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A comparison of air sampling methods for *Clostridium difficile* endospore aerosol

Casey W. Cooper · Kathleen A. N. Aithinne · Evan L. Floyd · Bradley S. Stevenson · David L. Johnson

Abstract The airborne dissemination of *Clostridium difficile* (*C. difficile*) endospores (spores) in healthcare environments is documented in multiple studies. Once airborne, spores have the potential for transport on air currents to other areas. This study compared the methods in the collection of *C. difficile* spore aerosol. This study determined the relative efficiency of commonly used bioaerosol air sampling methods when characterizing airborne *C. difficile* spore concentrations. Air samplers evaluated in this study were the AirTrace slit-to-agar impactor, AGI-30 impinger, SKC BioSampler impinger, and a 47-mm mixed cellulose ester (MCE) filter cassette. Non-toxigenic *C. difficile* spores were nebulized into an enclosure contained in a biological safety cabinet. Side-by-side air samples were drawn from the enclosure. The slit-to-agar impactor, successfully used in previous studies to collect airborne spores, served as the reference method. Relative efficiency for the 47-mm MCE filter cartridge was higher than the slit-to-agar impactor (mean 136.6%, 95% CI 124.7–148.5%). Efficiencies of the impingers were similar and were low (mean 4.13%, 95% CI 2.27–5.99%). Impingers failed to maintain culturability of *C. difficile* spores during sampling. This study is the first to compare the efficiencies of commonly used bioaerosol sampling methods to collect airborne *C. difficile* spores. Filter air sampling provided the greatest collection of airborne spores. Slit-to-agar air sampling may underestimate the number of airborne spores present. Impinger air sampling could significantly underestimate the actual number of airborne *C. difficile* spores present or fail to detect airborne spores.

Keywords *Clostridium difficile* · Air sampling · Aerosol · Airborne dissemination · Filter · Impinger

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1 Introduction

1.1 Background

Healthcare-acquired *Clostridium difficile* infection (CDI) is a significant burden to US healthcare facilities

and their patients, with an estimated 453,000 incident CDI and 29,000 deaths in 2011 (Lessa et al. 2015). Of these nearly half million cases, an estimated 293,300 were healthcare-associated with 107,600 hospital onset, 104,400 nursing home onset, and 81,300 post-outpatient care onset. CDI is the leading cause of gastroenteritis-associated death, causing 14,000 deaths in 2007 alone (Hall et al. 2012), was responsible for an estimated $4.8 billion in excess healthcare costs for acute care facilities in 2008 (Dubberke and Olsen 2012), and has become one of the most common healthcare-acquired infections in the USA (Magill et al. 2011).

CDI patients experience multiple episodes of watery diarrhea each day, and both *C. difficile* bacterial endospores (spores) and vegetative cells are shed in stool. Spore concentrations in symptomatic CDI patients may be from 10^4 to 10^6 spores per gram of stool (Kim et al. 1981; Naaber et al. 2011), and vegetative cell concentrations may be an order of magnitude more abundant (Jump et al. 2007). Whereas vegetative cells die off rapidly in the environment on dry surfaces (Jump et al. 2007), spores are resistant to environmental degradation and will survive in the environment for long periods of time (Gerding et al. 2008). The spores are infectious, and CDI transmission is believed to be primarily via hand contact with the patient and contaminated environmental surfaces during patient care (McDonald et al. 2018).

Environmental contamination of CDI patient care rooms and adjacent areas with *C. difficile* spores is a recognized contact transmission risk factor, which has been demonstrated in numerous studies conducted since the 1980s (Kim et al. 1981; McDonald et al. 2018). Culturable spores have been collected from surfaces in treatment rooms with symptomatic patients, asymptomatic patients, and even patients with no evidence of *C. difficile* colonization. Recent clinical practice guidelines for CDI prevention and control include: accommodate CDI patients in a private room with a dedicated toilet if possible, or cohort CDI patients if insufficient rooms are available; gown and glove upon entry to a CDI patient room and during patient care; perform hand hygiene before and after CDI patient contact; continue precautions for at least 48 h after diarrhea has resolved; perform terminal room cleaning with a sporicidal agent; and consider daily cleaning with a sporicidal agent during outbreaks or in hyper-endemic settings (McDonald

et al. 2018). Because CDI is only considered a contact transmission risk, negative pressure isolation in an engineered Airborne Infection Isolation Room is not included.

These practice guidelines implicitly assume that spore contact risk is limited to the patient, fomites in the patient room, and room surfaces; however, several studies have shown that *C. difficile* spores are intermittently aerosolized during patient care activities such as bedding changes, toilet flushing, and patient feeding or meal delivery. These spores can remain airborne for extended periods (Aithinne et al. 2018; Best et al. 2010, 2012; Roberts et al. 2008). These "droplet nuclei" spore aerosols could then travel with air currents and may contaminate environmental surfaces remote from the patient room. This airborne transport and deposition may provide additional, and likely unsuspected, opportunities for contact transmission. However, few studies have been successful in isolating *C. difficile* from air samples in healthcare environments (Roberts et al. 2008; Best et al. 2010, 2012).

Commonly used bioaerosol sampling methods include liquid impingement (e.g., SKC BioSampler and AGI-30 impingers), filtration (e.g., mixed cellulose ester and gelatin filters), and direct-to-agar impaction (e.g., MB2 and N6 multi-hole impactors, AirTrace slit-to-agar impactor) (Willeke and Macher 1999). Filtration is the simplest of the air sampling methods. A filter with support pad is placed in a cassette that is connected to a calibrated air sampling pump. The air flow rate may be selected from within a range of allowable values determined by the filter media and filter area. Filters may be extracted into a liquid for subsequent spread plating on agar-filled culture dishes, or placed directly on the agar surface. Gelatin filters will dissolve into the agar, whereas mixed cellulose ester (MCE) filters must absorb agar media in sufficient amounts to support colony development. Liquid impingers direct a high-velocity jet of particle-laden air onto the surface of collection fluid. The jet is forced to change directions abruptly, and the particles' inertia causes them to strike the liquid and be captured. Whereas the AGI-30 has a single nozzle oriented normally to the liquid surface, the BioSampler has three nozzles oriented at an angle to the liquid surface. The angled nozzles are intended to reduce stress on captured organisms and minimize re-aerosolization of captured organisms from the

collection fluid. After sampling, an aliquot of the collection fluid (diluted as necessary) may be spread onto agar-filled culture dishes; alternatively, the fluid may be filtered and the filter was then placed on agar for incubation. The slit-to-agar impactor directs a high-velocity stream of air through a narrow slit and impinges it onto the surface of an agar-filled culture dish, much like a knife preparing to cut into the radius of a birthday cake. The sheet of air must make an abrupt 90° turn, causing entrained particles to impact on the agar due to their inertia. The agar plate is slowly rotated under the inlet slit, so that particles will deposit over the agar surface area covered by the moving line of impingement. Collection plates from jet-to-agar or slit-to-agar impactors are directly incubated. To date, no studies have been reported that compared the relative performance of these methods when sampling for airborne *C. difficile* spore aerosol, and only one of these (slit-to-agar impaction) has been successful in detecting airborne *C. difficile* in a healthcare environment (Best et al. 2010).

Characterization of airborne *C. difficile* spore concentrations is needed to fully perform risk assessments for the transport of *C. difficile* in healthcare environments. It is therefore of interest to determine which sampling methods are most efficient for determining *C. difficile* spore aerosol presence in healthcare environments so that strategies can be developed for quantifying spore aerosol generation sources and rates during patient care, characterizing spore migration patterns in the care environment, and developing more effective strategies for minimizing transmission risk from patient contact with aerosol-transported spores.

1.2 Goals of this investigation

Our goal was to determine the relative efficiency of inexpensive and commonly used bioaerosol air sampling methods compared to the more expensive (but known to be successful) slit-to-agar method when characterizing airborne *C. difficile* spore concentrations. We compared, under controlled conditions, the relative capture efficiencies of commonly used collection devices based on liquid impingement, slit-to-agar impaction, and filtration. Our hypothesis was that the sampling efficiencies of filter- and impinger-based sampling methods would be comparable to those of the more expensive slit-to-agar method successfully used in previous studies.

2 Methods

2.1 Spore suspensions

All experiments were performed using a non-toxigenic strain of *C. difficile* (ATCC 700057, Microbiologics, St Cloud, MN). Spore suspensions were prepared after the method of Aithinne et al. (2018). Briefly, source organisms were placed in 500 mL of brain–heart broth and incubated anaerobically at 37 °C for 10 days. This extended incubation time ensured depletion of broth nutrients, resulting in sporulation. The resulting spore suspension was heat-shocked at 80 °C for 20 min to remove any remaining vegetative cells. The spore suspension was then mixed and separated into 50-mL aliquots to be centrifuged for 15 min at $5000g$. For each aliquot, supernatant broth was decanted, and the pelleted spores were re-suspended in sterilized water and were pelleted again via centrifugation for 15 min at 5000 g. The washing step was repeated three more times, and the final suspensions were refrigerated at 4 °C until needed.

2.2 Air samplers

The bioaerosol samplers evaluated were the AGI-30 liquid impinger (Ace Glass, Vineland, NJ), BioSampler liquid impinger (SKC, Eighty-Four, PA), AirTrace rotating plate slit-to-agar impactor (Particle Measuring Systems, Boulder, CO), and mixed cellulose ester (MCE) filters in conductive cassettes (MilliporeSigma, Burlington, MA) (Fig. 1). As previously noted, the liquid impingement and filtration-based devices are commonly used for bioaerosol sampling (Willeke 1999). MCE filters were included both because they are commonly used and because Xu et al. (2013) found that airborne bacteria in indoor environments could be cultured from MCE filters placed directly on ChromAgar® media. MCE filters were also shown to be compatible with ChromAgar media for enumerating *C. difficile* spores in water when placed directly onto the media surface (Aithinne et al. 2018). Gelatin filters were excluded due to their tendency to dry out during sampling (Macher and First 1984) and because its potential effect on *C. difficile* culturing on ChromAgar® was unknown. The rotating plate slit-to-agar impactor was included as the reference device because of its success in isolating airborne
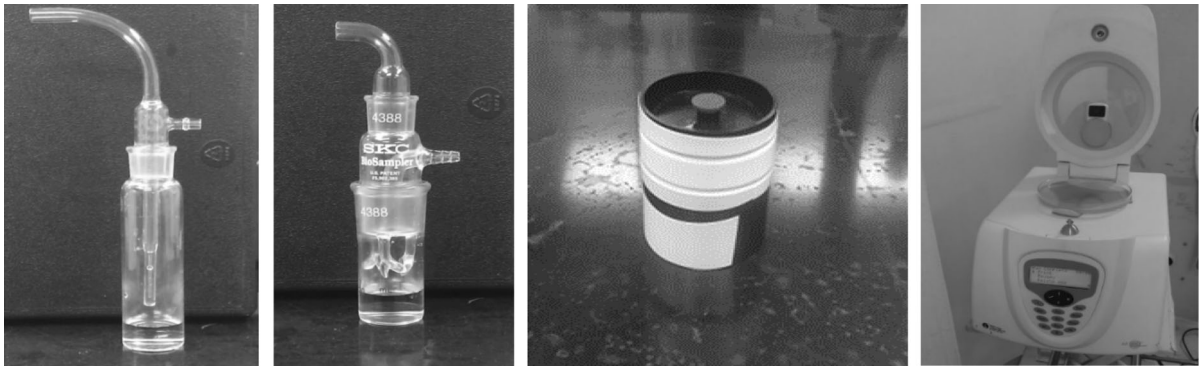
Fig. 1 Air samplers used in the study included (left–right) the AGI-30 impinger, BioSampler impinger, 47-mm MCE filters in conductive cassettes, and AirTrace slit-to-agar rotating plate impactor (not to scale)

C. difficile in recent studies (Best et al. 2010, 2012; Aithinne et al. 2018).

Low suspension concentrations were expected, so in this work we chose to filter the undiluted impinger liquid to maximize our detection limit and minimize potential variability due to inhomogeneous suspensions. Distilled water or phosphate-buffered saline is typically used as the collection fluid. PBS was selected as the impinger fluid due to its demonstrated effectiveness at preserving *C. difficile* spores in long-term storage up to 56 days without the loss of viability (Freeman and Wilcox 2003). The total colonies counted divided by the total volume of air sampled provides an estimate of the average air concentration during the sampling period, expressed as colony-forming units per cubic liter of air sampled (CFU/L). Both impingers were sampled at 12.5 L/min $\pm$ 10% and were filled with 20-mL sterile PBS (Sigma-Aldrich, St. Louis, MO) per manufacturer recommendations. Under these conditions, both types of impingers have been shown to have an absolute capture efficiency for particles 1 μm aerodynamic diameter of approximately 80–90% for the AGI-30 when sampling 1.1-μm *Bacillus cereus* spores (Grinshpun et al. 1997) or 1.0-μm inert particles (Willeke et al. 1998) and approximately 96% for the BioSampler sampling 1.0-μm inert particles (Willeke et al. 1998). Larger size particles up to 4 μm size have similar or higher absolute capture efficiencies for both types of impingers (Kesevan et al. 2010). The physical size of *C. difficile* spores is in the range at approximately 1–1.5 μm length and 0.5–0.7 μm diameter (Snelling et al. 2010). With a dry spore density of approximately 1.42 g/mL (Tisa et al. 1982), this

results in an orientation-averaged aerodynamic diameter in the range of 1.42–1.87 μm (Johnson et al. 1987).

The AirTrace impactor was sampled at a measured air flow rate of 25.5 L/min $\pm$ 10%. Under these conditions, the impactor has a high sampling efficiency for particles of *C. difficile* spore size (Hinds 1999).

We used 45-mm-diameter, 0.45-μm pore-size MCE filters (MilliporeSigma, Burlington, MA) that allowed sampling at the same rate as the slit-to-agar impactor, i.e., 25.5 L/min. After sampling, filters were placed directly onto agar-filled culture plates for incubation and counting, with average air concentrations again expressed in CFU/L. This technique of direct placement of an MCE filter onto agar was used in the collection of environmental bacteria by Xu et al. (2013). Post-sampling, the conductive filter cartridges were decontaminated with a 10% sodium hypochlorite bleach solution, rinsed, and reused with fresh filters. To verify effective decontamination, a blank filter was incorporated in each day of trials.

Clostridium difficile-selective ChromAgar® chromogenic agar (ChromAgar, Paris, France) was used in the impactor plates and in the plates used to culture air sample filters and filters used to recover spores from impinger liquid. This agar contains antibiotics to inhibit other organisms, as well as a reagent that causes the *C. difficile* colonies to fluoresce under ultraviolet light. The media also wicks efficiently into MCE filters when they are placed on the agar surface, which is necessary for the filter culture technique. All plates were anaerobically incubated at 37 °C for 24 h,

and colonies were counted under 365-nm UV illumination per manufacturer specifications.

2.3 Experimental apparatus for side-by-side air sampling

University of Oklahoma Health Sciences Center Institutional Biosafety Committee approval was obtained before conducting any experiments with *C. difficile*. Side-by-side air sampling with liquid impingers, the slit-to-agar impactor, and MCE filters was conducted in an aerosol containment chamber. A 75-L volume transparent plastic aerosol containment chamber was constructed, into which *C. difficile* spore aerosol could be generated using a 3-jet Collison MRE-type air jet nebulizer (Model CN-24, Mesa Labs, Butler, NJ). The chamber was placed within a Type II biological safety cabinet (BSC) to provide secondary containment and HEPA-filtered air for the containment chamber. Aerosol was sampled from the chamber by multiple samplers simultaneously, providing side-by-side comparisons. Due to space limitations, experiments were conducted in blocks with the slit-to-agar impactor and the two impingers as one combination, and the slit-to-agar impactor and two MCE filter cassettes as the other combination. The slit-to-agar impactor served as the "reference sampler" against which the impingers and filters were compared.

The impingers or filter cassettes could be placed inside the chamber, but due to its size the slit-to-agar impactor had to be placed outside the chamber with a 85-cm-long, 12.5-mm ID Tygon® 3606 tube conducting aerosol from the chamber to the instrument's inlet. This setup is similar to that used in previous air sampling studies by Best et al. (2012) utilizing a slit-to-agar impactor with air sample supplied by a Tygon 3606 inlet tube. The potential for losses to tubing walls and bends was assessed through penetration calculations and found to be negligible (1%) for the tubing size and flow conditions used in this work. The impingers, or the MCE filters, were placed to either side of the impactor tube inlet. A 47-mm MCE filter cassette was placed in line between the impinger exhaust and the air pump to capture any spores sampled but not captured, or captured and re-aerosolized. The impinger samplers were operated at 12.5 L/min ± 10% air flow rate and the impactor at 25.5 L/min ± 10%. The filter samplers were operated at 25.5 L/min to match the flow rate of the slit-to-agar impactor. Total air sampled from the chamber was thus approximately 53 L/min for the impinger trials and 76.5 L/min for the filter trials. The nebulizer air flow rate to the chamber was 10 L/min, so an additional 43 L/min of HEPA-filtered makeup air was drawn into the chamber from the BSC interior via relief holes in the chamber walls for the impinger trials and 66.5 L/min for the filter trials. Aerosol generated into the chamber was mixed by an 80-mm-diameter circular air fan. Uniform aerosol distribution across sampling points was verified in a series of nine trials using three MCE filter cassettes placed at different locations in the chamber, for which one-way ANOVA on these side-by-side measures showed no significant difference in indicated concentration at the three sampling locations (data not shown). Nevertheless, impinger location left or right of center for the two impinger types was alternated between impinger trials.

Four different AGI-30 samplers and three BioSamplers were used over the series of trials. The sampler flow rates were verified to be within 10% of their 12.5 L/min design flow. After each impinger trial, the impinger collection fluid was filtered through a 47-mm-diameter, 0.45-μm pore-size MCE filter, which was then placed directly onto a 65-mm-diameter ChromAgar® culture plate and anaerobically incubated at 37 °C for 24 h. The agar readily wicked into the filter matrix. Due to the low concentrations of culturable spores in the impingers, this census method provided a lower limit of detection than would be possible by surface plating a 0.1-mL aliquot. Filters from air filtering sample trials were also placed directly on ChromAgar® plates for culturing.

Sampling efficiencies were assessed relative to the slit-to-agar impactor, which has been shown to be capable of culturing *C. difficile* from healthcare environment air samples (Best et al. 2010). Air sampling time in all trials was 10 min, beyond which excessive impinger fluid losses might occur. For each trial, *C. difficile* air concentrations indicated by the two impingers or by the MCE filters were divided by the concentration indicated by the slit-to-agar impactor to provide measures of relative sampling efficiency.

2.4 Evaluation of impinger culturability retention and re-aerosolization

To evaluate the retention of culturable *C. difficile* spores by impinger air samplers, AGI-30s and BioSamplers were seeded with concentrated spore suspensions in PBS. Concentrations ranged from 500 to 1200 CFU/mL and the impingers drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min ± 10%. 0.1-ml aliquots of the impinger fluid, collected before and after the impinger operation, was spread onto a ChromAgar®-filled culture plate and anaerobically incubated for 24 h. Culturability retention was determined as a ratio of the number of culturable organisms in the impinger before and after impinger operation, as calculated from the colony counts, plated volume, and fluid volumes before and after operation.

2.5 Assessment of impinger re-aerosolization potential

We conducted an additional experiment to assess re-aerosolization, which is known to occur to some extent in liquid impingers due to fluid agitation (Grinshpun et al. 1997; Kesevan et al. 2010). We placed an MCE filter cassette in line between a BioSampler impinger and its air pump, seeded the impinger fluid with concentrated spore suspension at concentrations ranging from 500 to 1200 CFU/mL, and drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min ± 10% as before. Spore concentrations in the impinger fluid were assessed by surface plating dilutions of 0.1-mL aliquots taken immediately after seeding, and again after the 10-min air flow period. Downstream air filters were cultured on agar plates as before. Liquid volumes in the impingers were measured at initial seeding and again after impinger operation. Total culturable organisms present before and after impinger operation were calculated from the colony counts, plated volumes, and pre- and post-operation fluid volumes.

3 Results

Example of slit-to-agar and filter culture plates is shown in Fig. 2. *C. difficile* colonies were well defined and easily identified under UV illumination. No non-fluorescent colonies of other types were apparent on the plates, thus verifying the absence of contamination in the test chamber.

Nine sets of slit-to-agar versus impinger trial data and ten sets of slit-to-agar versus air filter trial data were collected. Slit-to-agar plate counts ranged from 30 to 73 (mean 49, median 49) for the slit-to-agar versus filter trials, while air filter counts ranged from 31 to 107 (mean 67, median 64). Slit-to-agar plate counts ranged from 67 to 150 (mean 108, median 106) for the slit-to-agar versus impinger trials, while impinger counts ranged from 0 to 4 for the AGI-30 and 0 to 5 for the BioSampler. Total counts for the two impingers ranged from 0 to 9 (mean 4, median 4).

Total CFU counts on the in-line exhaust filters for the two impingers ranged from 3 to 14 (median 9), which together sampled at approximately the same rate as the slit-to-agar impactor. This total was less than 10% of the culturable spores drawn into the two impingers, as estimated from corresponding slit-to-agar samples. This suggested, consistent with previous studies (Grinshpun et al. 1997; Willeke et al. 1998), that only minor penetration and re-aerosolization losses may have occurred in the impingers.

All slit-to-agar (reference) colony counts were nonzero, so when calculating relative efficiency a zero value in the numerator indicated an observed relative efficiency less than 1 over the slit-to-agar count. However, these are censored values. Six of the 18 impinger relative efficiency values had zero colonies (3 from each impinger type), so we compared impinger efficiencies first by using the nonparametric Mann–Whitney U test. The test showed no significant difference in the impinger relative efficiencies. We then assigned the values of ½ the LOD for each censored data point, i.e., ½ of 1/(slit-to-agar count) for the trial and compared the impinger mean efficiencies using the two-sample t test after assessing normality. The t test also failed to show a significant difference in the impinger relative efficiencies ($p > 0.05$), and the data were pooled for the two impingers. The pooled collection efficiency was then 4.1% (95% CI 2.27–5.99%) relative to the slit-to-agar sampler.

For the trials in which two MCE filters sampled side-by-side, with each's location randomized across trials, the lack of a position-related difference was verified by conducting a paired-sample t test. The pair differences for the ten filter trials were approximately normally distributed as shown by the Shapiro–Wilk

Fig. 2 Representative MCE filter (left) and slit-to-agar impactor plate (right)
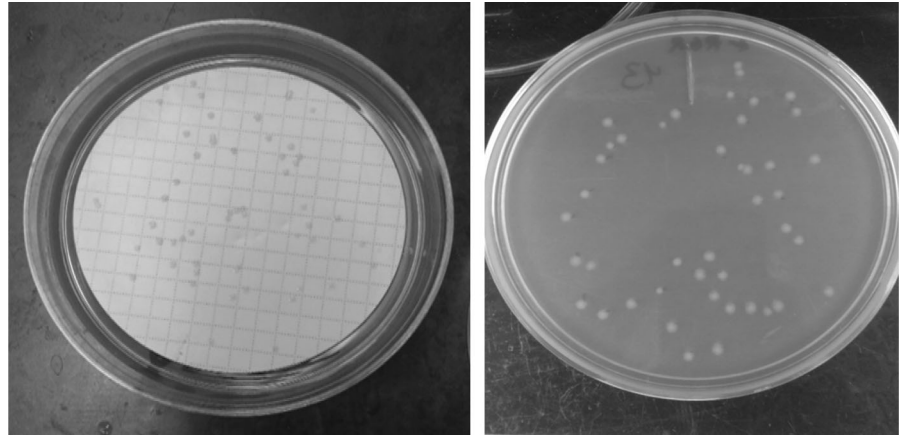
test ($p = 0.45$). The paired-sample t test failed to show a significant positional difference in the paired filter measures ($p > 0.05$), indicating good mixing in the chamber, and the filter measures were pooled. In contrast to the extremely low impinger relative efficiencies, pooled sampling efficiency values for MCE filters relative to the slit-to-agar sampler ranged from 89.0 to 189.6%. Mean relative sampling efficiency, compared to the slit-to-agar impactor, was 136.6% (95% CI 124.7–148.5%). Sample means and standard errors of the mean (SEM) are shown in Fig. 3.

In the 20 trials to assess the potential for re-aerosolization of spores from impinger suspensions,

with 10 trials per impinger type, the culturable spore concentrations were seen to decrease by a mean of 89.96% for the AGI-30 and 89.51% for the BioSampler. While some CFUs were seen on the in-line filters placed downstream of the impingers, these accounted for less than 0.35% of the spores in the original suspension.

4 Discussion

These results demonstrate substantial and surprising differences in the sampling efficiencies of liquid

Fig. 3 Air sampler efficiencies (mean ± SEM) relative to the slit-to-agar impactor
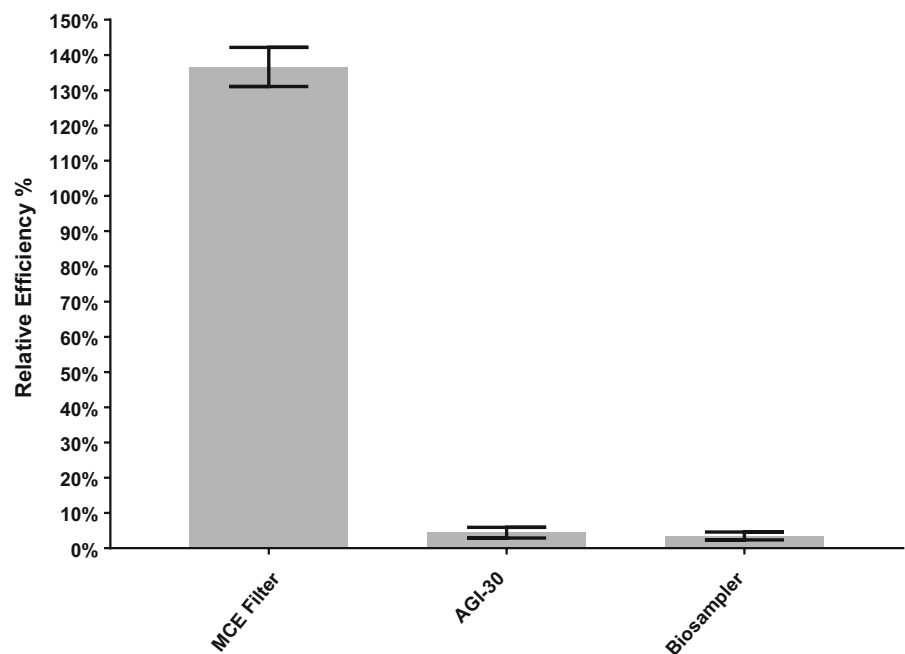
impinger, slit-to-agar impactor, and MCE filter sampling methods when sampling airborne *C. difficile* spores under these conditions. The impingers were particularly ineffective. This suggested that *C. difficile* spores drawn into the impingers were either not captured in the liquid, were captured but became re-aerosolized by fluid turbulence, or were captured but rendered non-culturable by the collection sampling conditions. However, in-line filters on impinger samples demonstrated only minor penetration through these devices. *C. difficile* spores are approximately 1.4–1.9 μm in orientation-averaged aerodynamic size, and particles of this size are collected with high efficiency in all four collectors used. Our experiments demonstrated that spores were indeed captured in the impingers and were not re-aerosolized in significant numbers; rather, spores were rendered non-culturable by the impinger operation.

Sampling stresses that might affect culturability include osmotic shock, oxygen toxicity, desiccation, and mechanical stresses. All of these are known to affect vegetative organisms during air sampling (Kesevan et al. 2010) but are not generally associated with effects on bacterial endospores. Indeed, spores of most types, including *C. difficile*, have been shown to survive in the environment for long periods while subjected to desiccation, light exposure, atmospheric oxygen, and temperature extremes (Kim et al. 1981; Edwards et al. 2016). The extremely poor recovery of *C. difficile* spores from impinger fluid in these trials is in sharp contrast to the recovery of *Bacillus atrophaeus* spores, used as a surrogate for anthrax spores, when sampled using liquid impingers (Kesevan et al. 2010). The influences of each of these stressors on *C. difficile* culturability during air sampling by impaction, impingement, or filtration are an area deserving of additional research.

5 Study limitations

The scope of this study was necessarily limited to a selection of the air sampling methods commonly used for the bioaerosol assessment. We examined the *C. difficile* spore bioaerosol sampling efficiency of the two most commonly used liquid impinger bioaerosol samplers, one type of impactor and one type of filter medium (MCE). We used one impinger fluid (sterile PBS), one sampling duration, and one set of culturing conditions. We also used a non-toxigenic strain of *C. difficile* as a surrogate for toxigenic *C. difficile* strains and prepared and aerosolized the spores in essentially pure suspensions. Whether naturally occurring toxigenic organisms aerosolized from other media (e.g., when flushing a contaminated toilet) and sampled and cultured by other methods would have similar susceptibility to sampling stresses is unknown. The particular mechanisms affecting the culturability of captured *C. difficile* spores, and whether other endospore types might be affected, are also unknown. These and associated questions will require further study.

6 Conclusions

This study was the first to compare air sampling devices and methodologies in the sampling of a laboratory-generated aerosol of *C. difficile* spores. Surprisingly, impinger bioaerosol sampling as conducted in this work was not effective for characterizing airborne *C. difficile* spore concentrations. The two impingers had equivalent performance, which was extremely poor compared to both the slit-to-agar impactor and MCE filters. Therefore, impinger bioaerosol sampling could routinely fail to detect the presence of aerosolized *C. difficile* as demonstrated by the absence of *C. difficile* in 6 of 18 impinger trials despite the presence of numerous colonies on the slit-to-agar plate. Of the three methods, filter-based sampling using MCE filters with direct culture yielded the highest estimates of airborne spore concentrations and indicated that air sampling of *C. difficile* by slit-to-agar impaction, the most practiced method, may actually underestimate airborne concentration and thus fail to fully assess the airborne transport risk in healthcare settings. Filtration-type air samplers are far more widely available to health and safety professionals and industrial hygienists. Air sampling via filtration is also less costly and much simpler than slit-to-agar sampling in terms of equipment requirements, consumables use, and sample storage. The mechanism by which *C. difficile* spores were damaged by impinger operation, and the rate at which damage occurs for captured spores are presently unknown. However, similar damage has not been observed in *B. atrophaeus* endospores. Additional research in each of these areas is needed. This study also demonstrated the

effectiveness of *C. difficile*-selective chromogenic agar in the analysis of air samples using multiple air sampling media.

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