Shiga-like toxigenic Escherichia coli (*Escherichia coli*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for shiga-like toxigenic *escherichia coli*.

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1. Introduction

E. coli producing verotoxin were first recognised as a cause of bloody diarrhoea and HUS in the early 1980's. Key determinants of disease were ability to colonise the gut and production of verocytotoxins, similar to Shiga toxin, giving them their early name of Shiga-like toxins, SLT hence Shiga–like Toxigenic *E. coli* (SLTEC). An alternative name was VT for Verotoxins, hence Verocytotoxigenic *E. coli,* (VTEC), reflecting their biological activity. To reduce confusion Calderwood et al (2) proposed the name, Shiga toxigenic *E. coli* (STEC), since the toxins (Stx 1&2) belong to the Shiga toxin family. All designations continue to be used although SLTEC is less common. For simplicity, STEC is the term used in this long case definition.

Testing is complicated by the difficulty of recognising STEC in a background of commensal *E. coli.* Most of the early STEC isolated from outbreaks and sporadic cases belonged to sero group O157. STEC belonging to the O157 clone characteristically do not ferment sorbitol, distinguishing them from the vast majority of commensal *E. coli,* and colonies can be easily recognised on indicator media containing sorbitol. Culture on sorbitol MacConkey-based medium remains the most commonly employed method to screen for STEC overseas. Chromogenic media designed specifically to detect this clone are also available.

STEC infection has been notifiable in all Australian states and territories since 2001. Until recently, methods to detect these organisms were perceived as being beyond the capabilities of most diagnostic laboratories and requests were usually referred to a public health laboratory. Some laboratories performed a preliminary screen on sorbitol-containing indicator medium to select for non-sorbitol fermenting *E. coli* O157, which were then referred to a reference laboratory for definitive

identification. This latter approach remains the most common detection method in USA and New Zealand where O157 has been considered to cause the vast majority of infections. In Australia, however, serotypes other than *E. coli* O157 have been implicated in approximately half the reported cases of STEC infections including the mettwurst outbreak in South Australia in 1995 (14). Therefore methods targeting virulence factors rather than serotype determinants are recommended for detecting STEC in Australia and elsewhere where non-O157 STECS are common.

Vero cell culture will allow detection of sorbitol fermenting STEC as well as O157. However this method is slow and only available to laboratories with cell culture facilities. Fortunately, the introduction of commercial EIA kits for toxin detection and the increased use of in-house NAA (nucleic acid amplification) techniques in larger laboratories has revolutionised the way STEC diagnostics can now be performed (5, 8, 10, 12). PCR is also effective later in the course of disease when numbers of STEC in stools may drop to below the limit of detection and isolation on primary plate culture.

The DNA sequences of STx 1& 2 have been known for more than a decade and are popular targets for diagnostic PCR assays. The toxins are immunogenic and immunological assays to detect them in human specimens, animal and food samples have been developed. Other STEC virulence factors targeted for diagnostics include enterocyte attachment and effacing (*eae*) and EHEC haemolysin A (*hly* A), markers for the locus of enterocyte effacement (LEE) pathogenicity island, and the STEC megaplasmid, respectively.

A recent report from OzFoodNet (4), documenting STEC screening practices used by laboratories in Australia confirmed that both the methods used and the types of specimens screened vary significantly. Owing to the cost of testing, laboratories are highly selective in which specimens to investigate; most screen bloody diarrhoeal specimens only or if a specific request is received. Therefore the authors conclude that the burden of disease may be much higher than the notifications suggest.

This long case definition aims to describe the methods presently available for screening specimens and confirming the identity of STEC and to provide guidance for laboratories, which might wish to introduce tests for STEC.

2. Laboratory diagnosis/tests

2.1 Screening Strategies

Isolation and presumptive identification of STEC O157 in the clinical diagnostic laboratory is relatively straightforward because of its inability, in most cases, to ferment sorbitol, with definitive identification achieved by serotyping and demonstrating one or both of the toxin genes *stx* 1 and *stx* 2. For sorbitol fermenting serotypes, isolation and presumptive identification presents more of a challenge and either a toxin test or NAA for virulence factors is essential.

All presumptive STEC isolates should be referred to a reference laboratory for definitive identification and subtyping.

2.1.1 Media

Faecal specimens may be cultivated directly to the following media and incubated at 35-37 o C: the media selected will depend on the detection strategy chosen.

- sorbitol MacConkey (SMAC) for the detection of non- sorbitol fermenting STEC (predominantly *E. coli* O157),
- cefixime-tellurite sorbitol MacConkey (CT-SMAC) which is more sensitive than SMAC
- Chromogenic agar
- washed sheep blood agar (WSBA) and enterohaemorrhagic *E. coli* (EHEC) agar may be used to demonstrate the weak haemolysis produced by the EHEC *hly* gene, which is present in approximately 89% of non-O157 STEC.
- an enrichment broth such as EC broth. (an additional broth may be incubated at 42 o C to select for *E. coli*)

2.1.2 Tests

Presumptive identification of STEC colonies may be achieved by several methods:

A Serotype-Specific Methods:

- **Non-sorbitol fermenting colonies** isolated from faecal cultures on sorbitol MacConkey (SMAC) or CT- SMAC agar may be presumed to be STEC after biochemical confirmation as *E. coli* and recognition of the O157 antigen by slide agglutination or latex agglutination tests.
- **Chromogenic agars** may also be useful for this purpose. S TEC O157 colonies growing on Rainbow agar, (Biolog Inc., Hayward) produce a black or grey colony due to the glucuronidase-negative, galactosidase-positive phenotype, whereas commensal *E. coli* grow as pinkish/gray colonies.
- **A commercial serotyping kit** utilising dried, sensitised latex particles in a card format (Dryspot Seroscreen, Oxoid) has recently become available. This provides a rapid screening test for the STEC serotypes most commonly isolated from serious human disease (O26, O91, O103, O111, O128, O145 and O157). Single factor sensitised latex particles are also available with this system (Dryspot Serocheck, Oxoid). This list doesn't include O113, which is a common cause of serious disease.
- **Immunomagnetic separation** of a specific serotype may be required to concentrate the bacteria prior to culture when a screening test indicates the presence of STEC but in numbers too low to be detected by standard isolation procedures. Briefly, magnetic beads coated with antisera to serotype-specific LPS are mixed with broth cultures or a suspension of growth from a plate. The b eads are then separated by a magnetic field, washed and plated and the resultant colonies tested. A commercial magnetic bead preparation (Dynabeads anti- *E. coli* O157, Dynal Oslo, Sweden) significantly improves the isolation of O157 from food samples and human and animal faeces. This technique is particularly useful during outbreaks when the implicated serotype is known.

B Virulence Factor-Specific Methods

- **NAA** can be used to detect virulence genes in individual colonies, sweeps of cultures on solid media or enrichment broths after incubation. (2.3) The results should be confirmed by isolation of STEC colonies.
- **Toxin production** may be detected by EIA or Vero cell assay (2.4). These tests may be performed on isolated colonies, culture sweeps or enrichment broths after incubation. Non-specific cytoxity due to faecal matter in primary enrichment may occur. Sensitivity of toxin tests on colonies may be affected by diffusion of toxin into the agar.
- **Enterohaemorrhagic *E. coli* (EHEC) agar** may be used to demonstrate the weak haemolysis produced by the EHEC *hly* gene, which is present in approximately 89% of STEC. An *E. coli* colony that is weakly haemolytic after 24 hours but not after 4 hours incubation on EHEC agar should be further investigated as a presumptive STEC.

2.1.3 Suitable specimens

Faeces, rectal swabs, urine are suitable specimens; sweeps on MacConkey agar are preferred to selected single colonies as the STEC may well be in the minority.

2.1.4 Test sensitivity

Serotype-specific tests have a very low sensitivity compared with PCR or toxin testing.

(See 2.2 and 2.3 for NA and toxin detection tests).

SMAC culture has a demonstrated sensitivity of 50-80% for detection of *E. coli* O157:H7 compared with Vero cell toxin testing (5). Chromogenic agars do not offer significantly improved sensitivity for isolation of *E. coli* O157.

2.1.5 Test specificity

Varies depending on the test used see 2.1.6.

2.1.6 Interpretation/Pitfalls

- Non-sorbitol fermenting colonies isolated on CT-SMAC are not necessarily *E. coli.* Other genera will grow on this medium, including *Morganella morganii* and *Hafnia alvei,* for example, which may also agglutinate *E. coli* O157 latex reagents. Thus it is important to confirm the identity of the isolate biochemically, and to titrate a boiled suspension against a known serotype O157 control to confirm the serotype.
- Isolates of *E. coli* O157 may be sorbitol fermenting (6), particularly when isolated from travellers from Continental Europe. Sorbitol fermenting strains of O157 have also been isolated in Scotland and Australia. The inability of STEC O157 to produce the enzyme b -D-glucuronidase, is not shared by sorbitol fermenting strains. In addition, these strains are sensitive to the presence of tellurite in CT-SMAC agar and will not grow on this medium. They must be isolated using procedures for non-O157 STEC (see 2.1.2).
- Some strains of STEC O157 hydrolyse urea and may be misidentified.

- Some strains of *Citrobacter freundii* and *Citrobacter braakii* express a molecule similar to O157 lipopolysaccharide on their cell wall which reacts with *E. coli* O157 latex reagents and will titrate to the same value as true STEC O157. They do not however, exhibit an appropriately sized product when PCR methods are used to demonstrate this serotype.
- Other genera such as Verotoxigenic *C freundii* (16) have been implicated in bloody diarrhoea and HUS cases.
- Rarely, strains of *E. coli* O157 will be isolated from human faecal samples that are negative for the toxin genes but carry the accessory virulence factors *eae* and EHEC *hly* A. These may represent strains that have lost the toxin-encoding phage(s) *in vitro.* Although these isolates are not, by definition, STEC, they should be reported to the clinician with an explanation that the patient may later develop similar sequelae to those seen following STEC infection due to the production of Shiga toxin(s) *in vivo.*
- Isolation of definitively identified STEC from a case of bloody diarrhoea or HUS is highly predictive in populations where the incidence of disease is low. The negative predictive value is not presently available. There have been many reports in the literature of HUS cases, even ones associated with outbreaks, which failed to yield VTEC, despite extensive microbiological studies. There have even been reports of patients developing antibodies to STEC, which were not isolated (1).

2.1.7 Suitable acceptance criteria

An STEC is an isolate of *E. coli* that carries one or both of the genes encoding the production of Stx1 or Stx2. A subset of these organisms, enterohaemorrhagic *E. coli* (EHEC) are defined as STEC that carry the accessory virulence factors *eae* and/or EHEC *hly* A and are capable of causing serious human disease.

2.1.8 Suitable internal controls

For health and safety reasons, toxin-producing control strains should be used only for those tests for which the demonstration of toxin production makes their use unavoidable.

Commercial latex agglutination kits for *E. coli* O157 are supplied with positive and negative controls. For confirmation of serotype by titration, a toxin-negative strain of *E. coli* O157 should be used, for example NCTC 12900, type O157:H7. This strain can also be used as a control for the demonstration of typical haemolysis on EHEC plates.

Note: There is a low infective dose for humans and laboratory acquired infections have been reported.

2.1.9 Suitable test validation criteria

STEC - the demonstration of Shiga toxin or Shiga toxin genes in an isolate that has been confirmed biochemically as *E. coli.*

EHEC – in addition to the criteria above, the demonstration of the accessory virulence factors *eae* and/or EHEC *hlyA*

2.1.10 Suitable external QC programme

There is no external quality control programme at present for the detection of STEC in specimens.

2.2 Nucleic acid amplification assays

Nucleic acid amplification (NAA) methods can rapidly determine the presence of genes encoding determinants for serotype and virulence factors such as Stx, attachment effacing factors or haemolysin in microbiologically complex samples without prior amplification by culture. They have been used to screen faeces and foodstuffs directly or can be used to screen cultures.

Primers targeting the toxin genes should be designed to detect all variants of *stx* 1&2 present in the geographical area. Sequences of *stx* 1 do not vary significantly unlike *stx* 2 which has at least 3 variants (*stx* 2, *stx* 2c, *stx* 2e) (7)

2.2.1 Tests

A. Direct Detection

Commercial NA kits are not routinely used in Australia for the direct detection of STEC in faeces. In-house methods are based on real time (TaqMan) technology or standard PCR followed by identification of product using gel electrophoresis. Most NA assays used in Australia are based on the PCR method of Paton and Paton (13), which targets a conserved region found in both *stx* 1 and s *tx* 2 followed by hybridisation of specific probes for each of the toxin genes. Variants of the method use a second round amplification with specific primer sets for each toxin (differentiation of product size after gel electrophoresis is not possible as products are too similar in size - 213 and 215bp). Real time assays can deliver a result within 24 hours of the specimen being received.

NA extracts positive for toxin genes can then be tested for the presence of accessory virulence factors found in STEC, including *eae* and EHEC *hly* A - markers for the locus of enterocyte effacement (LEE) pathogenicity island and the STEC megaplasmid, respectively, and the *rfb* region which specifies serotype. Presently, sequences for the O157, O111 and O113 *rfb* regions are commonly used in Australia. Multiplex PCR assays containing primers for these targets and alternate toxin gene sequences are often used to provide supplementary information about specimens, which have been found to be positive by the initial screening PCR.

The main impediment to the routine use of NA methods is the laborious nature of the NA extraction procedure directly from faeces and food. Less inhibitory extracts are obtained if the samples are inoculated into broth and extractions performed after overnight incubation either onsite or after transportation to a reference laboratory. This process suits the work-flow of a diagnostic laboratory where broth cultures may be set up over the course of the day or evening as the specimen arrives in the laboratory. Only a small amount of specimen (about the size of a match head) should be inoculated into the broth so that there is adequate dilution of inhibitory faecal material. With practice, inhibition can be reduced to close to zero.

B. NA Screening of isolates

Many laboratories use NA methods to screen for STEC after culture on either selective or nutrient agar plates (see 2.1.1). Commonly, PCR for toxin genes is performed on a rough NA extract of a

sweep of the growth. If the sweep is positive, separate colonies are selected for confirmation by PCR or dot blot assay using specific toxin gene sequences as probes. PCR-positive isolates should always be referred to a reference laboratory for definitive identification.

2.2.2 Suitable specimens

Faeces from cases of HUS or bloody diarrhoea. U rine may be appropriate if a UTI is concomitant with HUS and the patient is constipated.

Samples associated with cases of HUS or bloody diarrhoea, such as food stuffs.

2.2.3 Test sensitivity

There are very few published studies comparing the sensitivity of PCR to other methods of detecting STEC in specimens.

Direct NAA detection of the toxin genes in a suitable specimen is extremely sensitive if the initial NA extraction procedure is effective in removing inhibitory substances. The limit of sensitivity of the TaqMan system approached 10-100 organisms in a background of 10 9 enteric organisms in seeding experiments using LB broths from negative human faecal specimens. However, due to the high sensitivity of PCR, a positive result does not imply the organism will be isolated. If a sample of food implicated in an outbreak or specimen from an HUS case is positive by PCR up to 1000 colonies may need to be screened to find a positive colony on subculture.

2.2.4 Test specificity

Very high specificity if target sequences are well chosen. Isolation of STEC colonies from PCR positive samples/specimens should always be attempted.

2.2.5 Predictive values

Negative predictive value will depend on the inhibition rate and the primers, which should be designed to detect all variants present in the geographical area. Positive predictive value of an assay for the toxin genes is influenced by the primer design, specimen quality and test sensitivity.

Some strains of STEC O157 are PCR positive for *stx* but do not produce detectable toxin *in vitro.*

2.2.6 Suitable acceptance criteria

This will depend on the PCR method.

Qualitative NA tests are considered positive if a PCR product of the appropriate size is present following gel electrophoresis and negative control lanes are clear. All positive PCR products should be verified by second round PCR, dot blot analysis, restriction enzyme digest or sequencing to confirm the result.

Using the TaqMan detection system, a specimen is considered positive for the ST gene/s when the log increase of PCR product crosses the threshold (Ct value) before 35 cycles. A Ct value of more than 35 cycles is generally considered to be negative. Very weak positives with Ct values of up to 37 cycles may be discernible if the increase in fluorescence is sufficiently steep.

2.2.7 Suitable internal controls

Ideally an internal inhibition control should be included in each specimen tube. In practice specimens are analysed for inhibition by the inclusion of a separate specimen tube spiked with positive control DNA at two concentrations, one of which should be near the lower limit of detection. Inhibitory specimens are identified by the absence of a PCR product of the appropriate size in this sample after gel electrophoresis or absence of a fluorescent signal in the case of real-time PCR.

Applied Biosystems now has an internal control that can be spiked into samples. However, TaqMan protocols can only detect four dyes, which are presently used at maximum capacity (ie two test probes, an internal fluorescent dye and the quencher).

Positive and negative control organisms should be included in each run. The positive control is commonly a previously characterised STEC isolate, positive for *stx* 1 & 2 genes. The negative control is commonly an ATCC strain such as *E. coli* ATCC 25922, which is non-toxigenic.

2.2.8 Suitable test validation criteria

- Confirmation of NAA screen for toxin genes by multiplex PCR for other virulence factors and *rfb* region
- Isolation of STEC from positive specimens.
- Qualitative PCR runs may be validated by the amplification of serial ten-fold dilutions of the positive control. Reactions must be within one dilution of the established endpoint for validation of the results.

2.2.9 Suitable external QC programme

None available at present

2.3 Toxin detection

The gold standard assay for the presence of toxin in faecal specimens and isolates remains Vero cell culture. However several commercial toxin kits are now available including two EIA kits; ProsPecT Shiga Toxin *E. coli* (STEC) Microplate assay (Alexon-Trend, Ramsey, Minn.) and Premier EHEC immunoassay (Meridian Bioscience, Inc., Cincinnati), both of which have been registered by the US FDA for use on stool specimens directly or on overnight broth culture. Two other toxin assays, VTEC – RPLA and Duopath Verotoxin (DV) are recommended only for use on colony sweeps or isolates. Neutralisation tests in Vero cell cultures have shown that there is little, if any, crossreactivity between antibodies raised against each of the toxin types.

2.3.1 Tests

A. Vero cell culture

Cell-free filtrates of enrichment broth cultures are applied to freshly split monolayers of Vero cells and observed for cytopathogenicity following 48-72 hours incubation. Neutralisation tests, other antibody-based tests such as EIA or nucleic acid testing should be performed to differentiate cytopathogenicity due to Shiga-toxin production from non-specific toxic effects that may be seen in monolayers especially following inoculation of enrichment broths from primary faecal specimens. This specialised

test is unlikely to be available in diagnostic laboratories, and results are not available for 48-72 hours, making rapid diagnosis impossible.

B. EIA

Commercial EIA kits (ProsPecT, Alexon-Trend and Premier EHEC, Meridian Diagnostics) are readily available. They are specific, reasonably sensitive and easy to use, although expensive. Results are available in 1.5-2 hours. As with all EIA assays, care must be taken during the washing process to avoid false positives. Some isolates of STEC do not produce detectable levels of toxin *in vitro,* and require confirmation by PCR. The detectable toxin concentration can be increased by incubating the cell pellet obtained after incubation in broth culture with 0.1mg/ml polymyxin B.

C. Other Toxin kits

The VTEC –RPLA (Oxoid; Verotox-F, Denka Seiken) is a reverse-passive latex agglutination assay which differentiates between Stx1 and Stx2 and also quantifies the amount of toxin present. Bettelheim (1) has successfully adapted the Denka Seiken kit to be cost effective for screening multiple colonies.

Duopath Verotoxin (DV) immunochromatographic test was originally intended to confirm STEC isolates from foods. The DV test uses colloidal gold-labelled monoclonal antibodies to “trap” any Stx1 and Stx2 present in samples as they migrate over a membrane. A positive result appears as a red line within 10 minutes. Like the VTEC–RPLA the DV test is recommended for testing colony sweeps or isolates rather than primary faecal broth cultures (11)

2.3.2 Internal controls

Toxin tests should be controlled using positive and negative controls. In the case of commercial EIA and RPLA kits these will normally be supplied. For Vero cell culture, a toxin-producing strain should be included in duplicate in each batch, together with a heat-treated aliquot of the broth culture under test and an uninoculated well for each test organism.

2.3.3 Sensitivity

The sensitivity of all toxin detection assays is influenced by the number of STEC organisms in the sample, the total amount and potency of the toxin produced and the degree to which the toxin is released from the cells (12). Sensitivity of toxin tests performed on growth taken directly from an agar plate may be reduced by diffusion of the toxin into the agar.

Generally the sensitivity of EIA directly on faecal extracts is much lower than Vero cell culture but is comparable if used to test primary faecal cultures supplemented with polymyxin B and mitomycin to enhance release of toxins. Both are highly sensitive and specific compared with SMAC culture. Reports vary of the sensitivity of toxin tests compared with PCR for virulence genes. One study (15) found conventional light cycler-based PCR to be more sensitive than a commercial EIA kit (RIDASCREEN Verotoxin ELISA, R-Biopharm, Darmstadt, Germany) and Vero cell culture.

2.3.4 Specificity

As with all EIA assays, care must be taken during the washing process to avoid false positives. Some isolates of STEC O157 do not produce detectable levels of toxin *in vitro,* and require confirmation by PCR.

2.4 Definitive characterisation of STEC/VTEC

All presumptive STEC must be referred to a reference laboratory for definitive identification and subtyping for epidemiological purposes.

2.4.1 Identification

Definitive identification of STEC isolates requires demonstration of the toxin by EIA or Vero cell culture (2.4) or detection of virulence genes by PCR (2.3). Multiplex PCR for the *stx* genes should also include primers for the accessory virulence factors *eae* and EHEC *hly* A.

2.4.2 Serotyping

Toxin-positive isolates should be serotyped as a minimal epidemiological test. Isolates presumed to be serotype O157 by latex agglutination must be confirmed by titration of a boiled suspension. The H7 antigen may be identified by latex agglutination or by tube agglutination following serial passage through semi-solid medium to enhance motility. PCR methods may also be used to demonstrate the H7 antigen. In some isolates, the H7 PCR may be positive, but motility has been lost. These are reported as [H7].

A comprehensive STEC reference service is provided by the Microbiological Diagnostic Unit, Melbourne, although State PHLN laboratories may have facilities to perform at least “O” serotyping of local STEC isolates. Full O: H serotyping is required to characterise STEC to appreciate the significance of non-O157 STEC and strains of O157 having “H” antigens other than H7.

2.4.3 Subtyping

PFGE using *Xba* 1 is commonly used to subtype serotypes of STEC.

Phage typing of O157 isolates is performed by Microbiological Diagnostic Unit, Melbourne.

2.4.4 External QC programme

There is no external quality control programme at present for the identification of STEC, other than the Enter-Net programme for serological identification of *E. coli,* run from the WHO Collaborating Centre for *E. coli,* Copenhagen. Certain of the isolates provided will be STEC serotypes, although these isolates are toxin-negative for laboratory safety reasons.

2.5 Serological diagnosis

A humoral immune response may be mounted against a variety of STEC antigens including toxins, intimin and structural proteins. Of these, the most reliable marker of disease is demonstration of a rise or fall in antibodies to LPS (12). Unfortunately the response is serotype-specific and may be a result of infection with a non-toxigenic strain, reducing the test's usefulness unless the most likely infecting

serotypes are known. Serology, however, has been useful in confirming the cause of HUS cases where culture of stool has not yielded an STEC.

Assays are commonly based on EIA or passive haemagglutination and may measure antibodies in serum or IgM and IgA in saliva (9). A commercial latex agglutination kit (Oxoid Ltd, Basinstoke, UK) has been shown to give comparable results to EIA and Western Blotting but is available for O157 only (3). Microbiological Diagnostic Unit, Melbourne University, offer a tube agglutination assay for the most common serotypes isolated in Australia.

3. PHLN Laboratory definition

Definitive criteria

1. Isolation of Shigatoxigenic (Verotoxigenic) *E. coli* from faeces

2. Detection of Shiga (Vero) toxin genes in bloody faeces by a validated NA assay able to identify specific toxin sequences.

Suggestive criteria

Detection of Shiga (Vero) toxin in diarrhoeal stool.

4. Recommended reading

There are several reviews of detection methods for STEC (see below) and one recent publication by Humana Press, which has contributions from many of the main workers in this area:

E. coli: Shiga Toxin Methods and Protocols in Methods in Molecular Medicine, 73 (2004) Ed. Philpott D and Ebel F. Human Press Inc., Totowa, NJ.

Bettelheim, KA, Beutin, L. (2003) Rapid laboratory identification and characterization of verocytotoxigenic (Shiga toxin producing) *Escherichia coli* (VTEC/STEC). J App. Microbiol. **95** :205-217. (REVIEW)

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