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CLINICAL THERAPEUTICS

Optimization of an Assay To Determine Colonization Resistance to *Clostridioides difficile* in Fecal Samples from Healthy Subjects and Those Treated with Antibiotics

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ABSTRACT A healthy, intact gut microbiota is often resistant to colonization by gastrointestinal pathogens. During periods of dysbiosis, however, organisms such as *Clostridioides difficile* can thrive. We describe an optimized *in vitro* colonization resistance assay for *C. difficile* in stool (CRACS) and demonstrate the utility of this assay by assessing changes in colonization resistance following antibiotic exposure. Fecal samples were obtained from healthy volunteers ($n = 6$) and from healthy subjects receiving 5 days of moxifloxacin ($n = 11$) or no antibiotics ($n = 10$). Samples were separated and either not manipulated (raw) or sterilized (autoclaved or filtered) prior to inoculation with *C. difficile* ribotype 027 spores and anaerobic incubation for 72 h. Different methods of storing fecal samples were also investigated in order to optimize the CRACS. In healthy, raw fecal samples, incubation with spores did not lead to increased *C. difficile* total viable counts (TVCs) or cytotoxin detection. In contrast, increased *C. difficile* TVCs and cytotoxin detection occurred in sterilized healthy fecal samples or those from antibiotic-treated individuals. The CRACS was functional with fecal samples stored at either 4°C or −80°C but not with those stored with glycerol (12% or 30% [vol/vol]). Our data show that the CRACS successfully models *in vitro* the loss of colonization resistance and subsequent *C. difficile* proliferation and toxin production. The CRACS could be used as a proxy for *C. difficile* infection in clinical studies or to determine if an individual is at risk of developing *C. difficile* infection or other potential infections occurring due to a loss of colonization resistance.

KEYWORDS *Clostridioides difficile*, antibiotics, colonization resistance, dysbiosis

The burden of *Clostridioides difficile* (previously *Clostridium difficile*) infection (CDI) is substantial (1, 2). Asymptomatic carriage of *C. difficile* varies markedly according to risk factors, notably antibiotic exposure and hospitalization of individuals (3–5). For CDI to occur, *C. difficile* spores must reach the colon, germinate, and produce toxin. Within a healthy gut microbiota, colonization resistance to *C. difficile* is maintained, and so *C. difficile* proliferation and toxin production in the colon are unlikely to occur. In contrast, in a dysbiotic gut microbiota, such as that caused by broad-spectrum antibiotic treatment, *C. difficile* can colonize, proliferate, and cause disease (6, 7).

Investigating *C. difficile* colonization resistance can be labor-intensive. Current methods include clinical trials (8, 9), animal models (10, 11), and *in vitro* continuous models (12, 13), all of which are extremely time-consuming and expensive. Simple models that can quickly determine if colonization resistance to *C. difficile* is present or absent are needed; however, few studies have used batch culture-based models to assess colonization resistance in humans (14, 15). Borriello and Barclay demonstrated the vegeta-

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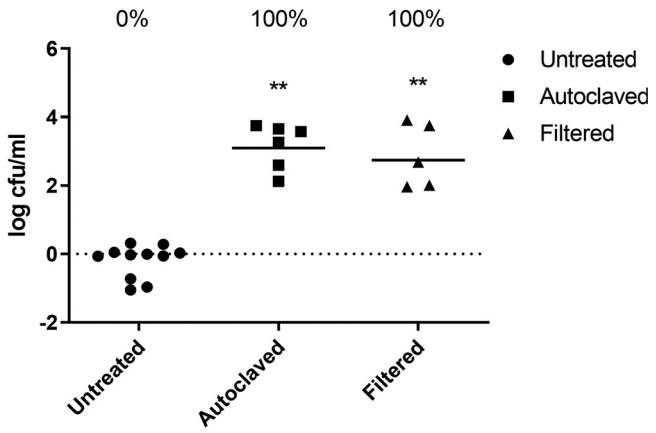

FIG 1 Δ change in *C. difficile* total viable counts (geometric means) over 72 h in fecal samples from healthy individuals that had been untreated (raw [R]) or sterilized (S) by autoclaving or filtration. Individual data points with geometric means are presented. Numbers above bars indicate the percentages of toxin-positive samples. **, $P < 0.01$ (untreated versus autoclaved, $P = 0.002$; untreated versus filtered, $P = 0.006$; autoclaved versus filtered, $P = 1.0$) ($n = 11$ [untreated], $n = 6$ [autoclaved], and $n = 5$ [filtered]).

tive growth of *C. difficile* in the stools of individuals who had received antibiotics but not those of healthy individuals, although this was not demonstrated longitudinally in the same subject (14). They also showed that sterilization of healthy fecal samples by autoclaving or by filter sterilization led to a loss of colonization resistance to *C. difficile*, with subsequent *C. difficile* growth and sporulation.

Building on the study by Borriello and Barclay, we have developed and examined the utility of an *in vitro* colonization resistance assay for *C. difficile* in stool (CRACS), using *C. difficile* spores of a ribotype 027 strain. We have determined the effects of fecal storage conditions on the performance of the assay and successfully used the optimized assay to assess the change in colonization resistance of healthy human subjects following antibiotic treatment.

RESULTS

Stool samples from healthy volunteers ($n = 6$) were used to confirm the utility of this assay and investigate optimal sterilization techniques and storage conditions. The optimized assay was then validated using stool samples from healthy volunteers before and after treatment with either moxifloxacin ($n = 11$) or no antibiotic ($n = 10$).

Effects of sterilization techniques on colonization resistance. The CRACS assesses colonization resistance by comparing the growth of *C. difficile* in raw versus sterilized feces as measured by change in total viable counts (Δ TVC). We wanted to compare sterilization by autoclaving with sterilization by filtration for use in this assay. Sterilizing feces by either autoclaving or filtration resulted in a loss of colonization resistance to *C. difficile*, with mean total viable count (TVC) increases of 3.4 $\log_{10}$ CFU/ml and 2.7 $\log_{10}$ CFU/ml, respectively, after 72 h of incubation. Colonization resistance remained in the raw samples, where there was no observed increase in the *C. difficile* TVC (0 $\log_{10}$ CFU/ml) (Fig. 1). Similar patterns were observed for spore populations; there was no increase in spore counts in raw samples, while autoclaved and filtered feces yielded increases in spores of 1.9 $\log_{10}$ CFU/ml and 1.0 $\log_{10}$ CFU/ml, respectively (see Fig. S1 in the supplemental material). Cytotoxin was not observed in raw samples but was universally detected in the autoclaved and filtered feces (Table 1).

Effect of fecal sample storage on colonization resistance. In order for this assay to be clinically useful, the effect of sample storage prior to processing must be investigated, and optimal storage conditions must be determined. We therefore investigated a range of different storage conditions. In the raw fecal samples, there was no effect of sample storage on *C. difficile* Δ TVCs (Fig. 2a) or spore counts (Fig. S2A) at 72 h, and cytotoxin was not detected (Table 1). In autoclaved samples, the storage temper-

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TABLE 1 Toxin results (percentages of cytotoxin-positive samples at 72 h) of the experiments shown in Fig. 1 to 4

Storage condition or patient group	% cytotoxin-positive samples (no. of positive samples/total no. of samples)[a]				Associated figure
	Raw	Autoclaved	Filtered	BHI broth and taurocholic acid	
Storage conditions					
Fresh	0 (0/11)	100 (6/6)	100 (5/5)		Fig. 1
4°C	0 (0/5)	100 (3/3)	100 (3/3)		Fig. 2
−80°C	40 (3/5)	100 (3/3)	100 (3/3)	100 (3/3)	Fig. 2
−80°C with 12% glycerol	0 (0/5)	66.6 (2/3)	0 (0/3)	100 (3/3)	Fig. 2
−80°C with 30% glycerol	0 (0/5)	0 (0/3)	0 (0/3)	0 (0/3)	Fig. 2
First freeze-thaw	0 (0/3)	66.6 (2/3)	66.6 (2/3)		Fig. 3
Second freeze-thaw	0 (0/3)	100 (3/3)	100 (3/3)		Fig. 3
Patient group					
Moxifloxacin day 1	9 (1/11)		100 (11/11)		Fig. 4
Moxifloxacin day 6	45 (5/11)		91 (10/11)		Fig. 4
Control day 1	0 (0/10)		100 (10/10)		Fig. 4
Control day 6	0 (0/10)		90 (9/10)		Fig. 4

[a]Shading indicates that the experiment was not performed.

ature did not affect the Δ TVC. *C. difficile* TVC increased by 2.7 $\log_{10}$ CFU/ml and 3.1 $\log_{10}$ CFU/ml for samples stored at 4°C and −80°C, respectively (Fig. 2b); similar effects were seen for the spore counts (Fig. S2B). However, the presence of glycerol affected *C. difficile* growth in autoclaved samples, with the Δ TVC between 0 and 72 h being ~1.5 $\log_{10}$ CFU/ml and 2.0 $\log_{10}$ CFU/ml lower in 12% and 30% glycerol-containing samples, respectively, than in non-glycerol-treated samples stored at −80°C (Fig. 2b). Cytotoxin detection was also reduced in samples with added glycerol (Table 1). Differences due to storage were even more apparent in the filtered fecal samples. Samples stored at −80°C resulted in a high Δ TVC compared with those held at 4°C. When samples were stored in glycerol, there was no increase in the *C. difficile* TVC, which was lower than those in samples stored at 4°C ($P = 0.026$) and −80°C (Fig. 2c). In addition, there was no detection of cytotoxin in samples stored with glycerol. Growth of *C. difficile* in brain heart infusion (BHI) broth with taurocholic acid (TCA) was not

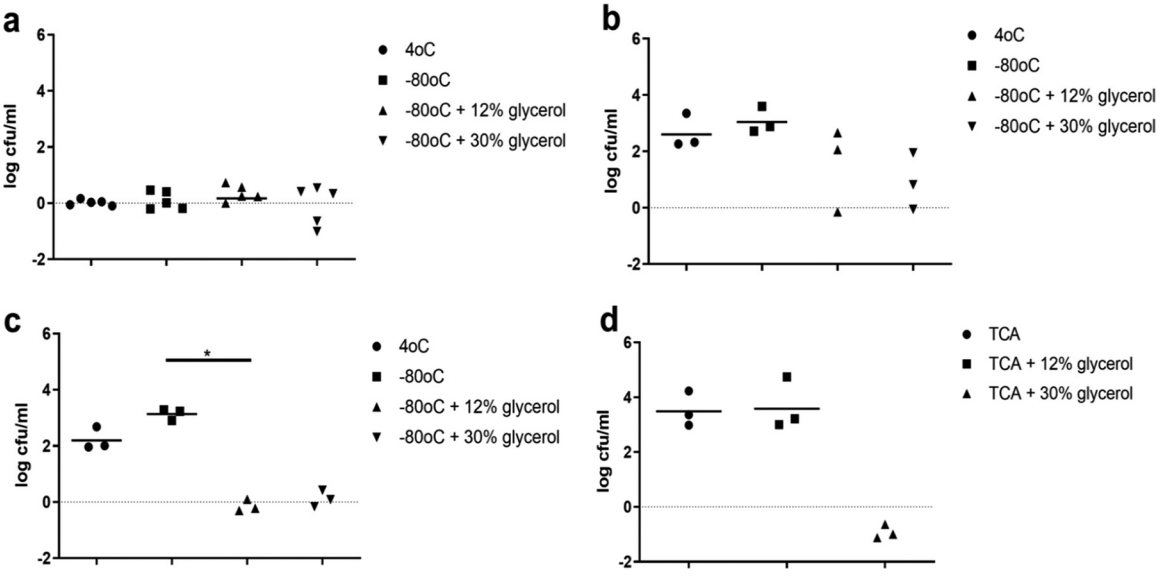

FIG 2 Impact of storage of healthy fecal samples on Δ change in geometric mean *C. difficile* total viable counts over 72 h. (a) Raw fecal samples ($n = 5$). (b) Autoclaved fecal samples ($n = 3$). (c) Filtered fecal samples ($n = 3$). Fecal samples were stored at 4°C, −80°C, −80°C with 12% glycerol, or −80°C with 30% glycerol. (d) Δ change in geometric mean *C. difficile* total viable counts over 72 h following growth in BHI broth with taurocholic acid with and without glycerol ($n = 3$). Individual data points with geometric means are presented. *, $P = 0.026$.

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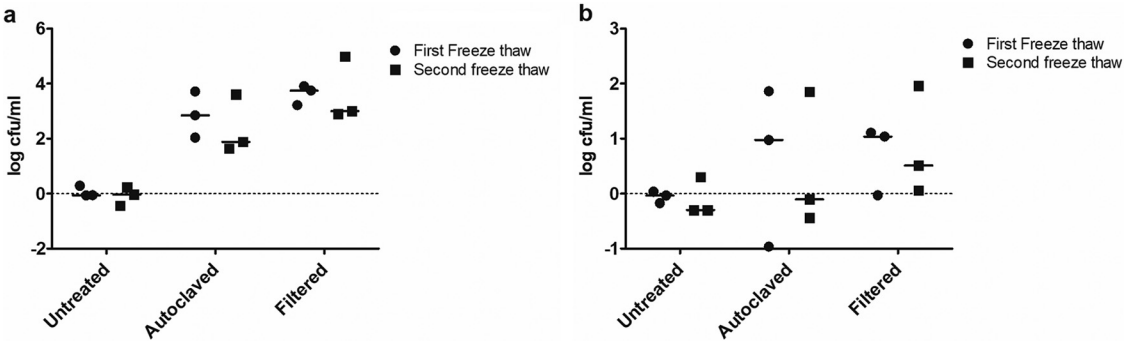

FIG 3 Effect of multiple freeze-thaw cycles on Δ change in geometric mean *C. difficile* total viable counts and spore counts over 72 h ($n = 3$). (a) Total viable counts; (b) spore counts. Individual data points with geometric means are presented.

affected by 12% glycerol; however, with 30% glycerol, there was a lower TVC than under the other conditions, and no cytotoxin was detected (Fig. 2d and Table 1).

Effect of freeze-thaw cycles. Two freeze-thaw cycles did not affect *C. difficile* TVCs (Fig. 3a) or cytotoxin levels (Table 1). Colonization resistance was maintained in raw samples (Δ TVC approximately 0.1 $\log_{10}$ CFU/ml) but was lost in the autoclaved and filtered samples (TVCs increased by 2.4 $\log_{10}$ CFU/ml and 3.6 $\log_{10}$ CFU/ml, respectively). In contrast, freeze-thaw cycles altered the spore counts (Fig. 3b).

Use of the CRACS to investigate the effect of antibiotics on colonization resistance. The CRACS allows the measurement of colonization resistance against *C. difficile* to be determined for individual fecal samples. We used the assay on stool samples from a clinical study to determine whether antibiotic exposure affected colonization resistance to *C. difficile*. Healthy individuals were given the fluoroquinolone antibiotic moxifloxacin for 5 days or no antibiotics (control). Fecal samples were provided before and after treatment, and colonization resistance to *C. difficile* was evaluated. Samples were raw or sterilized by filtration. Colonization resistance in stool samples taken before the beginning of treatment (day 1) did not differ between the treatment and control groups; no increase in TVCs was observed in raw samples (Fig. 4a), but an increase of ~3.2 $\log_{10}$ CFU/ml was observed in sterilized samples (Fig. 4b). After 5 days of antibiotic treatment (day 6), differences were observed between the treatment and control groups. Colonization resistance was maintained in the control group but lost in antibiotic recipients; raw samples taken after antibiotic treatment showed an increase in the TVC of 2.4 $\log_{10}$ CFU/ml ($P = 0.007$) (Fig. 4a), which was associated with an increase in the proportion of samples with detected cytotoxin (Table 1). Filter-sterilized samples did not differ, irrespective of the treatment group or time point; colonization resistance was lost on all occasions, with a TVC increase

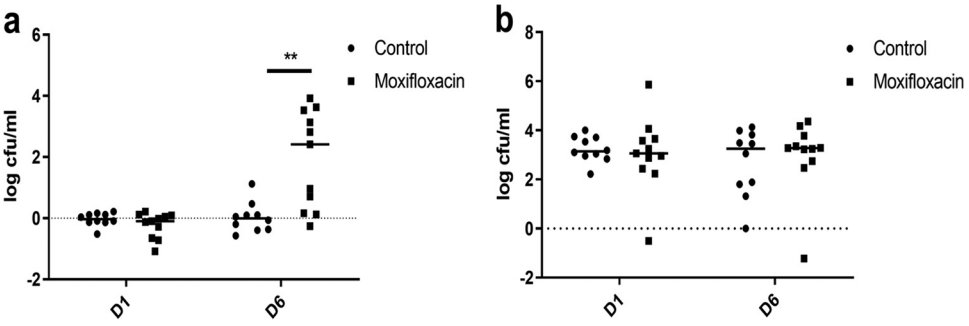

FIG 4 Effect of 5 days of antibiotic or control treatment on Δ change in geometric mean *C. difficile* TVCs over 72 h. (a) Raw samples; (b) sterilized samples. **, $P = 0.006$ by a Wilcoxon rank sum test ($n = 11$ for the antibiotic treatment group; $n = 10$ for the control group). Individual data points with geometric means are presented.

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of ~3.2 $\log_{10}$ CFU/ml for all samples (Fig. 4b) and a high percentage of toxin detection (Table 1).

DISCUSSION

We have demonstrated that the CRACS can be used to determine whether colonization resistance to *C. difficile* expansion is intact in human stool samples. When raw, healthy fecal samples are inoculated with *C. difficile* spores, spore germination, *C. difficile* proliferation, and cytotoxin production do not occur. In contrast, when fecal samples are sterilized by either autoclaving or filtration, spores germinate, *C. difficile* proliferates, and cytotoxin is detected.

Our data build on the study by Borriello and Barclay (14), who used untreated and sterilized fecal samples from different individuals and found that colonization resistance is lost in sterilized samples. We have confirmed this finding, undertaken comparative studies to create a reproducible optimized assay that can be used on fecal samples from human clinical studies, and used the optimized assay to demonstrate longitudinally in healthy subjects that colonization resistance is lost following fluoroquinolone treatment. In previous studies by both Borriello and Barclay and Horvat and Rupnik (14, 15) investigating colonization resistance of human stool samples, batch cultures were inoculated with *C. difficile* vegetative cells. Here, we have chosen to inoculate batch cultures with spores. In preliminary work (data not shown), we found inoculations of both vegetative cells and spores to work in this assay; however, as spores notably represent the primary infective/transmission form of *C. difficile* (16) and are more straightforward to prepare and manipulate *in vitro*, we found it advantageous to use spores.

Importantly, we have also shown that the CRACS can be used to evaluate fecal samples from individuals with potential dysbiosis and provide a measure of colonization resistance. In individuals treated with the fluoroquinolone antibiotic moxifloxacin for 5 days, there was a significant reduction in colonization resistance compared to a fecal sample obtained in the 24 h prior to the first antibiotic administration (Fig. 4). Interestingly, our data appear to show a binary distribution, with approximately half of samples taken following 5 days of moxifloxacin therapy supporting large increases in *C. difficile* growth and the other half yielding results that resemble those of control samples. The intestinal microbiota varies between individuals, and individualized responses to antibiotic exposure have been reported (17, 18). Therefore, variation in the alterations to the gut microbiota in our participants would be expected. While microbiota diversity is thought to be important, and various microbiota changes have been linked to CDI (19), the exact components conferring colonization resistance are as yet unclear. Our assay is designed to give a qualitative but not a quantitative assessment of colonization resistance. Thus, according to our assay and supported by clinical observations, in some patients, microbiota disruption following antibiotic exposure leads to a loss of colonization resistance, while in others, it does not.

Our results are in line with those of Horvat and Rupnik (15), who observed a loss of colonization resistance in dysbiotic fecal samples. Crucially, our data suggest that this assay may also be an effective proxy measurement for the risk of *C. difficile* infection prior to or during antibiotic treatment.

We have examined potential confounders relating to fecal sample storage, which are important in understanding the utility of this assay, including the desire to batch test and examine specimens retrospectively. Notably, the storage temperature had limited effects on the assay; raw samples from healthy individuals did not support *C. difficile* germination, growth, or toxin production, irrespective of the storage conditions. Some variation due to storage temperature was observed in the sterilized portions of the assay (which can be used as a positive control); greater *C. difficile* proliferation occurred in sterilized samples stored at −80°C than in those stored at 4°C. The storage temperature of fecal samples has been shown to have little effect on microbial populations, and their amplitudes are modest in comparison with intersubject variability (20–22). In contrast, sample storage in glycerol, a common cryoprotectant, was

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detrimental in terms of CRACS performance. Following glycerol storage, no *C. difficile* germination, growth, or toxin production was observed in the sterilized portions of the assay, contrary to expectation and to those samples stored without glycerol. In addition, when inoculated into BHI broth in the presence of TCA and glycine, known inducers of germination (23), no increases in the *C. difficile* TVC were observed when 30% glycerol was used for storage, although *C. difficile* growth was unaffected by 12% glycerol. The reasons for this are unknown, but it seems clear that fecal samples should be stored without glycerol prior to analysis using the CRACS or other colonization resistance assays reliant on *C. difficile* growth. One or two freeze-thaw cycles were not detrimental to the maintenance or loss of colonization resistance when assessing *C. difficile* germination and growth (total viable counts), but spore counts were affected. The reason for this is not clear, as *C. difficile* spores have been demonstrated to be resistant to many different environmental stressors (24). Additionally, the spore counts did not follow the patterns of the TVC or cytotoxin titer (Fig. 2 and Table 1), something that was already observed after the first freeze-thaw cycle.

A key advantage of the CRACS is that it is relatively quick and inexpensive to perform. We recommend that for the assay to function optimally, a sterilization technique should be used as a control, and fecal samples should be stored at −80°C without glycerol. One or two freeze-thaw cycles will not affect the results of the assay. Continuous culture models have been used repeatedly to demonstrate the capacity of spores to induce CDI and to assess the effectiveness of therapeutics (12, 13, 25). While these models closely simulate *in vivo* conditions and indeed are clinically reflective, they are time-consuming, technically demanding, of low throughput, and expensive.

This study is limited, as the optimized assay has been validated only on stool samples from a relatively small number of healthy volunteers in a clinical study ($n = 21$). We plan to further validate this assay with larger numbers of subjects from a wider range of clinical studies. Additionally, it is difficult to determine the extent to which this assay truly reflects colonization resistance in patients, as controlled exposure of patients to *C. difficile* spores is not possible. Prospective studies are problematic. Only a low number of patients receiving antibiotics go on to develop CDI, so such studies would need to be extremely large (and expensive) to garner meaningful data. However, we believe that some measure of clinical reflectivity could be achieved with retrospective studies or by examining particular subsets of patients such as those undergoing fecal microbiota transplantation (FMT), targeted restoration therapy, or microbiota-sparing treatments.

Interest in microbiota-sparing antibiotics and microbiota-restoring treatments is high, and multiple clinical studies are under way. Currently, the only way to assess CDI risk in such trials is to determine what proportion of subjects go on to develop *C. difficile*-mediated disease. This will always be a very low number given the clinical dynamics of CDI, and therefore, extremely large clinical trials are required to show any difference in CDI rates. Microbiome analyses that use parameters such as microbiota diversity, richness, or the presence/absence of specific components are therefore used as proxy measurements. However, as the exact components and factors responsible for *C. difficile* colonization resistance are unknown, the utility of these analyses is limited. We have demonstrated that the CRACS can be used to rapidly assess the presence of colonization resistance within fecal samples from both healthy individuals and those receiving antibiotics. We believe that this assay could be used as a proxy for CDI risk, adding important data to clinical studies, particularly small early-phase studies and those investigating the effectiveness of preventative approaches.

MATERIALS AND METHODS

Study design. The CRACS provides a measure of colonization resistance against *C. difficile* for a given stool sample. *C. difficile* germination and proliferation are expected in sterilized stool samples, whereas the germination and proliferation of *C. difficile* in raw stool samples depend on the colonization resistance of the stool sample. In this study, we have established the premise of this assay, optimized the conditions for this assay, and then validated the assay using samples from a clinical study. The assay and different conditions evaluated in this study are shown in Fig. 5.

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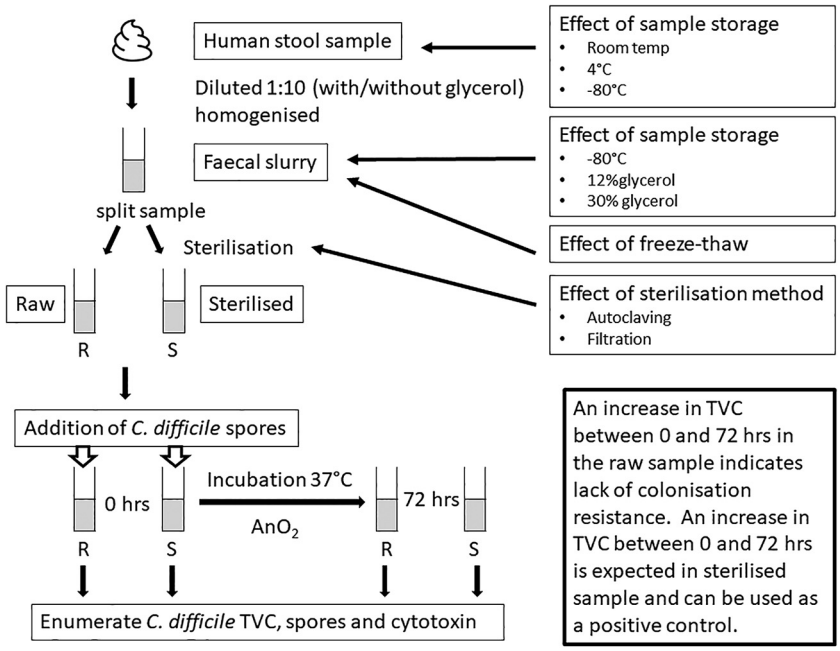

FIG 5 Manipulation of stool samples undergoing the CRACS. The different experimental conditions investigated during the optimization of the assay are indicated in boxes on the right.

During the optimization phase of this study, healthy volunteers over the age of 18 years ($n = 6$) with no history of antibiotic receipt in the last 3 months were recruited to provide anonymous fecal samples. The sample size was determined to ensure that at least three individual samples were tested under each condition (sterilization technique, storage temperature, use of glycerol, and effect of freeze-thaw).

In order to validate the optimized assay, we used fecal samples from a phase 1 clinical study (Da Volterra, unpublished data). Healthy volunteers provided fecal samples before and after treatment with either moxifloxacin ($n = 11$) or no antibiotic ($n = 10$). All available fecal samples from this study were used.

Fecal samples. Unless otherwise stated, fecal samples were provided by healthy volunteers (aged 18 to 65 years) with no history of antibiotic therapy in the past 3 months. Sample production kits included an AnaeroGen W-Zip compact generator system (Oxoid, Hampshire, UK), which maintained the samples in an anaerobic environment prior to processing. Samples were confirmed negative for *C. difficile* by both culture on Brazier's CCEYL agar (cefoxitin [8 mg/liter], cycloserine [250 mg/liter], and lysozyme [5 mg/liter] supplemented with 2% lysed defibrinated horse blood) and screening for the presence of glutamate dehydrogenase (GDH) antigen using the C.Diff Chek-60 enzyme immunoassay (DS2; Magellan Biosciences Dynex) before use. Collection of fecal samples was approved by the University of Leeds Research Ethics Committee (reference numbers MREC15-070 and MREC17-011).

The CRACS. The assay and the different conditions evaluated for optimization are outlined in Fig. 5. Fecal samples were diluted in prereduced phosphate-buffered saline (PBS) (1:10 [wt/vol]), homogenized in a stomacher, and filtered through muslin to remove any large particulate matter. Each sample was processed in raw and sterilized forms (Fig. 5). In an anaerobic environment, samples were inoculated with *C. difficile* ribotype 027 spores (1:100 [vol/vol] dilution of a spore preparation containing 5 $\log_{10}$ CFU/ml) and incubated anaerobically at 37°C for 72 h. At 0 and 72 h postinoculation, *C. difficile* total viable counts (TVCs), spore counts, and cytotoxin titers were measured. An observed increase in TVCs and toxin production in raw aliquots at 72 h compared to 0 h postinoculation indicates a lack of colonization resistance.

C. difficile clinical strains of ribotypes 027, 078, and 014 were initially tested in raw and autoclaved feces. No strain-to-strain differences were observed in TVCs or spore counts, although the *C. difficile* ribotype 027 strain yielded slightly higher cytotoxin titers (data not shown). This finding and the high clinical significance/prevalence of ribotype 027 (26) led to the selection of this strain for the assay.

Enumeration of *C. difficile* total viable counts, spores, and cytotoxin. Total viable counts were enumerated by serial 10-fold dilution, plating onto Brazier's CCEYL agar, and anaerobic incubation for 48 h. Spore counts were enumerated by the same method following 1 h of incubation in an equal volume of 100% ethanol. Cytotoxin levels were measured using the Vero cell cytotoxicity assay as described previously (27, 28). Briefly, sample supernatants were applied at a 1:10 dilution to a Vero cell monolayer in duplicate, 10-fold serially diluted, and incubated at 37°C in 5% CO_2 (Precision 190; LEEC). Cell rounding was assessed relative to positive and negative controls at 24 and 48 h using an inverted microscope. *C. difficile* toxin was confirmed by the presence of ≥80% cell rounding that was neutralized by *Clostridium sordellii* antitoxin (1:100 [vol/vol]) (Prolab Diagnostics, Bromborough, UK).

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CRACS optimization. (i) Effects of sterilization techniques on colonization resistance. Fecal slurries were separated into equal-sized aliquots. Each aliquot was either maintained anaerobically overnight (raw), autoclaved at 121°C for 15 min (autoclaved), or centrifuged at 16,000 × *g* for 10 min prior to filter sterilization through a 0.22-μm syringe filter (filtered) prior to anaerobic incubation and use in the CRACS (Fig. 5). Sterilized samples were plated onto nutrient agar and fastidious anaerobe agar (FAA; Oxoid, UK) and incubated aerobically and anaerobically to evaluate sterility. Sporadic colonies were observed but not counted.

(ii) Effect of fecal sample storage on colonization resistance. To determine the effects of different storage conditions on colonization resistance, fecal samples were stored for 16 to 24 h at room temperature (RT) (anaerobic), 4°C (4°C), −80°C (−80°C), −80°C with 12% glycerol (12% glycerol), and −80°C with 30% glycerol (30% glycerol) prior to use in the CRACS (Fig. 5). Samples stored with glycerol were diluted to the same level as those without glycerol (1:10), homogenized using a stomacher, filtered through muslin, and stored immediately. As required, samples were defrosted thoroughly, at room temperature, prior to processing as raw and sterilized aliquots. Additionally, to determine whether the concentrations of glycerol used had an effect on *C. difficile* germination and growth, *C. difficile* spores (1:100 [vol/vol]) were inoculated into BHI broth (supplemented with 0.1% taurocholate plus 0.4% glycine [23]) containing 0%, 12%, or 30% glycerol and incubated anaerobically for 72 h prior to enumeration of *C. difficile* spores, vegetative cells, and toxin, as described above.

(iii) Effect of multiple freeze-thaw cycles on colonization resistance. Fresh fecal samples were stored anaerobically for 16 to 24 h at room temperature and frozen at −80°C. Fecal samples were thawed, diluted, homogenized using a stomacher, and separated into two 2-g aliquots (as described above). One aliquot was processed for the CRACS immediately, and the other was frozen at −80°C for processing at a later date. These samples were processed using both autoclaving and filtration sterilization techniques as discussed above.

Use of the CRACS to investigate the effect of antibiotics on colonization resistance. Fecal samples from healthy individuals treated with either the fluoroquinolone antibiotic moxifloxacin or a placebo were provided by Da Volterra (Paris, France). Briefly, healthy volunteers (aged 18 to 60 years) with no history of antibiotic therapy in the past 3 months, and devoid of fecal *C. difficile* colonization, either were treated with oral moxifloxacin at 400 mg once a day (Izilox; Bayer HealthCare) for 5 days (treatment group) or did not receive antibiotics (control group). Fecal samples were collected within the last 24 h before the first drug administration (day 1) and the first 24 h following the last drug administration (day 6), homogenized using a stomacher, aliquoted, and kept frozen at −80°C until processing for the CRACS using the filtration sterilization technique described above. Total viable counts and toxin production were measured, but spore counts were not. Treatment of subjects and collection of samples were approved by the ANSM (Agence Nationale de Sécurité des Médicaments) and the Comité de Protection des Personnes Sud-Est IV and registered under ID-RCB identifier 2015-A01899-40 and at ClinicalTrials.gov under identifier NCT02917200; all subjects had agreed to the use of samples for such ancillary biological analyses as informed in their consent form (29).

***C. difficile* spore preparation.** The *C. difficile* PCR ribotype 027 strain 210 (BI/NAP1/toxinotype III) used here was isolated during an outbreak of CDI at the Maine Medical Center (Portland, ME, USA) and supplied courtesy of Robert Owens (formerly at Maine Medical Center). The *C. difficile* ribotype 078 (R10725) and ribotype 014 (R11446) strains were sourced from the Anaerobic Reference Unit, Cardiff, Wales. *C. difficile* was cultured from −80°C stocks on Brazier's CCEYL agar (cefoxitin [8 mg/liter], cycloserine [250 mg/liter], and lysozyme [5 mg/liter] supplemented with 2% lysed defibrinated horse blood) and checked for purity after 48 h of anaerobic growth at 37°C. Growth was harvested on a sterile swab, transferred to 40 prereduced Columbia blood agar (CBA) plates, and incubated anaerobically at 37°C for 14 days. Growth from the CBA plates was resuspended in 50% ethanol in saline and vortexed for approximately 5 min. The spore preparation was enumerated on Brazier's CCEYL agar and standardized to approximately 5 $\log_{10}$ CFU/ml.

Data analysis. All data presented are the Δ changes in the geometric means over 72 h (geometric mean at 72 h minus geometric mean at 0 h). All data were analyzed with SPSS version 23 and presented on graphs produced in GraphPad Prism version 5. Due to the sample size often being less than 10, all data were treated as nonparametric and analyzed using a Kruskal-Wallis test with Dunn's pairwise comparison and Bonferroni correction where applicable. If there was a single comparison, a Wilcoxon rank sum test was performed.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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