

APPENDIX B

LABORATORY METHODS

Central Public Health Laboratory, Laboratories Branch, Ontario Ministry of Health and Long-Term Care, Toronto.

Isolation and Identification of Enteric Pathogens

Stool specimens were collected into Cary–Blair Transport medium and shipped to the laboratory. The specimens were tested for the presence of enteric pathogens including *Salmonella sp.*, *Shigella sp.*, *Campylobacter sp.*, *Yersinia sp.*, *Escherichia coli* O157:H7, and *Aeromonas sp.*, using standard laboratory procedures briefly described below¹.

The specimens were inoculated onto MacConkey Agar (MAC), Sorbitol-MacConkey Agar (SMAC), Salmonella Shigella Agar (SS), Xylose-Lysine-Desoxycholate Agar (XLD), Charcoal Selective Medium (CSM), Cefsulodin-Irgasan-Novobiocin Agar (CIN), Ampicillin Tween 80 Agar (AT 80), Selenite Broth (SEL) and Liquid Enrichment Medium (LEM).

MAC, SMAC, SS, XLD, and AT 80 were incubated at 36°C, in air, for 24 hours and then examined for enteric pathogens using a plate microscope. SS was re-incubated for an additional 24 hours and re-examined. CSM was incubated at 42°C in a tri-gas (5% Oxygen, 10 Carbon dioxide, 85% Nitrogen) incubator and examined using a plate microscope after 24 hours and 48 hours incubation. CIN was incubated at 32°C in air for 24 hours and examined using a plate microscope. SEL was incubated at 36°C in air for 24 hours and sub-cultured onto SS, which was then incubated at 36°C in air and examined after 24 hours and 48 hours incubation. LEM was incubated at 42°C in a tri-gas incubator and sub-cultured to CSM, which was incubated at 42°C in a tri-gas incubator for 24 hours and examined.

Suspicious colonies were screened using biochemical tests and Gram stained as appropriate.

***E. coli* O157:H7 Identification**

Isolates which gave biochemical reactions suggestive of *E. coli* were screened by slide agglutination using in-house prepared *E. coli* O157 antisera. A rapid agglutination in the antiserum (15-30 seconds) and no agglutination in saline was presumptive evidence of *E. coli* O157. The presence of the H7 antigen was determined using the tube agglutination technique and in-house prepared antisera¹.

Verotoxin Detection (ELISA)

Verotoxin production from direct stool preparations and pure culture isolates was detected using the ProspecT STEC Microplate Assay (Alexon-Trend, Ramsey, MN) according to the manufacturer's protocol.

***Campylobacter sp.* Identification**

Suspicious colonies were tested for oxidase production using filter paper impregnated with 1%NNNN-tetramethyl-p-phenylenediamine dihydrochloride or aqueous aminodimethylaniline oxalate. The catalase test was performed using 3% hydrogen peroxide. Oxidase and catalase positive colonies were Gram stained using a modified technique, which requires counterstaining for 3 minutes with safranin. *Campylobacter sp.* appeared as tiny Gram negative curved, “s” shaped or gull-winged rods. The hippurate test was performed to determine the species of *Campylobacter*. A loopful of growth from a single colony was inoculated into 0.4 mL 1% aqueous sodium hippurate and was incubated in a water bath at 37°C for 2 hours. 0.2 mL of Ninhydrin solution was slowly added to form an overlay and re-incubated at 37°C for 10 minutes. Isolates that were hippurate positive were identified as *Campylobacter jejuni*. *Campylobacter coli* were identified by determining susceptibility to naladixic acid (30 mcg disc) and cephalothin (30 mcg disc) and by the indoxyl acetate test.

Pulsed Field Gel Electrophoresis (PFGE)

For molecular typing with PFGE method, all *E. coli* O157:H7 strains associated with Walkerton outbreak were cultured on Columbia Agar overnight at 37°C. Cells were then immobilized in 1.0% low melting-point agarose plugs and subsequently lysed in 2 mL lysis buffer containing 1 mg/ml protease K at 55°C for 16-20 hrs. After extensive washing, all agarose plugs containing genomic DNA of *E. coli* O157:H7 were restricted with endonuclease XbaI enzyme at 37°C overnight. One ATCC strain was used as internal PFGE quality control strain and its genomic DNA was prepared using identical procedures described in the text for outbreak strains.

Pulsed-field gel electrophoresis was performed using CHEF DRIII system (Bio-Rad Laboratories, Mississauga, Ontario) with the following parameters: 0.5X TBE buffer, initial switch time 2.2 seconds, final switch time 54.2 seconds, switch angle 120°C, voltage 6Volts/cm, buffer temperature 14°C, and total run time 24 hr.

Following gel electrophoresis, the agarose gel was stained in water containing 0.5 µg/ml for one hour and digital image obtained using Fluo-S-MultiImager system (Bio-Rad Laboratories, Mississauga, Ontario). *E. coli* O157 strains that shared identical PFGE pattern are considered genetically indistinguishable from each other. The predominant PFGE pattern observed for Walkerton outbreak was arbitrarily designated as Pattern A as reported in the patient's line list table. Strains that exhibited one band difference from pattern A are designated as pattern A1. Strains that showed more than three band difference from pattern A are designated as C, D, E, F, etc².

Polymerase Chain Reaction (PCR)

Direct detection of O157, H7, and verotoxin genes were carried out using an in-house polymerase chain reaction (PCR) method. Total genomic DNA was extracted from the original water samples and enrichment broth, using both manual (Qiagen Tissue DNA Kit, Mississauga, Ontario, Canada) and automated methods (NucliSens Extraction System, Organon Teknika, Scarborough, Ontario, Canada). PCR amplifications were carried out in 100 µl volumes, according to the method by Ganon *et al* and Paton *et al* using the GenAmp 9600 Thermocycler (PE Applied Biosystem,

New Jersey, USA) ^{3,4,5}. PCR-amplified DNA was detected using the gel electrophoresis technique.

Coliforms and *Escherichia coli* Enumeration by Membrane Filtration

Samples were stored at 4°C prior to analysis. Samples were mixed by shaking 25 times through a one-foot arc in 10 seconds. 100 ml of the samples was poured into a Millipore™ filtration apparatus containing a 0.45 µm porosity, Millipore™ EZ-Pak membrane filter. Vacuum was applied to the apparatus to filter the water. After the filtration was complete, the membrane filter was removed from the filtration apparatus with flamed forceps and placed on DC agar.

DC agar was formulated as follows (per liter): Difco FC base 39.5 g/L; Lactose (Sigma-Aldrich Canada), 10.0 g/L; 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Diagnostic Chemicals Ltd.), 0.20 g/L; Neutral Red (Sigma Aldrich Canada), 0.08 g/L; Sodium Cefsulodin (Marcor Development Corp.) 1.20 ml of a 10 mg/ml solution. After boiling and cooling to 45 to 50 °C, the pH of the media was verified to be 7.2 ± 0.2. The final medium was poured into 140 mm diameter petri plates and stored at 2 - 4°C for up to 2 weeks.

After filtration, the DC agar plates with filters were incubated at 35 ± 1.0°C for 22 to 24 hrs. All pink to red and blue to blue-green colonies were counted and recorded as coliform colonies. All blue to blue-green colonies were also counted and recorded as *Escherichia coli* colonies. Coliform and *Escherichia coli* were reported as up to 80 per 100 ml. When there were more than 80 colonies, > 80 was reported or overgrown when the membrane filters were confluent with colonies.

Presence/Absence (P/A) Test for Coliforms and *Escherichia coli*

Samples were stored at 4°C prior to analysis. Samples were mixed by shaking 25 times through a one foot arc in 10 seconds. 100 ml of water was aseptically transferred to 50 ml of P/A broth (Difco) in a 250 ml glass bottle. The bottles were incubated at 35 ± 1.0°C for 72 hrs.

The P/A bottles were examined daily for turbidity, effervescence (gas bubbles) and a change in colour . If the P/A broth changed from purple to yellow at any time during the 72 hour incubation period, the test is recorded as positive.

A yellow or acid reaction in the broth is strong evidence that coliform bacteria are in the sample.

To confirm the presence of *Escherichia coli*, Lauryl Sulfate Tryptose broth with 4-methylumbelliferyl-β-D-glucuronide (LST-MUG) was inoculated with the positive P/A broth. LST/MUG was prepared by adding 35.6 g of Difco Lauryl Sulfate Tryptose broth and 0.1 g 4-methylumbelliferyl-β-D-glucuronide (Diagnostic Chemicals Ltd) to 1 liter of demineralized water and mixing. A pH of 6.8 ± 0.2 was verified and the LST/MUG was dispensed into 20 mm x 150 ml screw capped test tubes containing Durham vials at 10 ml per tube.

The LST/MUG broths were incubated at $35 \pm 1.0^{\circ}\text{C}$ for 48 hrs. LST/MUG broths were then examined for fluorescence under long wave UV light (UVP Inc., Model UVGL-58) in a dark environment. If fluorescence was observed, *Escherichia coli* was recorded as being present.

Isolation of *Escherichia coli* 0157:H7 from Water by Immunomagnetic Separation

Filtration units (Millipore™) that was sterilized for a minimum of two minutes in a UV sterilizer (Millipore™) were used. Filtration units were placed on a filtration manifold (Millipore™). A 0.45 μm porosity membrane filter (Millipore™) was inserted into each filtration unit with flamed forceps.

The samples were shaken 25 times through a one- foot arc in 10 seconds.

Up to 1 liter of water was filtered was filtered through the membrane filter. The membrane filter was removed from the filtration unit with flamed forceps and placed into a bottle containing 100 ml of 0.1 % peptone broth (Difco). The 1% peptone enrichments were incubated $35 \pm 1.0^{\circ}\text{C}$ for 22 - 24 hrs.

Immunomagnetic Separation was performed using the following method:

- (1) Label one microcentrifuge tube for each enrichment.
- (2) Remove magnetic plate and load the labeled microcentrifuge tubes into the Dynal Magnetic Particle Concentrator® for microtubes (MPC-M).
- (3) Resuspend Dynabeads® anti E. coli 0157 by vortexing. Add 20 μl of Dynabeads® into each microcentrifuge tube.
- (4) Add 1 ml of pre-enrichment into each tube and close.
- (5) Incubate at room temperature for 10 minutes with mixing on the Dynal® rotating device.
- (6) Remove microcentrifuge tubes from rotating device. Insert the magnetic plate into the MPC-M. Invert to concentrate the beads against the side of the MPC-M. Allow three minutes for this to occur.
- (7) Aspirate and discard supernatant and any liquid remaining in the cap.
- (8) Remove the magnetic plate from the MPC-M.
- (9) Add 1 ml of washing buffer (PBS-Tween) and invert the MPC-M three times to re-suspend the beads.
- (10) Repeat steps 5 to 8.

(11) Repeat steps 5 to 7.

(12) Re-suspend the bead bacteria complex into 100 µl of PBS-Tween and mix on the Dynal rotating device.

(13) Transfer 50 µl of re-suspended beads onto a CT-SMAC and a SMAC plate respectively. Spread the bacteria-bead complex on ¼ of the plate with a swab and dilute further by streaking with a loop.

(14) Incubate plates at 35°C for 18 to 24 hrs. Examine plates and pick colonies as described above under *Confirmation of Isolates from SMAC and CT-SMAC*

Confirmation of Isolates from SMAC and CT-SMAC

Sorbitol MacConkey Agar was incubated for up to 24 hrs. Ten sorbitol negative colonies were picked and streaked plates were prepared on Blood agar for purity. The following tests were set up from the purity plates: TSI slant, ONPG-PA-M, Simmons citrate, Sorbitol, urea, cellibiose and a Blood plate at room temperature for pigment production.

In addition to the above biochemical screen, the slide agglutination test was performed using the following method:

Divide a clean glass slide into two compartments with a wax pencil.

To one compartment add a drop of O157 antiserum; to the other, add a drop of saline.

Using an inoculating wire or loop, transfer a small amount of growth from the purity plate. Emulsify growth in saline first and then in the second compartment containing antiserum. The resulting suspensions should be turbid or milky in appearance.

Rock slide back and forth for up to 15 seconds and observe for granular-like agglutination.

A rapid agglutination in the antiserum and no agglutination in saline is presumptive evidence of *Escherichia coli* O157.

False positives (including weak reactions) may occur with the slide agglutination test. Some *Enterobacter hermanii* strains are positive.

Typical *Escherichia coli* O157:H7 give the following reactions:

TSI	A/A, H ₂ S -, Gas
Citrate	Negative
ONPG	Positive
Motility	Positive
Sorbitol	Negative

Urea	Negative
Cellibiose	Negative
Blood at R/T:	Nonpigmented
Antisera	Positive

Isolates were confirmed as described in the *E. coli* O157:H7 identification section, in this appendix.

Isolation of *Campylobacter* from Water

Filtration units (Millipore™) that were sterilized for a minimum of two minutes in a UV sterilizer (Millipore™) were used. Filtration units were placed on a filtration manifold (Millipore™). A 0.2 µm porosity membrane filter (Millipore™) was inserted into each filtration unit with flamed forceps.

The samples were shaken 25 times through a one- foot arc in 10 seconds.

Up to 1 liter of water was filtered through the membrane filter. The membrane filter was removed from the filtration unit with flamed forceps and placed into a Zip-Lock bag (4inch by 6 inch) containing 100 ml of Rosef's Broth*. The bags were then flushed three times with a gas mixture containing 5% oxygen, 10% carbon dioxide and 85% nitrogen. The gas was delivered by inserting into one corner of the Zip-lock bag a sterile 8 inch glass rod connected to a gas cylinder and allowing the bag to expand.

The enrichment broths were incubated with shaking at $42 \pm 0.2^{\circ}\text{C}$ for 48 ± 2 hs. After incubation, a bacteriological loop was used to prepare streak plates on *Campylobacter*-Charcoal Selective Medium (CSM) for isolation of bacteria from the Rosef's broth. The CSM plates were incubated microaerophilically in a CO₂ incubator for 48 ± 2 hrs.

After incubation, plates were examined with a stereomicroscope at 10X magnification. Plates were examined for typical *Campylobacter* colonies. Typical colonies that were oxidase positive were examined by gram stain and wet mount. Colonies that were gram negative, spiral "s" shaped or gull winged form were identified as detailed in the *Campylobacter sp.* Identification Section in this Appendix.

***Rosef's Broth**

Peptone, 10 g/L; Lab Lemco powder (Oxoid), 8.0 g/L; Yeast Extract, 1.0 g/L; NaCl, 5.0 g/L; Resazurin, 16 ml of a 0.025% solution per liter. In addition, after autoclaving add 0.25 ml FBP solution and 2 ml VTP solution.

FBP solution prepared by combining per 100 ml: Ferrous sulfate, 10g; Sodium metabisulfite, 10.0 g; Sodium pyruvate, 10.0 g.

VTP solution is prepared by combining the following in 1 liter of distilled water: Vancomycin, 0.5g; Trimethoprim, 0.25g; Polymyxin B sulfate, 5000 IU.

National Laboratory for Enteric Pathogens, National Microbiology Laboratory, Winnipeg, Manitoba

Phage typing Methodology for *E. coli* O157:H7

E. coli O157:H7 strains isolated from human, environment and animals associated with outbreak were received at National Laboratory for Enteric Pathogens, Health Canada, Winnipeg. All strains were stored at -70EC and working cultures were maintained on nutrient agar (Oxoid LTD., Basingstoke, Hants England) slopes.

Phage-typing of *E. coli* O157:H7 was performed using standard techniques described previously^{6,7}. Briefly, strains were plated on nutrient agar and incubated for 18 hours at 37EC. A single smooth colony was inoculated in 4.5 ml of DIFCO phage broth (pH 6.8) and incubated at 37EC for 2.5 hours in a shaking water bath. The bacterial cultures were inoculated by flooding DIFCO Phage Agar Plate to form a smooth lawn. A panel of 16 phages at their routine test dilution (RTD) were spotted on the bacterial lawn and the plates were allowed to dry. The plates were incubated at 37EC for 18 hours before examination for Lytic patterns.

Serotyping Methodology for *Campylobacter jejuni*

Serotyping of *Campylobacter jejuni* ssp. *jejuni* was performed by:

- slide agglutination with live bacteria using crude and absorbed antisera for the detection of heat-labile (HL) antigens (Lior Scheme)⁸ and
- passive haem-agglutination with soluble antigen and antisera for the detection of heat stable (O) antigens (Penner Scheme)⁹.

All cultures were grown on Mueller-Hinton agar (Oxoid Ltd., London, England) containing 5% sheep blood for 48 h at 37EC in a gas mixture of 5% O₂, 10% CO₂ and 85% N₂. Slide agglutinations were performed on glass slides to detect HL antigens using live *Campylobacter* cultures and antisera prepared at the National Laboratory for Enteric Pathogens (NLEP)⁸.

The passive haem-agglutination technique for the detection of O antigens was performed using heated extracts, sensitized sheep erythrocytes and antisera in microtitre plates⁹.

Phage typing Methodology for *Campylobacter jejuni*

The phage typing of *Campylobacter jejuni* was performed as described by J. A. Frost¹⁰. The phages were propagated by modified soft agar overlay technique¹¹. The bacterial strains were inoculated into 5 ml of Brain Heart Infusion broth supplemented with 10 mmol MgSO₄ and 1 mmol CaCl₂(CBHI). Bacterial suspensions were adjusted to a cell density equivalent to MacFarland No.1 and incubated for 4 h at 37EC to prepare overlay suspensions. Agar overlay plates were prepared using Nutrient Broth (Oxoid CM 67) supplemented with 10 mmol Mg SO₄, 1 mmol CaCl₂ and 0.7 % agar (Oxoid L11) as the basal medium and CBHI containing 0.7 % agar (Oxoid L 11) as the overlay. The phage reactions were examined against dark background using 10× hand lens and recorded as degrees of lysis using standard nomenclature¹⁰.

Methodologies for PFGE and VT-Typing

PFGE was performed using the method of Barrett et al. ¹² as modified to correspond with the PulseNet standardized method 13 (4). Bacterial cell concentrations were adjusted to a reading of 0.68 - 0.72 as measured in #2057 Falcon tubes with a Dade Microscan Turbidity meter. Plugs were made, washed, and digested according to the standard protocol. Electrophoresis was carried out in 1 % agarose gels made using SEAKEM GOLD agarose (Mandel Scientific Co., Ltd., Guelph, ON). The PFGE buffer was 0.5 × Tris-Borate-EDTA (TBE) made from 5 × TBE buffer concentrate (Sigma-Aldrich Canada, Ltd., Oakville, ON). Switch times were 2.2 to 54.2 s and the run time was 20 h. Gels were run at a temperature of 14°C and a voltage of 6 V/cm in a CHEF-DR III PFGE unit (Bio-Rad, Mississauga, ON, Canada). Gels were stained in 0.5 µg/ml ethidium bromide and the DNA was visualized with an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA). All isolates tested were analyzed using *Xba* I, while selected isolates were analyzed using *Bln* I as the second enzyme. Patterns were labelled by designating the organism (EC = *E. coli*), the enzyme (XA = *Xba* I; BN = *Bln* I), and a unique identifier (.0001 etc.). Any pattern with one or more bands different was given a unique identification number. PFGE results were interpreted in accordance with the criteria described previously ^{13, 14}.

Detection of Verotoxins

The Vero cell cytotoxicity and neutralization assays for the detection of Verotoxins produced by *E. coli* strains were performed as previously described ^{15,16}. Sera for neutralization was produced in the National Laboratory for Enteric Pathogens. PCR for detection of VT1 and VT2 was done according to the protocol of Meng et al. ¹⁷. VT2v was detected and variants were typed using the PCR-RFLP method of Tyler et al. ¹⁸.

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