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Fecal specimens for *Clostridium difficile* Diagnostic Testing are Stable for up to 72 hours at 4°C

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Abstract

Background: *Clostridium difficile* testing for stool specimens transported from remote geographic locations is a challenge due to long transit times that are often at room temperature. The impact of storage at room temperature versus 4°C on *Clostridium difficile* diagnostic tests during transport of stool samples has not been well studied

Methods: This study assessed the impact of storage at room temperature versus 4°C for up to 72 hours on the stability of glutamate dehydrogenase antigen, Toxin A and B antigens, toxigenic culture and cytopathic effect testing. Twelve diagnostic stool samples that were tested on the day of collection and shown to be *C. difficile* toxin positive were used for this study. Sample aliquots of each stool were stored at room temperature and 4°C and testing was repeated at 24, 48 and 72 hours.

Results: The glutamate dehyrdogenase antigen and toxigenic stool culture tests were shown to be 100% reproducible at room temperature and 4°C for up to 72 hours. Toxin A and B antigen deteriorated to 70% by 72 hours at room temperature but was 90% reproducible if held at 4°C. The cytopathic effect assay was 90% reproducible by 72 hours at room temperature and 4°C.

Conclusions: Based on our data we recommend that for laboratories receiving stool samples from remote regions where transit may be prolonged that glutamate dehydrogenase antigen screening followed by nucleic acid amplification testing may be a feasible diagnostic algorithm.

Keywords: Glutamate dehydrogenase antigen; Cytotoxin; Toxin A; Toxin B; Culture

Introduction

Disease caused by *Clostridium difficile* may be community onset or hospital onset [1-6]. It has become one of the most common hospital-acquired-infections (HAIs) and causes significant patient morbidity and mortality, especially in elderly patients [2,4,5,7]. Discrepancies in *C. difficile* detection rates using nucleic acid amplification tests (NAAT) and antigen, culture and cytopathic effect (CPE) tests have been reported [2,6,8,9]. Indeed Leslie et al. [8] reported that this may primarily be due to differences in the load of organisms in stool (very low load in asymptomatic carriage) and suggested that determining the viable count for *C. difficile* may be needed to differentiate between asymptomatic colonization versus true disease. In order to reduce the spread of this infection in healthcare facilities guidance documents recommend rapid turn-around-time (TAT) for *C. difficile* diagnostic tests [2,7]. To ensure rapid TAT of diagnostic results from stool sample s it is necessary to use a rapid diagnostic test NAAT is recommended by Cohen et al. [7], Baron et al. [2], Martinez et al. [4] and ensure transit time is minimized. The recent Infectious Diseases Society of America (IDSA) guideline states that stool for *Clostridium difficile* toxin testing should be submitted at room temperature within two hours to the diagnostic laboratory [2]. The 2010 clinical practice guideline [7] indicates that stools for *C. difficile* toxin testing should be submitted to the microbiology laboratory promptly. Neither of these guidelines provides any information on what impact more prolonged stool specimen transport at room temperature (RT) or refrigeration temperature has on the sensitivity of various *C. difficile* diagnostic testing methods. Modi et al. [10] reported that diagnostic toxin testing was not adversely affected by storage of stools at room temperature (RT) for up to 13 hours. No prospective comparative evaluation of different storage temperatures was undertaken in this study. Freeman et al. [11] reported that up to 5 days storage at RT or frozen at -20°C [even with repeated freeze-thaws] had no impact on the ability to recover toxigenic *C. difficile* by culture. They also reported only a slight loss of sensitivity over time when stored 5 days at 4°C, however, when stored frozen, repeated freeze-thawing of stool samples resulted in significant loss of sensitivity for the CPE assay. Weese et al. [12] had previously reported that toxin was stable at -20°C for 30 days in stool samples from horses, but that culture detection of stool stored aerobically at 4°C was possible for only 2.5 days. NAAT has been reported in manufacturers' product inserts to provide reliable detection of toxigenic *C. difficile* from stool samples for up to 5 days storage at 4°C.

For patients in rural healthcare facilities and for diagnostic laboratories without molecular diagnostics, the impact that storage conditions and prolonged transport may have on culture, antigen detection and cytopathic effect testing has not been well studied.

Longtin et al. [13] found that there are dramatically different *C. difficile* HAI rates depending on the test method used and questioned how best to standardize diagnostic testing for this pathogen to ensure HAI rates were comparable. Any comparative analysis of diagnostic testing requires an understanding of the impact that stool stability over various transport times and conditions has on the diagnostic tests used.

The key objective of this study was to use clinical stool samples to evaluate the impact of prolonged transport at RT or 4ºC on diagnostic tests for *C. difficile* including; toxigenic culture, glutamate dehydrogenase(GD) antigen testing, Toxin A and B antigen test and CPE testing for Toxin B.

Materials and Methods

All stool samples submitted for *C. difficile* toxin testing at a large tertiary care hospital were processed by the Clinical Microbiology diagnostic laboratory as per the diagnostic test manufacturers' recommended procedure using the three-step diagnostic algorithm reported by Alfa et al. [14]. Those stools that were positive for the presence of glutamate dehydrogenase (GD) using the C.DIFF QUIK CHEK® kit (TECHLAB, Blacksburg, VA) and also positive for the presence of Toxin A/Toxin B when tested by the TOX A/B QUIK CHEK® kit (TECHLAB) were included in the study providing they were tested on the same day the stool sample was collected and there was a sufficient volume of stool to perform all the parallel tests (i.e. at least 3 mLs of stool). Stool samples were transferred into six aliquots of 0.5 mLs each. Three stool aliquots were stored at 4ºC and three stool aliquots were stored at room temperature. At 24, 48 and 72 hours after the original diagnostic testing was performed, the stool aliquots from each storage temperature was tested for GD antigen, Toxin A/B antigen, cytopathic effect (CPE), and culture (with subsequent NAAT testing of any *C. difficile* isolates for toxin A and B genes).

CPE assay

A 200 uL sample of stool was mixed with 800 uL of diluent and then centrifuged at 14,000 rpm (16,000 x g) in a refrigerated microfuge for five minutes. The supernatant was passed through a 0.45 µm syringe filter; this filtrate was used for the CPE assay using Human Foreskin Fibroblast cells (Inter Medico, Markham, ON) and anti-toxin following the manufacturer's instructions for use.

Culture

The pellet from the sample used for the CPE test was re-suspended in 200 uL of diluent and an equal volume of 95% ethanol was added. The tube was gently rocked for one hour at room temperature then centrifuged at 14,000 rpm (16,000 x g) in a refrigerated microfuge for five minutes, the supernatant discarded and the pelleted material used to inoculate *Clostridium difficile moxalactam norfloxacin* (CDMN) agar (Oxoid Company, Nepean, ON). The inoculated plates were incubated up to 5 days at 35-37 °C anaerobically. All suspect *C. difficile* colonies were confirmed to be *C. difficile* by; gram stain (large, Gram positive bacilli with sub-terminal swollen spores), ultra-violet fluorescence of colonies grown on trypic soya agar supplemented with 5% sheep blood (BA) plates (i.e. suspect colonies were sub-cultured from CDMN plate onto BA plates and colonies exposed to ultra-violet light), Proline test (positive), and the presence of the typical "horse manure" smell. All confirmed *C. difficile* isolates were stored frozen at -70°C. These isolates were confirmed to be toxigenic using NAAT testing as described by Alfa et al. [15].

Results

All stool samples submitted that were GD positive and Toxin A/B positive on the day of collection were included in this study providing that there was sufficient volume of sample to perform all tests. Table 1 summarizes the results of the stability comparison of *C. difficile* toxin positive stool samples that were stored at 4°C and RT. Each sample was assessed for GD antigen, Toxin A and B antigens, CPE and toxigenic culture diagnostic tests after 24, 48 and 72 hours and the results compared to the original diagnostic results.

	Time [Hours] stool held from collection prior to testing					
Temperature and Test Method:	24 Hours		48 Hours		72 Hours	
4°C[1]	Number tested[3]	% Positive:	Number tested[3]	% positive	Number tested[3]	% positive:
GD antigen	12	100.00	12	100.00	10	100.00
Toxin A/B antigen	11	91.67	12	100.00	9	90.00
CPE	11	91.67	11	91.67	10	100.00
Culture	12	100.00	12	100.00	10	100.00
Room Temperature[2]	N=12					
GD antigen	12	100.00	12	100.00	10	100.00
Toxin A/B antigen	11	91.67	11	91.67	7	70.00
CPE	11	91.67	10	83.33	9	90.00
Culture	12	100.00	12	100.00	10	100.00

[1]The average temperature [standard deviation] over 72 hours was 4.58°C [0.42].

[2]The average temperature [standard deviation] over 72 hours was 21.56°C [0.72].

[3]There were some samples lost during testing, so the number of samples tested at each time point varies.

Table 1: Stability of GD antigen, Toxin A and B antigen, CPE and toxigenic culture testing on stool samples stored at 4°C versus room temperature over 72 hours

Discussion

Our data demonstrated that the GD antigen and toxigenic culture test results were 100% stable over 72 hours of aerobic storage of stool samples at RT or 4°C. Although GD antigen is stable at RT, our data demonstrated that Toxin A/B antigen detection deteriorates to 70% when stool samples were stored aerobically at RT for 72 hours. This supports Dubberke et al. [6] caution that toxin deteriorates when stool is held at room temperature. Furthermore, our data clarifies the time-frame within which transport at RT will cause deterioration. Both the Toxin A and B antigen assay and the CPE assay showed some variability, however, at least 90% of the test results were stable up to 72 hours at 4°C.

Modi et al. [10] reported that antigen detection was stable for up to 13 hours at RT but indicated that prospective studies were needed to confirm their findings. Our study confirms and extends Modi et al. findings by showing that GD antigen, Toxin A/B antigen, toxigenic culture and CPE are all >90% reproducible when stool samples are stored at 4°C for up to 72 hours. Ideally, specimen transport should be as short as possible and within a facility or a city it is feasible to expect transport times to be within a few hours. However, as microbiology laboratories continue to consolidate, transport times from remote regions becomes a concern. Our data is the first to demonstrate that when transported at 4°C for up to 72 hours the GD antigen, Toxin A/B antigen, toxigenic culture and CPE tests will be >90% reproducible compared to these same tests performed on the day of stool collection. For remote regions the transport may be at RT rather than 4°C and our data demonstrated that under these transport conditions, detection of Toxin A/B antigen will not be reliably detected. As such we suggest that for microbiology diagnostic laboratories receiving stool specimens from remote regions that may be up to 72 hours in transit they could reliably use the GD antigen screening test but that toxin A/B antigen detection would not be recommended. Since CPE and toxigenic culture are time consuming the NAAT would be a better alternative method to Toxin A/B antigen detection. Although NAAT testing could be done on all samples, it is important to consider the cost of testing and impact of detection of asymptomatic carriers, in which case GD screening followed by NAAT testing of GD positive stools as described by Longtin et al. [13] may be a good algorithm. Longtin et al. [13] indicated that rates of *C.difficile* [i.e. cases/10,000 admissions] may vary as much as 50% between NAAT and a multi-step algorithm using GD antigen, Toxin A/B antigen and CPE testing. Furthermore, they indicated that cases detected by NAAT alone are less likely to present with complications within 30 days of their diagnosis. They recommend that standardization is needed to ensure rates of *C. difficile* disease can be compared between different centres. We would also recommend that transport conditions and duration of transport prior to diagnostic testing also needs to be taken into consideration as there is variability in the impact of transport conditions on different test methods.

A weakness of this study is that we did not quantitate the cfu/gram of stool over time, rather, we only assessed if any level of viable *C. difficile* could be detected. Other weaknesses were that only 12 diagnostic stool samples were tested and that NAAT was not part of this comparative study. The manufacturers' product inserts indicate that stool should be tested as soon as possible, but that NAAT can be used for stool samples for up to 5 days when stool is stored at 4°C. There is no data that the authors are aware of regarding the impact of RT storage on the reproducibility of NAAT.

In summary our study demonstrated that if stool samples are held at 4°C they are stable [>90% reproducible results] for all diagnostic tests assessed for up to 72 hours. However, if stool samples are held at room temperature, the Toxin A/B antigen testing is only stable for 48 hours [>90% sensitive] and by 72 hours is only 70% sensitive. Based on our findings, we would recommend that for stool samples received from remote geographic locations where transport is at room temperature and may be up to 72 hours, that a combination of GD antigen screening combined with NAAT be used to ensure optimal sensitivity.

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