospitals and in daycare centres for young children.¹ The most severe clinical disease is usually seen in children and the elderly and a significant mortality has been observed in several outbreaks. Outbreaks have

been associated with beefburger meat and unpasteurised milk. 0157 VTEC have been isolated from cattle in several countries including Britain,² suggesting that cattle are a reservoir of infection in man. Examination of meats in North America showed that 0157 VTEC were present in samples of beef, pork, poultry and lamb but, so far, VTEC of serogroup 0157 have not been isolated from food in Britain.^{1,2}

Laboratory diagnosis

The methods used to provide evidence of VTEC infection can be divided into the following categories: isolation of VTEC including *E coli* 0157; demonstration of specific Vero cytotoxin; and presence of antibodies with VT neutralising ability or antibodies to *E coli* 0157 lipopolysaccharide (LPS). Techniques for the isolation of VTEC and particularly strains of serogroup 0157 and for the detection of VT are described in detail with a brief description of methods primarily used in the reference laboratory.

SPECIMENS

The appearance of the faeces is variable, from soft specimens to frank blood. The absence of red blood cells or the presence of leucocytes in faeces does not exclude VTEC infection. The collection and testing of early faecal specimens is most important. Previous studies have shown that 0157 VTEC are rapidly cleared from the gut with a low recovery of the organism from specimens tested seven or more days after the onset of symptoms.³

Tests for 0157 VTEC

Most VTEC belonging to serogroup 0157 do not ferment sorbitol within 24 hours of incubation whereas about 95% of *E coli* from faecal samples are prompt sorbitol fermenters under these conditions.⁴ However, in a recent report from Germany 0157 VTEC strains fermented sorbitol within 24 hours.⁵ The prevalence of such strains is unknown but they would be discarded using the standard screening method described here.

MacConkey agar plates with 1% D-sorbitol instead of lactose are used to screen faecal specimens for non-sorbitol fermenting (NSF)

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colonies of *E coli*. These colonies are then tested for agglutination with an 0157 antiserum or with an 0157 latex agglutination kit. Any colonies that give agglutination must be confirmed as *E coli*. NSF strains of serotype 0157:H16 that do not produce VT have been reported.⁶ In view of these findings it is recommended that presumptive 0157 isolates should be sent to a reference laboratory and tested for VT production and for flagellar antigens.

PROCEDURE

Faecal specimens are resuspended in an equal volume of phosphate buffered saline (PBS). Suspension of the sample may not be required for liquid specimens, but in some cases faecal samples require dilution up to 10 fold. Where possible up to 1 g of faeces should be examined. Samples are streaked out on to sorbitol MacConkey agar (SMAC) and the plates are incubated at 37°C for 18 hours. VTEC of serogroup 0157 produce NSF colonies that are small, round, smooth and may look greyish (fig 1). Each colonial type of NSF colony is selected for testing as possible *E coli* 0157; a total of five to 10 colonies is usually examined.

Colonies are emulsified in a drop of saline on a glass slide and then mixed with one drop of 0157 antiserum or 0157 specific latex (see Appendix). The slide is rocked and observed for agglutination; a positive test is indicated by rapid clumping and clearing of the solution. False positive results should be excluded by testing colonies with a non-0157 antiserum. Some commercial kits have a negative control reagent.

CONFIRMATION

NSF colonies that agglutinate with the 0157 antiserum must be confirmed as *E coli* using a set of biochemical tests. These tests should exclude any non-*E coli* that give false positive agglutination tests with the 0157 antiserum. *Escherichia hermannii* is biochemically and sero-

logically similar to *E coli* 0157⁷ and is positive in most screening tests for *E coli* 0157, although at least one latex reagent (Pro-Lab Diagnostics) does not cross-react with *E hermannii*. However, *E coli*, unlike *E hermannii*, does not ferment cellobiose and does not grow in the presence of potassium cyanide. Strains of *E hermannii* producing Vero cytotoxin have not been identified. Another characteristic of 0157 VTEC is that, with very few exceptions, they do not produce β glucuronidase, whereas most other *E coli* are positive in this test.^{3 5 8} Testing for β glucuronidase activity has therefore been proposed to aid the identification of 0157 VTEC.⁸ The hydrolysis of 4-methylumbelliferyl- β -D-glucuronide (MUG) to produce a fluorescent compound is a commonly used method and can be performed by different techniques. The preferred method in our laboratory⁹ is as follows:

The MUG reagent is prepared by diluting 100 mg MUG (Sigma) in 100 ml distilled water containing 2 drops of Triton X-100 (Sigma). The solution is filter sterilised (0.45 μ m Minisart MNL; Sartorius) and stored at 4°C. Drops of the MUG reagent are applied to circles of filter paper (Whatman No 1) in Petri dishes to obtain even distribution of the reagent, and air dried. These dishes are wrapped in aluminium foil and refrigerated until used. Cultures for testing are grown on blood agar plates (Oxoid No 2 nutrient broth containing 5% vol/vol unwashed horse blood solidified with 2% agar). Growth from a single colony is applied to the paper to form a small patch. At least 16 strains can be tested on each 8.5 cm paper. The growth is moistened with a drop of saline near each patch and the plate is incubated at 37°C for 20 minutes. The papers are examined for fluorescence using ultraviolet light in a darkened room (fig 2).

IMPROVED MEDIUM FOR THE ISOLATION OF 0157 VTEC

A recent paper has reported the inclusion of cefixime and rhamnose into sorbitol MacCon-

Figure 1 Sorbitol MacConkey agar plate showing colourless colonies of *E coli* 0157.

Figure 2 Paper method to test β glucuronidase activity of strains of *E coli* showing fluorescence of 4-methylumbelliferyl- β D-glucuronide (MUG).

key agar (CR-SMAC) to improve the detection of VT producing *E coli* 0157.¹⁰ Using tests on agar plates, 0157 VTEC do not ferment rhamnose, whereas 60% of non-sorbitol fermenting *E coli* belonging to other serogroups ferment rhamnose. In contrast to these results on agar, 0157 VTEC do ferment rhamnose within one day in standard tube sugar fermentation tests.^{3, 11} Cefixime is included as it is more active against *Proteus* spp than against *E coli* and *Proteus* strains account for about 15% of NSF. The use of CR-SMAC rather than SMAC showed a significant improvement in selectivity for the isolation of 0157 VTEC.¹⁰ In a study of 1763 samples 397 required testing for *E coli* 0157 using SMAC but only 176 needed to be investigated using CR-SMAC. In the same study strains of *E hermannii* fermented rhamnose and were therefore not confused with 0157 VTEC.

Cefixime (Cyanamid, Gosport, Hants) is dissolved in ethanol (25 mg in 10 ml) and diluted further in broth. The CR-SMAC medium is prepared by adding cefixime (0.05 mg/l) and 0.5% rhamnose to sorbitol Mac-Conkey agar. Faecal samples are streaked out on to the medium and the plates are incubated at 37°C overnight. Non-fermenting colonies are selected and tested as described above with an 0157 antiserum or 0157 latex reagent.

A special agar medium containing sorbitol and MUG has been developed (see Appendix). A disadvantage of using a medium containing MUG is that it can be difficult to identify a glucuronidase negative colony when it is surrounded by a number of fluorescing colonies.

COLONY IMMUNOBLOTTING

Another method used for testing for presence of *E coli* 0157 is that of colony immunoblotting. Bacterial growth to be tested can be in the form of spotted cultures in a grid pattern on an agar plate, streaked out growth on a plate or a plate with several hundred separated colonies from a single sample (GA Willshaw, personal communication).

An 82 mm diameter membrane (Hybond C extra; Amersham) is marked with an orientation line and placed on the plate with bacterial growth for 5 minutes. The membrane is rubbed gently with a glass spreader to ensure good contact with the surface and then peeled off and placed in a plastic box containing PBS with 5% dried milk. This is shaken for 30 minutes at room temperature. This blocking buffer is poured off and discarded by autoclaving. The alkaline phosphatase conjugated 0157 antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) is diluted 1 in 1000 with blocking buffer. For eight membranes, 20 ml of diluted antibody reagent is prepared. The membranes are transferred to a plastic bag so that the sides with bound antigen face outwards. The diluted antibody reagent is added and the bag is sealed and incubated with shaking for two hours at room temperature. The membranes are removed and washed three times for 10 minutes in PBS at room temperature. After rinsing the membranes in

alkaline phosphatase buffer (0.1M TRIS-HCl, 0.1M NaCl, 0.05M MgCl₂) the substrate is added: 69 µl nitroblue tetrazolium chloride (Sigma Chemical) and 54 µl of 5-bromo-4-chloro-3-indolyl phosphate (Northumbria Biologicals, Cramlington, Northumberland) per 15 ml of alkaline phosphatase buffer for eight membranes. A positive control gives a dark blue coloration in 5 to 10 minutes and samples or spots that react similarly may indicate the presence of *E coli* 0157. The reaction is stopped after 20 minutes by washing the membranes in distilled water. The membranes are blotted dry and stored in the dark. Any colonies that appear to be *E coli* 0157 must be confirmed biochemically and serologically (see above) and tested for VT production.

A recent paper has described a rapid sandwich enzyme linked immunosorbent assay (ELISA) for the detection of 0157 VTEC in food.¹² In this test a polyclonal 0157 antibody is used as the capture antibody and a monoclonal antibody, specific for VTEC belonging to serogroups 0157 and 026, as the detection antibody. The reagents will be available in kit form in the near future.

Tests for presence of Vero cytotoxin (VT)

Vero cytotoxin present in faeces can be detected directly by its cytotoxic effect on Vero cells. Alternatively, VT production by *E coli* strains isolated from the faeces can be examined, either by testing culture filtrates or, in a rapid screening method, by testing live cultures. In some early studies of VT production bacteria were grown in iron restricted media and such growth conditions may increase production of VT1, but not VT2. For routine testing, concentrations of VT are adequate in ordinary broth media as described below. A considerable amount of VT is not liberated into the medium but remains cell bound. It can be released by sonication, with the use of a French press or by polymyxin treatment, and these techniques have been used for preparing large quantities of VT. To detect VT production by clinical strains these techniques are not necessary. However, a method using polymyxin extraction for the screening of sweeps of colonies from faeces for VT production is described. Some workers have used HeLa cells for the detection of VT but this cell line cannot be recommended as variants of VT2 are known that do not have a cytotoxic effect on HeLa cells.

Several ELISAs have been described for the detection of VT but as the reagents are not commercially available the methods are not described in detail here. Some ELISAs bind VT to glycolipids containing a terminal α -D-Gal-(1→4)-D-Gal; purified globotriosyl ceramide (Gb₃), lyso-Gb₃, and hydatid cyst fluid have been used.^{13, 14} Other ELISAs have used monoclonal antibodies against VT to bind the toxin initially.¹⁵ In both assay systems bound toxin is then detected using monoclonal or polyclonal antiserum against VT, followed by an appropriate alkaline phosphatase-

labelled goat antiserum and then the enzyme substrate. In general, these tests have not proved to be as sensitive as the Vero cell test. In addition, as the toxins show considerable variation in their antigenicity and binding properties (even within the VT2 class of toxins) care must be taken in the choice of reagents if the aim is to detect all VT producing strains from a clinical specimen.

TESTS WITH CULTURE SUPERNATANTS

The method used is a modification¹⁶ of the original method of Konowalchuk *et al.*¹⁷

Preparation of culture supernatant

Trypticase soy broth (BBL, Becton Dickinson, Cowley, Oxfordshire), 10 ml, in a 250 ml flask is inoculated with the bacterial strain to be tested. The flask is incubated at 37°C for 18–24 hours with shaking (120 rpm). The culture is then centrifuged ($17\,000 \times g$ for 30 minutes), with cooling if possible, and the supernatant fluid sterilised by filtration using a pore size of $0.45\ \mu\text{m}$. This filtrate is used directly for VT tests. A sample of the culture supernatant is also heated at 100°C for 15 minutes.

Vero cell test

Monolayers of Vero cells are prepared in 96-well tissue culture plates. A $25\ \mu\text{l}$ portion of the test filtrate is added to duplicate wells without changing the medium. Control filtrates should also be included. A sterility check of the filtered supernatant is useful as growth of any remaining bacteria will result in death of the monolayer. After the addition of the filtrate the plates are covered and incubated at 37°C.

Cells round up and become detached in the presence of VT. The monolayers can be examined after one day for early cytotoxic effects using an inverted microscope (figs 3A and C). However, final readings should be made after the plates have been incubated for three to four days (compare figs 3C and 3E). For ease of viewing and to obtain a permanent record the monolayer can be stained (compare figs 3A and 3B, 3C and 3D, and 3E and 3F). The tissue culture medium is removed and the cells fixed with methanol for 5 minutes. The methanol is removed and Giemsa stain (5% w/v in phosphate buffer) added. After 45 minutes the monolayer is washed three times with distilled water and air dried. The cells are examined microscopically.

To obtain toxin titres two-fold or five-fold dilutions in tissue culture medium can be tested. Toxin titres are expressed either as the highest dilution that causes any cytotoxic effect in the monolayer or more usually as the dilution at which 50% detachment of cells in the monolayer occurs. A unit of VT is defined as the amount present in this dilution.

TEST USING FAECAL PREPARATION

Faecal specimens can be examined directly for the presence of VT.¹⁸ The specimen is centrifuged; a microcentrifuge is suitable using a screw capped tube for the sample. The supernatant fluid is filtered and tested for VT as

described above for culture supernatants. The addition of PBS to the specimen is often necessary, followed by thorough mixing, to obtain a sufficiently liquid sample. This dilution is considered in the final estimation of any toxin titre.

TEST OF COLONY SWEEPS AFTER POLYMYXIN EXTRACTION

As an alternative to testing individual colonies, broth inoculated with sweeps—that is, loopfuls of confluent bacterial growth from agar media—can be examined. To increase the sensitivity of the test it is recommended that VT obtained after polymyxin release is determined.¹⁹ For this, sweeps from MacConkey agar plates are inoculated into 20 ml volumes of Penassay broth (antibiotic medium No 3: Difco Laboratories) and incubated for 5 hours at 37°C with shaking. This broth culture is centrifuged at $10\,000 \times g$ for 10 minutes and the supernatant fluid discarded. The cell pellet is resuspended in 1 ml PBS (Dulbecco A; Oxoid) containing polymyxin B (0.1 mg/ml and incubated at 37°C for 30 minutes. The suspension is centrifuged at $10\,000 \times g$ for 10 minutes and the supernatant fluid is filtered and tested for VT as described above. When artificial mixtures of known VT positive and VT negative colonies were tested in this way VT could be detected when the proportion of VT positive organisms was at least 1.25%. The specificity of the toxin can be determined as described below.

If faeces are screened by this method, only those that are positive need to be examined further, if necessary, for VT producing colonies.

SPECIFICITY OF THE TOXIN

To confirm that cytotoxic effects on Vero cells are indeed due to the presence of VT (or VTs) additional tests are needed. These are particularly important in the examination of VT in faeces. Preferably, neutralisation tests using antisera against VT1 or VT2 should be used, but these antisera are not commercially available. VT tests on a portion of the sample heated at 100°C for 15 minutes should be negative, confirming the heat lability of the toxin. The specimens can also be tested on a cell line which is not sensitive to VT, such as Y1 mouse adrenal tumour cells.¹⁸ A faecal preparation that kills Y1 cells to the same titre as Vero cells has to be considered negative for VT, which if also present at a lower titre would not be detected in the presence of the other uncharacterised toxin. Vero cells are also sensitive to the heat labile enterotoxin (LT) produced by some strains of *E. coli*. LT is not cytotoxic but causes rounding of the cells after 24 hours.¹⁷ This is unlikely to be mistaken for VT but as LT also causes rounding of Y1 cells this allows the toxins to be clearly differentiated.

To perform neutralisation tests¹⁸ $25\ \mu\text{l}$ volumes of appropriate dilutions of the preparations to be tested are added to 0.2 ml volumes of complete Vero cell tissue culture medium in a 96-well plate. At least two series are prepared

Figure 3 Tests of culture filtrates of VT+ or VT- strains of *E. coli* on Vero cell monolayers. These were examined by phase contrast microscopy or after staining at the times indicated. 3A VT- 1 day unstained; 3B VT- 1 day stained; 3C VT+ 1 day unstained; 3D VT+ 1 day stained; 3E VT+ 4 day unstained; 3F VT+ 4 day stained.

so that one can be kept as a control without antiserum. Anti-VT serum (25 μ l) is added to each well of a series. This can be anti-VT1, anti-VT2, or both together. The plates are incubated at 37°C for one to three hours and then placed at 4°C overnight. The medium is carefully removed from a monolayer of Vero cells that has been prepared earlier and the contents of the neutralisation wells transferred into them. The plates are incubated and read as above. The dilution of antiserum used is determined in preliminary experiments so as to contain at least 20 units. A unit is defined as the amount present in the highest dilution of antiserum which neutralised 1–5 units of VT.

SIMPLIFIED TEST USING LIVE BACTERIAL CULTURES²⁰
A monolayer of Vero cells growing in a 96-well tissue culture plate is prepared. The colonies to be tested are grown in 0.5 ml trypticase soy broth at 37°C for 18 hours without shaking. The tissue culture medium is

carefully removed from the cells. Live bacterial culture (50 μ l) is added to a test well. After 5–10 minutes the medium and bacteria are removed and the cells washed once with PBS. Tissue culture medium (0.2 ml) with penicillin, streptomycin, and gentamicin (final concentration of 40 μ g/ml) is then added. The plate is covered again, incubated at 37°C, and examined for cytotoxic effects as described above. Filters, a centrifuge and facilities to shake cultures are not needed for this test. Results are very clear but it is not possible to test the specificity of the toxic effect.

MAINTENANCE OF VERO CELLS

To maintain Vero cells monolayers are washed twice with 10 ml Dulbecco's PBS. Trypsin in Versene buffer (5 ml) is added and poured off after 1 minute. The monolayers are then incubated at 37°C until cells begin to detach. Medium 199 (5 ml) is then added and the cells are resuspended. Volumes (2 ml) of resus-

pended cells are added to 12 ml of growth medium in a tissue culture flask and incubated at 37°C. A CO₂ incubator is not necessary. This procedure is repeated weekly. Vero cells may become less sensitive to VT after prolonged subculture and this should be monitored by including titrations of supernatants of control VT producing strains.

PREPARATION OF MONOLAYERS

For the VT test monolayers of Vero cells are prepared in 96-well plastic plates of tissue culture grade. A portion of the resuspended cells after digestion with trypsin is diluted in the complete tissue culture medium to obtain a final concentration of about 5×10^4 cells/ml. Counting in a haemocytometer is recommended but usually a 1 in 20 dilution is satisfactory. The diluted suspension (0.2 ml) is distributed in each well of a 96-well tissue culture plate. The plate is covered and incubated for two to three days at 37°C in an atmosphere of 5% CO₂ – 95% air. However if plates are sealed by pressure sensitive film this special atmosphere is not needed.

MAINTENANCE OF Y1 CELLS

Maintenance of Y1 cells and preparation of monolayers are as described for Vero cells except for the use of Ham's F10 medium instead of Medium 199.

REAGENTS AND MATERIALS

Requirements for tissue culture

The cell lines and all materials for tissue culture can be obtained from ICN Flow, High Wycombe, Buckinghamshire, unless otherwise indicated. For convenience the tissue culture media and many other reagents can be purchased in solid or liquid form. Careful reference should be made to the formulations given. Glutamine is usually added to the tissue culture media immediately before their use, but the formulations should be consulted to see whether NaHCO₃ is already included or has to be added.

- 1 Cell line Vero, African green monkey kidney, ATCC No. CCL81.
Cell line Y1, mouse adrenal cortex tumour, ATCC No. CCL79.
- 2 Dulbecco's phosphate buffered saline, *without calcium and magnesium*.
Trypsin (2.5% w/v) is diluted 10 fold in Versene buffer for use.
EDTA (Versene) buffer (0.02% w/v) in 0.85% saline.
L-glutamine solution (200 mM).
Amphotericin B (Fungizone) (stock solution 250 µg/ml).
Penicillin and streptomycin (stock solution 5000 IU/ml and 5000 µg/ml, respectively).
Gentamicin (stock solution 10 or 50 mg/ml).
Fetal bovine serum.
Donor horse serum.
Medium 199 (Modified with Earle's salts).
Ham's F10 medium.
- 3 Plastic materials from numerous stockists including ICN Flow, Nunc Life Technologies, Glasgow and Falcon, Marathon Labo-

ratory Supplies, London NW10 7JP.

96-well tissue culture grade plastic plates with lids.

75 cm² plastic tissue culture flasks.

Vero cells are grown in Medium 199 (Modified) with Earle's salts. To 100 ml are added 10 ml fetal bovine serum and 0.5 ml glutamine solution. If not already incorporated, NaHCO₃ (2.2 g/l) must also be added. Penicillin-streptomycin (2 ml) solution to reduce bacterial contamination and 0.1 ml Amphotericin B to reduce fungal contamination may be added if required.

Y1 cells are grown in Ham's F-10 medium. To 100 ml are added 12.5 ml donor horse serum, 2.5 ml fetal bovine serum and 0.5 ml glutamine. If not already in the formulation NaHCO₃ (1.2 g/l) must be added. Penicillin-streptomycin (2 ml) solution to reduce bacterial contamination and 0.1 ml Amphotericin B to reduce fungal contamination may be added if required.

DNA probe tests for VTEC

The genes encoding production of VT1 and VT2 have been cloned and probes for the detection of these genes were developed.^{20, 21} The use of such probes will detect all VTEC and not only strains of serogroup O157. In addition to the polynucleotide probes from the cloned genes, synthetic oligonucleotide probes for the detection of different VT genes have also been developed. Amplification of part of the VT gene, using the polymerase chain reaction, has also been used to test for the presence of VTEC. The present description of DNA probe tests will be restricted to the use of non-radioactively labelled polynucleotide probes as these methods are the most applicable to a wide range of laboratories. Full details of the methods have been published elsewhere.²² Probe fragments are prepared from recombinant plasmids and labelled by the random primer method with digoxigenin-11-dUTP (Boehringer Corporation Limited, Mannheim, Germany). Unincorporated nucleotides are removed using QIAGEN-tip5 (DIAGEN, Dusseldorf) and labelled probe is stored at -20°C. The target DNA is prepared in one of the following ways.

- 1 Faecal suspensions are spotted directly on to nylon membranes (82 mm in diameter, Hybond-N, Amersham) supported on MacConkey agar and the plates are incubated overnight at 37°C.

- 2 In order to test a large number of colonies, specimens are resuspended in an equal volume of PBS, and 0.1 ml samples of 10 fold dilutions are spread on MacConkey agar plates. Several hundred well separated colonies on a single plate are replicated using velvet on to a nylon membrane placed on agar and the replicated colonies are grown for about five hours. Alternatively, growth from streaking a faecal specimen on a MacConkey plate can be replicated on to a nylon membrane.

- 3 For testing of purified *E coli* isolates nutrient broth cultures after overnight incubation at 37°C are spotted on a nylon membrane in a grid pattern and grown for about five

hours. Up to 50 test strains and a positive and negative control are tested on a single membrane.

Membranes are prepared for hybridisation by placing on a series of Whatman 3 MM papers saturated with the following solutions: 10% sodium dodecyl sulphate (5 minutes), lysis solution containing 0.5M NaOH and 1.5M NaCl (5–10 minutes), neutralising solution containing 1.5M NaCl and 0.5M TRIS-HCl, pH8.0 (5 minutes), and finally with $2 \times$ SSPE (5 minutes). $2 \times$ SSPE contains 0.3M NaCl, 20 mM NaH_2PO_4 , and 2mM EDTA. Membranes are dried and the DNA is bound by baking for two hours at 80°C or placed on an ultraviolet transilluminator (wavelength 302 nm) for four minutes. The procedures for hybridisation and detection of homology between probe and target have been described in detail previously.²² Results of DNA probe tests with polynucleotides show whether strains carry the genes for production of VT1, VT2, or both toxins.

Tests in the reference laboratory

Strains presumed to be VTEC belonging to serogroup O157 or other serogroups should be sent to a reference laboratory for confirmation and further characterisation.

SEROTYPING

Full serotyping of VTEC requires the facilities of a reference centre as investigation with antisera against 173 O antigens and 56 H antigens is needed. VT production has been reported in strains belonging to many different serotypes.^{1,2} Strains of O157 are most common, usually with the flagellar antigen H7; non-motile strains have also been reported and this may be a useful distinguishing feature.^{11,23} Some VTEC belong to classic enteropathogenic serogroups such as O26, O55, O111 and O128. Therefore, strains of these serogroups isolated from cases of haemorrhagic colitis or HUS or outbreaks of diarrhoea should be sent to a reference laboratory for further tests.

VT TYPE

Two major types of Vero cytotoxin VT1 and VT2 have been defined. VT1 is virtually identical to Shiga toxin produced by *Shigella dysenteriae* type 1. VT2 is antigenically distinct from VT1 and Shiga toxin. A number of variants of VT2 have also been defined and they are found in strains of both human and animal origin.^{1,22} Neutralisation experiments with specific VT antisera can be used to determine the type of VT. However, such tests cannot detect strains that produce both VT2 and a variant of VT2. Specific oligonucleotide probes have been developed to identify the genes of the different VT2 variants. VT genes can be differentiated using different primers in PCR amplifications. These variations in VT genes should provide a useful basis for the subdivision of VTEC strains in future studies.

BIOTYPING

The biochemical properties of VTEC are in general those characteristic of *E. coli*. Occa-

sional strains unable to ferment lactose have been reported. As described above, most O157 VTEC are unusual in their inability to ferment sorbitol or to produce β glucuronidase. These tests have been suggested as aids in the confirmation of O157 VTEC. A few O157 VTEC with other unusual properties have been reported including indole negative or urease positive strains and strains able to use citrate.²³ These characteristics could prove useful markers and care must be taken not to eliminate such atypical strains of *E. coli* in the early investigation of faecal samples. Although different biotypes of O157 VTEC with respect to fermentation patterns have been reported, most workers have considered the tests too irreproducible to be of use in the differentiation of this group.

MULTILOCUS ENZYME ELECTROPHORESIS

Strains of *E. coli* O157 have been included in multilocus enzyme electrophoresis studies of the genetic relatedness of *E. coli*.²⁴ Many enzymes were examined for differences in their rate of migration and this was used to assess the clonal relations of the strains. Using 19 enzyme systems the O157:H7 strains were shown to be unrelated to O157 strains belonging to 11 other H types. O157 VTEC (either H7 or non-motile) isolated in the USA from outbreaks or sporadic cases of haemorrhagic colitis or from healthy cattle formed a group of very closely related clones. It was noted that this group was characterised by the presence of a distinct fast migrating electromorph of aspartate aminotransferase and this property was suggested as a useful marker for the recognition of the group.

PHAGE TYPING OF *E. COLI* O157

A phage typing scheme for VT producing strains of *E. coli* O157 was developed in Canada for epidemiological investigations.²⁵ The scheme which uses 16 phages now recognises 66 types (H Lior, personal communication). Twenty four phage types have been identified in Britain although most strains belong to phage types 1, 2, 4 and 49.²

PLASMID ANALYSIS

Plasmid analysis of O157 VTEC can be used to identify strains in outbreaks and sporadic cases of infection.^{2,3,11} Virtually all O157 VTEC isolates carry a plasmid with a molecular weight of about 60×10^6 but certain strains carry additional plasmids. Identification of phage type, VT type, and plasmids provides a very useful combination for the characterisation of O157 VTEC.

SERODIAGNOSTIC TESTS

Studies in Canada showed that patients with VTEC infection developed rising titres of VT neutralising antibodies and this was used to diagnose VTEC infection in additional patients when other evidence was lacking.¹ Patients with known O157 VTEC infection develop rises in antibody titre to O157 LPS. High titre serum antibodies to O157 LPS can also be detected in patients when it is not possible to

isolate 0157 VTEC.²⁶ Subjects in the control group were negative for such antibodies and it was concluded that testing sera from patients with HUS and haemorrhagic colitis is a very useful method to provide evidence of infection by *E coli* 0157. An ELISA is used to screen sera with confirmatory tests, where necessary, using immunoblotting.²⁶

Conclusion

Tests for 0157 VTEC based on the use of sorbitol MacConkey agar, or improved media, combined with an 0157 antiserum remain the most practical for clinical laboratories. Further studies by the reference laboratory are required to confirm the 0157 strains and provide characterisation of the isolates for epidemiological investigations. Where tests for 0157 VTEC are negative, specimens should be tested for VT or VTEC using toxin tests, immunological methods, or DNA probes. Such tests are hampered at present by the lack of widely available commercial reagents. Examination of sera for antibodies to 0157 LPS has proved very useful and should be investigated in appropriate cases when tests for 0157 VTEC are negative.

Appendix

- 1 Suppliers of *E coli* 0157 Latex tests or specific 0157 antiserum.
 - (a) Laboratory of Microbiological Reagents, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT.
 - (b) Oxoid Ltd, Wade Road, Basingstoke, Hampshire.
 - (c) Pro-Lab Diagnostics Ltd, Wirral, Merseyside.
 - (d) Mercia Diagnostics Ltd, Mercia House, Broadford Park, Shalford, Guildford, Surrey.
 - (e) Difco Laboratories Limited, PO Box 148, Central Avenue, East Molesey, Surrey KT8 0JE.
 - (f) Kirkegaard & Perry Laboratories Inc., 2 Cessna Court, Gaithersburg, Maryland, 20879, USA.
UK Distributor: Dynatech Laboratories Ltd., Daux Road, Billingshurst, Sussex RH14 9SJ.
- 2 Suppliers of MacConkey Sorbitol agar.
 - (a) Oxoid Ltd.
 - (b) Difco Laboratories.
- 3 Suppliers of MacConkey Sorbitol MUG agar.

Biolife, Viale Manza 272, 20128, Milan, Italy.

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