APPENDIX C

Canadian Nosocomial Infections Surveillance Program

Laboratory Surveillance Protocol

Surveillance for *Clostridium difficile* associated diarrhea (CDAD) within Acute-Care Institutions

March 1, 2007-April 30, 2007

Version 3, Feb. 13, 2007

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Introduction

Clostridium difficile associated diarrhea (CDAD) is the most frequent cause of nosocomial infectious diarrhea in industrialized countries[1-3], affecting more than 300,000 hospitalized patients yearly in the United States[4, 5]. Clinical manifestations range from asymptomatic colonization, to severe diarrhea, pseudomembranous colitis (PMC), toxic megacolon and death[6]. Recently, several hospitals in Quebec have experienced dramatic increase in the incidence, severity and relapses associated with CDAD[7-10]. This finding prompted the Canadian Nosocomial Infection Surveillance Program (CNISP) to conduct a six month study that was carried out on stool samples collected from Nov. 1, 2004 to April 30, 2005. The results of that study are currently being analysed to:

1. Determine the burden of illness on patients and health care facilities through nosocomial CDAD acquisition.
2. Collect patient-specific non-nominal information to describe the demographics and epidemiology of those who are acquiring CDAD.
3. Determine the antimicrobial susceptibility profile of *C. difficile* strains.
4. Characterize the molecular subtypes of *C. difficile* strains across Canada and analyse data to compare to epidemiological data to identify epidemic strains and/or hypervirulent strains.
5. To determine prospectively if increase in outcomes (mortality and morbidity associated with CDAD) compared to 1997 data[11].

In 2006 CNISP decided to carry out yearly ongoing CDAD surveillance at all its sites including laboratory studies on stool samples collected for a two month period.

Laboratory Protocol for the CNISP Site

Numerous laboratory protocols exist for the identification of *C. difficile* from stool. In Canada, almost all clinical laboratories report a case of *C. difficile* associated diarrhea (CDAD) by the identification toxins present in the stool either by cytotoxic tissue culture assays or commercial tests bases on immunologic methods and bypass the isolation of the organism. However, to accomplish objectives 3 and 4 stated above, a *C. difficile* isolate will be required for the study. Since most laboratories do not have the capacity/funding to carry out this procedure, we have devised a protocol, which should minimize the burden at the individual laboratories.

Each laboratory will be sent:

1). Pre-labeled 2 ml cryovials in storage boxes.

2). An equal number of empty storage boxes for storing the vials at -20^{o}C.

3). A reporting form to record laboratory information which will include a column with numbers corresponding to the pre-labeled vials i.e. **CHEC numbers**.

Methodology

The study will take place over a two month period from March 1, 2007-April 30, 2007.

1). Each laboratory will use their current laboratory procedures to diagnose stools from diarrhetic patients (potentially CDAD) for the presence *C. difficile* toxin(s).

2). Potential CDAD stools should be held at 4 ^{o}C degrees for no longer than 48 h while the confirmatory tests are conducted.

3). Once a stool specimen is confirmed as positive for *C. difficile* toxin(s), remove a prelabeled cryovial (has a CHEC # on it) from the supplied box (can be stored on the bench) and dispense 2 ml of the watery stool into the vial.

4). ***Immediately*** store the vial containing the stool sample ***at -20 ^{o}C*** degrees in a similar storage box (we supply).
Note: It is extremely important to freeze the sample as soon as possible. The viability of C. difficile decreases over time in stool even when stored at 4 ^{o}C. It may become difficult to isolate a C. difficile from a stool which has been held longer than 48 h at 4^{o}C.

5). Record the information requested on the supplied form. You may include your Laboratory Identification Number (LIN), if there is one, for the specimen. ***Note: the LIN and/or CHEC # will be used to match this specimen with the corresponding patient information collected by the hospital infection control team. It is imperative that the number you record can be cross-referenced to the patient number.***

6). Sites with a large number of samples may want to ship send them to us in two batches, one at the end of March and one in May after the end of the study period. Smaller sites may want to batch all samples and ship them to us in May. Ship the boxes and forms to National Microbiology (NML) ***on dry ice*** to the address below.

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1015 Arlington St.
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Tel: 204-789-2133
Use FedEx billing number: 2299-8435-7

Note: The samples MUST be shipped on DRY ICE to avoid thawing during transport and the shipment should be made on a Monday or Tuesday to ensure the specimens are not held in transit over a weekend.

This concludes the CNISP CDAD laboratory protocol for the clinical laboratory.

For your interest we have included the *C. difficile* isolation and identification protocol (Appendix 1), information on the molecular studies to be done (Appendix 2).

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APPENDIX 1

Laboratory Protocol for the for the Isolation and Identification of *Clostridium difficile* from Stool at the National Microbiology Laboratory
(kindly provided by Dr. Michelle Alfa, St. Boniface Hospital, Winnipeg)

1) Thaw stool sample in minimum time possible and transfer 1 ml into a sterile centrifuge tube. Return remaining sample in cryovial to –70°C freezer.

2) Add 1 ml of 95% ethyl alcohol to 1 ml of stool sample. Cap tube.

3) Homogenize sample using a vortex mixer. Alternatively, the sample may be mixed in a haematology/chemistry rotating mixer.

4) Leave at room temperature for 30-60 minutes (35-40 minutes is usually sufficient).

5) Centrifuge at 3500 rpm for 5 minutes.

6) Decant alcohol into a discard bucket with suitable disinfectant.

7) Using a sterile swab, culture sediment to pre-reduced *C. difficile* Moxalactam Norfloxacin agar (CDMN, Oxoid). Streak to obtain isolated colonies.

8) Incubate the CDMN plate anaerobically at 35°C until there is visible growth (up to 72 hours).

9) If no growth in 72 hours, repeat steps 1-8. Negative plates are also checked daily and held for seven days before being discarded.

10) If growth occurs, describe morphology of all colony types present. *C. difficile* appears as a large, non-haemolytic grey-white colony with irregular edges, a dull surface, and slightly raised when viewed under a dissecting microscope. However, colony appearance may be atypical due to the presence of antibiotics. *C. difficile* also has a distinctive horse stable odour.

11) Perform a Gram stain on each colony type present. *C. difficile* appears as large Gram-positive to Gram-variable even-sided rods. Spores are not usually seen on selective media, but may be seen on subcultures on non-selective media and are typically oval subterminal.

12) Isolate each colony type showing Gram morphology suggestive of *C. difficile* to Brucella agar supplemented with haemin and vitamin K (BAK)(or brain heart infusion agar supplemented with yeast extract, cysteine, haemin and vitamin K (BHIA)).

13) Incubate the plates anaerobically at 35°C for 48 hours.

14) If culture appears pure, check for bright yellow-green fluorescence under long-wave UV light. Some *C. difficile* may not fluoresce under UV light. If colony morphology is typical of *C. difficile*, perform latex agglutination test to confirm. Note: It is important to only perform latex agglutination on colonies that have morphology typical of *C. difficile* since *C. glycolicum* (which is a non-fluorescent colony) will agglutinate in the latex test.

15) Perform culture confirmation using Microscreen *C. difficile* Latex Agglutination Kit.

16) Subculture a single colony to a fresh BAK plate incubated anaerobically, for further testing and stock maintenance. At the same time, perform aerotolerance testing on one-quarter blood agar plates incubated in air, in CO2, and under microaerophilic conditions to confirm that the isolate is an anaerobe. Incubate plates at 35°C for 48 hours.

17) Using a sterile applicator stick, scrape colonies from the single-colony subculture and emulsify into 10% skim milk. Using the same stick, inoculate a blood agar plate and incubate anaerobically to check for purity. Label skim milk vial and store at –70°C.

18) From the same plate (step #17), inoculate a single colony to pre-reduced brain heart infusion (BHI) broth and incubate at 35°C anaerobically overnight or until visibly turbid. Broth culture is used for PFGE analysis and to make DNA for PCR analysis.

19) Report culture positive for *C. difficile*, if an organism is isolated that is an anaerobic Gram-positive spore-forming bacillus with typical colony morphology that produces a horse stable odour, fluoresces a bright yellow-green under long-wave UV light, and positive for latex agglutination using the Microscreen kit.

APPENDIX 2

Laboratory Studies

- *Antimicrobial susceptibility testing:* Minimum inhibitory concentrations to metronidazole, vancomyicin, clindamycin, a number of fluroquinolones, and others antimicrobials gauged to be important will be determined using agar dilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines for anaerobic bacteria susceptibility testing [12], and/or other widely accepted methods (i.e. Etest strips).
- *Molecular* subtyping of *C. difficile:* A number of molecular typing methods have been developed for subtyping strains of *C. difficile* [13]. Pulsed-field gel

electrophoresis (PFGE) is currently the "*Gold Standard"* for subtyping many bacterial species and has been utilized for subtyping *C. difficile* in numerous studies. Previous studies have shown that PFGE is one of the most discriminatory methods available for subtyping *C. difficile* which is important for determining nosocomial transmission or confirming potential cases of relapse [14-16]. Although certain strains of *C. difficile* have been reported to be untypable using PFGE, recent modifications to the protocol have improved typability [17]. The previous CNISP *C. difficile* surveillance study carried out from Nov. 1, 2004 to April 30, 2005, generated fingerprints from ~ 2000 isolates and with over 250 unique types assigned. Further, analysis allowed us to define several epidemic clusters of strains found throughout the country. All strains in the on-going surveillance will be subtyped by PFGE and their fingerprints compared to those in the existing database. Analysis will help give us a detailed understanding of the molecular epidemiology of this organism in Canada. This data may identify potential clonal populations with the capacity for increased virulence.

- *PCR analysis of C. difficile strains:* Two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB), have been recognized as the main virulence factors of *C. difficil*e and the genes encoding these toxins are localized to a well-defined chromosomal region termed the pathogenicity locus (PaLoc)[19]. Some strains harbour a third toxin called binary toxin located in a location different from the PaLoc. Toxigenic strains that posess the binary toxin may have an increased virulence. PCR analysis will be used to confirm any isolated organism is *C. difficile* (presence of the triose phosphate isomerase *tpi* housekeeping gene), confirm toxigenic status (A+B+, A-B+), and if binary toxin is present (subunit b *cdtB* gene of binary toxin). In addition PCR for the putative negative regulator gene *tcdC* will be carried out. Some strains carry 18, 36, or 39 bp deletions which can be visualized after gel electrophoresis of the amplicons with comparisons to controls. The NAP1/027 epidemic strain is known to carry an 18 bp deletion in its *tcdC* gene.