Method for the Isolation and Identification of *Escherichia coli* O157:H7 from Waters 1996

Methods for the Examination of Waters and Associated Materials

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The method described in this booklet is based on a report produced by Strathclyde Water Services entitled "Detection of toxin producing strains of *Escherichia coli*" and funded by the Department of the Environment (DOE contract EPG 1/9/22, March 1995).

Performance data have not been established and users should satisfy themselves that the method is validated before being used routinely.

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Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, groundwater, river and seawater, waste water and effluents as well as sewage sludges, sediments and biota. In addition, short reviews of the more important analytical techniques of interest to the water and sewage industries are included.

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In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

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The preparation of booklets in the series 'Methods for the Examination of Waters and Associated Materials' and their continuous revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is managed by the Drinking Water Inspectorate. At present there are nine working groups, each responsible for one section or aspect of water quality analysis. They are:

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Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D WESTWOOD
Secretary

5 August 1995

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with The Health and Safety at Work etc Act 1974 and any regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1988 SI 1988/1657. Where particular or exceptional hazards exist in carrying out the procedures described in this booklet then specific attention is noted. Numerous publications are available giving practical details on first aid and laboratory safety and these should be consulted and be readily accessible to all analysts. Amongst such publications are those produced by the Royal Society of Chemistry, namely 'Safe Practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory, 5th edition, 1992'; by Member Societies of the Microbiological Consultative Committee, 'Guidelines for Microbiological Safety, 1986, Portland Press, Colchester'; and by the Public Health Laboratory Service 'Safety Precautions, Notes for Guidance'. Another useful publication is produced by the Department of Health entitled 'Good Laboratory Practice'.

Method for the Isolation and Identification of *Escherichia coli* O157:H7 from Waters

1 Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC), first described in 1977,[1] are now recognised as a major cause of haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS).[2] HUS is now the most common cause of renal failure in children in North America and in England and Wales.[3,4]

The serogroup associated with human illness is *Escherichia coli* O157:H7 (*E. coli* O157:H7), accounting for over one-half of all VTEC isolates from humans.[5] This serogroup has been isolated from cattle in the United Kingdom,[6-8] but only rarely from foods.[7,8] In North America, VTEC have been isolated from beef, beef products and raw milk, and these commodities have been put forward as possible sources of human infection.[9]

Serogroup O157:H7 was first identified as a cause of human illness in 1982 when it was shown to be associated with two food-related outbreaks of HC in the USA.[9] Several additional outbreaks have since been reported.[10-13] Foodborne, waterborne and person-to-person outbreaks of *E. coli* O157:H7 have been recognised.[14]

Waterborne infections with *E. coli* O157:H7 have occasionally been implicated.[15-17] However, recovery of the organism from environmental samples is often difficult because of the altered physiological state that bacteria sometimes develop in order to survive hostile environments.[18]

In a recent study of verocytotoxigenic *E. coli* O157 isolates, ten out of 39 strains were found to ferment sorbitol. No satisfactory methods for their detection have been developed. Additionally, there are numerous non-O157 verocytotoxigenic *E. coli* serotypes which have been associated with HC and HUS, most of which ferment sorbitol and therefore would not be detected using current procedures

2 Hazards

E. coli O157:H7 are pathogenic to humans. All cultures and stages of cultivation should be handled in specified areas with the utmost care by properly trained personnel. All equipment should be sterilized before and after use. Cultures should not be kept longer than is necessary and should be sterilized prior to discarding. Due regard should be paid to relevant Codes of Practice for Safety in Microbiology Laboratories.[19,20]

All cultures, Eppendorf tubes and agglutination cards should be discarded into suitable autoclave bags and sterilized at 121°C for 20 minutes. Glassware should be autoclaved before washing.

Fluids from the immunomagnetic separation (IMS) procedure should be carefully dispensed into a suitable disinfectant.

Glass pipettes and slides should be dispensed into "contaminated sharps" bins.

IMS equipment should be wiped over with an appropriate disinfectant after use.

3 Definition of *E. coli* O157:H7

E. coli are gram-negative motile rods, catalase positive, oxidase negative and facultatively anaerobic. Most O157:H7 strains do not ferment sorbitol. Also, they are ß-glucuronidase negative and grow poorly at 44°C, but otherwise are biochemically similar to other *E. coli* serotypes.

4 Scope

The method described is for the qualitative isolation of *E. coli* O157:H7 from fresh waters only.

5 Principle

Concentration of water samples by membrane filtration (MF) followed by enrichment culture in a selective broth with incubation at 37°C for 6 hours. Concentration of the organism using IMS, by attachment to antibody-coated paramagnetic beads, which are then separated from the broth using a magnetic field. The IMS concentrate is plated out onto a selective agar to produce individual colonies. Subculture of characteristic colonies to media for biochemical and serological confirmation.

6 Sampling

6.1 Natural waters and effluents.

Procedures and apparatus used for the bacteriological sampling of aquatic situations as described in Report 71 [21] are applicable. As with other pathogens, the longer the time interval between sampling and examination, the lower the likelihood of recovery. Samples should be examined as soon as possible after collection. Exposure of the sample to daylight will increase attenuation and may be bactericidal. It is essential that chlorinated samples should be dechlorinated at the time of collection. The presence of toxic metals will adversely affect bacterial populations and may subsequently limit the success of the culture techniques described. Competition from other organisms may also reduce isolation efficiency.

6.2 Information to be supplied with samples.

Sufficient information should accompany the sample. This may include;

(i) the source;
(ii) the sampler's identification;
(iii) the date and time of sampling; and
(iv) the exact location where the sample was taken.

If the sample was taken from a river, stream, lake or reservoir, additional information may include the depth at which the sample was taken, whether taken from the side or middle of the water mass, whether the water level was above or below average and whether the sample was taken after heavy rainfall, flooding or during drought.

7 Analytical procedure

Step	Procedure	Notes
7.1	**Membrane filtration methods**	
7.1.1	A suitable volume of water should be filtered, notes a and b.	(a) Due to the retention properties of cellulose acetate membranes it is generally only possible to filter relatively clear waters using standard laboratory equipment. (b) Pressurised MF systems using larger diameter membranes can be used to filter more turbid waters. The larger membranes should be aseptically cut into smaller pieces using sterile scissors and forceps to facilitate transfer to the enrichment broth.
7.2	**Enrichment culture in vancomycin cefixime cefsulodin (VCC) broth**	
7.2.1	Transfer the filter membrane to 20 ml of VCC broth (A1). Close cap and shake well. Incubate the broth at 37 $\pm$ 1.0°C for six hours.	

Step	Procedure	Notes
7.3	**Immunomagnetic separation**	
7.3.1	Eppendorf tubes should be labelled and placed in a magnetic particle concentrator (MPC). Remove the magnetic plate.	
7.3.2	Re-suspend the antibody-coated paramagnetic beads, for example using a whirl-mixer and pipette 20 μl into each tube.	
7.3.3	Add 1 ml of well-mixed enrichment broth to a correspondingly labelled tube and close the cap. Mix well. For turbid waters, any gross particulates should be allowed to settle after the enrichment broth has been added.	
7.3.4	Place the tubes on a rotary mixer, set at 30 rpm, and mix at room temperature for 30 minutes.	
7.3.5	Insert the magnetic plate into the MPC, and load the tubes into the MPC. Allow to stand for 3 minutes. Invert the MPC 3 times to collect beads located inside the cap and to further concentrate the beads on the side of the tube.	
7.3.6	Carefully aspirate and discard the supernatant as well as the remaining liquid in the cap of the tube. Remove the magnetic plate from the MPC.	
7.3.7	Add 1 ml of phosphate buffered saline (PBS) Tween 20 solution (see appendix A4) to each tube, close the cap and immediately invert the MPC three times to re-suspend the beads.	
7.3.8	Repeat steps 7.3.5 to 7.3.7.	
7.3.9	Repeat steps 7.3.5 and 7.3.6.	
7.3.10	Re-suspend the bead-bacteria complex in 50 μl of PBS Tween 20 solution, and mix, note c.	(c) The whole of the concentrate can now be cultured on a solid medium. The bacteria usually remain viable while bound to the beads and will multiply to form colonies if nutritional requirements are provided.[22]
7.4	**Isolation on cefixime tellurite sorbitol MacConkey (CTSM) agar**	
7.4.1	Confirm the presence of *E. coli* O157:H7 by isolating the organisms in pure culture for final identification. Pipette the re-suspended 50 μl IMS concentrate onto CTSM agar, and plate out for single colonies, note d. After incubation of the plates at 37 $\pm$ 1.0°C for 18 to 24 hours, *E. coli* O157:H7 strains will typically produce colourless colonies, 2-3 mm diameter, with dark centres. Most other colonies which appear on this medium are pink due to fermentation of sorbitol.	(d) It should be noted that overgrowth of the characteristic colonies may occur unless careful attention is paid to the plating out technique.

Step	Procedure	Notes
7.5	**Serological testing**	
7.5.1	CTSM plates should be examined both by reflected and transmitted light using a hand lens (about 8-fold magnification) if necessary. All sorbitol non-fermenting colony types are subcultured to nutrient agar to provide sufficient bacterial growth for confirmation of purity, serological and biochemical testing. Suspect colonies can be picked with a straight wire, using a stabbing motion, to avoid touching adjacent colonies.	
7.5.2	After incubation of the nutrient agar plates at 37 $\pm$ 1.0°C for 18 to 24 hours, individual colonies may be tested for agglutination using a commercially available *E. coli* O157 latex agglutination kit. (Slide agglutination with antisera may also be satisfactory). The appropriate control reagents should be used to ensure the specificity of any reactions.	
7.5.3	The problem of autoagglutination, ie agglutination in the absence of *E. coli* O157 antiserum, may be overcome by heat treatment of the isolate. A turbid normal saline suspension of a colony is prepared in a glass vial and heated at 100°C in a water bath for ten minutes. After cooling, a drop of the suspension is used to repeat the agglutination test, note e.	(e) Due to the tendency of some other members of the family Enterobacteriaceae to cross-react with *E. coli* O157 antiserum, a biochemical profile of the isolate should also be obtained.
7.6	**Biochemical testing**	
7.6.1	Commercially available biochemical identification systems suitable for Enterobacteriaceae may be used to confirm the isolate. Serogroup O157:H7 differs from typical *E. coli* by failing to ferment sorbitol or to produce β-glucuronidase, and by fermenting dulcitol and raffinose. The inoculum for the test is prepared from the nutrient agar subculture, preferably from an individual colony. A separate culture for a purity check is prepared on nutrient agar, using the same colony. This culture can also be used to double-check the serological reaction of the isolate. Complete identification and confirmation should be carried out.	

Appendix A: Media and Reagents

A1 Vancomycin Cefixime Cefsulodin (VCC) Broth [7]

Buffered peptone water supplemented with vancomycin (8 mg/l), cefixime (0.05 mg/l) and cefsulodin (10 mg/l).

A1.1 Buffered peptone water

Peptone	10	g
Sodium chloride	5	g
Disodium hydrogen phosphate (anhydrous)	3.5	g
Potassium dihydrogen phosphate (anhydrous)	1.5	g
Water	1000	ml

pH 7.2 ± 0.2

Bottle in litre volumes and autoclave at 121°C for 15 mins.

A1.2 VCC supplement

Vancomycin hydrochloride	800	mg
Cefixime powder	80	mg
Cefsulodin (sodium salt)	1	g
Water	100	ml

Dissolve cefixime in 20 ml of ethanol, with occasional mixing. Dissolve the other compounds in 100 ml of distilled water and add 1.25 ml of cefixime solution. Sterilise the supplement by filtration through a 0.22 μm membrane filter, and distribute in 1 ml aliquots. Store the aliquots frozen until required.

A1.3 For use

Aseptically add 1 ml of solution A1.2 to 1 litre of solution A1.1. Distribute in 20 ml volumes in sterile screw-capped containers. The complete medium may be stored for up to one week at 4°C without deterioration.

The use of VCC broth has recently been reported [23,24] to be too inhibitory with low numbers of *E. coli* O157:H7. Furthermore, incubation at 42°C has been shown to improve the selectivity of the isolation procedure.

A2 Cefixime tellurite sorbitol MacConkey (CTSM) agar [25]

Sorbitol MacConkey agar supplemented with cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l).

A2.1 Sorbitol MacConkey agar

Peptone	20	g
Sorbitol	10	g
Bile salts No.3	1.5	g
Sodium chloride	5	g
Neutral red	30	mg
Crystal violet	1	mg
Agar	15	g
Water	1000	ml

pH 7.1 ± 0.2

Steam to dissolve and bottle in 1 litre volumes. Autoclave at 121°C for 15 mins.

A2.2 Cefixime-tellurite supplement

Cefixime powder	80	mg
Potassium Tellurite (hydrate)	250	mg
Water	100	ml

Dissolve cefixime in 20 ml of ethanol, with occasional mixing. Dissolve the tellurite in 100 ml of distilled water and add 1.25 ml of cefixime solution. Sterilise the supplement by filtration through a 0.22 μm membrane filter, and distribute in 1 ml aliquots. Store the aliquots frozen until required.

A2.3 For use

Melt 1 litre of solution A2.1 in a boiling water bath and allow to cool to 45°C. Aseptically add 1 ml of solution A2.2 and mix by swirling to avoid the formation of bubbles. Distribute 15 ml volumes into 90 mm vented petri dishes. The agar plates can be stored for up to 1 week without deterioration.

A3 Nutrient Agar Slopes and Plates

Beef extract	1	g
Yeast extract	2	g
Peptone	5	g
Sodium chloride	5	g
Agar	15	g
Water	1000	ml

pH 7.4 $\pm$ 0.2

Steam to dissolve and bottle in suitable volumes. Autoclave at 121°C for 15 minutes.

A4 Phosphate buffered saline (PBS) Tween 20 solution

Sodium chloride	80	g
Potassium chloride	2	g
Disodium hydrogen phosphate (anhydrous)	11.5	g
Potassium dihydrogen phosphate (anhydrous)	2	g
Polyoxyethylene-sorbitan monolaurate (Tween 20)	0.5	ml
Water	1000	ml

pH 7.3 $\pm$ 0.2

Steam to dissolve and bottle in suitable volumes. Autoclave at 121°C for 15 minutes.

A5 Latex Agglutination Kits

A range of *E. coli* O157:H7 latex agglutination kits are commercially available. Ensure that the kits contain adequate control reagents.

Appendix B: Apparatus

B1 In addition to the normal glassware and other equipment available in a bacteriology laboratory, the following extra items may be necessary:

B1.1 Sterile screw-capped bottles—1 litre volume, for sampling.

B1.2 Membrane filtration units—300–500 ml capacity funnels. Alternatively, a pressurised membrane filtration system may be used.

B1.3 Sterile cellulose acetate membrane filters—for example, 47mm diameter, 0.45μm pore size. Larger diameter membranes may be used with pressurised filtration equipment.

B1.4 Sterile syringe filter holders—0.2μm, disposable, for sterilisation of antibiotic solutions.

B1.5 Magnetic particle concentrator (MPC)—suitable for use with Eppendorf tubes.

B1.6 Rotary mixer—for end-over-end mixing, suitable for use with Eppendorf tubes; can be set at 30 rpm.

B1.7 Paramagnetic beads—coated with antibody to *E. coli* O157:H7.

B1.8 Eppendorf tubes.

B1.9 Whirl mixer.

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