Detection of unusual strains of *Clostridium difficile* from the environment in Western Australia

Shivaperumal Nirajmohan[1], Knight Daniel R[2], Androga Grace O[3], Chang Barbara J[1], Riley Thomas V[1, 2, 4, 5]

[1]The University of Western Australia, Faculty of Health and Medical Sciences, Nedlands, Western Australia, Australia; [2]Murdoch University, Medical, Molecular and Forensic Sciences, Murdoch, Western Australia, Australia; [3]PathWest Laboratory Medicine, Department of Diagnostic Genomics, Nedlands, Western Australia, Australia; [4]PathWest Laboratory Medicine, Department of Microbiology, Nedlands, Western Australia, Australia; [5]Edith Cowan University, School of Medical and Health Sciences, Joondalup, Western Australia, Australia

INTRODUCTION

Chromogenic agar, such as ChromID *C. difficile* agar, is widely used for the isolation of *Clostridium difficile* from clinical and environmental sources[1]. The agar, containing the chromogenic substrate esculin or its derivative 3,4-cyclohexenoesculetin-glucoside (CHE-glucoside), which can be hydrolysed to give glucose and esculetin. The latter forms an insoluble complex with ferric citrate in the agar resulting in black colonies, which can be used for presumptive identification for *C. difficile*[2]. The hydrolysis of esculin depends on the presence of the relevant *β*-glucosidase /esculinase[2, 3].

Clostridium difficile clade 3 ribotype (RT) 023 strains that fail to produce black colonies on ChromID *C. difficile* agar (bioMerieux) have been reported[2], and an apparently new variant strain of *C. difficile* that produces only toxin A was found in a patient with diarrhoea in France[4]. We have recently isolated strains of *C. difficile* from the environment in Western Australia (WA) with similar characteristics. The objective of this study was to characterise these strains, referred to as 'white colony-producing *C. difficile* or WCD' from here on. We hypothesised that a putative *β*-glucosidase gene might be lacking in WCD, including RT023.

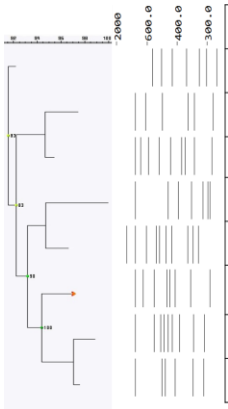
MATERIALS AND METHODS

Nineteen WCD previously isolated from home garden specimens were included in the study **(Fig 1).** MALDI-TOF MS was used to confirm the identity of these isolates[5]. Isolates were characterised by PCR ribotyping and toxin genes detection. A cell cytotoxicity assay was performed to detect a cytopathic effect (CPE) on Vero cells, and the strains were tested for the susceptibility to erythromycin, vancomycin, clindamycin, moxifloxacin, amoxicillin-clavulanate, rifaximin, metronidazole and fidaxomicin. Motility was detected with a sloppy agar stab culture[6]. An in-house PCR was performed to detect the presence of a putative *β*-glucosidase gene.

The novel primers, *putativeF* (Forward: 5'-GGAGGTTGGTTAGTTTTAGA-3' [20 bp]) and *putativeR* (Reverse: 5'-AGGATACCATTCTTGAGCT-3' [19 bp]), were designed to detect the presence of the putative *β*-glucosidase gene (amplicon size, 1131 bp) using the NCBI Primer-BLAST[7]. Whole-genome sequencing was performed on three selected variant strains of *C. difficile* from different suburbs in WA[8,9].

RESULTS and DISCUSSION

- MALDI-TOF MS analysis confirmed all strains as *C. difficile* (Mean cut-off scores of 1.93 ranging from 1.74 – 2.11).
- 5/19 isolates were toxigenic, all of which carried only the *tcdA* gene ($A^+B^-CDT^-$) and were a novel RT QX597 **(Fig 2).**



Ribotype	Toxin profile	Sample No.				No.	Post codes
		Soil	Compost	Manure	Shoes		
Novel	A-B-CDT-	1	-	-	-	1	6152
125	A-B-CDT-	3	4		-	7	6059, 6163, 6014, 6151, 6024
QX 637	A-B-CDT-	2	-	-	-	2	6152
QX 639	A-B-CDT-	2	-	-	-	2	6010, 6009
Novel	A-B-CDT-	1	-	-	-	1	6059
QX 597	A+B-CDT-	4	-	-	1	5	6006, 6054, 6024
023*	A+B+CDT+	NA	NA	NA	NA	NA	NA
QX 638	A-B-CDT-	1	-	-	-	1	6009

Fig 2. PCR ribotyping patterns and toxin gene profiles for 19 WCD isolated from the environment of Perth, WA. The dendogram was generated using a neighbour-joining tree and Pearson correlation (optimization, 5%; curve smoothing, 1%).

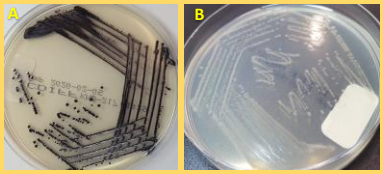


Fig 1. *C. difficile* on ChromID *C. difficile* agar

(A) Black colonies produced by an esculin hydrolysis positive strain (RT078)
(B) Non-black colonies produced by an esculin hydrolysis negative WCD

- No CPE was seen on Vero cells 24 h post-treatment with toxin filtrate, as Vero cells are more sensitive to toxin B[10] **(Fig 3).**

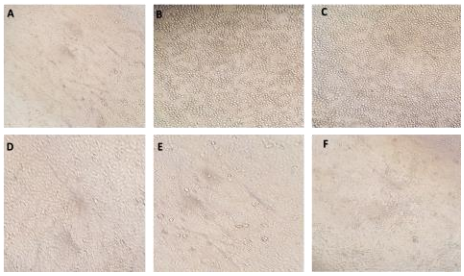


Fig 3. Detection of TcdA and TcdB in Vero cells 24 h post-treatment with 72 h culture filtrate at 37°C with 5% CO_2 (A) Untreated Vero cells, (B) TcdB induced CPE in Vero cells (VPI 10463), (C) TcdB induced CPE in Vero cells (ATCC 43600), (D) Treated Vero cell with purified control toxin A (TGCBiomics, Bingen, Germany) (E) Treated with garden isolate (A+B-CDT-) HGP30, (F) Treated with garden isolate (A+B-CDT-) HGP05.

- All isolates were susceptible to all antimicrobials tested except one isolate (toxin A positive) which was resistant to clindamycin (MIC = 8 mg/L).
- While seven isolates including five *tcdA*-positive strains were highly motile (mean projection length = 7 mm), three isolates appeared to have average motility with a projection length of 3 mm.
- All WCD isolates, including RT023, were negative for putative *β*-glucosidase gene by PCR, indicating that this may be a determinant for the utilization of esculin in all esculin-hydrolysing strains.
- The three sequenced strains belonged to the novel multi-locus sequence type 632, which belongs to the evolutionarily divergent clade C-III, and cgSNP analysis showed these strains shared a recent evolutionary history **(Fig 4, Table 1 & 2)**. All three genomes were positive for full-length *tcdA* but negative for *tcdB* and binary toxin genes.

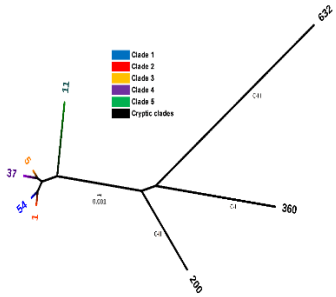


Fig 4. Global phylogenetic context of ST632 (QX597). MLST phylogeny based on concatenated allele sequences for ST632 (cryptic clade C-III) and well characterised representatives of MLST clade 1 (ST54, RT012), clade 2 (ST1, RT027), clade 3 (ST22, RT023), clade 4 (ST37, RT017), clade 5 (ST11, RT078), as well as other cryptic clades C-I (ST360) and C-II (ST200). Scale shows the number of substitutions per site.

Table 1. Core genome SNP analysis

	HGP14	HGP30	HGP78
HGP14		58	68
HGP30	58		54
HGP78	68	54	

Table 2. MLST and summary of AMR, phage, and virulence gene content

Sample	ST	Clade	PaLoc genes	AMR genes	Prophages
HGP14 HGP30 HGP78	632	C-III	*tcdA+, tcdB-*	AMR - neg	ΦMMPO3, ΦMMPO1, ΦCDHM19

CONCLUSIONS

- This study provides insights into the isolation and identification of WCD from environmental samples.
- A putative *β*-glucosidase gene was present in all strains except WCD indicating that this gene is the determinant for esculin hydrolysis phenotype.
- WCD from environmental samples could be overlooked when using ChromID *C. difficile* agar, leading to false-negative results.
- Further investigation is required to study the occurrence of the putative *β*-glucosidase gene in a wider range of RTs and putative *β*-glucosidase gene knock-out studies to compare with the wild type will aid to confirm the gene encoded esculin hydrolysis in *C. difficile* together with the virulence of this variant strain in laboratory animals.

REFERENCES

1. Eckert, C. *et al.* 2013 *J Clin Microbiol* 51, 1002-4.
2. Connor, M.C. *et al.* 2016 *J Clin Microbiol* 54, 1404-5.
3. Edberg, S. C. *et al.* 1985 *J Clin Microbiol* 22, 437.
4. Monot, M. *et al.* 2015 *Sci Rep* 5, 15023.
5. Schulthess, B. *et al.* 2013 *J Clin Microbiol* 51, 1834-40.
6. Hong, S. *et al.* 2019 *Anaerobe* 102066.
7. https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi
8. Knight, D. R. *et al.* 2019 *MBio* 10, e00446-19.
9. Knight, D. R. *et al.* 2017 *Front Microbiol* 7, 2138.
10. Kuehne, S. A. *et al.* 2010 *Nature* 467, 711.