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A Systematic Evaluation of Methods to Optimize Culture-Based Recovery of *Clostridium difficile* From Stool Specimens

Tiffany Hink[1], **Carey-Ann D. Burnham**[2,3], and **Erik Dubberke**[1]
[1]Department of Medicine, Washington University School of Medicine, St. Louis, MO

[2]Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO

[3]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO

Abstract

With the increasing prevalence of *Clostridium difficile* infection among hospitalized patients, a clear understanding of *C. difficile* epidemiology is needed to evaluate current prevention policies, and to create new and effective policies. To determine the epidemiology of *C. difficile*, the most sensitive methods for detection of *C. difficile* are required. The purpose of this study was to systematically assess multiple methods to determine the most sensitive method to recover *C. difficile* from stool and rectal swabs. Stool samples from healthy asymptomatic individuals that were collected and confirmed to be culture negative for *C. difficile* were spiked with known concentrations of *C. difficile* ranging from 10^4 CFU of *C. difficile* per ml of stool to 10 CFU per ml. Two sets of experiments (A and B) were performed and each involved multiple combinations of untreated and treated stool/rectal swab specimens and selective and non-selective broth and agar. Overall, recovery of *C. difficile* was increased with the use of an initial broth enrichment followed by plating to solid medium. The most sensitive method of *C. difficile* detection for both stools and swab specimens was heat shock prior to inoculation of cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB, Anaerobe Systems, Morgan Hill, CA) followed by isolation from pre-reduced TSA II with 5% sheep blood (BAP, BBL BD and Co., Sparks, MD). Identifying the most sensitive method of recovery will allow for further study of asymptomatic *C. difficile* carriers and their role in the epidemiology of *C. difficile*.

Keywords

C. difficile; Asymptomatic carrier; Broth enrichment; Alcohol shock; *C. difficile* culture

1. Introduction

Clostridium difficile is the most frequent infectious cause of antibiotic and healthcare associated diarrhea, and the incidence and severity of *C. difficile* infection (CDI) has significantly increased in recent years. Although the amount of data available is growing, the

*Corresponding Author: Erik Dubberke, MD, MSPH 660 S Euclid Ave., Box 8051 St. Louis, MO 63110 edubberk@dom.wustl.edu Tel: (314) 454-8293 Fax: (314) 454-5392.

epidemiology and transmission dynamics of *C. difficile* remain poorly understood [1]. This is concerning because a complete understanding of *C. difficile* epidemiology is necessary to critically assess the efficacy of current CDI prevention measures, and to develop and study novel prevention measures [2,3]. To effectively define *C. difficile* epidemiology, all sources of *C. difficile* need to be accurately identified, with recovery of the organism for molecular typing. This necessitates sensitive methods for detection of *C. difficile*.

Multiple methods to culture *C. difficile* have been described in the literature [4-11]. These studies have often been limited to two or three different variations in specimen processing or culture techniques. To our knowledge, there are no comprehensive comparisons of multiple culture methods. Past and recent research indicates asymptomatic carriers may be an important source of *C. difficile* in the health care setting [12-15]. Insensitive culture methods may bias results because asymptomatic carriers may have low numbers of organism per gram of stool. The objective of this study was to systematically evaluate multiple methods to identify the most sensitive method to recover *C. difficile* from stool and rectal swab specimens.

2. Materials and Methods

2.1. Study Overview

To identify the most sensitive method to culture *C. difficile* from stool and rectal swabs, stool from asymptomatic individuals that did not grow *C. difficile* was spiked with known concentrations of *C. difficile*. The final concentrations of *C. difficile* in the spiked stool ranged from 10^4 CFU of *C. difficile* per ml of stool down to 10 CFU per ml. Two sets of experiments were conducted. The first set of experiments (Experiment A) involved multiple permutations of untreated stool/rectal swab specimens, treated specimens (heat or ethanol shock), initial inoculation into selective or non-selective broth or direct plating onto selective or non-selective agar, and for broth specimens with growth, plating onto selective or non-selective agar with or without treatment (heat or ethanol) (Figure 1). For the second set of experiments (Experiment B), the most promising methods from experiment A were repeated, including some additional modifications of those methods (Figure 2). Three rounds were conducted for all experiments and all dilutions of *C. difficile*.

2.2 Stool specimens

Stool specimens were collected from four healthy, community dwelling donors not on antimicrobials, and frozen at −20°C. Prior to freezing, the specimens were diluted with 1X PBS to a paste-like consistency and divided into 9 mL aliquots. Before conducting the experiments, the stools were cultured for *C. difficile* to confirm the absence of *C. difficile* from the specimens. Prior to experiment A, the donor stool was plated to cycloserine-cefoxitin fructose agar with horse blood and taurocholate (CCFA-HT, Anaerobe Systems, Morgan Hill, CA) and incubated under anaerobic conditions for 2 days, after an ethanol shock procedure. After experiment A, residual stool from experiment A and the stool for experiment B were heat shocked at 80°C for 10 minutes and then transferred to cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB-TAL broth, Anaerobe Systems, Morgan Hill, CA). They were incubated under anaerobic conditions at 35°C and

monitored for growth for 7 days. All of these control specimens were negative for *C. difficile* using these culture conditions. The same stool specimen was used for all of experiment A. In experiment B, stool from a different donor was used for each round of the experiment.

2.3 Culture methods

2.3.1 General procedure—The *C. difficile* strain ATCC 9689 was grown for 4 days on TSA II with 5% sheep blood (BAP, BBL BD and Co., Sparks, MD). A 0.5 McFarland suspension of organism was made and serial dilutions were made with 1X PBS. At the time the stool specimens were spiked, the dilutions were also plated onto BAP to confirm the number of CFU/ml of *C. difficile*. The stool specimen was vortexed for 30 seconds after *C. difficile* was added to homogenize the bacterial suspension within the stool. For each round of experiments, an unspiked stool specimen served as a control.

Both stool and swab specimens were cultured for *C. difficile*. Flocked swabs (BD Eswab, Sparks, MD) were inserted into the stool specimen to saturation and transferred back into the Amies transport media within the Eswab device. The swab/media was vortexed for 30 seconds prior to culture. Approximately 1 mL of the swab transport media was inoculated into broth or a large drop was plated directly onto solid agar. For stool specimens, approximately 1 ml of stool was inoculated into broth or a large drop was plated directly onto solid agar.

2.3.2 Culture media—The solid media included: BAP, cycloserine-cefoxitin fructose agar with lysozyme (CCFAL, Anaerobe Systems, Morgan Hill, CA), and CCFA-HT (Anaerobe Systems, Morgan Hill, CA). Broth media included: chopped meat broth (CMB, Anaerobe Systems, Morgan Hill, CA) and CCMB-TAL (Anaerobe Systems, Morgan Hill, CA). The BAPs were pre-reduced prior to use. All other media were supplied pre-reduced by the manufacturer.

2.3.3 Specific procedures

Ethanol shock procedure: 1 mL of stool, swab liquid, or pellet from the broth media was added to 1 mL of ethanol. The mixture was vortexed, and then incubated at room temperature for 45 to 60 min. Following incubation, the mixture was centrifuged at 2500 X *g* for ten minutes. The liquid was decanted off and the pellet was then transferred to broth or solid media. In experiment A, after the ethanol pre-treatment of the swab liquid, a large drop was added to each piece of solid media or 100 uL was added to broth without centrifugation.

Heat shock procedure: 1 mL of stool, swab liquid, or pellet from the broth media was placed in a micro-centrifuge tube and vortexed. The tube was placed in a heat block at 80°C for 10 minutes. The entire specimen or pellet was then added to broth media or solid media, depending on its position in the procedure.

Direct plating procedure: Using a Pasteur pipette (Fisher Scientific, Pittsburgh, PA), a large drop of the stool, swab liquid, or pellet from the broth media was plated to solid media. The plates were then streaked out for isolation using a 4 quadrant pattern. Growth on solid

media was recorded in a semi-quantitative fashion (0=no growth, 1=growth in first quadrant only, 2=growth in first and second quadrants, 3=growth in first, second, and third quadrants, and 4=growth in all four quadrants).

2.3.4 Identification of *C. difficile*—Means for identification of growth varied upon the medium type in question. With the chopped meat broth, turbidity was suggestive of growth. Turbidity and a change of the broth color from pink to yellow signified growth in CCMB. Broth was examined for growth daily for 5 days in experiment A, while broth was examined on days 1, 2, 3, and 7 in experiment B. To determine if the growth in the broth was *C. difficile*, a portion of broth was subcultured to solid media. The solid media were observed at 48 hours for growth of flat, spreading colonies characteristic of *C. difficile*. On CCFA-HT and BAP, grey colonies were sought, whereas on CCFAL, *C. difficile* colonies were yellow or clear. After growth on solid media, suspicious colonies were subcultured to BAP for isolation. The identification of *C. difficile* was confirmed by Gram stain, spot indole (Remel, Lenexa, KS) and PROLINE Disk (Remel, Lenexa, KS).

3. Results

3.1 Experiment A

Results were recorded when growth was first observed. Overall, *C. difficile* grew better in the methods that utilized initial broth enrichment followed by plating to solid medium, rather than direct plating to solid medium (Tables 1 and 2). In addition, it was typical that growth in the broth enrichment would be visible within 24 h, while growth on the solid medium was not typically observed until at least 48 h of incubation. The method of stool treatment (none, heat, ethanol) produced similar recovery amounts throughout the experiment, with a decrease in growth as the concentration of *C. difficile* decreased. The least sensitive methods for recovery from swabs were direct plating methods and inoculation of CMB. The methods that utilized CCMB provided the best recovery from stools and swabs, but recovery was lowest when ethanol shock was performed after CCMB inoculation with the swab specimens.

3.2 Experiment B

After completing experiment A, it was determined that plating growth from broth to only one selective agar would be adequate for recovery of *C. difficile* as there was no difference in recovery of *C. difficile* following subculture of the broth. Therefore, CCFA-HT was not used in experiment B. CCFA-HT is slightly more costly than CCFAL, and with its color change in the agar, *C. difficile* was more easily recognized on CCFAL. The first round results for experiment B confirmed recovery of *C. difficile* from CCFAL and BAP after CCMB enrichment was identical, but the BAP was less expensive. Therefore, after the first round of experiment B, it was determined CCFAL was no longer needed after broth enrichment.

Results were recorded at 3 time points: first visible growth, day 3 of incubation, and day 7 of incubation. Most of the specimens were visibly positive within 24 hours for all dilutions, except the 10 CFU per ml from two of the three rounds where more grew within 48 hours.

Heat shock of stool prior to CCMB inoculation produced growth into the fourth quadrant for all dilutions and rounds of the experiment, yielding the best recovery of *C. difficile* (Table 3). The untreated stool method was the second best method, but growth decreased as the concentration of *C. difficile* in the specimen decreased. Ethanol shock prior to CCMB inoculation demonstrated the lowest sensitivity for *C. difficile* recovery from stools. The most sensitive method for swabs was also heat shock prior to CCMB inoculation. *C. difficile* growth was noted in the fourth quadrant for all rounds of the experiment, except for round 1 for the two lowest dilutions, where no growth was noted. CCMB inoculation followed by ethanol shock provided the least sensitive recovery for swabs. The higher concentrations yielded some growth, but no growth was observed with the 10 CFU/mL suspension.

4. Discussion

After performing two sets of experiments that involved various combinations of specimen processing and culture methods, heat shock followed by inoculation into CCMB, then plating growth onto BAP proved to be the most sensitive and most cost effective method for recovery of *C. difficile* from both stool and swab specimens. This study was systematic and comprehensive. Known quantities of *C. difficile* were used to spike *C. difficile* free stool. This was done, rather than obtaining stools from patients suspected with CDI or diarrhea-free hospitalized patients, to avoid potential for type 2 error if *C. difficile* was not recovered. It also allowed for an assessment of the limit of detection of the different methods. In all, 69 different permutations were evaluated. This ensured the most sensitive and cost effective method was identified. Both stool and swab specimens were evaluated. This was done because in epidemiological studies it may be necessary to obtain a rectal swab from a study subject if he/she is unable to have a bowel movement at the appropriate time.

Most studies that have evaluated different methods to culture *C. difficile* have been narrow in scope, often limited to one or two variations in *C. difficile* recovery techniques. The literature also supports our finding that the use of enrichment broth is more sensitive than direct plating to agar [4,6]. This study included both selective and non-selective media, in addition to comparing recovery from broth and agar. Although heat shock and ethanol shock have been included in prior culture method comparison studies, it is not common for both methods to be directly compared [4,6,7,9]. In addition, comparing the impact of heat or ethanol shock both before and after inoculation into broth has not been previously published. Marler et al. did compare both methods [7]. In their study, the difference between heat shock and ethanol shock was not significantly different, whereas heat shock was determined to be more sensitive in our study.

This is perhaps due to the fact that they plated to pre-reduced BAP after ethanol shock, while we inoculated selective broth after ethanol shock and heat shock. Also, we utilized a standard approach of inoculating each specimen with a known concentration of *C. difficile*, but the Marler study used stools submitted to the clinical microbiology lab for *C. difficile* testing. By including selective and non-selective agar after enrichment broth, it was determined the selective agar is not necessary for optimization of sensitivity. This was an important finding because BAP is approximately six times less expensive than the selective agar included in this study. Conversely, there was better recovery from selective broth than

non-selective broth even after heat or ethanol shock. This suggests suppression of the small number of organisms that survive the shock procedure is important to optimize *C. difficile* recovery with broth enrichment.

There are some limitations to this study. To ensure the stool specimens were available at the time the *C. difficile* isolates were ready for the experiment and free of *C. difficile* prior to spiking the specimens, it was necessary to collect the specimens ahead of time and freeze them. It is possible some portion of the fecal flora that inhibits *C. difficile* growth did not survive the freezing, thus enhancing the recovery of *C. difficile*. Also, specimens from only four donors were used. Fecal flora varies from person to person, and one person's fecal microbiome may be better at inhibiting growth of or overgrowing *C. difficile* than another person's. However, the most sensitive method identified included a heat shock of the specimen followed by inoculation into selective broth. Any flora that may inhibit recovery of *C. difficile* are likely killed or inhibited by these measures. In our experience analyzing the specimens in this study, overgrowth of other fecal flora did not appear to be an impediment to recovery of *C. difficile* using the methods we evaluated. Preparation of the samples may have affected the results of this study if mixing of the stool and dilutions was not sufficient, although care was taken to prevent this, and the results of the study were reproducible across replicates. The less sensitive culture methods trended to lower *C. difficile* recovery as the concentration of *C. difficile* in the specimen decreased; suggesting that inadequate homogenization of the specimen was not a problem. Results from LOD B were better than the results from similar methods in LOD A. It is unlikely this is due to the stool specimens or the mixing of specimens based on consistent results over three rounds per experiment. Also, the dilutions were plated to BAP before mixing with stool to confirm the concentration of *C. difficile* in each suspension. The variance in results may be due to the learning curve that is involved in the completion of the experiments or the fact that LOD B was performed by a laboratory technician with more experience than the one who performed LOD A. Only one ATCC strain of *C. difficile* was used in both experiments. It is possible recovery of *C. difficile* may be impacted by the colonizing strain. To our knowledge, this has not been previously identified as an issue.

5. Conclusions

In summary, the most sensitive method for the detection of *C. difficile* from stool and swab specimens was heat shock followed by inoculation into CCMB, then isolation of *C. difficile* from pre-reduced BAP. Studies on the role of asymptomatic *C. difficile* carriers on the epidemiology of *C. difficile* are needed [3,15]. Recovery of *C. difficile* isolates is necessary for investigations to allow for molecular typing and tracking the transmission of the organism. Culture methods with sub-optimal recovery of *C. difficile* will bias the results of epidemiological studies and lead to erroneous conclusions. In addition, identifying the optimal culture method is important because having a standardized process would simplify comparison of results across studies.

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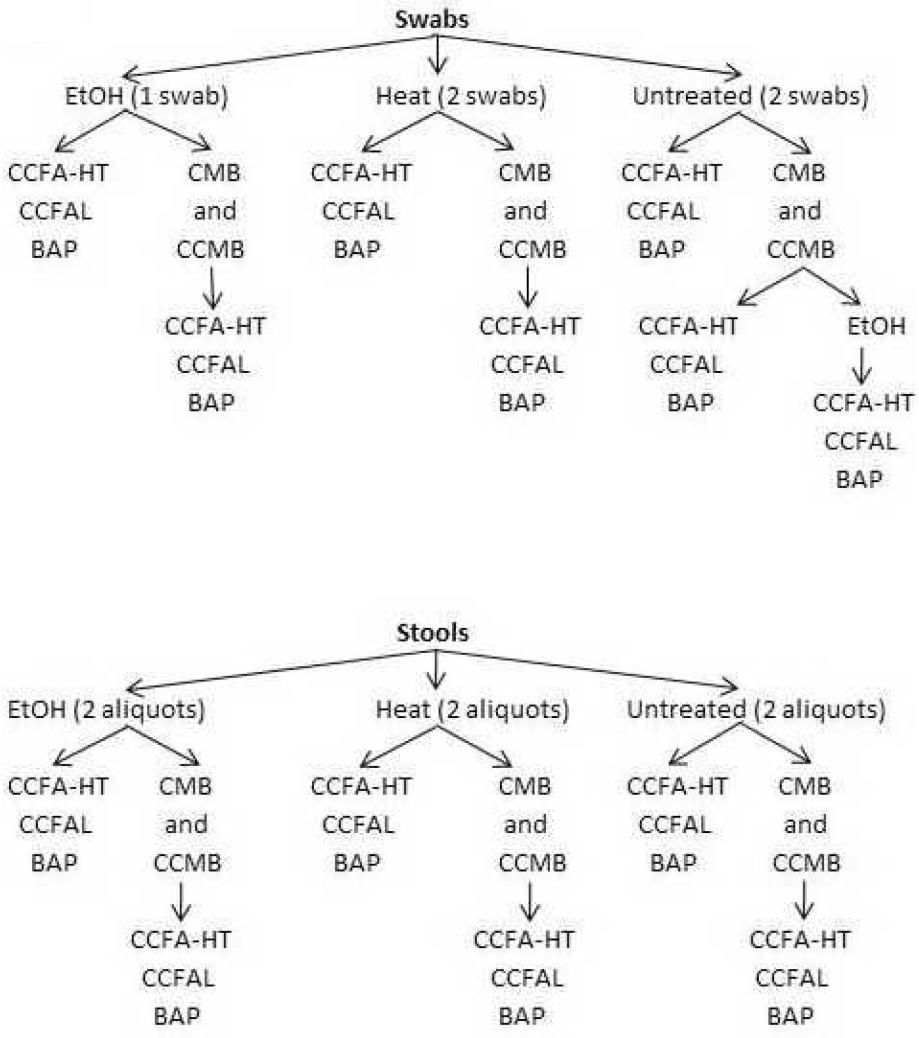

Figure 1.

Design of Experiment A. Stool and swab specimens were used. Untreated and treated specimens were inoculated into selective or non-selective broth or plated onto selective or non-selective media. If there was growth after pretreatment or from an untreated stool, the broth was plated onto solid media. If the swab was not pretreated, growth was plated directly to solid media and treated with ethanol prior to plating to solid media. CCMB: cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine, CCFAL: cycloserine-cefoxitin fructose agar with lysozyme, CCFA-HT: cycloserine-cefoxitin fructose agar with horse blood and taurocholate, BAP: TSA II with 5% sheep blood, H: heat shock, E: ethanol shock, CMB: chopped meat broth.

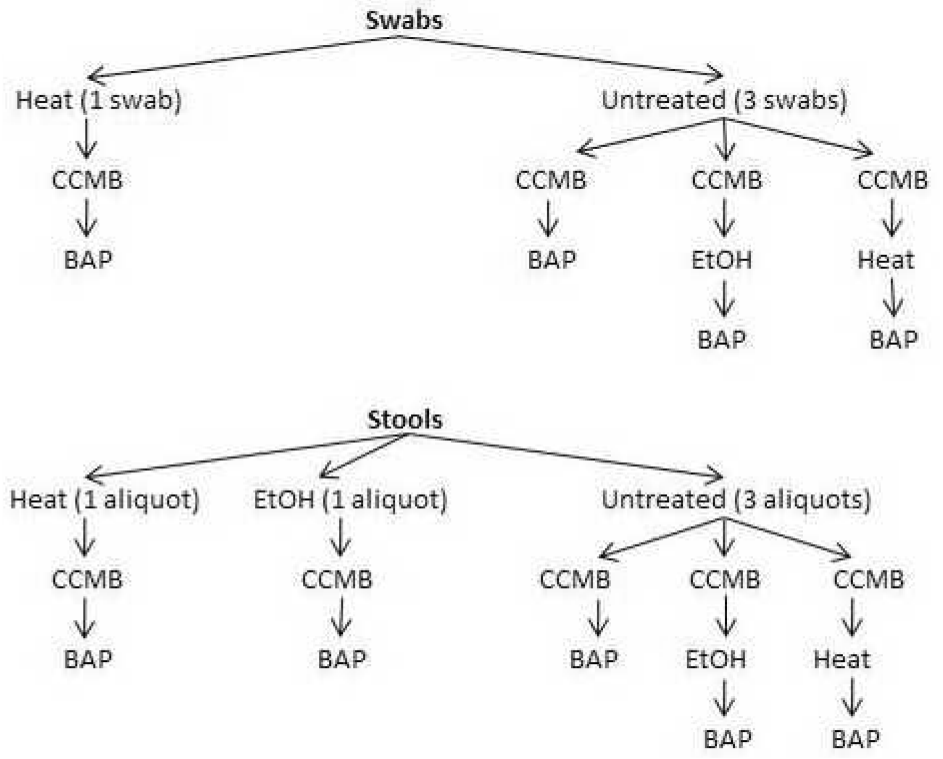

Figure 2.

Design of Experiment B. Stool and swab specimens were used. CCMB was inoculated directly or after pretreatment with an ethanol or heat shock. If there was growth after pretreatment, the specimen was plated onto BAP. If the specimen was not pretreated, growth was either directly plated onto BAP or treated with an ethanol shock or heat shock prior to plating to BAP. CCMB: cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine, BAP: TSA II with 5% sheep blood, H: heat shock, E: ethanol shock.

Table 1

Results of experiment A with stool specimens. Cultures were semi-quantitative (0=no growth, 4 = growth on all four quadrants). Results reported are the mean and median semiquantitative growth results at the two lowest dilutions of *C. difficile* in stool. Order of abbreviations indicates the order of events in processing the specimen and the media used.

***C. difficile* concentration**	**10^2 CFU/ml**		**10 CFU/ml**	
Culture method	**Mean**	**Median**	**Mean**	**Median**
Cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB) enrichment methods				
CCMB, CCFAL	2.7	4	1.3	0
CCMB, CCFA-HT	4	4	1.3	0
CCMB, BAP	3.3	4	1.3	0
H, CCMB, CCFAL	2.7	4	0	0
H, CCMB, CCFA-HT	2.7	4	1.3	0
H, CCMB, BAP	2.7	4	1.3	0
E, CCMB, CCFAL	2.7	4	0	0
E, CCMB, CCFA-HT	2.7	4	0	0
E, CCMB, BAP	2.3	3	1.3	0
Chopped meat broth (CMB) enrichment methods				
CMB, CCFAL	0	0	1.3	0
CMB, CCFA-HT	0	0	1.3	0
CMB, BAP	1.3	0	0	0
H, CMB, CCFAL	1.3	0	0	0
H, CMB, CCFA-HT	1.3	0	0	0
H, CMB, BAP	0	0	0	0
E, CMB, CCFAL	1.3	0	1.3	0
E, CMB, CCFA-HT	0	0	0	0
E, CMB, BAP	0	0	0	0
Direct plate methods				
CCFAL	0	0	0	0
CCFA-HT	0	0	0	0
BAP	0	0	0	0
H, CCFAL	0.3	0	0	0
H, CCFA-HT	0	0	0	0
H, BAP	0	0	0	0
E, CCFAL	0	0	0	0

C. difficile concentration	10^2 CFU/ml		10 CFU/ml	
Culture method	**Mean**	**Median**	**Mean**	**Median**
Cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB) enrichment methods				
E, CCFA-HT	0.3	0	0	0
E, BAP	0	0	0	0

CCMB: cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine, CCFAL: cycloserine-cefoxitin fructose agar with lysozyme, CCFA-HT: cycloserine-cefoxitin fructose agar with horse blood and taurocholate, BAP: TSA II with 5% sheep's blood agar, H: heat shock, E: ethanol shock, CMB: chopped meat broth

Table 2

Results of experiment A with swab specimens. Cultures were semi-quantitative (0=no growth, 4 = growth on all four quadrants). Results reported are the mean and median semiquantitative growth results at the two lowest dilutions of *C. difficile* in stool. Order of abbreviations indicates the order of events in processing the specimen and the media used.

***C. difficile* concentration**	**10^2 CFU/ml**		**10 CFU/ml**	
Culture method	**Mean**	**Median**	**Mean**	**Median**
Cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB) enrichment methods				
CCMB, CCFAL	2.7	4	0	0
CCMB, CCFA-HT	3.3	4	0	0
CCMB, BAP	2.7	4	0	0
H, CCMB, CCFAL	1.3	0	0	0
H, CCMB, CCFA-HT	1.3	0	1	0
H, CCMB, BAP	1.3	0	0	0
E, CCMB, CCFAL	2	2	1	1
E, CCMB, CCFA-HT	2	2	0	0
E, CCMB, BAP	1.5	1.5	0	0
CCMB, E, CCFAL	0	0	0	0
CCMB, E, CCFA-HT	0	0	0	0
CCMB, E, BAP	0	0	0	0
Chopped meat broth (CMB) enrichment methods				
CMB, CCFAL	1.3	0	0	0
CMB, CCFA-HT	1.3	0	0.3	0
CMB, BAP	0	0	0	0
H, CMB, CCFAL	1.3	0	1.3	0
H, CMB, CCFA-HT	1.3	0	1.3	0
H, CMB, BAP	0	0	0	0
E, CMB, CCFAL	0	0	0	0
E, CMB, CCFA-HT	0	0	0	0
E, CMB, BAP	0	0	0	0
CMB, E, CCFAL	0	0	0	0
CMB, E, CCFA-HT	0	0	0	0
CMB, E, BAP	0	0	0	0
Direct plate methods				
CCFAL	0	0	0	0

C. difficile concentration	10^2 CFU/ml		10 CFU/ml	
Culture method	**Mean**	**Median**	**Mean**	**Median**
Cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB) enrichment methods				
CCFA-HT	0	0	0	0
BAP	0	0	0	0
H, CCFAL	0	0	0	0
H, CCFA-HT	0	0	0	0
H, BAP	0	0	0	0
E, CCFAL	0	0	0	0
E, CCFA-HT	0	0	0	0
E, BAP	0	0	0	0

CCMB: cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine, CCFAL: cycloserine-cefoxitin fructose agar with lysozyme, CCFA-HT: cycloserine-cefoxitin fructose agar with horse blood and taurocholate, BAP: TSA II with 5% sheep blood, H: heat shock, E: ethanol shock, CMB: chopped meat broth.

Table 3

Results of experiment B. Cultures were semiquantitative (0=no growth, 4 = growth on all four quadrants). Results reported are the mean and median semiquantitative growth results at the two lowest dilutions of *C. difficile* in stool. Order of abbreviations indicates the order of events in processing the specimen and the media used.

***C. difficile* concentration**	**10^2 CFU/ml**		**10 CFU/ml**	
Stool Method	**Mean**	**Median**	**Mean**	**Median**
H, CCMB, BAP	4	4	4	4
E, CCMB, BAP	0	0	0	0
CCMB, BAP	4	4	2.2	4
CCMB, H, BAP	2.4	3	1.3	0
CCMB, E, BAP	0.9	0	0.4	0

***C. difficile* concentration**	**10^2 CFU/ml**		**10 CFU/ml**	
Swab Method	**Mean**	**Median**	**Mean**	**Median**
H, CCMB, BAP	2.7	4	2.7	4
CCMB, BAP	2.6	4	2.1	3
CCMB, H, BAP	0.9	0	0.3	0
CCMB, E, BAP	0.9	0	0	0

CCMB: cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine, BAP: TSA II with 5% sheep's blood agar, H: heat shock, E: ethanol shock.