Public Health Agency of Canada | Agence de la santé publique du Canada

National Integrated Enteric Disease Surveillance Program

Sample Collection, Preparation & Laboratory Methodologies

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Canada

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1. Sample Collection

Retail Meat

C-EnterNet's retail surveillance component involves a random and systematic approach to derive estimates of the pathogen levels on raw meat available to the consumer at the grocery store level. The sampling framework is based on a census of the retail grocery store outlets operating within the sentinel site. This includes large and medium sized chain stores as well as independently owned butcher and market shops. Each week, three chain stores and one independent outlet are randomly selected from the store census. A C-EnterNet field sampler anonymously visits the selected stores and purchases one ground beef, one chicken breast and one pork chop package. The meat package purchased is required to be fresh (not previously frozen) and at least 500-600 g to ensure sufficient amount of sample for bacterial testing. Twelve meat samples per week are collected with a total of 600 samples per year (50 weeks/year sampling). Samples are shipped at refrigeration temperature (4-6ºC) to a laboratory for primary isolation, enumeration and subtyping.

Agriculture

C-EnterNet's agriculture surveillance component involves the sampling of food-animal operations (swine, beef, dairy and poultry) within the sentinel site. Farm selection and visitation is performed through a contracted third party to ensure producer confidentiality. In general, 30 farms for each commodity are randomly selected for a single visitation during the calendar year. Each month, approximately 2 farms per commodity are visited. At each farm visit, a short farm management questionnaire is administered and manure samples are collected.

Three pooled fresh fecal samples representing different age or production groups and a pooled stored manure sample are collected at each visit. In total, 120 samples are collected per year for each commodity. Samples are shipped in coolers at refrigeration temperatures (4-6ºC) for primary isolation and subsequent subtyping.

Fresh Fecal Sampling Protocol

Fresh fecal samples from five individual animals are collected and put into one large plastic bag and gently mixed. A sterile scoop is used to transfer the pooled sample to specimen containers.

Stored Manure Sampling Protocol

a) Liquid Pit Sampling:

A sampling pole and bottle holder are used to collect three sub-samples from three locations around the pit, and if possible, up to two depths (i.e. the top 1/3, and mid depth of the storage), for a total of 6 sub-samples. The sub-samples are poured into a sampling bucket with a clean plastic liner and mixed to create the pooled sample.

b) Dry Pile Sampling:

A sterile sampling scoop is used to select sub-samples from five different locations in the stored manure area. These sub-samples are then added to a sampling bucket with a clean plastic liner bag and mixed to create a pooled sample.

Untreated Surface Water

C-EnterNet's water sampling and analysis framework includes a systematic sampling frame of the Grand River watershed in the sentinel site in the Region of Waterloo, Ontario. The Grand River watershed is sampled twice per month at five locations upstream of the regional drinking water intake. The approximate number of samples per year is 120. At each sample collection, field staff collect water samples using 1 L sterile sampling bottles - containing sodium thiosulfate - and an extendable sampling pole to collect from a fast flowing portion of the river.

Field staff deliver samples in a cooler to the laboratory for bacteriology testing. Samples (25 L) are also collected and filtered for protozoa analysis for the presence of parasites. Filters from positive samples are shipped to British Columbia Centre for Disease Control (BCCDC) for enumeration (USEPA Method 1623) and molecular genotyping (*Cryptosporidium* and *Giardia*).

Human

C-EnterNet's human stool samples are collected through the existing passive surveillance system in Ontario. A passive surveillance system is based on case presentation to physicians in which a stool specimen is requested and then submitted to a private hospital or public health laboratory for testing of enteric pathogens that are reportable in the province. A physician can request three types of stool samples for analysis (i.e. parasite and ova, culture and sensitivity, or viral). For the case to be captured by the public health reporting system, either the laboratory or the physician must report the positive isolation directly to a local health authority, who will then report the case to the province, and subsequently that case is reported to the national level.

C-EnterNet works with the provincial public health laboratory system, three private laboratories and the regional hospital laboratory that serve Sentinel Site 1, to ensure that the positive isolations of reportable enteric pathogens that have been initially identified are forwarded to the Ontario Central Public Health Laboratory and the Public Health Agency of Canada's National Microbiology Laboratory in Winnipeg for subtyping.

An enhanced standardized questionnaire for endemic enteric disease cases was among the first new epidemiological tools to result from C-EnterNet's partnership with the local public health unit at its pilot sentinel site in the Region of Waterloo, Ontario. The questionnaire, originally based on the one used at the CDC FoodNet site at the Minnesota Department of Health, is designed to secure additional risk factor information for each enteric case, to support further epidemiological investigation and C-EnterNet's surveillance. The questionnaire data are manually entered into iPHIS and EpiData for further analysis.

2. Sample Preparation

Retail Meat

1. Meat packages are removed from the cooler and placed on a clean surface. The external meat package surface is wiped with sterile gauze and 70% ethanol. The meat package is then aseptically opened using sterilized scissors. The external surface of the wrapping is prevented from touching the meat samples to avoid possible contamination of meat from outside sources.

2. Sterile tongs are used to remove the meat samples from its package. Several pieces of meat are cut from different areas of the meat sample to make a representative sample weighing 50 g. This is placed into a prepared stomacher bag with selective enrichment broth specific to each of the following primary detection methods:

    a. 50 g of meat sample into 450 ml of Buffered Peptone Water (BPW) used for primary isolation of:
        - VTEC
        - *E. coli*/Coliform counts
        - *Salmonella* spp.

    b. 50 g of meat sample into 250 ml of Bolton Broth used for primary isolation of :
        - *Campylobacter* spp.

    c. 50 g of meat sample into 450 ml of *Listeria* Enrichment Broth (LEB) for primary isolation of:
        - *Listeria monocytogenes*

    d. 50 g of PORK meat sample into Luria Bertani-Bile Salt Irgasan (LB-BSI)
        - *Yersinia enterocolitica*

3. Samples are then massaged or stomached in a stomacher bag for 2 minutes.

Agriculture

1. Sample containers are placed on a clean surface, and then weighed.
2. Sample containers are then carefully opened to avoid spillage due to gas build-up during transport.
3. Sterile tongue depressors are used to mix samples gently and to transfer the samples into prepared stomacher bags with selective enrichment broth specific to each of the following primary detection methods:

Swine samples

a. 25 g of fecal sample into 100 ml of Buffered Peptone Water (BPW).
   5 ml of the BPW mix for primary isolation of:
   - *Yersinia enterocolitica*

b. More BPW is added to the remaining BPW mix to obtain a 1:10 dilution which is used for primary isolation of
   - Salmonella spp.
   - *E. coli* 0157:H7
   - *Campylobacter* spp.
   - Original fecal sample preservation

c. 1 g of fecal sample into 9 ml (1:10) of Bolton broth for primary isolation of:
   - *Campylobacter* spp.

Bovine and poultry samples

a. 10 g of fecal sample into 90 ml (1:10) of Buffered Peptone Water (BPW) used for primary isolation of:
   - Salmonella spp.
   - *E. coli* 0157:H7
   - *Campylobacter* spp.
   - Original fecal sample preservation

b. 1 g of fecal sample into 9 ml (1:10) of Bolton broth for primary isolation of:
   - *Campylobacter* spp.

4. Massage or stomach samples in a stomacher for 30 sec.

Untreated Surface Water

1. Bacterial analysis is started within 24 hrs of sample collection.
2. Using proper aseptic techniques, the appropriate amount of water is removed from the sample broth, filtered through a 0.45µm filter and the filter membrane is then placed into selective enrichment specific to each of the following primary detection methods:

   a. 500 ml water for *Yersinia enterocolitica*
   b. 500 ml water for *Salmonella* spp.
   c. 1000 ml water for *Campylobacter* spp.
   d. 500 ml water for *E. coli* 0157
   e. 100 ml water for *E. coli* enumeration

3. Laboratory Methodologies

3.1 Campylobacter

Retail Meat

Culture Detection

Samples are processed and enriched in Bolton Broth at 42°C. Following 48 hrs incubation at 42°C under microaerophilic conditions, samples are streaked onto selective agar plates. Suspect colonies are identified using microscopy. Typical *Campylobacter* colonies are confirmed and speciated using biochemical methods.

Agriculture

Culture Detection

Method # 1 (implemented in 2008)

Bovine and poultry fecal samples are processed by transferring 10 g of feces in 90 ml of Buffered Peptone Water (BPW). For porcine fecal samples 25 g of feces are transferred in 225 ml of BPW. Then 1 ml of the BPW mix is transferred in 9 ml of Hunt's Enrichment Broth (HEB) and incubated in a microaerophilic atmosphere at 35°C for 4 hrs. After this first incubation, 36 µL of sterile cefoperazone is added to the HEB. Tubes are then incubated in microaerophilic condition at 42°C for 20 to 24hrs. A loop of the incubated HEB is then plated onto modified cefoperazone charcoal deoxylate agar (mCCDA) agar plate. Plates are incubated at 42°C in microaerophilic conditions for 72 hrs. Suspect colonies are further tested using a second mCCDA plate, gram stain, dark field microscopy, and Mueller Hinton Blood (MHB) agar. Typical *Campylobacter* colonies are confirmed using catalase and oxidase tests. Hippurate hydrolysis and indoxyl acetate hydrolysis tests and cephalothin susceptibility tests are performed to speciate suspected *Campylobacter* into *C. jejuni, C. coli and C. lari*.

Method #2 (utilized in 2006-2007)

Samples are processed by transferring 1g of manure into 9 ml of Bolton broth. Samples are incubated at 42°C for 48 hrs in microaerophilic conditions, and plated onto modified cefoperazone charcoal deoxylate agar (mCCDA) agar plate. Plates are incubated at 42°C in microaerophilic conditions for 24 to 48 hrs. Suspect colonies are further tested using a second mCCDA plate, gram stain, dark field microscopy, and Mueller Hinton Blood (MHB) agar. Typical *Campylobacter* colonies are confirmed as in method #1.

Untreated Surface Water

Culture Detection

A 1000 ml river water sample is passed through a 0.45 µm filter membrane. The filter is placed in Bolton broth, incubated at 42°C for 48 hrs. The broth is then plated onto modified cefoperazone charcoal deoxylate agar (mCCDA) agar and incubated in microaerophilic conditions at 42°C for 2 to 5 days. Colonies from mCCDA plates are observed microscopically, and presumptive colonies plated on Mueller Hinton Blood

(MHB) agar and incubated under microaerophilic conditions at 37°C for 24 to 48 hrs. Presumptive *Campylobacter* colonies are then confirmed by Gram stain, catalase, oxidase, slide agglutination and polymerase chain reaction (PCR) tests. Species identification is determined using hippurate hydrolysis, indoxyl acetate hydrolysis and cephalothin/nalidixic acid susceptibility.

Molecular Detection

Quantitative PCR for *Campylobacter* identification in river water samples uses primers designed by Lubeck et al (2003) together with a probe designed by the University of Waterloo (UW) NSERC Chair in Water Treatment research group. The assay is specific for the 16S rRNA gene of *C. jejuni*, *C. coli* and *C. lari*.

Subtyping (Retail, Agriculture, Water)

Molecular Comparative Testing and PFGE

A subset of *Campylobacter* isolates are sent to the Public Health Agency of Canada's National Microbiology Laboratory (NML) for comparative molecular testing and PFGE testing.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *Campylobacter* is performed by the LFZ AMR Laboratory, Saint-Hyacinthe Unit using a broth microdilution Sensititre ™ plate (Trek Diagnostic System Ltd.). Isolates are prepared for testing by streaking colonies on Mueller Hinton agar containing 5% lysed horse blood and incubating them in a microaerophilic atmosphere for 24 hrs at 42°C. Isolated colonies are then mixed in 5 ml of Sensititre™ cation adjusted Mueller Hinton broth with TES buffer (CAMHBT) and adjust to 0.5 MacFarland. One hundred µl of this broth is then mixed with 11 ml of CAMHBT with lysed horse blood, which is then inoculated in a Sensititre™ Campy plate. After 24 hrs incubation in a microaerophilic atmosphere plates are read visually using Sensitouch™ giving minimum inhibitory concentrations (MIC) values. The following are the antimicrobials tested: azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin and tetracycline. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) breakpoints are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

Enumeration - Most Probable Number (MPN)

The Most Probable Number (MPN) method is used for retail samples that are positive for *Campylobacter*. This method estimates the number of *Campylobacter* species per millilitre or gram of sample using three tube MPN dilutions. Fifty grams of meat are stored under microaerophilic conditions at 4 ºC for the MPN test. Positive samples are then retrieved, and added to Hunts Enrichment Broth (HEB); processed and serial dilutions are prepared. Tubes are incubated at 36°C for 4 hrs and following incubation cefaperazone is added to each sample tube and incubated at 42°C for 24 hrs under microaerophilic conditions. Then, each sample is streaked onto selective agar plates. Suspect colonies are identified using microscopy. Typical *Campylobacter* colonies are confirmed using biochemical methods. MPN is calculated using the table from the FDA Bacteriological Analytical Manual (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

Human

LAB A

Specimens are collected and mixed with Enteric Plus transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto Karmali blood-free agar. Plates are incubated at 42°C for 48 hrs in microaerophilic conditions using a CO_2-N_2-O_2 percentage ratio of 10:80:10. Grey suspect colonies are sub-plated onto sheep blood agar for speciation testing. In some cases the original Karmali plate is used for biochemical testing. Isolates are subject to hippurate, catalase, TSI for H2S testing and cephalothin and nalidixic acid susceptibility testing.

LAB B

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample which is streaked for isolated colonies onto *Campylobacter* blood-free agar. Plates are incubated at 42°C for 48 hrs in microaerophilic conditions using a CO_2-N_2-O_2 percentage ratio of 10:85:5. Suspect colonies are subcultured onto *Campylobacter* blood-free agar or sheep blood agar for speciation testing. In some cases the original Campylobacter blood-free plate is used for biochemical testing. Isolates are subject to hippurate, oxidase, and gram stain testing.

LAB C

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample which is streaked for isolated colonies onto *Campylobacter* blood-free agar. Plates are incubated at 42°C for 48 to 72 hrs in microaerophilic conditions. Suspect colonies are subcultured onto a blood agar plate for speciation testing. In some cases the original *Campylobacter* blood-free plate is used for biochemical testing. Isolates are subject to hippurate, oxidase, and gram stain testing.

LAB D

Specimens are collected in a container with Cary-Blair transport media and mixed by the patient before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto *Campylobacter* blood-free agar. Plates are incubated at 42°C for 36 to 60 hrs in microaerophilic conditions using Oxoid Campygen pack®. Suspect colonies are subcultured onto a blood agar plate for speciation testing. Isolates are tested by hippurate, oxidase, and gram stain.

London Regional Public Health Laboratory-LRPHL

Stool samples are collected and mixed with Cary-Blair transport medium at the time of collection. Once received in the laboratory, a sterile swab is used to obtain a representative sample, which is streaked for isolated colonies onto *Campylobacter* Selective Medium (CSM). Plates are incubated at 42°C for 18 to 48 hrs in microaerophilic conditions using a CO_2-N_2-O_2 percentage ratio of 10:85:5. Suspect colonies are subcultured onto a TSA-blood agar plate for speciation testing. Isolates are tested for hippurate hydrolysis, oxidase and catalase production and gram stain reaction.

Toronto Regional Public Health Laboratory - TRPHL

Specimens are collected and mixed with Cary-Blair transport media before arriving at the Laboratory. Once at the laboratory, a portion of the specimen solution is inoculated in CSM, streaked for isolated colonies and incubated at 42°C for 18 to 48 hrs. A liquid enrichment broth (LEM) is also inoculated and incubated at the same temperature for 18 to 24 hrs. A loopful of the enrichment solution is streaked for isolated colonies onto Charcoal Selective Medium (CSM) agar and incubated at 42°C for 18 to 48 hrs. All inoculated media (plates and enrichment broth) are incubated under microaerophilic conditions using a CO_2-N_2-O_2 percentage ratio of 10:85:5. Suspect colonies are subcultured onto three Columbia Blood Agar plates and incubated for 18 to 48 hrs at different temperatures for speciation testing. The first Columbia Blood Agar plate is incubated at 25°C (aerophilic), the second plate at 35°C and the third plate at 42°C (microaerophilic). Isolates are subject to Hippurate hydrolysis, Oxidase production test, Gram stain testing, Catalase test, Indoxyl acetate hydrolysis test and Nalidixic acid/Cephalothin susceptibility testing. Additional tests to aid in identification may include: Nitrate Reduction Test, Urea hydrolysis, H_2S (TSI) production and tolerance to 1% glycine.

Subtyping (Human)

Campylobacter species isolates other than *C. jejuni and C. coli* are tagged as a C-EnterNet sample and sent to TRPHL for further testing. All *Campylobacter* isolates are tagged with a C-EnterNet label and further classified at TRPHL using antimicrobial susceptibility testing. Also, a sub sample of *Campylobacter* isolates are sent to NML for experimental comparative molecular subtyping.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *Campylobacter* is performed by TRPHL using the Etest method (AB BIODISK, Sweden). An appropriate number of isolated colonies are emulsified from the overnight growth in Mueller Hinton broth to produce 0.5 McFarland turbidity. Using a sterile swab, the suspension is spread onto the entire surface of Mueller Hinton agar with 5% lysed horse blood (MHLHB) plate. The inoculum is left to dry (10-15 minutes). Two different appropriate antimicrobial Etest strips are placed with forceps in an anti-parallel fashion on the swabbed agar surface of the MHLHB. The plates are then incubated at 37°C in a microaerophilic atmosphere for 48 hrs. The Etest is read at the point in which the zone of inhibition intersects the Etest strip.

Quality control: For every new lot of media and Etest strip package used, QC is performed using *C. jejuni* ATCC 33560, *E.coli* ATCC 25922 and *S. aureus* ATCC 29213.

The following are the antimicrobials tested: azithromycin, ciprofloxacin, chloramphenicol, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline. CIPARS breakpoints are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

3.2 Salmonella

Retail Meat

Culture Detection

Retail meat samples are screened by a modification of MFLP-75 primary isolation method of the *Compendium of Analytical Methods, Health Protection Branch, and Methods of Microbiological Analysis of Food, Government of Canada* is utilized. In order to minimize possible false negative results, a secondary enrichment followed by Modified Semi-solid Rappaport Vassiliadis (MSRV) plating. This method is applicable to viable, motile *Salmonella* species found in meat products.

Samples are pre-enriched in a non-selective broth (buffered peptone water) at 35°C One millilitre is transferred to 9 ml of tetrathionate brilliant green (TBG) broth. An MSRV plate is inoculated with TBG broth and incubated at 42°C for 24 hrs. Suspect colonies are further screened and confirmed using biochemical methods.

Agriculture

Culture Detection

A modification of MFLP-75 primary isolation method of the *Compendium of Analytical Methods, Health Protection Branch, Methods of Microbiological Analysis of Food, Government of Canada* is used for the detection of *Salmonella* in manure. *Salmonella* spp. can be detected in Modified Semi-solid Rappaport Vassiliadis (MSRV) media at a temperature of 42°C. In order to minimize possible false negative results, a secondary enrichment followed by MSRV plated is conducted in parallel.

Samples are pre-enriched in a non-selective broth (buffered peptone water) at 35°C. In parallel, 0.1ml of the pre-enrichment broth is inoculated into MSRV. Samples are incubated at 42°C for 24 to 72 hrs and examined at 24 hr intervals. Suspect colonies are further screened on MacConkey, Triple Sugar Iron (TSI) and Urea agar plates. Further testing of presumptive colonies is performed with the Kovac's Indole test and the slide agglutination test using the *Salmonella* O antiserum Poly A-I and V1.

Untreated Surface Water

Culture Detection

A 500 ml river water sample is passed through a 0.45 µm filter membrane. The filter is placed in a non-selective pre-enrichment broth (buffered peptone water) at 37°C for 24 hrs. Pre-enrichment broth is then added to selective enrichment broths (Rappaport Vassiliadis and Tetrathionate) and incubated at 42°C for 24 hrs. From each selective enrichment broth, samples are transferred to Xylose Lysine Tergitol 4 (XLT4) and Brilliant Green (BG) agar. Presumptive colonies are further screened using Lysine Iron Agar (LIA), catalase, oxidase and Gram stain reactions, and confirmed using the O.B.I.S. *Salmonella* kit (Oxoid).

Molecular Detection

The quantitative PCR method used in the UW NSERC Chair for Water Treatment lab uses the primers, probe and conditions for *Salmonella* as described by Rodriguez-Lazaro *et al* (2003), which is based on a Taqman assay targeting the *invA* gene of *Salmonella spp*. This method is specific for all *Salmonella* species, including *S*. Enterica subspecies I, II, III and IV, and *S*. Bongori (subspecies V).

Subtyping (Retail, Agriculture, Water)

Serotyping

Salmonella isolates are transferred on agar slants to the Public Health Agency of Canada - Laboratory for Foodborne Zoonoses (LFZ), Office International des Epizooties Reference Laboratory for Salmonellosis, *Salmonella* Typing Laboratory Guelph, Ontario for serotyping and phagetyping. Briefly, the serotyping procedure involves determining the O or somatic antigens of the *Salmonella* isolates using slide agglutination Ewing (1986). The H or flagellar antigens are identified using a microtechnique Shipp and Rowe (1980) that employs microtitres plates. The antigenic formulae of Le Minor and Popoff (2001) are used to identify and name the serovars.

Phagetyping

The standard phagetyping technique described by Anderson and Williams (1956) is followed. *Salmonella* Enteritidis isolates are phagetyped with typing phages obtained from the International Centre for Enteric Phage Typing (ICEPT), Central Public Health Laboratories, Colindale, UK Ward (1987) via the Enteric Diseases Program, National Microbiology Laboratory (NML), Public Health Agency of Canada, Winnipeg, Man. The phage typing scheme and phages for *Salmonella* Typhimurium developed by Callow (1959) and further extended by Anderson (1964) and Anderson et al. (1977) were obtained from the ICEPT via the NML. The *Salmonella* Heidelberg phagetyping scheme and phages were supplied by the NML (Demczuk et al., 2003).

PFGE Testing

PFGE testing is performed at the Laboratory for Foodborne Zoonoses, Guelph (*E. coli* Lab) according to standardized protocols developed by PulseNet USA and in use by PulseNet Canada. For *Salmonella*, two restriction enzymes, *XbaI* and *BlnI*, are used to obtain two separate patterns for each strain. The resulting tiff files are uploaded to the national database at the NML to obtain the national designation for the two separate patterns.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is performed by the CIPARS Guelph Laboratory at the Laboratory for Foodborne Zoonoses using the Sensititre® Automated Microbiology System (Trek Diagnositic Systems Ltd.). *Salmonella* isolates are prepared by streaking the cultures onto Mueller Hinton Agar for isolated colonies, and then for growth, and incubated for 18 to 24 hrs at 35°C. Each culture is prepared in Sensititre® demineralized water to a 0.5 McFarland standard and then inoculated into Sensititre® Mueller Hinton Broth with TES. The NARMS Gram negative plate with the CMV1AGNF panel is inoculated with the broth suspension and is incubated for 18 hrs at 37°C. Each plate is then read by the Sensititre® AutoReader to determine the minimum inhibitory concentration. The following are the antimicrobials tested: amikacin, ampicillin,

amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, trimethoprim/sulfamethoxazole. CIPARS breakpoints are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

Enumeration - Most Probable Number (MPN)

The Most Probable Number method is only used for retail samples positive for *Salmonella* from the detection method. This method estimates the number of *Salmonella* species per ml or g of sample using three tube MPN dilutions. Fifty millilitres of the original processed sample is stored for MPN testing to prepare serial dilutions. Tubes are incubated at 35°C for 24 hrs. After incubation, 1 ml of each tube is transferred to TBG broth, and 0.2ml of potassium iodine is added to each new tube and incubated at 42°C for 24 hrs. Two different selective media plates are streaked and incubated at 35°C for 24 hrs. Suspect colonies are confirmed with biochemical methods. The MPN is calculated using the MPN table from the FDA Bacteriological Analytical Manual (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

Human

LAB A

Specimens are collected and mixed with Enteric Plus transport media before arriving at the laboratory. Once at the laboratory, the Enteric Culture Manual method for *Salmonella* spp. is used. A sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto MacConkey agar and *Salmonella-Shigella* (SS) agar. Samples are incubated at 35°C for 18 to 24 hrs. Once at the laboratory, a portion of the homogenized sample is inoculated into Selenite F broth and incubated at 35°C for 12 hrs. The broth is subcultured onto an SS agar plate and incubated for 18 to 24 hrs. Suspect colonies are identified by non-lactose fermenting (NLF) colonies with or without H_2S production on the SS plate. Sheep blood agar or MacConkey agar is used for *Salmonella* spp confirmation with triple sugar iron (TSI), OPM, antisera agglutination and Microscan biochemical panel of tests. All *Salmonella* species are sent to TRPHL for species identification.

LAB B

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a portion of the specimen solution is inoculated in Selenite F broth and incubated at 35°C for 12 hrs. At the same time, a Hektoen plate is streaked using the original specimen incubated at 35° for 18 to 24 hrs. After incubation, using a sterile swab, the enrichment solution is streaked for isolated colonies onto Hektoen agar. Samples are incubated at 35°C for 18 to 24 hrs. Non-lactose fermenting (NLF) colonies with or without H_2S production on Hektoen are screened using urea, SIM and PYR. For confirmation testing, the original Hektoen plate, or a freshly subcultured sheep blood agar, is used for testing suspect isolates. Confirmation tests include Vitek 2 ID system and antisera latex agglutination test. All *Salmonella* species are sent to TRPHL for identification. Susceptibility testing is performed on all sterile sites, as per CLSI M100 using Viteck2 and Nalidixic KB. For stool samples, susceptibility testing is only performed on *S.* typhimurium and *S.* paratyphi. For other *Salmonella* species, susceptibility testing is done if the individual is less than 3 months of age or older than 65 year of age.

LAB C

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a portion of the specimen solution is inoculated in Selenite F broth and incubated at 36°C for 18 to 24 hrs. Using a sterile swab, the enrichment solution is streaked for isolated colonies onto Hektoen agar and MacConkey agar. Samples are incubated at 36°C for 48 hrs. Suspect colonies are identified by non-lactose fermenting (NLF) colonies with H_2S production. For confirmation testing, a freshly subcultured CLED plate is used. Confirmation tests include Urea, TSI, ONPG, SIM, Phoenix ID system and an agglutination test.

LAB D

Specimens are collected in a container with Cary-Blair transport media and mixed by the patient before arriving at the laboratory. Once at the laboratory, a portion of the specimen is inoculated in Selenite F broth and incubated at 37°C for 8 to 12 hrs. At the same time, a Hektoen plate is streaked using the original specimen solution and incubated at 37°C for 18 to 24 hrs. After incubation, using a sterile swab, the enrichment solution is streaked for isolated colonies onto Hektoen agar and incubated at 37°C for 18 to 24 hrs. Non-lactose fermenting (NLF) colonies with or without H_2S production are screened with TSI, SIM and Urea tests. Confirmation tests include Enterotube or the Vitek card automated ID system, PYR, Indole and an agglutination test performed from a blood agar plate. Isolates are forwarded to PHL for monotyping.

London Regional Public Health Laboratory- LRPHL

Stool samples are collected and mixed with Cary-Blair transport medium at the time of collection. Once received in the laboratory, a portion of the specimen is inoculated onto MacConkey Agar, XLD Agar, and *Salmonella-Shigella* (SS) Agar and streaked so as to obtain isolated colonies. A Selenite F broth is also inoculated and all media are incubated at 36°C for 18 hrs. After incubation the Selenite F enrichment broth is sub-cultured and streaked for isolated colonies onto SS agar. Suspect colonies are non-lactose fermenting (NLF) colonies with or without H_2S production. Typical NLF colonies are screened using TSI, ONPG-PAM and PYR strips. Confirmation is performed on TSA Blood Agar sub-cultures of screen positive isolates using API biochemical strips and slide agglutination using *Salmonella* polyvalent antiserum.

Toronto Regional Public Health Laboratory-TRPHL

Specimens are collected and mixed with Cary-Blair transport media before arriving to the Laboratory. Once at the laboratory, a portion of the specimen is inoculated in MacConkey Agar, XLD Agar and *Salmonella-Shigella* Agar (SS) and Selenite F Broth. The MacConkey Agar, XLD Agar and Selenite F broth are incubated at 35°C for 18 to 24 hrs. The SS Agar is incubated at the same temperature for 18 to 48 hrs. After incubation, a loopful of the Selenite F Broth is streaked for isolated colonies onto *Salmonella-Shigella* Agar (SS) and incubated for the same time and environmental conditions as the primary SS Agar. Suspect colonies are identified by non-lactose fermenting (NLF) colonies with or without H_2S production. Typical colonies are screened using TSI, ONPG-PA-M with indole strip, Lysine decarboxylase and PYR test. For confirmation, a freshly subcultured Blood Agar plate, MacConkey plate and motility plate (Gard plate) are used for testing isolates. Comfirmation tests include an agglutination test (for somatic and flagellar antigens) and a biochemical panel of tests.

Subtyping (Human)

As part of C-EnterNet's enhanced public health passive surveillance system, all isolates are submitted to the Toronto Regional Public Health Laboratory (TRPHL) for serotyping, phagetyping where appropriate, antimicrobial susceptibility testing and pulse-field gel electrophoresis (PFGE).

Serotyping

The serotyping method is based on the methods of Farmer (2003), Ewing (1986) and Popoff (2001).

Phagetyping

Phagetyping is performed at the NML, Winnipeg, in the Enteric Diseases Program lab.

PFGE testing

One-day (24 to 48 hrs) Standardized Laboratory Protocol for the Molecular Subtyping of Enteric Pathogens by Pulsed Field Gel Electrophoresis (PFGE) and Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed Field Gel Electrophoresis (Center for Disease Control and Prevention Manual – PNL 05) are followed for the PFGE testing of *Salmonella* isolates.

Antimicrobial susceptibility Testing

Salmonella isolates are forwarded to the National Laboratory for Enteric Pathogens (NLEP) for antimicrobial susceptibility testing, according to the Sensititre®: automated micro-dilution AMR test.

The following are the antimicrobials tested: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole. CIPARS breakpoints are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

3.3 Pathogenic *E. coli*

Retail Meat

Detection Assay

All meat samples are screened for the presence of verocytotoxins using the verocell assay. This method is based on the MFLP-89 VTEC detection in the *Compendium of Analytical Methods, Health Protection Branch, Methods of Microbiological Analysis of Food, Government of Canada*. Fifty grams of meat sample is processed in 450 ml buffered peptone water (BPW) incubated at 35°C for 24 hrs and 1 mL is transferred into 9 ml of MacConkey broth. After incubation 100µl is transferred into brain heart infusion (BHI) broth and further incubated at 35°C for 6 hrs. The verocell assay is prepared and incubated at 35°C for 48 hrs. Microtitre plates are examined microscopically for cytotoxic activity. Confirmation of positive isolates is performed using biochemical methods.

E. coli / Coliform: Detection and Counts

All meat samples are tested using the 3M Petrifilm™ *E. coli* / Coliform count plate. Meat samples are processed in 450 ml BPW; one millilitre is transferred onto Petrifilm ™ and incubated at 35°C for 48 hrs. Bacterial colonies are counted and CFU/ml is calculated.

Agriculture

Culture Detection

All fecal samples are tested for the presence of *E. coli* 0157 using the Dynabead™ Anti-*E. coli* 0157 test. This method is based on the MFLP-90 and MFLP-80 Identification of *E. coli* 0157 by Dynabead™ anti-*E. coli* 0157 method of the *Compendium of Analytical Methods, Health Protection Branch, Methods of Microbiological Analysis of Food, Government of Canada*. Ten grams of fecal sample is pre-enriched in 90 ml BPW, homogenized and 1 ml is transferred to a 9 ml Brilliant Green Bile broth selective enrichment. The sample is incubated at 35°C for 6 hrs on a shaker. After incubating, 0.1 ml of sample is added to Dynabeads and further incubated at 25°C for 6 hrs on a shaker. The enrichment mixture is prepared for immunomagnetic separation (IMS) and plated onto three selective media (Rainbow agar, Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) agar and CHROMagar O157). Samples are incubated at 35°C for 24 hrs and examined. Suspect colonies are further tested for a Tryptic Soy Blood agar slant using MacConkey broth and EC broth +MUG. Positive isolates are confirmed with slide agglutination using 0157 antisera.

PCR Testing for confirmation of: H7

Presumptive *E. coli* 0157:H7 isolates are sent to The Escherichia coli Laboratory, (OIE reference laboratory), Faculté de médecine vétérinaire of University of Montreal for: H7 testing using polymerase chain reaction (PCR).

Untreated Surface Water

Culture Detection

A 500 ml river water sample is passed through a 0.45 µm filter membrane. The filter is

then placed in EC broth, and incubated at 37°C for 4 hrs. Novobiocin (20 mg/L final) is added, and the broth is further incubated at 42°C for 24 hrs. After incubation, 1 mL of broth is processed using Dynabead anti-*E. coli* O157 immunomagnetic separation method (Dynal) and plated onto two selective media, Cefixime Tellurite Sorbitol MacConkey (SMAC-CT) agar and Chromagar O157 (Oxoid). Plates are incubated at 42°C and examined at 24 and 48 hrs. Suspect colonies are further tested using the *E. coli* 0157 Dry Spot test kit (Oxoid). Confirmation is conducted using a Gram stain, an oxidase test, and Biolog identification.

Molecular Detection

The assays for *E. coli* O157:H7 include the stx-1 primer/probe set described by Sharma and Dean-Nystrom (2003); the stx-2 primer/probe set described by Iijima et al. (2004), and the eae primer/probe set described by Ram and Shanker (2005). The eae assay specifically detects the O157:H7 serotype, by targeting the eae gene encoding intimin. The primer/probe combinations described by Iijima et al. (2004) and Ram and Shanker (2005) were found to have the same specificity as those described by Sharma and Dean-Nystrom (2003), but the assays result in a better detection limit using positive control strains.

Subtyping (Retail, Agriculture, Water)

PFGE Testing

PFGE is performed at the Laboratory for Foodborne Zoonoses, Guelph (*E. coli* Lab) according to standardized protocols developed by PulseNet USA and in use by PulseNet Canada. For *E. coli*, only one restriction enzyme, *XbaI*, is used to obtain the PFGE pattern. The resulting tiff files are uploaded to the national database located at NML in Winnipeg to obtain the national designation for the two separate patterns.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is performed using a broth microdilution Sensititre™ ARIS Automated Microbiology System (Trek™ Diagnostic Systems Ltd.). Isolates are prepared for testing by streaking specimens onto Mueller Hinton agar. Isolated colonies are tested to a standard panel of antimicrobials identified by the National Antimicrobial Resistance Monitoring System (NARMS). Minimum inhibitory concentrations (MIC) values from the NARMS CMV7CNCD panel of antimicrobials are identified by inoculating the sensititre plate wells with a 0.5 McFarland standard bacterial growth suspension in Mueller-Hinton broth. After 18 hr incubation, plates are read by the Sensititre plate reader. The following are the antimicrobials tested: amikacin, ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, trimethoprim/sulfamethoxazole. CIPARS breakpoints are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

Human

LAB A

Specimens are collected and mixed with Enteric Plus transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto Chromogenic O157 agar. Samples are incubated at 35°C for 18 to 24 hrs. Plates are examined for typical *E. coli* O157 colony morphology on both agar plates. One suspected colony is subcultured to a sheep blood agar if needed. Presumptive colonies are tested using the O157 Antisera and Microscan biochemical panel of tests. Confirmation of H7 is completed by TRPHL.

LAB B

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto Sorbitol MacConkey (SMAC) agar. Samples are incubated at 35°C for 24 hrs. Plates are examined for typical *E. coli* O157 colony morphology and one suspected colony is subcultured onto a blood agar plate if needed. Presumptive colonies are tested using the O157 Antisera, Latex agglutination and the Vitek 2 ID system. Isolates are forwarded to TRPHL.

LAB C

Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample which is streaked for isolated colonies onto Sorbitol MacConkey (SMAC) agar and Chromogenic agar. Samples are incubated at 36°C for 18 to 24 hrs. Plates are examined for typical *E. coli* O157 colony morphology on both agar plates. One suspected colony is picked from each plate and subcultured to a blood agar if needed. Presumptive colonies are tested using the Microgen O157 Antisera agglutination test and the Phoenix automated ID system.

LAB D

Specimens are collected in a container with Cary-Blair transport media and mixed by the patient before arriving in the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto Sorbitol MacConkey (SMAC) agar. Samples are incubated at 37°C for 18 to 24 hours. Plates are examined for typical *E. coli* non-sorbitol fermenting colony morphology. One suspected colony is subcultured to a fresh SMAC agar plate or blood agar if needed. Presumptive colonies are tested using the O157 Antisera agglutination test and the Vitek automated ID system. Isolates are forwarded to TRPHL for 'H' agglutination.

London Regional Public Health Laboratory-LRPHL

Stool samples are collected and mixed with Cary-Blair transport medium at the time of collection. Once received in the laboratory, a sterile swab is used to obtain a representative sample, which is streaked for isolated colonies onto Sorbitol MacConkey (SMAC) agar. Samples are incubated at 35°C for 18 to 24 hrs. Plates are examined for typical *E. coli* O157 non-sorbitol fermenting colony morphology. Suspected colonies are subcultured to fresh TSA-blood agar. Presumptive colonies are tested using the O157 Antisera agglutination test, TSI, ONPG-PAM and the API20E test.

Toronto Regional Public Health Laboratory-TRPHL

Specimens are collected and mixed with Cary-Blair transport media before arriving to the laboratory. Once at the laboratory, a portion of the specimen solution is inoculated in Sorbitol MacConkey Agar (SMAC), Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC) and MacConkey Broth. Inoculated media are incubated at 35°C for 18 to 24 hrs. After incubation, a loopful of the MacConkey Broth is streaked for isolated colonies onto Sorbitol MacConkey Agar (SMAC) and CT-SMAC Agar. Plates are incubated at 35°C for 18 to 24 hrs. Plates are examined for typical E. coli O157 non-sorbitol fermenting colony morphology and screened using TSI, ONPG-PA-M with indole strip, Lysine decarboxylase and Blood Agar plate. Presumptive colonies are tested using the E. coli O157 antisera for slide agglutination test. For confirmation, a freshly subcultured Blood Agar plate, SMAC plate and MacConkey plate are used for testing isolates. Confirmation tests include a series of tube agglutination for both the somatic (O157) and flagellar (H7) antigens and a biochemical panel of tests.

Subtyping (Human)

Once confirmed positive, one isolate from each positive specimen is tagged as a C-EnterNet sample and sent to TRPHL for H7 typing and PFGE testing.

PFGE testing

The one-day (24 to 48 hrs) Standardized Laboratory Protocol for the Molecular Subtyping of Enteric Pathogens by Pulsed Field Gel Electrophoresis (PFGE) and Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed Field Gel Electrophoresis (Center for Disease Control and Prevention Manual – PNL 05) protocols are followed.

3.4 Yersinia

Retail Meat

Culture Detection

Screening for *Yersinia enterocolitica* is performed on pork samples. Fifty grams of pork meat is processed in 450 ml of Luria Bertani-Bile Salt Irgasan (LB-BSI) broth, incubated at 12°C for 24 hrs and inoculated with 5ml of Irgasan solution. Samples are incubated at the same temperature for 48 hrs longer. Following incubation, LB-BSI is streaked onto selective agar plates and incubated at 30°C for 20 hrs. Suspect colonies are further screened and confirmed using biochemical methods.

Agriculture

Culture Detection

Screening for *Yersinia enterocolitica* is performed on swine manure samples. Twenty five grams of swine fecal sample is processed in 100 ml of BPW broth, homogenized and 5 ml is transferred to 100 ml of Irgasan Tircacillin Broth (ITCB). The sample is incubated at 24°C for 72 hrs. Following incubation, ITCB is streaked onto Cufsulodin-Irgasan Novobiocin (CIN) agar and incubated at 28°C for 48 hrs. Suspected colonies are further tested using TSI, MacConkey and Urea agar. Confirmation for *Yersinia enterocolitica* is performed using the API20E test. One presumptive isolate is sent to TRPHL for serotyping and PFGE and to LFZ AMR Laboratory, Saint-Hyacinthe Unit for antimicrobial susceptibility testing.

Untreated Surface Water

Culture Detection

A 500 ml river water sample is passed through a 0.45 µm filter membrane. The filter is added to modified Trypticase Soy Broth (mTSB) and incubated at 12°C for 4 hrs. Irgasan (4 mg/L final) is added, and the enrichment broth further incubated at 12°C for 48 hrs. Enrichment broth is plated onto Cefsulodin-Irgasan-Novobiocin (CIN) agar. Enrichment broth is also treated with a KOH/NaCl solution, and plated onto CIN agar. CIN plates are incubated at 28°C for 24 hrs. Presumptive isolates are further tested using catalase, oxidase, Gram stain, MacConkey agar, Simmon's citrate agar, Kliger's iron agar, Christensen's urea agar, and Biolog identification.

Molecular Detection

The quantitative PCR assay for *Yersinia enterocolitica* is described by Bhaduri et al. (2005) and targets the ail gene.

Subtyping (Retail, Agriculture, Water)

Serotyping, Biotyping and PFGE

Presumptive *Yersinia enterocolitica* isolates from the retail, agriculture and water samples are sent to TRPHL for speciation, serotyping and biotyping. The serotyping and biotyping methods for all *Yersinia* isolates are based on the methods of Wauters (1981), Wauters (1991), Bottone (1997), Bockemuhl (2003) and Aleksic (1984).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is performed on the *Yersinia enterocolitica* isolates by LFZ AMR Laboratory, Saint-Hyacinthe Unit using a broth microdilution Sensititre™ ARIS Automated Microbiology System (Trek™ Diagnostic Systems Ltd.). Minimum inhibitory concentrations (MIC) values from the NARMS CMV7CNCD panel of antimicrobials are identified by inoculating the sensititre plate wells with a 0.5 McFarland standard bacterial growth suspension in Mueller-Hinton broth. After 18 hrs incubation, plates are read by the Sensititre plate reader. The following are the antimicrobials tested: amikacin, ampicillin (Yersinia is always resistant to Ampicillin), amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, trimethoprim/sulfamethoxazole. CIPARS breakpoints for *E. coli* are used to determine resistance profiles and can be found at: http://www.phac-aspc.gc.ca/cipars-picra/analys_e.html.

Enumeration - Most Probable Number (MPN)

The Most Probable Number method is used for retail pork samples positive for *Yersinia enterocolitica* from the initial primary detection method. The MPN method estimates the number of *Yersinia* species per millilitre or gram of sample using three tube dilutions. Fifty millilitres of the original processed sample is stored for subsequent MPN testing and used to prepare serial dilutions. Tubes are incubated at 12°C for 24 hrs. After incubation, 100µl of an Irgasan solution is added to each tube and they are incubated at 12°C for 48 hrs. Then each tube is streaked onto selective agar plates and incubated at 30°C for 20 hrs. Suspect colonies are further screened and confirmed using biochemical methods. MPN is calculated using the MPN table in the FDA Bacteriological Analytical Manual (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

Human

LAB A

Specimens are collected and mixed with Enteric Plus transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto SS agar and incubated at 35°C for 18 to 48 hrs. Presumptive *Yersinia* spp. identified as small NLF colonies is further tested using Microscan, TSI, OPM and motility testing for confirmation. All *Yersinia* species are sent to TRPHL for typing.

LAB B

Yersinia enterocolitica testing is only performed routinely for children <12yrs of age. Specimens are collected and mixed with Cary-Blair transport media before arriving at the

laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample which is streaked for isolated colonies onto *Yersinia* selective agar (CIN) and incubated at 30°C for 24 hrs. Presumptive *Yersinia* spp. are identified as small, dark pink "bull's eye" colonies, and confirmation is done with Triple Sugar Iron (TSI) and the Vitek 2 ID system. All *Yersinia* species are sent to TRPHL for typing.

LAB C

Yersinia enterocolitica testing is only performed routinely for children <12yrs of age. Specimens are collected and mixed with Cary-Blair transport media before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample which is streaked for isolated colonies onto *Yersinia* selective agar (CIN) and incubated at room temperature for 48 hrs. Presumptive *Yersinia* spp. are identified as small, dark pink "bull's eye" colonies, and confirmation is done with TSI, Urea, SIM and the Phoenix automated ID system.

LAB D

Specimens are collected in a container with Cary-Blair transport media and mixed by the patient before arriving at the laboratory. Once at the laboratory, a sterile swab is used to obtain a homogenized sample, which is streaked for isolated colonies onto *Yersinia* selective agar (CIN) and incubated at 30°C for 24 hours. Presumptive *Yersinia* spp. are identified as small, dark pink "bull's eye" colonies, and confirmation is done with Catalase, Salicin and the Vitek automated ID system. Isolates are forwarded to TRPHL for typing.

London Regional Public Health Laboratory-LRPHL

Yersinia enterocolitica testing is only performed routinely for children <12yrs of age. Stool samples are collected and mixed with Cary-Blair transport media at the time of collection. Once received in the laboratory, a sterile swab is used to obtain a representative sample, which is streaked for isolated colonies onto Cefsulodin Irgasan Novobiocin (CIN) Agar medium and incubated at 25-32°C for 18 to 48 hrs. Presumptive *Yersinia* spp. appear as small, dark pink "bull's eye" colonies. Screening is performed using TSI, ONPG-PAM, and confirmation is performed on TSA Blood Agar isolates using API20E test strips. *Yersinia enterocolitica* may also be isolated from *Salmonella*/*Shigella* (SS) Agar.

Toronto Regional Public Health Laboratory-TRPHL

Specimens are collected and mixed with Cary-Blair transport media before arriving to the laboratory. Once at the laboratory, a portion of the specimen is inoculated in Cefsulodin-Irgasan-Novobiocin Agar (CIN) and streaked for isolated colonies. The CIN plate is incubated at 25-32°C for 18 to 24 hrs. Presumptive *Yersinia* spp. are identified as small, dark pink "bull's eye" colony and screened using TSI, ONPG-PA-M with indole strip, Lysine decarboxylase and Blood Agar plate. For confirmation, a freshly subcultured Blood Agar, MacConkey and Congo-Red Magnesium Oxalate Agar (CRMOX) are used for testing isolates. Confirmation tests include slide agglutination and a biochemical panel of tests.

Subtyping (Human)

Yersinia enterocolitica isolates are tagged with a C-EnterNet label and submitted to TRPHL for sero - and biotyping. The serotyping and biotyping methods for all *Yersinia*

isolates are based on the methods of Wauters (1981), Wauters (1991), Bottone (1997), Bockemuhl (2003) and Aleksic (1984).

Antimicrobial susceptibility testing is not performed on the human *Yersinia* isolates.

3.5 Listeria

Retail Meat

Culture Detection

Samples are processed in *Listeria* Enrichment Broth (LEB) and incubated at 30°C for 24 and 48 hrs. This method is based on the MFHPB-30 *Listeria monocytogenes* detection in the *Compendium of Analytical Methods, Health Protection Branch, Methods of Microbiological Analysis of Food, Government of Canada*. After enrichment incubation, 0.1ml of LEB is transferred to Modified Fraser Broth (MFB) and incubated at 35°C for 48 hrs. MFB inoculum is streaked onto selective agar plates, incubated at 30°C for 24 to 48 hrs Suspect colonies are screened and confirmed using biochemical methods and microscopy.

Agriculture

Culture Detection

Samples are processed by transferring one part fecal sample into nine parts *Listeria* Enrichment Broth (LEB) and incubated at 30°C for 24 to 48 hrs. This method is based on the MFHPB-30 *Listeria monocytogenes* detection in food method of the *Compendium of Analytical Methods, Health Protection Branch, Methods of Microbiological Analysis of Food, Government of Canada*. The complete method is performed for LEB broth after 24 hrs incubation and separately for 48 hrs incubated broth. After enrichment incubation, 0.1ml of LEB is transferred to Modified Fraser Broth (MFB) and incubated at 35°C for 48 hrs. MFB inoculums is streaked onto Oxford Agar (OXA Palcam agar and Rapid'L.mono agar plates (chromogenic agar) and incubated at 30°C for 24 to 48 hrs and suspect colonies are further tested on MHB agar. Biochemical tests are performed using the method MFHPB-30 list of tests as a guideline and include motility testing, mannitol, rhamnose, xylose sugar utilization and a Gram stain. Biochemical testing plates are incubated at 35°C for 48 hrs. Confirmation testing is done using slide agglutination with *Listeria* antiserum.

Subtyping (Retail, Agriculture)

Confirmation typing and PFGE

Listeria monocytogenes isolates are further tested using the PFGE methodology outlined by Graves (2001).

Enumeration- Most Probable Number (MPN)

The Most Probable Number method is only used for retail samples positive for *Listeria monocytogenes*. This method estimates the number of *Listeria* species per ml or g of sample using three tube MPN dilutions. Fifty millilitres of the original processed sample is stored for MPN testing and used to prepare serial dilutions. Tubes are incubated at 30°C for 48 hrs. After incubation, 100µl from each tube is transferred to 10 ml of MFB and incubated at 35°C for 24 hrs. Then, MFB inoculums are streaked onto selective agar plates and incubated at 30°C for 24 to 48 hrs. Suspect colonies are screened and confirmed using biochemical methods and microscopy.

MPN is calculated using the MPN from the FDA Bacteriological Analytical Manual (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

3.6 Giardia and Cryptosporidium

Agriculture

During the first year of sampling each commodity is tested for the presence of *Giardia* and *Cryptosporidium*. Fecal floats are prepared for DNA extraction and microscopy. A Two-step nested PCR analysis is conducted for *Giardia* and *Cryptosporidium*. For *Giardia*, round one primers used are Gia2029 and Gia2150c, followed by RH4R and RH11F in round two of testing. For *Cryptosporidium*, the round one primers are 1325R and 1325R, followed by 826F and 826R for round two of testing.

Untreated Surface Water

Microscopic Detection

Testing for *Giardia* and *Cryptosporidium* is performed on water samples twice per month by the BC Centre for Disease Control (BCCDC) Laboratory Services. A 25 litre river water sample is passed through a Filta-max filter (IDEXX) in the field, and the filter is then shipped to BCCDC and processed for parasite enumeration using USEPA Method 1623.

Molecular Typing

Cryptosporidium genotyping is done by removing the coverslips and mounting media from positive slides. Material is then removed from the entire well using four rinses with lysis buffer and scraping with a pipette tip. DNA is extracted by repeated freeze-thawing of oocysts and purified using the QIAGEN DNA Micro Kit. Nested PCR is done by targeting the small ribosomal subunit RNA (18S rRNA) gene with primary and secondary primers as described by Xiao *et al*. (1999). DNA sequencing of the secondary PCR product is used to confirm *Cryptosporidium* species/genotypes. Bidirectional sequence analysis is performed using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

Human

Gamma-Dynacare Medical Laboratories

Stool concentrates are prepared for microscopic identification by placing a drop of the concentrate on a glass slide, diluting it with saline or formalin if necessary, and placing a cover slip over top of the drop. Stool received unfixed are stained with modified acid fast/Iron Haematoxylin. The entire slide is scanned on low power (10x) looking for ova and larvae. Cysts and trophozoites are scanned using a 50x oil immersion objective. A 100x oil immersion objective scan is used to identify smaller cysts and trophozoites i.e. *Enteromonas hominis*. All organisms are measured with a calibrated ocular micrometer.

Toronto Regional Public Health Laboratory-TRPHL

SAF preserved fecal specimens are processed by the following method for ova and parasites including *Giardia* and *Cryptosporidium*

Routine processing of SAF-preserved fecal specimen involves the following steps: Firstly, an initial processing is carried out in which a portion of the specimen is filtered through gauze and washed twice with physiological saline to produce a sediment. From this sediment, a small amount is retrieved and mixed with gelatin adhesive to make a smear for iron-haematoxylin staining. Then, the remainder of the sediment is mixed with formalin, then di-ethyl ether and centrifuged to produce a concentrate after the plug, the aqueous and organic material are decanted. The stained smear and concentrate are used for the recovery of most helminth eggs/larvae and protozoan cysts/trophozoites including *Giardia*. In addition to these two items, directly from the specimen two air-dried smears are prepared. One is stained with the fluorescent stains auramine-rhodamine for the detection of *Cryptosporidium* and other coccidians. The second smear is stained with modified trichrome stain for microsporidia spores. Additional testing such as direct wet mounts and other concentration procedures are also performed depending on the parasite suspected.

3.7 Viruses

Agriculture

During the first year of sampling each commodity is tested for the presence of rotavirus and norovirus. Fecal samples are filtered and prepared for PCR analysis using the one-step RT-PCR reactions. Presumptive positives are gel purified and sequenced on the tested strands.

Fecal samples are suspended at 5% w/v in 0.9% NaCl, vortexed briefly and clarified through a combination glass fiber/PVDF 0.22 µM filter. RNA is extracted from a 140 µl sample of the resulting filtrate using the QIAamp viral RNA extraction kit according to the manufacturer's recommendations. Total RNA is extracted from 25 g meat samples using Tri reagent and Dynabeads coated with oligo-dT as described by Kinglsey and Richards (2001).

The RNA was used as a template for one-step RT-PCR reactions using Monroe region B primers (Anderson et al., 2003) or Rota1 and End9 primers (Gouvea et al., 1990; Jean et al., 2002) and the Qiagen One Step RT-PCR kit. 213 bp (norovirus) and 268 bp (rotavirus) amplicons are considered to be presumptively positive. They are gel purified using a QIAquick gel extraction kit and sequenced on both strands by DNA Landmarks.

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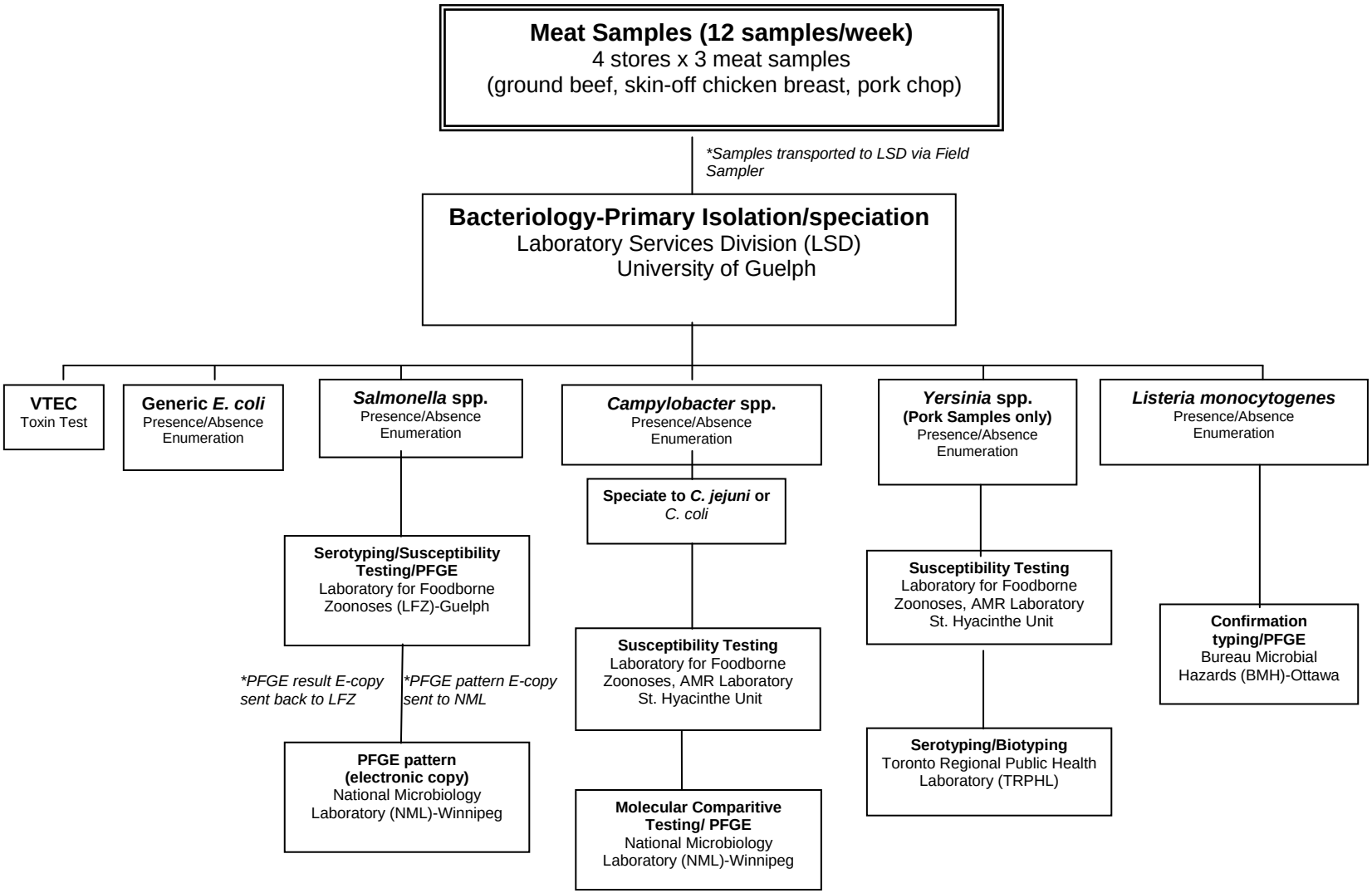
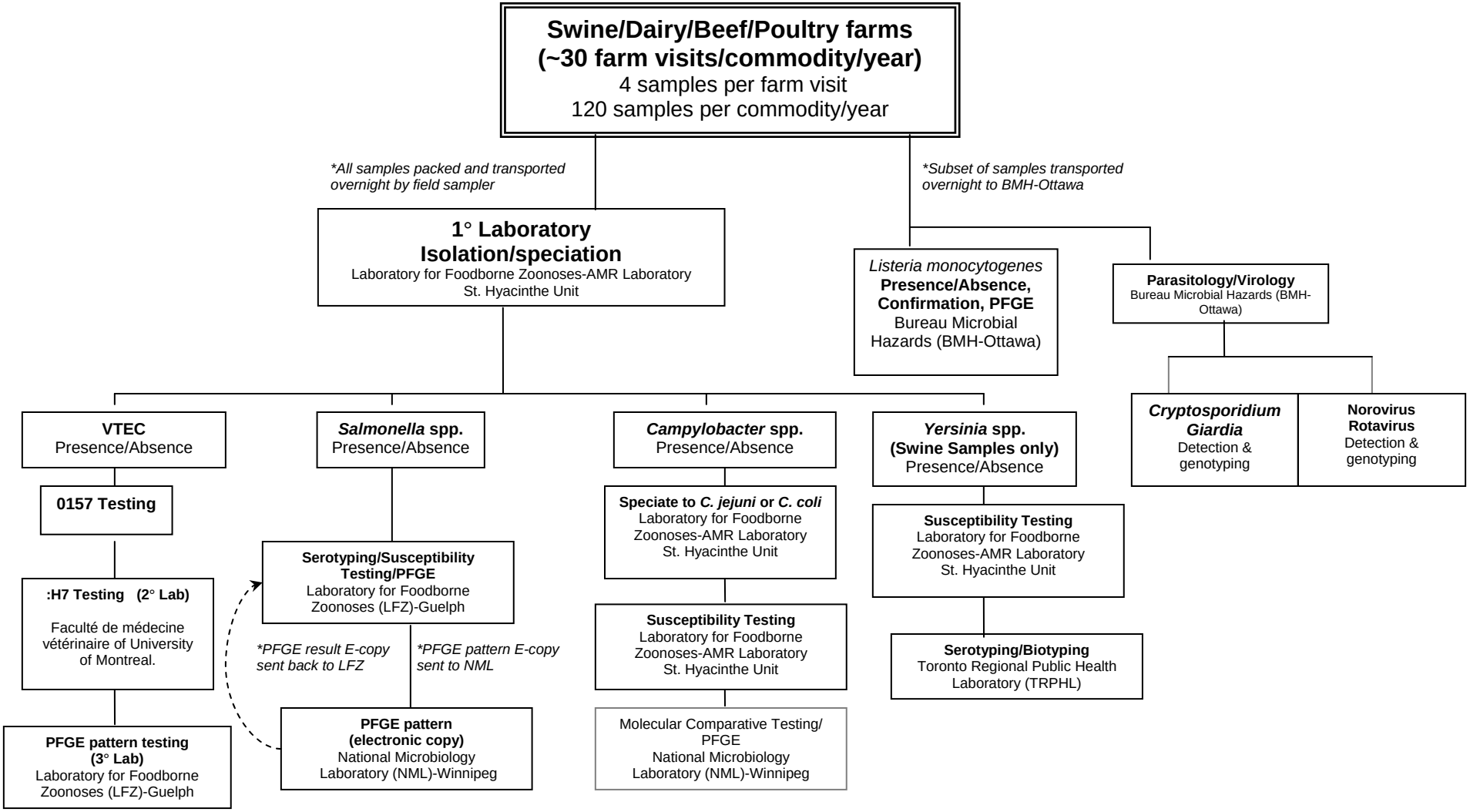
Appendix

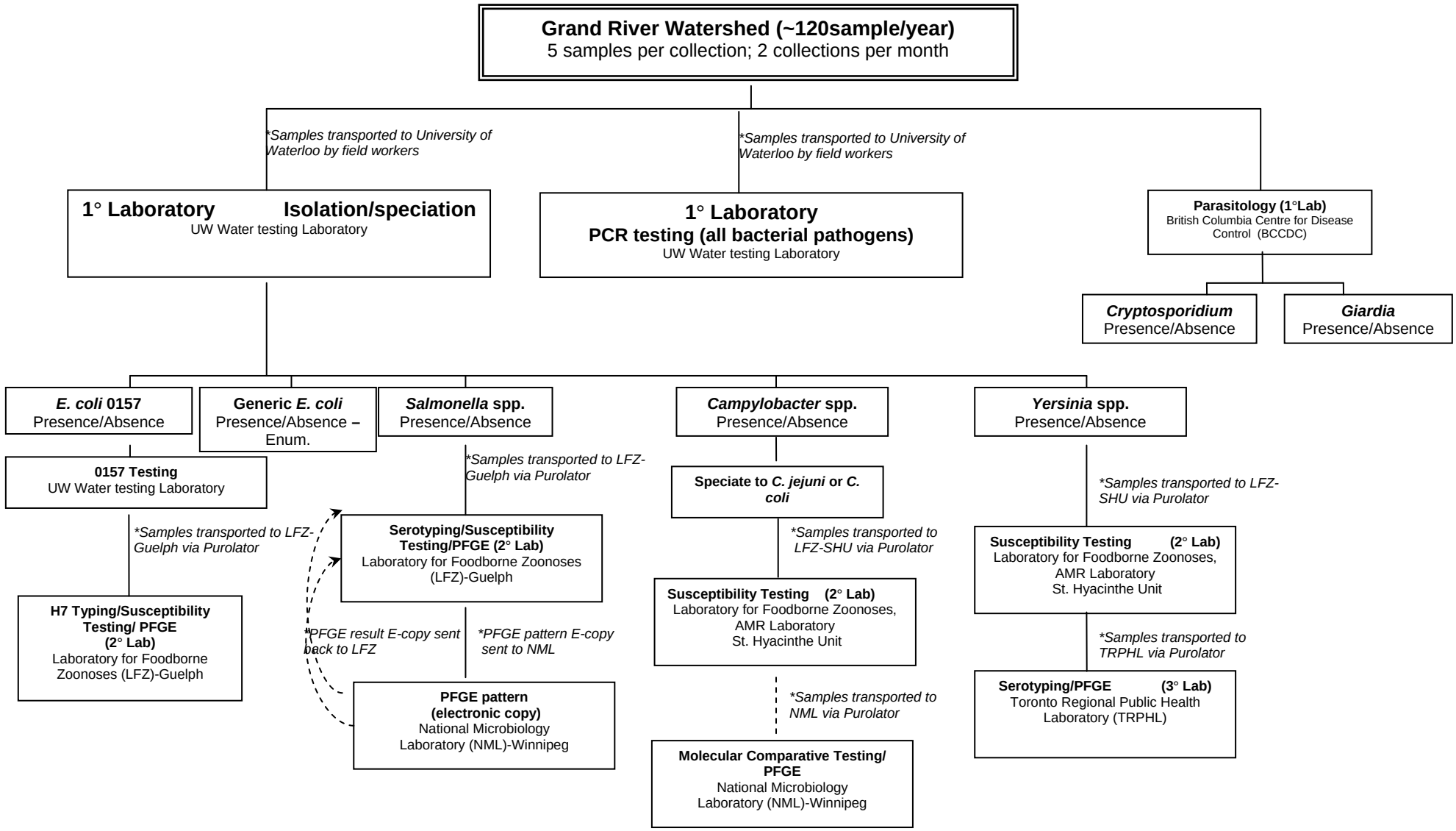
C-EnterNet Retail Sample Collection Overview



C-EnterNet Agriculture Sample Collection Overview



C-EnterNet Watershed Sample Collection Overview



C-EnterNet Human Sample Collection Overview