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Development of a Rapid-Viability PCR Method for Detection of *Clostridioides difficile* Spores from Environmental Samples

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Abstract

Clostridioides difficile is a common pathogen that is well known to survive for extended periods of time on environmental healthcare surfaces from fecal contamination. During epidemiological investigations of healthcare-associated infections, it is important to be able to detect whether or not there are viable spores of *C. difficile* on surfaces. Current methods to detect *C. difficile* can take up to 7 days for culture and in the case of detection by PCR, viability of the spores cannot be ascertained. Prevention of *C. difficile* infection in healthcare settings includes adequate cleaning and disinfection of environmental surfaces which increases the likelihood of detecting dead organisms from an environmental sample during an investigation. In this study, we were able to adapt a rapid-viability PCR (RV-PCR) method, first developed for detection of viable *Bacillus anthracis* spores, for the detection of viable *C. difficile* spores. RV-PCR uses the change in cycle threshold after incubation to confirm the presence of live organisms. Using this modified method we were able to detect viable *C. difficile* after 22 hours of anaerobic incubation in Cycloserine Cefoxitin Fructose Broth (CCFB). This method also used bead beating combined with the Maxwell 16 Casework kit for DNA extraction and purification and a real-time duplex PCR assay for toxin B and *cdd3* genes to confirm the identity of the *C. difficile* spores. Spiked environmental sponge-wipes with and without added organic load were tested to determine the limit of detection (LOD). The LOD from spiked environmental sponge-wipe samples was 10^4 spores/mL but after incubation initial spore levels of 10^1 spores/mL were detected. Use of this method would greatly decrease the amount of time required to detect viable *C. difficile* spores; incubation of samples is only required for germination (22 hours or less) instead of colony formation, which can take up to 7 days. In addition, PCR can then quickly confirm or deny the identity of the organism at the same time it would confirm viability. The presence of viable *C. difficile* spores could be detected at very low levels within 28 hours total compared to the 2 to 10-day process that would be needed for culture, identification and toxin detection.

Keywords

Clostridioides difficile; Spores; Environmental Sampling; Rapid Viability PCR

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1. Introduction

Clostridioides difficile is the most common cause of healthcare-associated diarrhea. In 2011 the burden was estimated at 453,000 infections and 29,000 deaths with 65.8% of the infections being considered healthcare-associated and 24.2% of onset occurring during hospitalization [1]. Most patients remain asymptomatic after colonization but often continue to shed *C. difficile* in their stool. In the healthcare setting this can be cause for concern since *C. difficile* spores can persist in the environment for 5 months [2–4]. Environmental contamination, especially high-touch surfaces in patient bathrooms and rooms can be sources of infection [5, 6].

In a recent study to determine bioburden on high-touch healthcare environmental surfaces *C. difficile* was recovered by culture from 15.7% of cleaned rooms [7]. While the range was low 64.7 - ≤ 1 CFU/100 cm^2 the human infectious dose of *C. difficile* is unknown and the presence of viable spores in a patient's environment after cleaning is cause for concern. In the hamster model the infectious dose is as low as 10 colony forming units (CFU) per animal and there was 100% disease and mortality at 100 CFU per animal [8]. Traditional methods to detect by culture can take up to 7 days, as *C. difficile* is a slow grower. Real-time PCR can detect *C. difficile* rapidly, but does not distinguish between live or dead cells [9]. This distinction is critical when assessing the threat to a patient's health during investigations of *C. difficile* infections and implementation of efficacious environmental disinfection procedures.

Rapid-viability PCR (RV-PCR) is a new method, recently developed by Lawrence Livermore National Labs (LLNL) for the rapid identification and viability assessment of *Bacillus anthracis* from environmental samples [10, 11]. This method involves extracting the spores or cells from a sampling device (swab or sponge-wipe), then performing two quantitative real-time PCR analyses on the eluent, with an incubation step in between the first and second analysis. The incubation time is shorter than that required time for traditional culture. The results of the two PCR analyses are compared, and if viable cells or spores were present, there should be several orders of magnitude more spores or cells after incubation, therefore a significant decrease in the C_T value on the post-incubation sample. Because of the enrichment step, the limit of detection (LOD) is improved at the same time.

Adapting the *B. anthracis* RV-PCR procedure developed by LLNL to detect viable spores from environmental sponge-wipe samples will decrease the amount of time required for confirmation of *C. difficile*-positive environmental samples, identification of toxin presence, and confirmation of the presence of viable spores.

2. Materials and Methods

2.1 Bacterial Strain and Culture Conditions

The ATCC reference strain of *C. difficile* ATCC 43598 (*tcdB* positive) was used for sample spiking and as the *tcdB* PCR positive control. For development of the real-time PCR primers and probes the nontoxigenic *C. difficile* ATCC 43593 reference strain, along with eight *tcdB* positive *C. difficile* environmental isolates and two nontoxigenic *C. difficile* environmental

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isolates were used (Table 1). The other anaerobe isolates used for PCR design were *Terrisporobacter glycolicus* (#2008298), *Clostridium novyi* (#2000073-2), *Clostridium perfrigens* (#20100347), and *Clostridium septicum* (#2007189) obtained from the CDC Anaerobe Reference Laboratory collection. Additional aerobic bacteria used to simulate background contaminants (dirty sample spiking) included: *Escherichia coli* (#4402-07), *Klebsiella pneumoniae* (#2001-22-18B), *Staphylococcus aureus* (ATCC 43300), *Acinetobacter baumannii* (#2008-23-01-01), and *Enterococcus faecium* (#93-18-73) from the Outbreak lab collection in the Clinical and Environmental Microbiology Branch within the Division of Healthcare Quality Promotion at CDC. All anaerobic strains were initially cultured on anaerobic blood agar (ABA; PathCon Labs, Norcross, GA) while aerobic bacteria were cultured on tryptic soy agar with 5% Sheep's Blood (TSAII; Becton Dickinson and Company, Franklin Lakes, NJ).

2.2 *C. difficile* Spore Preparation

Spore stocks of strains ATCC 43598 and 43593 were generated using an adapted procedure by Hasan *et al*. Briefly, spore stocks were produced by spreading vegetative cells grown in Reinforced Clostridial Medium onto ABA plates and incubating at 36°C for up to 10 days under anaerobic conditions before harvesting [12]. The spore suspensions were washed with phosphate buffered saline with 0.02% Tween® 80 (PBST) three times, and heat-treated at 65±2°C for 10 minutes. The spore suspensions were then purified by centrifugation in a 50% HistoDenz (Sigma-Aldrich, St. Louis, MO) solution, followed by three washes with cold PBST. Spore stock concentrations were evaluated microscopically and enumerated on cycloserine cefoxitin fructose agar containing horse blood and taurocholate (CCFA-HT; Anaerobe Systems, Morgan Hill, CA). The final purified spore stocks (10^9 spores/mL) were stored at 4°C for the duration of the study.

2.3 Sample Preparation and Processing

Prior to each RV-PCR experiment a new test suspension inoculum was made up from the purified *C. difficile* spore stock. Test suspension inoculum levels were 10^4, 10^3, 10^2, and 10^1 spores/mL. Ten-fold dilutions of the spore stock were made in PBST. Titers were checked by plating in triplicate onto CCFA-HT. All plates were incubated anaerobically for 48 h at 36°C.

Sterile pre-moistened sponge-wipes (3M™ Sponge-Stick (1.5" × 3") with neutralizing buffer, 3M, St. Paul, MN) were inoculated with 1 mL of spore suspension with 10^1 – 10^4 spores/mL; ten sponge-wipes per spore concentration. After the sponge-wipes were inoculated, they were placed into the storage bag and sealed. One sponge-wipe was spiked with 1 mL PBST as a negative control while 1 mL of the spore suspension was added to 90 mL PBST in a stomacher bag (Seward, Worthing, UK) as a positive control. All 12 samples were then placed at 4°C for 24 hours to simulate refrigerated shipping of the samples.

Sponge-wipes inoculated with spores suspended in PBST were considered to simulate clean samples i.e. a sample from a cleaned surface without organic soil or other organisms. To simulate dirty samples, artificial test soil (ATS; Healthmark Industries, Co., Fraser, MI) was used along with an environmental *E. coli* isolate as a background organism. ATS is a stock

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composition of purified bovine proteins (albumin, hemoglobin), amino acids, vitamins and carbohydrates which simulates the organic contaminants most likely to be found on a healthcare surface or medical device. *E. coli* was grown on TSAII overnight and suspended in buffered water (Butterfield's Buffer), then added to ATS for a titer of 10^5 CFU/ml. Ten sponge-wipes were inoculated with the desired *C. difficile* spore concentration and 10^4 CFU/mL *E. coli* in 200 μL of the ATS. The ATS-*E. coli* suspension was also added to the negative and positive control samples. All dirty sample sponge-wipes were placed at 4°C for 24 h to simulate refrigerated shipping of the samples.

After 24 h the sponge-wipes and positive control sample were removed from the refrigerator and processed as described by Rose *et al* [13]. Briefly, each sponge-wipe was aseptically removed from the sample bag and placed into a stomacher bag containing 90 mL PBST. The sponge-wipes were then homogenized in a circulating stomacher (Seward, Worthing, UK) for 1 min at 260 rpm. The positive control stomacher bag was immediately placed into the stomacher and homogenized. Two aliquots of 45 mL were then removed and concentrated by centrifugation for 20 min at 2700 × *g*. After centrifugation about 42 mL of eluent was removed from each tube and discarded. The remaining eluent was then vortexed and combined into one tube for a final eluent volume of approximately 6 mL. The eluent was then vortexed for 2 min with 10 sec bursts.

To quantify recovery, up to 2 mL eluent was removed and placed into a separate tube. Appropriate 1:10 serial dilutions in PBST were performed, with 100 μL aliquots spread plated in duplicate on CCFA-HT. If the initial inoculum was 10^2 spores/mL, 500 μL was also plated, and if inoculum was 10^1 spores/mL, 1 mL aliquots were filtered in duplicate and filters placed on CCFA-HT. All aliquots were incubated for 48 hrs at 36°C anaerobically before enumeration.

2.4 Rapid-viability PCR Protocol Development

The rapid-viability PCR method is based on testing samples at T_0 and at another time point after sample incubation. In order to determine the appropriate media for incubation of the sample eluent for recovery of *C. difficile* five different broth media were compared; *C. difficile*-Moxalatam & Norfloxacin +0.1% sodium taurocholate (CDMN) [14], *C. difficile* Brucella Broth (CDBB) [15], Cycloserine-cefoxitin-fructose broth (CCFB) [16], Thioglycolate Broth with Hemin, Vitamin K and no indicator (Thio; Anaerobe Systems), and Difco® Liver Infusion Broth (LIB; BD). *C. difficile* spores at a concentration of 10^2 spores/mL were spiked onto 2 sponge-wipes (3M) which were combined and processed as described in 2.3. Each broth was inoculated with 100 μL of eluate from the sponges (10 replicates each). The mean spore count in 100 μL was 3.8 spores (SD=1.9). The broths were then incubated in the anaerobe chamber (Coy Lab Products, Grass Lake, MI) and checked for turbidity/growth at 2 and 4 days.

After the best media was determined *C. difficile* growth curves in CCFB (minus phenol red, which was removed due to interference with optical density measurements) were conducted to determine the incubation time required; initial inoculum levels of 10^4 and 10^1 spores/mL were compared. Two sets of flasks containing 99 mL of pre-reduced CCFB were inoculated with 1 mL of either a 10^6 or 10^3 spores/mL suspension. The flasks were swirled to distribute

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the inoculum evenly and 3 mL was removed (T_0 sample) from each flask. The flasks were placed in an incubating anaerobe chamber (35°C) and one set of flasks were placed on a shaker table and shaken at low speed while the other was not shaken. At specific time points, the suspension was mixed well and 3 mL were removed from the flasks. One mL was used to measure optical density (OD) at 860 nm (DR/4000 Spectrophotometer; Hach, Loveland, CO) and another 1 mL was diluted in series and plated on brain heart infusion agar with horse blood and taurocholate (BHI-HT; Anaerobe Systems, Morgan Hill, CA). The BHI-HT plates were incubated anaerobically at 35°C for 48 h. To determine the T_0 sample concentration for the 10^1 inoculum level, 1 mL was filtered by membrane filtration onto 0.45 μm filters (Pall), in duplicate. The filters were rinsed with PBST then placed onto BHI-HT plates. The plates were then incubated anaerobically for 48 h at 36°C.

2.5 RV-PCR Sample Processing

For each clean and dirty sponge-wipe sample the remaining eluent after culture (~4 mL) was transferred into 0.45 μm AutoCup® filter funnels (Whatman, Maidstone, UK). Any spores in the eluent were then captured by the 0.45 μm filter within the filter cup using a vacuum manifold which can be seen in Fig. 1. Filters were washed with 7 mL of filter-sterilized 207 mM KH_2PO_4 buffer (pH 6.0) followed by 3 mL of filter-sterilized 25 mM KH_2PO_4 buffer (pH 7.2). The filter cups were then sealed at the bottom with a plastic quick-turn tube coupling cap (McMaster-Carr, Elmhurst, IL), 2.5 mL pre-reduced CCFB growth medium (without phenol red) was added, after which the filter cup tops were sealed with a CEP plug (Caplugs, Buffalo, NY). After vortexing for 10 min on a multi-tube vortexer (VWR, Radnor, PA), a 500 μL aliquot (T_0 sample) was removed and transferred to a 2 mL Lysing Matrix E bead beating tube (MP Biomedicals, Solon, OH) for DNA extraction and purification. The cups were loosely sealed on the top and placed into either an anaerobe chamber on a shaker platform, or a large anaerobic jar (Anoxomat, Advanced Instruments, Norwood, MA) which was sealed and converted to an anaerobic environment. If an anaerobic jar was used, it was placed into an incubating shaker. Incubation was for 22 hrs at 35°C and shaking was set at 75 rpm. Optimal incubation time was determined from growth curve experiments of 10^1 *C. difficile* spores/mL inoculated into CCFB to determine exponential phase. After incubation was complete, the filter cups were removed from the anaerobic jar, caps tightly sealed, and then placed back on the manifold to be vortexed for 10 min on the multi-tube vortexer. A 500 μL aliquot (T_{22}) was removed and placed into the bead beating tubes. An additional 500 μL was then spread plated onto CCFA-HT to confirm growth during the incubation step. The CCFA-HT plates were anaerobically incubated for 48 hrs at 35°C.

2.6 DNA Extraction and Purification

The sample aliquot bead beating tubes were placed on a vortex with a multi-tube adapter (MoBio, Boulder, CO) and vortexed for 10 min at high speed. The tubes were then centrifuged at 5000 rpm for 1 min. About 400 μL was then transferred into the Maxwell Casework LEV cartridge (Promega, Madison, WI). The Maxwell cartridges and elution tubes were set-up following the manufacturer's instructions and processed on the Maxwell 16 (Promega, Madison, WI). When extraction and purification was complete the elution tubes were stored at −20°C until needed for real-time PCR.

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DNA controls for *C. difficile* toxigenic and non-toxigenic strains were extracted by adding 500 μL of 10^8 spores/mL suspension to the bead beating tubes along with 200 μL PBST. The bead beating tubes were vortexed for 10 min and then centrifuged at 5000 rpm for 1 min. About 400 μL was then transferred into the Maxwell Cell SEV cartridge. The Maxwell cartridges and elution tubes were set-up following the manufacturer's instructions and processed on the Maxwell 16. When extraction and purification was complete the elution tubes were stored at −20°C until needed for real-time PCR. DNA controls for the other anaerobe species and Gram negative and positive reference strains were extracted using standard boil preparation procedures [17]. Concentrations of DNA were measured on the Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). Standard *C. difficile* spore DNA concentrations ranging from 35 ng/μL to 35 fg/μL were prepared in PCR-grade water. Eight 10-fold dilutions, ranging from 180 ng per 20-μL PCR to 180 fg per 20-μL PCR, were run with each PCR plate.

2.7 Real-time PCR and Data Analysis

Standardization of the duplex real-time PCR assay was done using nine *C. difficile* toxigenic isolates (*tcdB*) and three non-toxigenic isolates, along with four other anaerobic bacterial isolates, and five common aerobic bacteria found on hospital environmental surfaces (*A. baumannii*, *E. coli*, *K. pneumoniae*, methicillin-resistant *S. aureus*, and vancomycin-resistant *E. faecium*; Table 1).

Primers and Taqman probes for *tcdB* and *cdd3* are listed in Table 2. The *tcdB* probe was HEX-labeled [18] and the *cdd3* probe was FAM-labeled. Primer and probe concentrations were optimized so that the standard curves yielded R^2 values above 0.900. Five-microliters of template DNA was added to the duplex reaction containing each set of forward and reverse primers at concentrations of 500 nM (*tcdB*) and 400 nM (*cdd3*), probes at a concentrations of 200 nM (*tcdB*) and 250 nm (*cdd3*), and 10 μL 2x PerfeCTa FastMix II, Low ROX (Quanta Biosciences, Gaithersburg, MD) for a final volume of 20 μL. An initial denaturation cycle of 95°C for 10 min was followed by 40 cycles of 95°C for 1 min, 54°C for 30 sec, and 72°C for 1 min on the ABI7500FAST (Applied Biosystems, Foster City, CA).

PCR assay selectivity was tested for both *tcdB* and *cdd3* against eight *C. difficile* environmental toxin B positive isolates, 2 toxin negative environmental isolates, ATCC 43255 (toxin negative strain) and four other anaerobe species.; *T. glycolicus*, *C. novyi*, *C. perfringens*, and *C. septicum* (Table 1). Other pathogens were also tested; *A. baumannii*, *E. coli, K. pneumoniae*, *S. aureus*, and *E. faecium* (Table 1). All isolates were tested in triplicate. Specificity was determined by the C_T result for known negative isolates and sensitivity was determined by the threshold for known positive *tcdB* isolates and *C. difficile* isolates. Three toxin B positive control samples (ATCC 43598) and three negative controls (PCR-grade water) were run with each assay.

A C_T value ≤ 35.0 at T_0 was considered positive for detection of *C. difficile*. The criteria needed to consider the results positive for viable *C. difficile* spores with the RV-PCR method included a T_{22} $C_T \leq 35.0$ and a $\Delta C_T (C_T[T_0] - C_T[T_{22}])$ of ≥ 9.0 which represents a 3-log

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increase in DNA concentration after 22 hrs of incubation. If the PCR C_T was not determined at either time point, a C_T value of 40 was used for calculation purposes.

3. Results and Discussion

3.1 RV-PCR Protocol Development

Five different broths were compared for recovery of *C. difficile* (mean inoculum 3.8 spores, SD 1.9) from spiked sponge-wipe samples: CDMN, CDBB, CCFB, THIO, and LIB. After 2 days incubation, only the CCFB samples were positive for growth (all 10 replicates). After 4 days of incubation, the number of broth tubes that were positive for *C. difficile* were: 10/10 CCFB, 7/10 CDDB, 3/10 LIB, 0/10 CDMN, and 0/10 Thio. CCFB was determined to be the most reliable broth to use for rapid growth of *C. difficile* in this RV-PCR method.

Next, growth curve analyses were performed to determine the optimum incubation time between sample time points. At the lowest inoculum level of 30 spores/mL, the generation time was 1.17 h/gen and exponential phase was reached within 22 h (7.65×10^6 spores/mL) with shaking of the sample, without shaking it was 1.18 h/gen. With a higher starting inoculum level of 3.60×10^4 spores/mL, the generation time was 1.29 h/gen with shaking and 1.35 h/gen without shaking, exponential phase was reached earlier between 11 and 13 h for both. When PCR analysis was performed on the T_{22} samples 7/10 samples were positive when shaking was used whereas only 5/10 were positive without shaking. In order to determine the LOD, the longer incubation period of 22 h was chosen for the RV-PCR, however in the future it may be possible to decrease the incubation time and still obtain valid results as seen with *B. anthracis* [11]. In addition, the limit of detection (LOD) for standard PCR analysis of environmental eluates is approximately 10^4 spores per mL, since only 5 μl of eluate is analyzed. Improving the LOD is critical to enable better detection.

3.2 Real-time PCR Assay Standardization

The real-time PCR assay for this study focused on toxin B positive *C. difficile* because it is one of the two large Clostridial toxins that *C. difficile* produces and a principal virulence factor [19]. The assay selected for toxin B detection has an established Taqman real-time PCR primers and probe set for gene *tcdB*, which was tested against 3 reference *C. difficile* strains and 24 fecal samples from calves, and it distinguished *C. difficile* strains well from other anaerobes [18]. To differentiate between toxin B positive strains and other nontoxigenic *C. difficile* the *cdd3* gene (found in all *C. difficile* strains) PCR primers were modified using GenScript for real-time PCR and a Taqman probe was designed (Table 2). Further assessment of the sensitivity and specificity of both *tcdB* and *cdd3* was conducted against 8 *C. difficile* environmental toxin B positive isolates, 2 toxin negative environmental isolates and ATCC 43255 (a toxin negative strain). Additional anaerobic bacteria were tested: *T. glycolicus*, *C. novyi*, *C. perfringens*, and *C. septicum* (Table 1); along with other antimicrobial-resistant pathogens likely to be found on hospital surfaces: *A. baumannii*, *E. coli*, *K. pneumoniae*, MRSA, and VRE. Specificity was demonstrated for all toxin B positive strains which were correctly detected by the primer/probe combo after optimization. The other anaerobes were either not detected by PCR or produced C_T values > 35.0 (*C. septicum*). For all *C. difficile* strains *cdd3* was correctly detected and C_T values for the other

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anaerobes were all >35.0. All of the aerobic bacterial strains tested negative for both *tcdB* and *cdd3*. Primer/probe combinations were optimized individually and then tested together for any interference. No decreases were seen in C_Ts, R^2, or efficiency of either assay when run as a duplex assay. Fig. 2 shows the standard curve for the control *C. difficile* spores DNA with an n = 3 for each point and R^2 values of 0.9945 for *tcdB* and 0.9944 for *cdd3*. The sensitivity LOD for the assays was 3.38×10^{-5} ng for *tcdB* and 4.92×10^{-5} ng for *cdd3*. The presence of ATS was tested with varying levels of spores and no difference in C_T values were seen, indicating no interference by organic matter.

3.3 Detection of Live *C. difficile* Spores from Spiked Sponge-wipe Samples without Soil (Clean)

Sponge-wipes spiked with $10^4 – 10^1$ *C. difficile* spores alone showed differences in detection by real-time PCR dependent on initial spore concentration at T_0. Average C_T values at T_0 were <35.0 for only the 10^4 inoculum, and inoculum levels of $10^3 – 10^1$ spores resulted in C_T values of >35.0 or undetected for both assays (Table 3). This indicates that the LOD for the PCR assay is 10^4 spores/mL. Spore concentrations from the T_0 samples consistently agreed with the inoculum levels as determined by culture (CFU). After 22 h of incubation, however, all C_T values for the duplex assay at all inoculum levels were <35.0 except when the samples did not produce growth as confirmed by a negative culture result from the T_{22} samples. For the 10^3 and 10^2 inocula, 1/10 samples did not grow as confirmed by the T_{22} culture. Culture results from the T_0 sample verified that the samples were inoculated with viable spores; however, growth of the sample did not occur. This could have occurred because the aliquot taken from the sponge-wipe eluent for the RV-PCR incubation had too low of a concentration of spores. When applying the RV-PCR positive criteria to determine sensitivity of the procedure, the 10^4 and 10^1 spores/mL inoculum levels were 100% sensitive. The sensitivity was 90% for both 10^3 and 10^2 spores/mL inoculum level (Table 3). Specificity was 100% for all inoculum levels.

3.4 Detection of Viable *C. difficile* Spores from Spiked Sponge-wipe Samples with Soil (Dirty)

To simulate a healthcare surface sample, sponge-wipes were spiked with $10^4 – 10^1$ *C. difficile* spores/mL and a mixture of ATS with *E. coli*. While ATS was designed to simulate the soiling of medical devices, it also mimics what might be found on hospital surfaces, thus provides a good background organic soil for healthcare environmental surface samples. In addition, *E. coli* was chosen as a background organism since it is a common enteric bacteria. Since *E. coli* can also grow in an anaerobic environment, but not in CCFB, it was a negative control for the selectivity of CCFB for the RV-PCR procedure. As seen with the clean samples, differences in detection by real-time PCR were dependent on initial spore concentration at T_0 and average C_T values at T_0 were <35.0 only for 10^4 inoculum; for $10^3 – 10^1$ spores the C_T was either >35.0 or undetected for both assays (Table 3). This reinforces the fact that the LOD is 10^4 spores/mL and that the presence of organic material and other bacteria does not inhibit growth of the spores or the detection by PCR. Spore concentrations from the T_0 samples consistently agreed with the inoculum levels as determined by culture (CFU). After 22 h of incubation all C_T values for the duplex assay and all inoculum levels were < 35.0.

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When applying the RV-PCR positive criteria (ΔC_T(C_T[T_0] - C_T[T_{22}]) of ≥ 9.0), specificity and sensitivity at all inoculum levels were 100% (Table 4). The increased sensitivity when dirty samples are processed could be due to the ATS altering the pH, charge, or hydrophobicity of the sponge and/or the spore coat, thereby allowing better release from the sponge or by providing better dispersion of spores in the eluent as compared to PBST [20]. Sampling may occur before and after cleaning so some samples may be dirtier than others and some may include disinfectant residue. The presence of such residue was not tested in this study, however the sponge-wipes were pre-moistened with neutralizing buffer thus any disinfectant residue should be neutralized before the sample is processed and growth of the spores in the CCFB not inhibited. In the study by Shams *et al.* the same sponge-wipes were used to culture environmental surfaces for *C. difficile* and CCFB was used for qualitative recovery [7]. The surfaces were sampled after cleaning thus there was the likelihood of disinfectant residue but recovery of *C. difficile* was still achieved [7]. The impacts of residual disinfectant and surfactants, such as Tween 80, are currently unknown for PCR and it is possible that low concentrations could have an inhibitory effect on the PCR [21, 22]. However, use of the Maxwell Casework kit or another strategy for removing PCR inhibitors can mitigate this issue. Additionally, the level of background dust might be higher on some surfaces, and further studies could be conducted to determine if increased dust levels or other inhibitory materials impact growth or PCR. Letant *et al* found that when the Arizona test soil amount was increased from 0.25 g to 0.5 g, the RV-PCR method showed a slight underestimate of the number of *B. anthracis* spores, though not significantly different from the culture results [11]. The purpose of this method is to detect viable *C. difficile* spores from surfaces as part of an epidemiological investigation, most likely in a healthcare facility or home. Thus the amount of dust on the surfaces may vary but should not be excessive. Since most surfaces in healthcare facilities are frequently cleaned, a level such as used in this study is reasonable.

4. Conclusion

A rapid viability method was adapted to detect the presence and relative quantity of viable *C. difficile* spores from simulated clean and dirty environmental sponge-wipe samples. The protocol is summarized in Fig. 1. Briefly, sample eluents from processed sponge-wipes were filtered under vacuum in filter cups before adding CCFB growth media. The samples were vortexed and a T_0 sample aliquot was removed for DNA extraction and purification. The remaining sample was incubated anaerobically for 22 hrs and a second aliquot (T_{22}) was removed for DNA extraction and purification. Both sample aliquots were analyzed by real-time PCR and the change in C_T value between the two aliquots were used to determine the presence of viable spores recovered from the sponge-wipe sample. Detection of *C. difficile* at inoculum levels of 10^1 viable spores on a sponge-wipe was achieved within 28 h, using a duplex real-time PCR method that was able to confirm the presence of toxin B positive *C. difficile* along with non-toxigenic *C. difficile*. RV-PCR detection levels correlated well with traditional plate counts for samples containing 10^1 spores. The duplex real-time PCR LOD was 10^4 spores/mL. The presence of background organics (ATS), surfactants (Tween 80), and other organisms (*E. coli*) did not negatively influence detection. One limitation of this method is that the influence of disinfectants on this assay was not tested. The sponge-wipes

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are wetted with neutralizing buffer which should deactivate most disinfectants used on healthcare surfaces, but additional studies are needed to confirm that disinfectants would not adversely influence this assay.

This method greatly decreases the amount of time required to detect viable *C. difficile* spores; incubation of samples is only required for germination (22 hours or less) instead of colony formation, which can take 2 – 7 days. Real-time PCR can then quickly confirm or deny the identity of the organism at the same time it would confirm viability unlike current PCR methods used to detect toxins. Though quantitation was not possible below the 10^4 LOD, the presence of viable *C. difficile* spores can be detected at 10^1 spores per sample within 28 hours compared to the 2–10 day process needed for culture, identification and toxin detection. The results would be reported as $< 10^4$ viable spores present per sample. This procedure provides rapid detection of viable *C. difficile* spores in the environment during an epidemiological investigation and can potentially be used to determine if cleaning methods are adequate for disinfection of *C. difficile* spores. The study was limited to a laboratory simulation of clean and dirty samples; therefore, future studies will include field application of the protocol with surface sponge-wipe samples collected from environments where *C. difficile* contamination is suspected.

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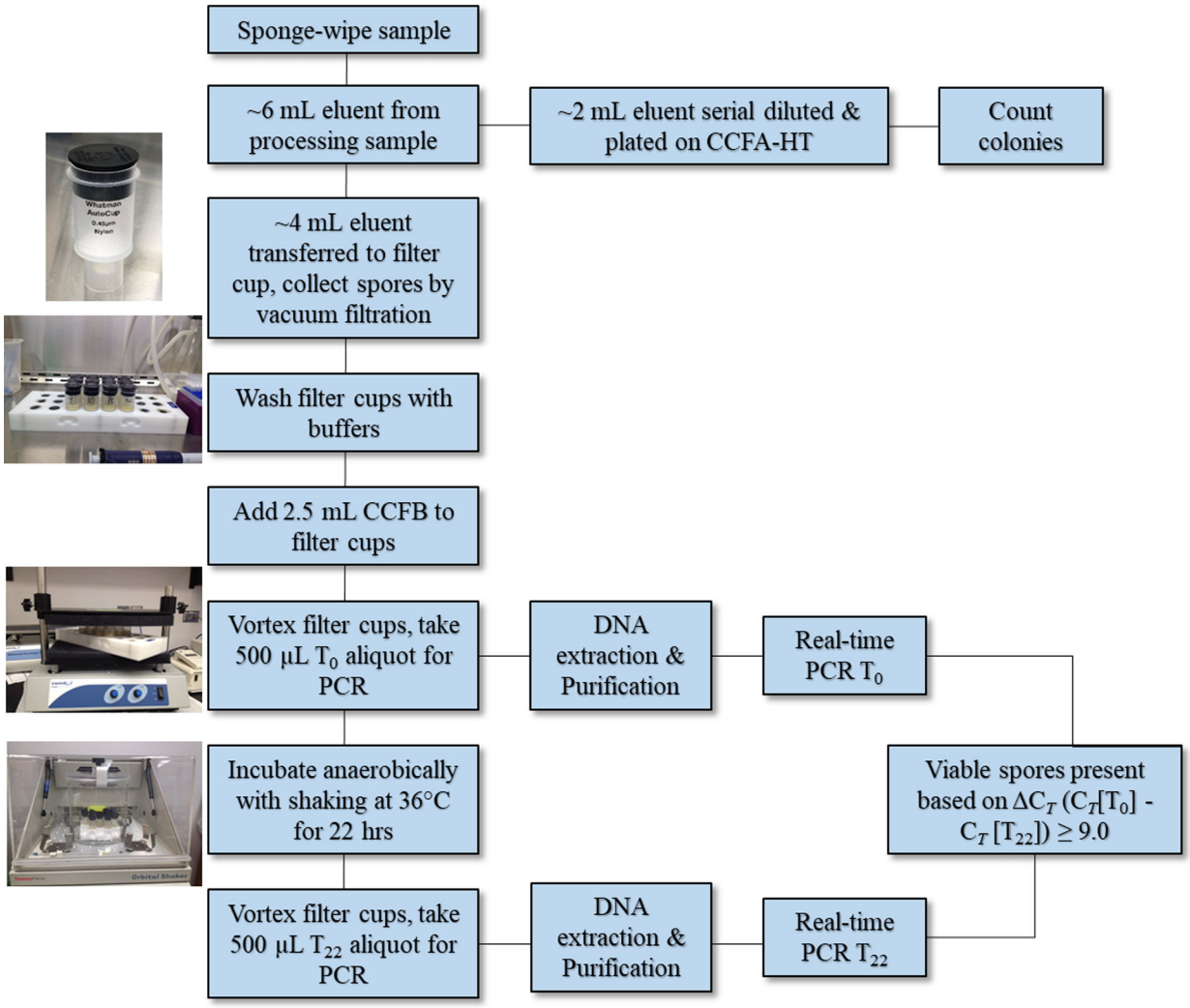

Fig. 1.
Summary of RV-PCR sample processing steps.

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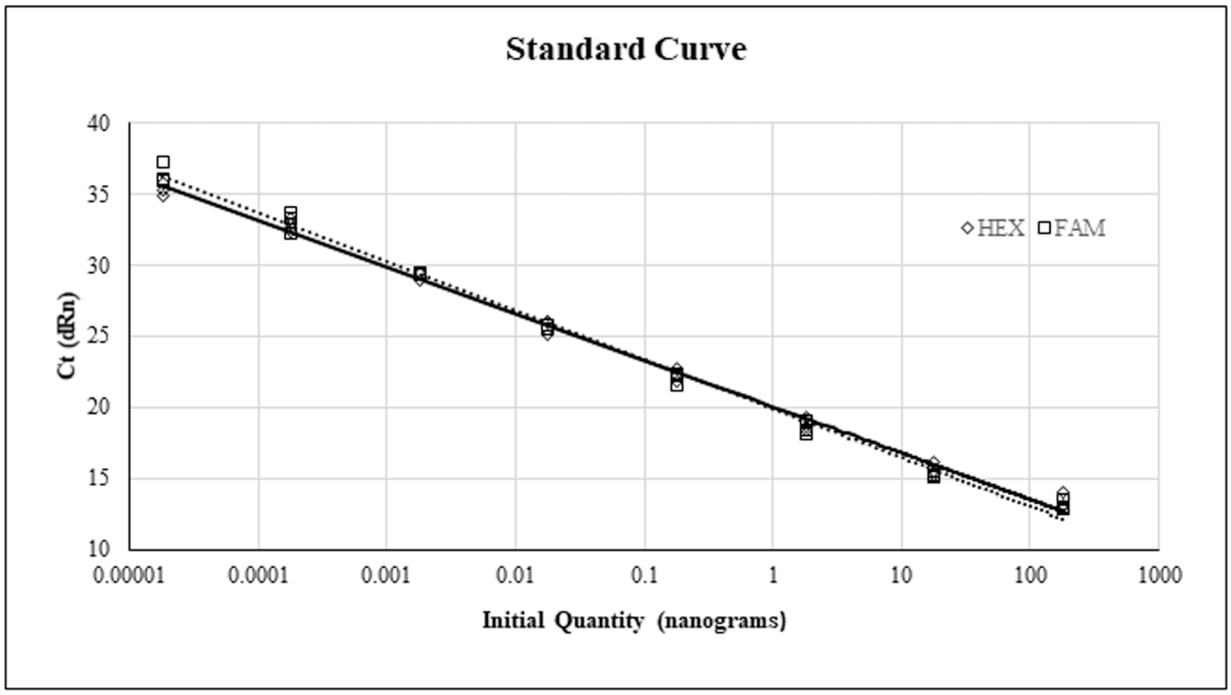

Fig. 2.

Log scale standard curves for detection of *tcdB* (HEX) and *cdd3* (FAM) from *C. difficile* DNA. HEX $R^2 = 0.9945$ and FAM $R^2 = 0.9944$. n = 3.

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Table 1.

Isolates used for PCR Standardization

Isolate	Source	tcdB	*cdd3*
Clostridioides difficile	Environmental Sample IL123	−	+
	Environmental Sample MD131	+	+
	Environmental Sample MN46-1	−	+
	Environmental Sample MN55-1	+	+
	Environmental Sample MN163	+	+
	Environmental Sample MN330B	+	+
	Environmental Sample MN526	+	+
	Environmental Sample GA72	+	+
	Environmental Sample GA102	+	+
	Environmental Sample GA107	+	+
	ATCC 43593	−	+
	ATCC 43598	+	+
Terrisporobacter glycolicus	Clinical isolate 2008298	−	−
Clostridium novyi	Clinical isolate 200073-2	−	−
Clostridium perfrigens	Clinical isolate 20100347	−	−
Clostridium septicum	Clinical isolate 2007189	−	−
Klebsiella pneumoniae	Environmental isolate 2001-22-18B	−	−
Staphylococcus aureus	ATCC 43300	−	−
Acinetobacter baumannii	Environmental isolate 2008-23-01-01	−	−
Enterococcus faecium	Reference isolate 4402-07	−	−

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Table 2.

Primer and Probe Sequences for *Clostridioides difficile* toxin B and *cdd3*

Target	Oligo	Sequence (5'−3')
tcdB	tcdB F	GGTATTACCTAATGCTCCAAATAG
	tcdB R	TTTGTGCCATCATTTTCTAAGC
	tcdB probe	HEX-ACCTGGTGTCCATCCATCCTGTTTCCCA-BHQ
cdd3	cdd3 F	ACTACCAATCATGCCAAATG
	cdd3 R	TGCTATCATTGCACTACACCT
	cdd3 probe	FAM-TGGATTGCAACTGTAATTCCAAATCA-BHQ

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Table 3.

Average C_T values (n = 10) and standard deviations (SD) at T_0 and T_{22} for Sponge-wipes inoculated with *C. difficile* spores without ATS+*E.coli* matrix (clean) and spores with ATS+*E.coli* matrix (dirty)

Sponge-wipe spike type	Spore Inoculum Concentration (Spores/mL)	T_0		T_{22}	
		tcdB	***cdd3***	***tcdB***	***cdd3***
Clean	10^4	32.75 (0.71)	33.02 (0.97)	13.37 (0.26)	13.24 (0.21)
	10^3	35.24 (0.57)	35.85 (1.39)[a]	15.65 (6.38)[b]	15.56 (6.71)[b]
	10^2	37.64 (0.08)[a]	ND[c] (−)	18.72 (4.53)[b]	18.60 (4.49)[b]
	10^1	ND (−)	38.53 (−)[a]	15.27 (1.09)	15.19 (1.07)
Dirty	10^4	32.34 (0.96)	33.61 (1.09)	13.49 (1.08)	13.97 (1.21)
	10^3	36.41 (1.06)	36.78 (1.67)[a]	14.70 (0.52)	14.11 (0.59)
	10^2	38.07 (−)[a]	37.83 (0.30)[a]	14.62 (0.62)	13.96 (0.71)
	10^1	39.32 (−)[a]	ND (−)	21.84 (1.44)	21.51 (1.45)

[a] Average and SD calculated for less than 10 samples due to no C_T value detected for 1 or more samples

[b] 1 sample negative at T_{22} by real-time PCR and culture

[c] ND indicates that no C_T was detected after 40 cycles for any of the 10 samples

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Table 4.

RV-PCR T_{22} positive criteria results (sensitivity and specificity)

Initial Spore Concentration (Spores/mL)	Sensitivity		Specificity	
	Clean	Dirty	Clean	Dirty
10^4	100% (10/10)[a]	100% (10/10)	100% (10/10)	100% (10/10)
10^3	90% (9/10)	100% (10/10)	100% (10/10)	100% (10/10)
10^2	90% (9/10)	100% (10/10)	100% (10/10)	100% (10/10)
10^1	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)

[a]Number of samples positive for *C. difficile* according to RV-PCR criteria