Optimisation of an assay to determine colonisation resistance to *Clostridioides difficile* in faecal samples from healthy subjects and those treated with antibiotics.

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Abstract

A healthy, intact gut microbiota is often resistant to colonisation by gastrointestinal pathogens. During periods of dysbiosis, however, organisms such as *Clostridioides difficile* can thrive. We describe an optimised *in vitro* Colonisation Resistance Assay for *C. difficile* in Stool (CRACS) and demonstrate the utility of this assay by assessing changes to colonisation resistance following antibiotic exposure. Faecal samples were obtained from healthy volunteers (n=6), and from healthy subjects receiving five days of moxifloxacin (n=11) or no antibiotics (n=10). Samples were separated and either not manipulated (raw), or sterilised (autoclaved or filtered) prior to inoculation with *C. difficile* ribotype 027 spores and anaerobic incubation for 72 hours. Different methods of storing faecal samples were also investigated in order to optimise the CRACS. In healthy, raw faecal samples, incubation with spores did not lead to increased *C. difficile* total viable counts (TVC) or cytotoxin detection. By contrast, increased *C. difficile* TVC and cytotoxin detection occurred in sterilised healthy faecal samples or those from antibiotic-treated individuals. The CRACS was functional with faecal samples stored at either 4^{o}C or -80^{o}C, but not with those with glycerol (12% or 30% v/v). Our data show that the CRACS successfully models *in vitro* the loss of colonisation resistance, and subsequent *C. difficile* proliferation and toxin production. 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 colonisation resistance.

Introduction

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 hospitalisation of individuals (3-5). For CDI to occur, *C. difficile* spores must reach the colon, germinate and produce toxin. Within a healthy gut microbiota, colonisation resistance to *C. difficile* is maintained, and so *C. difficile* proliferation and toxin production in the colon is unlikely to occur. By contrast, in a dysbiotic gut microbiota, such as that caused by broad spectrum antibiotic treatment, *C. difficile* can colonise, proliferate and cause disease (6, 7).

Investigating *C. difficile* colonisation resistance can be labour 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 colonisation resistance to *C. difficile* is present or absent are needed, however few studies have used batch culture based models to assess colonisation resistance in humans (14, 15). Borriello and Barclay demonstrated the vegetative 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. They also showed that sterilisation of healthy faecal samples by autoclaving, or by filter sterilisation led to a loss of colonisation 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* Colonisation Resistance Assay for *C. difficile* in Stool (CRACS), using *C. difficile* spores of a ribotype 027 strain. We have determined the effects of faecal storage

63 conditions on the performance of the assay, and successfully used the optimised assay to
64 assess the change in colonisation resistance of healthy human subjects following antibiotic
65 treatment.

66

Materials and Methods

Study design

The CRACS assay provides a measure of colonisation resistance against *C. difficile* for a given stool sample. *C. difficile* germination and proliferation is expected in sterilised stool samples, whereas the germination and proliferation of *C. difficile* in raw stool depends on the colonisation resistance of the stool sample. In this study, we have established the premise of this assay, undertaken to optimise 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 described in figure 1.

During the optimisation phase of this study, healthy volunteers over the age of 18 (n=6) with no history of antibiotic receipt in the last 3 months were recruited to provide anonymous faecal samples. Sample size was determined to ensure at least three individual samples were tested for each condition (sterilisation technique, storage temperature and use of glycerol, effect of freeze-thaw).

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

Faecal samples

Unless otherwise stated faecal samples were provided by healthy volunteers (aged 18-65 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 sample 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/L],

cycloserine [250 mg/L], lysozyme [5 mg/L], supplemented with 2% lysed, defibrinated horse blood) and by screening for the presence of GDH antigen using the C.DIFF CHEK-60 enzyme immunoassay (DS2, Megellan Biosciences Dynex) before use. Collection of faecal samples was approved by the University of Leeds Research Ethics Committee (Reference numbers: MREC15-070 and MREC17-011).

The CRACS assay

The assay and the different conditions evaluated for optimisation are outlined in figure 1. Faecal samples were diluted in pre-reduced PBS (1:10 w/v), homogenised in a stomacher and filtered through muslin to remove any large particulate matter. Each sample was processed in raw and sterilised forms (Fig. 1). In an anaerobic environment, samples were inoculated with *C. difficile* ribotype 027 spores (1:100 v/v of a spore prep containing 5 $\log_{10}$ CFU/mL), and incubated anaerobically at 37^{o}C for 72 hours. At 0, and 72 hours post inoculation, *C. difficile* total viable counts (TVC), spore counts and cytotoxin titres were measured. An observed increase in TVCs and toxin production in raw aliquots at 72 hours compared to 0 hours post inoculation indicates a lack of colonisation resistance.

C. difficile clinical strains of ribotypes 027, 078, and 014 were initially tested in raw and autoclaved faeces. No strain to strain differences were observed in TVC or spore counts, although the *C. difficile* ribotype 027 strain yielded slightly higher cytotoxin titres (data not shown). This finding and the high clinical significance/prevalence of ribotype 027 (16) 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 hours. Spore counts were enumerated by the same method following 1 hour incubation in an equal volume of 100% ethanol. Cytotoxin levels were measured using the Vero cell cytotoxicity assay as described previously (17, 18). Briefly, sample supernatants were applied 1:10 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 hours using inverted microscopy. *C. difficile* toxin was confirmed by the presence of ≥80 % cell rounding that was neutralised by *Clostridium sordellii* antitoxin (1:100 v/v) (Prolab Diagnostics, Bromborough, UK).

CRACS Optimisation

1. *Effect of sterilisation techniques on colonisation resistance*

Faecal slurries were separated into equal sized aliquots. Each aliquot was either maintained anaerobically overnight (raw), autoclaved at 121°C for 15 mins (autoclaved), or centrifuged at 16000g for 10 minutes prior to filter sterilisation through a 0.22 µm syringe filter (filtered) prior to anaerobic incubation and use in the CRACS assay (Fig 1). Sterilised 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.

2. *Effect of faecal sample storage on colonisation resistance*

To determine the effect of different storage conditions on colonisation resistance, faecal samples were stored for 16-24 hours at: room temperature (RT, anaerobic), 4°C (4°C), -80°C (-80°C), -80°C + 12% glycerol (12% glycerol), and -80°C + 30% glycerol (30% glycerol) prior to use in the CRACS assay (Fig 1). Samples stored with glycerol were diluted to the same level as those without (1:10), homogenised using a stomacher, filtered through muslin and stored immediately. As required, samples were defrosted thoroughly, at room temperature, prior to processing as raw, and sterilised aliquots. Additionally, to determine whether the concentrations of glycerol used had an effect on *C. difficile* germination and growth, *C. difficile* spores (1:100 v/v) were inoculated into BHI broth (supplemented with 0.1% Taurocholate + 0.4% glycine (Sorg and Sonenshein, 2008)) containing 0%, 12% or 30% glycerol and incubated anaerobically for 72 hours, prior to enumeration of *C. difficile* spores, vegetative cells and toxin as described above.

3. *Effect of multiple freeze-thaw cycles on colonisation resistance*

Fresh faecal samples were stored anaerobically for 16-24 hours at room temperature, and frozen at -80°C. Faecal samples were thawed, diluted, homogenised using a stomacher, and separated into two, 2 g aliquots (as above). One aliquot was processed through CRACS assay immediately and the other was frozen at -80°C for processing at a later date. These samples were processed using both autoclaving and filtration sterilisation techniques as discussed above.

Use of the CRACS assay to investigate the effect of antibiotics on colonisation resistance.

Faecal 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 18-60) with no history of antibiotic therapy in the past 3 months, and devoid of faecal *C. difficile* colonisation, were either treated with oral moxifloxacin 400 mg once a day (Izilox, Bayer HealthCare) for 5 days (treatment group), or did not receive antibiotics (control group). Faecal samples were collected within the last 24h before the first drug administration (D1), and first 24h following the last drug administration (D6), were homogenised using a stomacher, aliquoted and kept frozen at -80°C until processing for the CRACS assay using the filtration sterilisation technique described above. Total viable counts and toxin production was measured, but spore counts were not. Treatment of subjects and collection of samples, were approved by ANSM (Agence Nationale de Sécurité des Médicaments) and the Comité de Protection des Personnes Sud-Est IV, and registered as ID-RCB: 2015-A01899-40, and on Clinicaltrials.gov under number NCT02917200; all subjects had agreed the use of samples for such ancillary biological analysis as informed in their consent form (19).

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 Centre (Portland, ME, USA) and supplied courtesy of Dr Robert Owens (formally at Maine Medical Centre). The *C. difficile* ribotype 078 (R10725) and ribotype 014 (R11446) 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/L], cycloserine [250 mg/L], lysozyme [5 mg/L], supplemented with 2% lysed, defibrinated horse blood), and checked for purity after 48 hour anaerobic growth at 37°C.

Growth was harvested on a sterile swab and transferred to 40 pre-reduced Columbia Blood Agar (CBA) plates and incubated anaerobically at 37°C for 14 days. Growth from the CBA plates was re-suspended in 50 % ethanol in saline and vortexed for approximately 5 mins. The spore preparation was enumerated on Brazier's CCEYL agar and standardised to approximately 5 log10 CFU/mL.

Data analysis

All data presented is the Δ change in the geometric mean over 72 hours (geometric mean at 72h minus geometric mean at 0h). All data were analysed on 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 non-parametric and analysed using Kruskal-Wallis with Dunns pairwise comparison and Bonferroni correction where applicable. If there was a single comparison a Wilcoxon rank sum test was performed.

Results

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

Effect of sterilisation techniques on colonisation resistance

The CRACS assesses colonisation resistance by comparing growth of *C. difficile* in raw vs sterilised faeces. We wanted to compare sterilisation by autoclaving with sterilisation by filtration for use in this assay. Sterilising faeces by either autoclaving or filtration resulted in loss of colonisation resistance to *C. difficile,* with mean TVC increases of 3.4 $\log_{10}$ cfu/ml and

199 2.7 $\log_{10}$ cfu/ml, respectively, after 72 hours of incubation. Colonisation resistance
200 remained in the raw samples, where there was no observed increase in *C. difficile* TVC (0
201 $\log_{10}$ cfu/ml, Figure 2). Similar patterns were observed for spore populations; there was no
202 increase in spore counts in raw samples, while autoclaved and filtered faeces yielded
203 increases in spores of 1.9 $\log_{10}$ cfu/ml and 1.0 $\log_{10}$ cfu/ml, respectively (supplementary
204 Figure 1). Cytotoxin was not observed in raw samples, but was universally detected in the
205 autoclaved and filtered faeces (Table 1).

206

207 *Effect of faecal sample storage on colonisation resistance* In order for this assay to be
208 clinically useful, the effect of sample storage prior to processing must be investigated, and
209 optimal storage conditions determined. We therefore investigated a range of different
210 storage conditions. In the raw faecal samples, there was no effect of sample storage on *C.*
211 *difficile* Δ TVC (Figure 3A) or spores counts (supplementary Figure 2A) at 72 hours, and
212 cytotoxin was not detected (Table 1). In autoclaved samples, storage temperature did not
213 affect the Δ change in *C. difficile* TVC, which increased by 2.7 $\log_{10}$ cfu/ml and 3.1 $\log_{10}$
214 cfu/ml for samples stored at 4°C and -80°C, respectively (Figure 3B); similar effects were
215 seen in the spore counts (supplementary figure 2B). However, the presence of glycerol did
216 affect *C. difficile* growth in autoclaved samples, with the Δ change in TVC between 0 and 72
217 hours being ~1.5 $\log_{10}$ cfu/ml and 2.0 $\log_{10}$ cfu/ml less, in 12% and 30% glycerol containing
218 samples respectively when compared to non-glycerol treated samples stored at -80°C (Fig
219 3B). Cytotoxin detection was also reduced in samples with added glycerol (Table 1).
220 Differences due to storage were even more apparent in the filtered faecal samples.
221 Samples stored at -80°C resulted in a higher Δ TVC compared with those held at 4°C. When

samples were stored in glycerol, there was no increase in *C. difficile* TVC, which was lower than samples stored at 4°C ($p = 0.026$) and -80°C (Figure 3C). In addition, there was no detection of cytotoxin in samples stored with glycerol. Growth of *C. difficile* in BHI with taurocholic acid (TCA) was not affected by 12% glycerol, however with 30% glycerol there was a lower TVC compared with the other conditions, and no cytotoxin was detected (Figure 3D, Table 1).

Effect of freeze-thaw cycles

Two freeze-thaw cycles did not affect *C. difficile* TVC (Figure 4A) nor cytotoxin levels (Table 1). Colonisation resistance was maintained in raw samples (~-0.1 $\log_{10}$ cfu/ml) but was lost in the autoclaved and filtered samples (TVC increased 2.4 $\log_{10}$ cfu/ml and 3.6 $\log_{10}$ cfu/ml, respectively). In contrast, freeze-thaw cycles altered the spore counts (Figure 4B).

Use of the CRACS assay to investigate the effect of antibiotics on colonisation resistance.

CRACS allows a measure of colonisation resistance against *C. difficile* to be determined for individual faecal samples. We used the assay on stool samples from a clinical study to determine whether antibiotic exposure affected colonisation resistance to *C. difficile*. Healthy individuals were given the fluoroquinolone antibiotic moxifloxacin for 5 days, or no antibiotics (control). Faecal samples were provided before and after treatment, and colonisation resistance to *C. difficile* was evaluated. Samples were raw or sterilised by filtration. Colonisation resistance in stool samples taken before the beginning of treatment

(D1) did not differ between the treatment and control groups; no increase in TVCs was observed in raw samples (Figure 5A) but an increase of ~3.2 $\log_{10}$ cfu/mL was observed in sterilised samples (Figure 5B). After 5 days of antibiotic treatment (D6) differences were observed between the treatment and control groups. Colonisation resistance was maintained in the control group, but lost in antibiotic recipient; raw samples taken post antibiotic treatment showed an increase in TVC of 2.4 $\log_{10}$ cfu/ml ($p = 0.007$, Figure 5A), which was associated with an increase in the proportion of samples with detected cytotoxin (Table 1). Filter sterilised samples did not differ irrespective of treatment group or time point; colonisation resistance was lost on all occasions with a TVC increase of ~3.2 $\log_{10}$ cfu/ml for all samples (Figure 5B) and a high percentage of toxin detection (Table 1).

Discussion

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

Our data build on the study by Borriello and Barclay (1986) who used untreated and sterilised faecal samples from different individuals and found that colonisation resistance is lost in sterilised samples. We have confirmed this finding, undertaken comparative studies

to create a reproducible optimised assay that can be used on faecal samples from human clinical studies, and used the optimised assay to demonstrate longitudinally in healthy subjects that colonisation resistance is lost following fluoroquinolone treatment. In previous studies by both Boriello and Barclay and Horvat and Rupnik (2018) investigating colonisation 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* (20), 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 faecal samples from individuals with potential dysbiosis and provide a measure of colonisation resistance. In individuals treated with the fluoroquinolone antibiotic moxifloxacin for five days, there was a significant reduction in colonisation resistance compared with a faecal sample obtained in the 24h prior to the first antibiotic administration (Figure 5). 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, while the other half yield results that resemble control samples. The intestinal microbiota varies between individuals, and individualised responses to antibiotic exposure have been reported (21, 22). Therefore, variation in the alterations to the gut microbiota in our participants would be expected. Whist microbiota diversity is thought to be important, and various microbiota changes have been linked to CDI (23), the exact components conferring colonisation resistance are as yet unclear. Our assay is designed to give a qualitative but not a quantitative assessment of colonisation resistance. Thus, according to

289 our assay and supported by clinical observations, in some patients, microbiota disruption following
290 antibiotic exposure leads to loss of colonisation resistance, whilst in others, it does not.

291 Our results are in line with those of Horvat and Rupnik (2018) who observed a loss of
292 colonisation resistance in dysbiotic faecal samples. Crucially, our data suggest that this
293 assay may also be an effective proxy measurement for risk of *C. difficile* infection prior/
294 during antibiotic treatment.

295 We have examined potential confounders relating to faecal sample storage, which are
296 important in understanding the utility of this assay, including the desire to batch test and
297 examine specimens retrospectively. Notably, storage temperature had limited effects on
298 the assay; raw samples from healthy individuals did not support *C. difficile* germination,
299 growth or toxin production, irrespective of storage condition. Some variation due to
300 storage temperature was observed in the sterilised portions of assay (which can be used as
301 a positive control); greater *C. difficile* proliferation occurred in sterilised samples stored at -
302 80^{o}C compared to those stored at 4^{o}C. Storage temperature of faecal samples has been
303 shown to have little effect on microbial populations, and their amplitude are modest in
304 comparison with inter-subject variability (24-26). By contrast, sample storage in glycerol, a
305 common cryoprotectant, was detrimental in terms of CRACS performance. Following
306 glycerol storage, no *C. difficile* germination, growth or toxin production was observed in the
307 sterilised portions of the assay, contrary to expectation and to those samples stored without
308 glycerol. In addition, when inoculated into BHI in the presence of TCA and glycine, known
309 inducers of germination (27), no increases in *C. difficile* TVC were observed when 30%
310 glycerol was used for storage, although *C. difficile* growth was unaffected by 12% glycerol.
311 The reasons for this are unknown, but it seems clear that faecal samples should be stored

without glycerol prior to analysis using CRACS or other colonisation resistance assays reliant on *C. difficile* growth. One or two freeze-thaw cycles were not detrimental to the maintenance or loss of colonisation 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 (28). Additionally, the spore counts did not follow the patterns of the TVC, or cytotoxin titre (Figure 3, 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 sterilisation technique is used as a control, and faecal samples are stored at -80^{o}C without glycerol. One or two freeze-thaw cycles will not affect results of the assay. Continuous culture models have been used repeatedly to demonstrate the capacity of spores to induce CDI and assess the effectiveness of therapeutics (12, 13, 29). While these models closely simulate *in vivo* conditions and indeed are clinically reflective, they are time consuming, technically demanding, low throughput and expensive.

This study is limited, as the optimised assay has only been validated 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 colonisation 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, and 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 faecal 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 underway. 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 which use parameters such as microbiota diversity, richness or presence / absence of specific components are therefore used as a proxy measurement. However, as the exact components and factors responsible for *C. difficile* colonisation resistance are unknown, the utility of these are limited. We have demonstrated that CRACS can be used to rapidly assess the presence of colonisation resistance within faecal 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.

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463 Tables

464 **Table 1: Toxin results (Percentage of cytotoxin positive samples at 72 hours) of the experiments shown in figures 2-5. Grey boxes indicate**

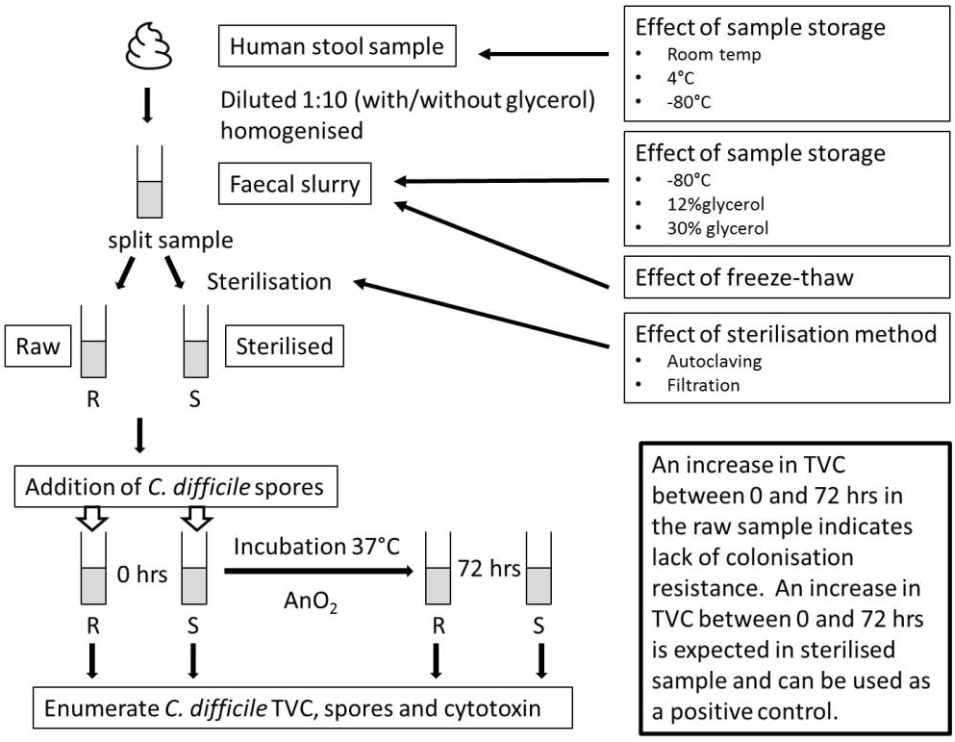
465 **experiment not performed.**

Storage condition or patient group	Raw (n)	Autoclaved (n)	Filtered (n)	BHI and taurocholic acid (n)	Associated figure
Fresh	0 (0/11)	100 (6/6)	100 (5/5)		2
4°C	0 (0/5)	100 (3/3)	100 (3/3)		3
-80°C	40 (3/5)	100 (3/3)	100 (3/3)	100 (3/3)	3
-80°C with 12% glycerol	0 (0/5)	66.6 (2/3)	0 (0/3)	100 (3/3)	3
-80°C with 30% glycerol	0 (0/5)	0 (0/3)	0 (0/3)	0 (0/3)	3
First freeze-thaw	0 (0/3)	66.6 (2/3)	66.6 (2/3)		4
Second freeze-thaw	0 (0/3)	100 (3/3)	100 (3/3)		4
Moxifloxacin D1	9 (1/11)		100 (11/11)		5
Moxifloxacin D6	45 (5/11)		91 (10/11)		5
Control D1	0 (0/10)		100 (10/10)		5
Control D6	0 (0/10)		90 (9/10)		5

466

467

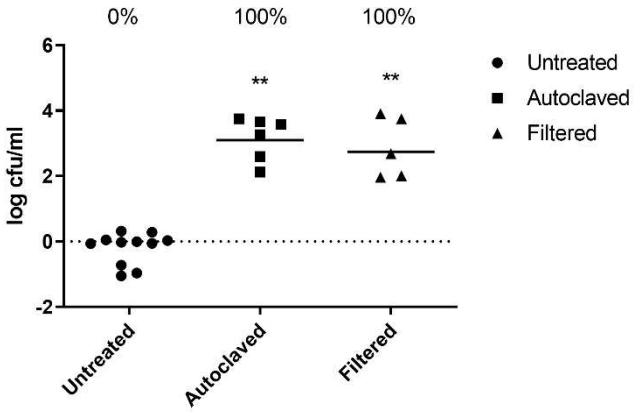
468 Figures



469

470 **Figure 1: Manipulation of stool samples undergoing CRACS. The different experimental**
471 **condition investigated during optimisation of the assay are indicated in boxes on the right.**

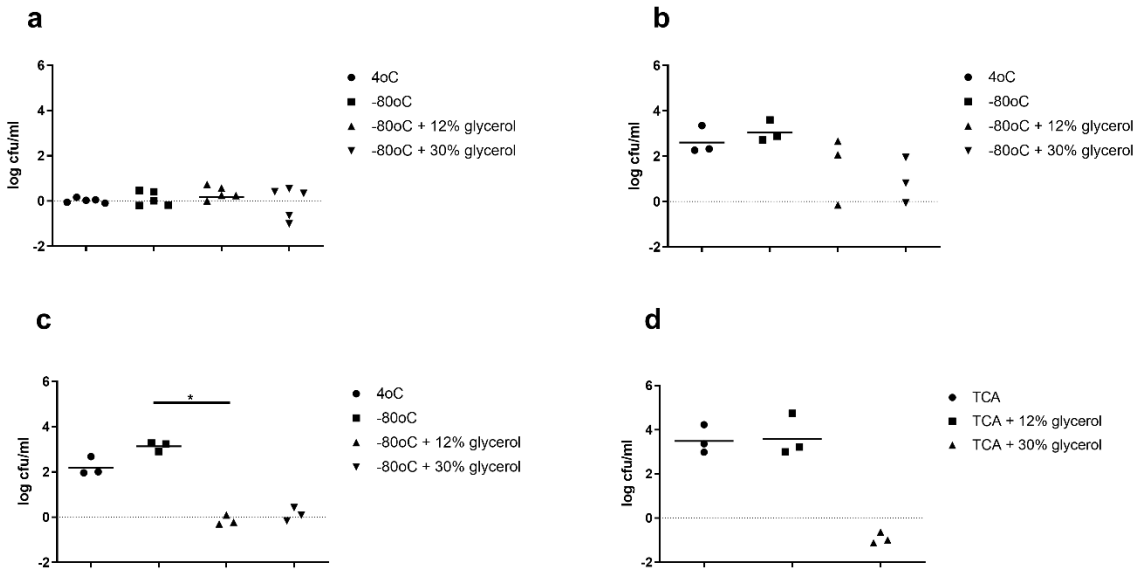
472



473

474 **Figure 2: Δ change in *C. difficile* total viable counts (geometric mean) over 72 hours in**
475 **faecal samples from healthy individuals which have been untreated (raw) or sterilised by**
476 **autoclaving or filtration. Individual data points, with the geometric mean are presented.**
477 **Numbers above bars indicate the percentage of toxin positive samples. ** p <0.01 where**

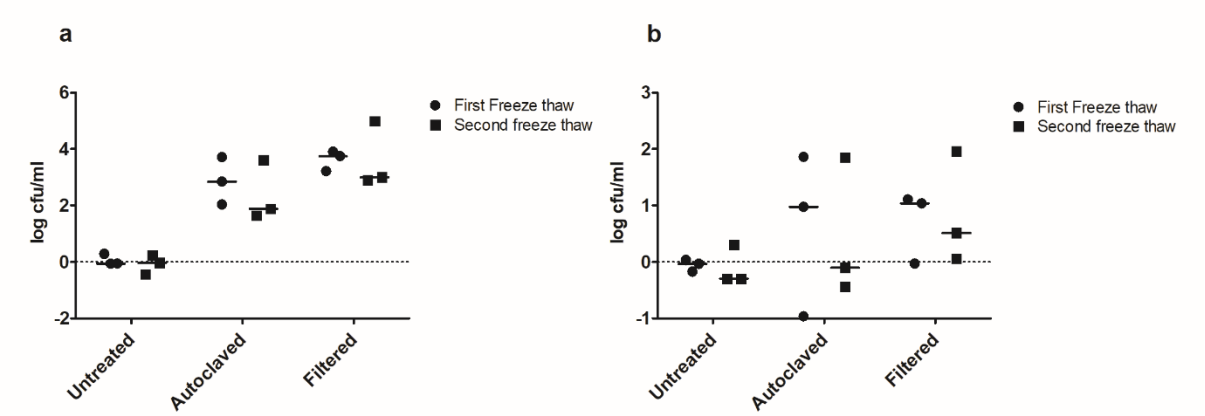
Untreated vs Autoclaved p = 0.002, Untreated vs Filtered p = 0.006, and Autoclaved vs Filtered p = 1.0. Untreated n = 11, Autoclaved n = 6, Filtered n = 5



481

482 **Figure 3: Impact of storage of healthy faecal samples on Δ change in the geometric mean**
483 ***C. difficile* total viable counts over 72 hours. (a). Raw faecal samples(n = 5), (b). Autoclaved**
484 **faecal samples (n = 3), (c). Filtered faecal samples (n = 3). Faecal samples were stored at**
485 **4°C, -80°C, -80°C with 12% glycerol or -80°C with 30% glycerol. Panel (d) shows Δ change in**
486 **the geometric mean *C. difficile* total viable counts over 72 hours following growth in BHI**
487 **broth with taurocholic acid with and without glycerol (n = 3). Individual data points, with**
488 **the geometric mean are presented. * indicates p = 0.026**

489



490

491 **Figure 4: Effect of multiple freeze-thaw cycles on Δ change in the geometric mean *C.***

492 ***difficile* total viable counts and spore counts over 72 hours (n = 3). (a); total viable counts,**

493 **(b); spore counts. Individual data points, with the geometric mean are presented.**

494

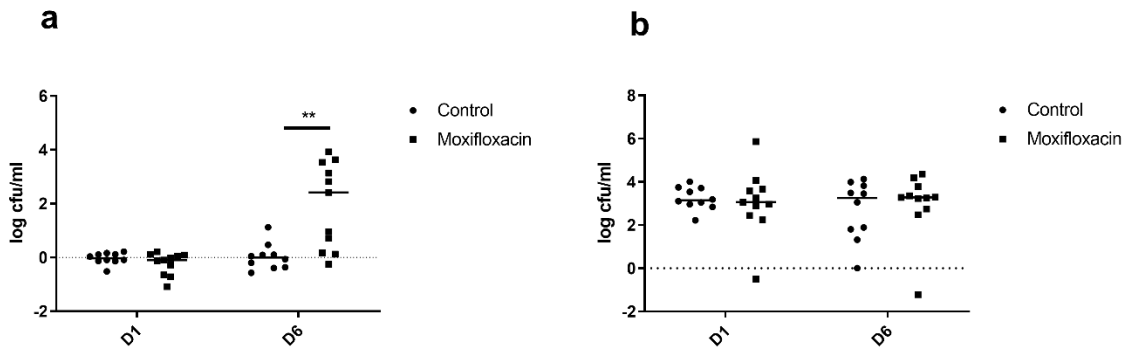


Figure 5: Effect of five days of antibiotic or control treatment on Δ change in the geometric mean *C. difficile* TVC over 72 hours. (a). Raw samples, (b). Sterilised samples. ** p = 0.006, with Wilcoxon rank sum test, n = 11 for antibiotic treatment group, n = 10 for control group. Individual data points, with the geometric mean are presented.