Generation of a low passage panel of *Clostridium difficile* reference strains

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INTRODUCTION

Clostridium difficile is a gram positive, spore-forming, obligate anaerobic pathogen. It is a healthcare acquired infection causing mild to severe forms of antibiotic associated diarrhoea, in serious cases causing Pseudomembranous colitis and Toxic megacolon. To facilitate preclinical research, BARDA required a panel of well characterised, low passage, high provenance, recent clinical isolates of *C. difficile*. PHE won a competitive international grant application to select and generate this panel, and this poster details the methods and results of the project.

METHODS

Panel selection The *C. difficile* Ribotyping Network (CDRN) was initiated in 2007, and comprises eight regional laboratories, of which Leeds CDRN Reference Laboratory (National Infection Service, Public Health England) is one. Starting from a database of 960 available clinical isolates held by the CDRN, including PCR-ribotypes 002, 020, 027 and 078 strains (the main ribotypes circulating clinically in UK and North America) four isolates were selected. Selection was based on severity of *C. difficile* infection (CDI), recent isolation (2015), and antibiotic resistance profile (**Figure 1**).

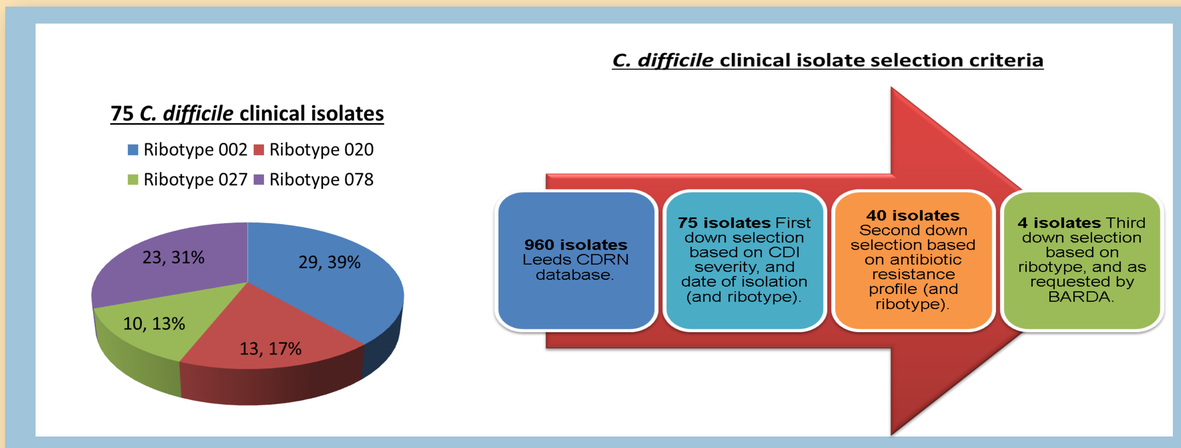

Figure 1. Above left: 75 *C. difficile* clinical isolates containing main ribotypes 002, 020, 027, and 078.

Above right: Isolate selection from 960 to 4 *C. difficile* clinical isolates.

Figure 2. Image of a MACS MG 1000 Anaerobic Workstation (Don Whitley Scientific) for handling *C. difficile* isolates.

Panel generation - The four selected isolates were cultured to produce spore Master Stocks and Working Stocks. *C. difficile* isolates were handled in an anaerobic environment (**Figure 2**). Master stocks were generated from liquid cultures (Edwards A *et al*, 2013). The process involved growing *C. difficile* in supplemented Brain-Heart Infusion (sBHI) broth for 10-14 days and harvesting into Nutrient Broth (NB) with 10% glycerol. Aliquots were frozen and stored at -80ºC (**Figure 3**). Working stocks were generated from agar plate cultures (Edwards A *et al*, 2013). *C. difficile* spore Master Stock was grown on Fastidious Anaerobic Agar with Horse Blood (FAA+HB) for 14-16 days and harvested into NB with 10% glycerol. Aliquots were frozen and stored at -80ºC (**Figure 4**).

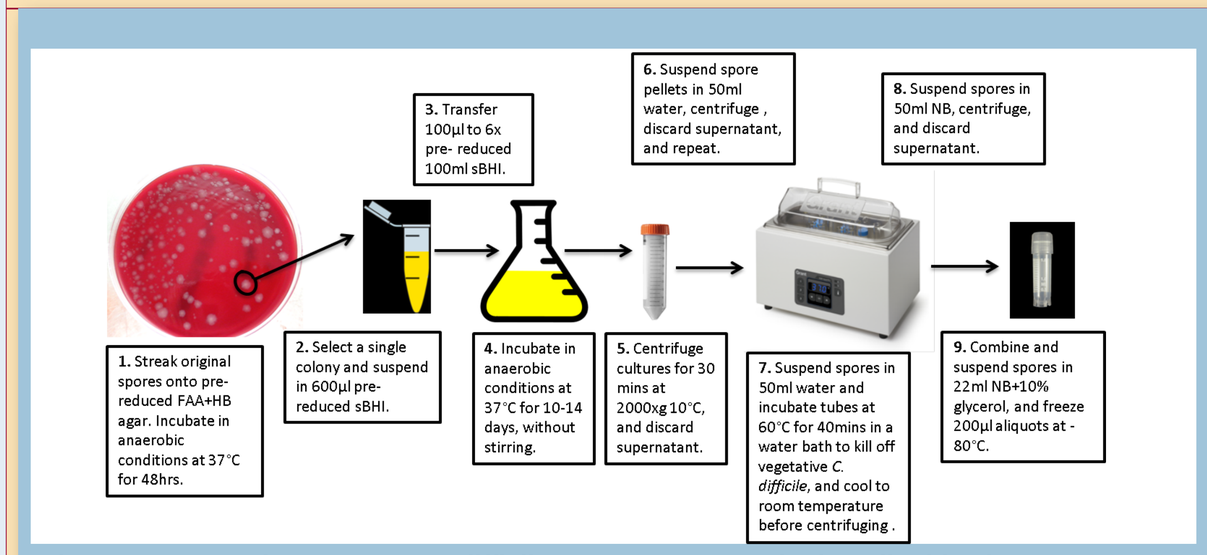

Figure 3. Protocol to generate *C. difficile* spore Master Stocks from liquid culture.

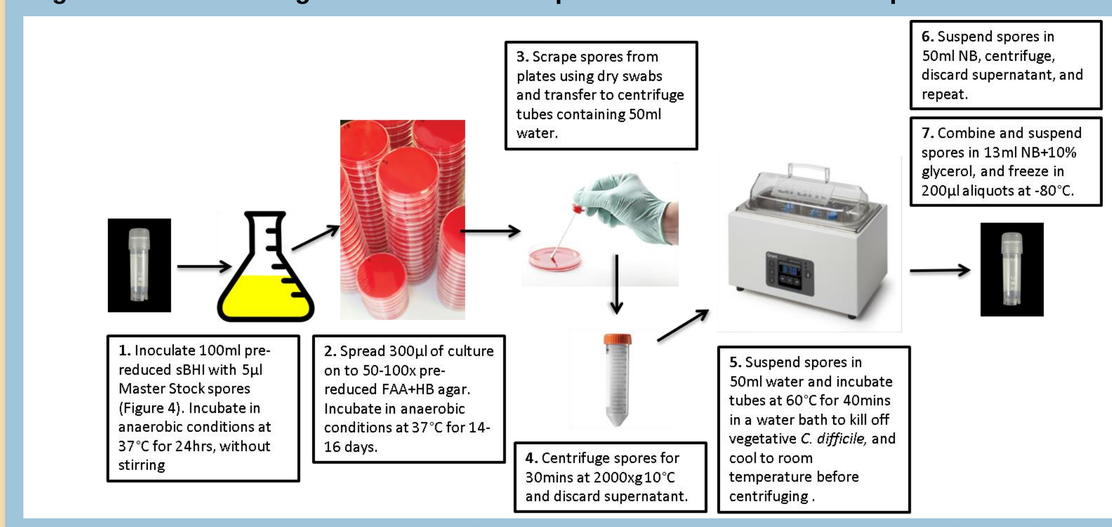

Figure 4. Protocol to generate *C. difficile* spore Working Stocks from agar plate culture.

RESULTS

Clinical isolate	Isolation date	Ribotype	Antibiotic MIC (mg/L)								
			MET	VAN	FDX	RIF	MXF	CLI	IPM	CAM	TGC
35829	07/01/2015	002	0.125	0.5	0.06	0.002	16	8	4	8	0.03
36877	16/02/2015	027	1	1	0.125	0.004	32	4	8	4	0.03
38047	30/03/2015	020	0.25	0.5	0.125	0.004	2	16	4	8	0.03
38157	06/04/2015	078	0.125	0.5	0.015	0.002	1	2	4	4	0.03

Figure 5. Information on 4 *C. difficile* clinical isolates including isolation date, ribotype, and antibiotic resistance profile (Antibiotics: MET – Metronidazole, VAN – Vancomycin, FDX – Fidaxomicin, RIF – Rifampicin, MXF – Moxifloxacin, CLI - Clindamycin, IPM – Imipenem, CAM - Chloramphenicol, and TGC – Tigecycline. Sensitivity interpretation: Green – Sensitive, Orange – Intermediate, and Red - Resistant) (Freeman J *et al*, 2015).

- Four *C. difficile* clinical isolates were selected for the production of Master Stock and Working Stock spores (**Figure 5**).
- The BARDA requirements to produce 5-8 sets of 10 Master Stock vials and 5 sets of 10 Working Stock vials of spores from each selected isolate was achieved.

Master and Working Stocks of spores of each isolate were characterised before shipping to a US holding facility for subsequent storage and dissemination for future use in BARDA grant awards.

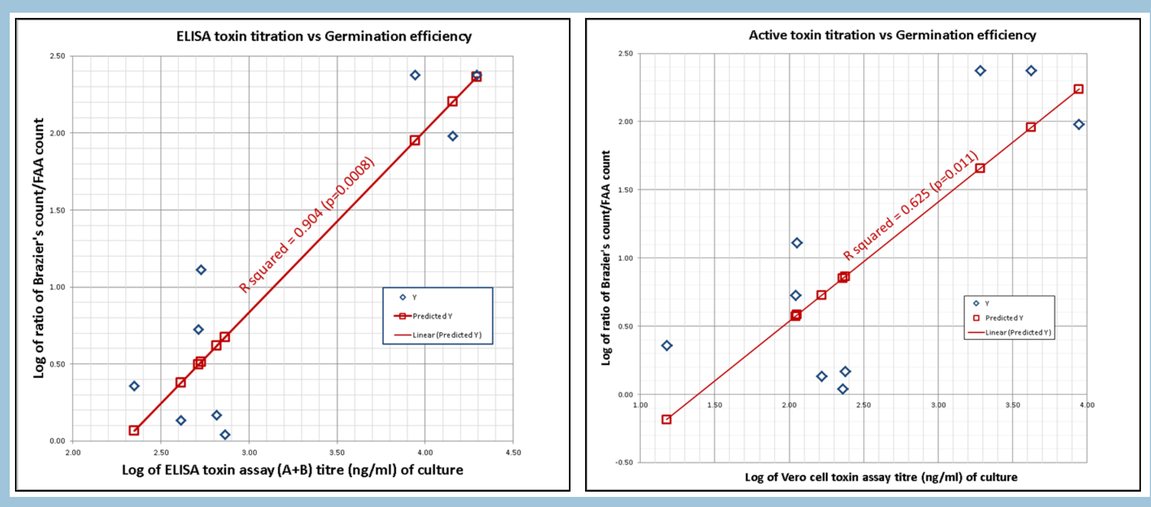

Figure 6. Left: Regression line of ELISA toxin titration against germination efficiency. Right: Regression line of active toxin titration against germination efficiency.

Determination of Master and Working stock spore viability (cfu/ml) was conducted on both Brazier's *C. difficile* Selective agar and FAA+HB agar. A distinctive pattern of spore dormancy (expressed at Brazier's/FAA count, **Figure 6**) was observed using the different growth media. Some spore lots required the addition of specific germinants or components found in Brazier's but absent in FAA for germination to occur. In some cases, only 1/250 spores grew on FAA. Assessment of toxin production was also conducted using ELISA and Vero cell bioassay. Significant regressions are shown in **Figure 6** between the ELISA toxin titre and spore dormancy as well as the toxin bioassay titration and spore dormancy. The complete characterisation data set is shown on our accompanying poster (Mathews D *et al.*, 2017).

Although the characteristics of only 9 sets of stocks have been analysed here, the apparent correlation between both ELISA and biologically active toxin titre with spore dormancy might suggest that these two characteristics are linked. This might be worth investigating further to verify and to determine why there is an apparent link.

CONCLUSIONS

Panel creation PHE has produced a well characterised, low passage high provenance, panel of recent clinical isolates of *C. difficile*.

Panel characterisation Characterisation involved viable counts (cfu/ml), purity, gram staining, toxin production measured by ELISA assay, and active toxin production measured by Vero cell–based cytotoxicity assay. In addition, whole genome sequencing was also performed on isolated genomic DNA (Mathews D *et al.,* 2017).

Panel availability This panel of strains will be distributed to future awardees of US government grants to research the development of prophylactics, treatments or therapies against infection and disease caused by *C. difficile.*

Future work 1 PHE is currently utilising the panel for *in vivo* preclinical trials of antibiotics.

Future work 2 It may be important to verify the preliminary finding regarding spore dormancy and toxin production using a larger number of strains.

ACKNOWLEDGEMENTS

This work was entirely funded by BARDA research grant HHSO10033003T RTOR-BIO-0019. Acknowledgements are made to the Leeds CDRN Reference Laboratory , Leeds Teaching Hospital and the University of Leeds which facilitated access and selection of all strains in the panel.

REFERENCES

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Mathews D *et al.* (2017) Phenotypic and genotypic characterisation of a reference strain panel of *Clostridium difficile*.