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MICROBIOLOGY

Comparison of ChromID C. difficile agar and cycloserine-cefoxitin-fructose agar for the recovery of *Clostridium difficile*

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Summary

Aim: The rapidly changing epidemiology of *Clostridium difficile* infection highlights the need for improved and continuing surveillance involving stool culturing to enable molecular tracking. Culture of *C. difficile* can be difficult and time consuming. In this report ChromID C. difficile agar (CDIF) was compared to cycloserine-cefoxitin-fructose-egg-yolk agar which contained 0.1% sodium taurocholate (TCCFA) as a germinant.

Results: All ribotypes of *C. difficile* tested ($n = 90$) grew well on CDIF within 24 h and most gave characteristic small irregular black colonies with a raised umbonate profile. Counts from standard suspensions of *C. difficile* at 24 h ($p < 0.005$) and 48 h ($p = 0.01$) were significantly higher on CDIF than on TCCFA. Similar results were achieved after alcohol shock. When temperature shock was used to differentiate vegetative cells and spores, the total number of culturable and vegetative cells on CDIF was significantly higher than on TCCFA (culturable cells, $p = 0.003$ at 24 h and $p = 0.002$ at 48 h; vegetative cells, $p = 0.0003$ at 24 h and $p = 0.0002$ at 48 h).

Conclusions: These data suggest that CDIF is a better medium for the recovery of vegetative *C. difficile* than TCCFA and equal to TCCFA for spore recovery.

Key words: chromID, chromogenic agar, clostridium difficile, isolation medium.

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INTRODUCTION

In the past 10 years, the epidemiology of *Clostridium difficile* infection (CDI) has undergone great change with the arrival and rapid dissemination of the epidemic strains PCR ribotype 027, or North American pulsed-field type 1 (NAP1), PCR ribotype 078 and PCR ribotype 017. Each epidemic strain has unique characteristics and has spread in a different manner. Ribotype 027 produces high *in vitro* levels of toxins A and B, as well as binary toxin, is less susceptible to fluoroquinolones, and infection results in higher rates of morbidity and mortality.[1,2] Ribotype 078 also produces more toxins A and B, and binary toxin, was susceptible to fluroquinolones initially, and causes similar rates of morbidity and mortality to ribotype 027.[3] The toxin A^-B^+ ribotype 017 often has high level resistance to clindamycin due to the presence of the *erm* (B) gene,[4] has caused several outbreaks in Europe and appears to be hyperendemic in Asia.[5–7] While all three epidemic ribotypes have been found in animals,[8,9] ribotype 078 predominates in the Northern Hemisphere. This ribotype is associated with pigs from the Netherlands and has only recently been isolated from humans with both community-acquired and healthcare-related disease recorded. Animal ribotype 078 isolates have a high degree of genetic relatedness to human ribotype 078 isolates in Europe.[3,10–12] This ribotype is now the third most commonly isolated from humans in Europe[13,14] but is rarely isolated in Australia and not to date from production animals (Squire MM, Knight DR and Riley TV, unpublished).

The rapidly changing epidemiology of CDI highlights the need for improved and continuing surveillance involving stool culturing to enable molecular tracking.[14–16] Most laboratories world-wide do not culture for *C. difficile* and instead use enzyme immunoassays to detect toxins A and/or B[15] or, increasingly, molecular tests.[17–19] Culturing *C. difficile* can be difficult and time consuming, and may not be clinically relevant due to the slow turnaround time. However, due to its sensitivity and specificity, *C. difficile* culture is the standard against which all other laboratory diagnostic tests are often measured.[20] In the Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), culture for *C. difficile* is recommended on cycloserine-cefoxitin-fructose-egg-yolk agar (CCFA)[21] to which a germinant, either lysozyme or taurocholate, has been added and which has been pre-reduced under anaerobic conditions.[20] CCFA has long been recognised as the first choice for the isolation of *C. difficile* and there are a number of variations of this medium with common changes being the reduction of antibiotic concentration to half that of the original formulation,[22] the inclusion of sodium taurocholate as a germinant[23] and the substitution of blood for egg yolk.[24] There are also a number of commercial varieties of CCFA available from various manufacturers who have used the original formulation as a basis for their own isolation medium.

The prototype of a new chromogenic agar from bioMérieux (France) for the isolation and identification of *C. difficile* was evaluated by Perry *et al.*[25] On this medium *C. difficile* colonies were grey or black against a clear background and could be isolated in 24 h from faecal specimens without the use of alcohol-shock treatment. Pure cultures of 10 distinct ribotypes gave significantly higher counts on the chromogenic agar than on five other media used for *C. difficile* culture which did not include CCFA, but did include the BBL and Oxoid equivalent

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Table 1 *Clostridium difficile* strains (90) with toxin profiles and ribotypes that were plated directly from −70°C onto CDIF agar

Strains	Source	Toxin profile	Ribotypes
En 13	Lake WA	$A^-B^-CDT^-$	AU208
AI 25	Bovine	$A^-B^-CDT^-$	AU179
AI 27	Bovine	$A^-B^+CDT^+$	AU147
AI 16	Porcine	$A^-B^-CDT^+$	238
AI 152, AI 185	Porcine	$A^-B^+CDT^+$	285, AU169
R 10 (IS58)	Unknown	$A^-B^-CDT^+$	033
HCD 31	Human Australia	$A^-B^-CDT^-$	AU096
HCD 48	Human Australia	$A^-B^-CDT^-$	AU150
ES 98	Human NSW	$A^+B^+CDT^+$	AU098
ES 22, ES 48, ES 102, ES 104, ES 120, ES 129, ES 144, ES 170	Human NSW	$A^+B^+CDT^-$	103, AU028, AU054, AU027, 255, AU057, 049, 043
ES 130, ES 166, WA 151	Human NSW	$A^-B^+CDT^+$	280, 281, 237
ES 145	Human NSW	$A^-B^-CDT^+$	288
ES 42, ES 138	Human NSW	$A^-B^-CDT^-$	287, 286
JIR 8532	Human Vic	$A^+B^+CDT^+$	AU090
JIR 8398, JIR 8523, JIR 8571, JIR 8552	Human Vic	$A^+B^+CDT^-$	AU173, AU155, AU191, AU097
ES 167, JIR 8572	Human Vic	$A^-B^-CDT^-$	AU071, AU174
Q 23	Human Qld	$A^+B^+CDT^-$	AU145
WA 107	Human WA	$A^+B^+CDT^+$	127
RPH 17, RPH 61, WA 24, WA 76, WA 111, WA 113, WA 118, WA 139, R 16	Human WA	$A^+B^+CDT^-$	013, 192, AU033, AU034, 018, AU017, 054, 192, AU201
WA 13	Human WA	$A^-B^+CDT^+$	291
RPH 77, RPH 97, RPH 98, RPH 118, RPH 128, RPH 133, WA 12, WA 13, WA 68, WA 75, WA 110	Human WA	$A^-B^-CDT^-$	039, 289, AU204, 125, 290, AU215, 239, 291, 009, 031, 051
NZ 3, NZ 9	Human New Zealand	$A^-B^-CDT^-$	AU136, AU104
R 59	Human ECDC	$A^+B^+CDT^+$	131
R 32, R 33, R 34, R 38, R 39, R 40, R 41, R 43, R 45, R 47, R 48, R 49, R 50, R 52, R 53, R 54, R 55, R 56, R 58	Human ECDC	$A^+B^+CDT^-$	001, 002, 003, 014, 015, 020, 023, 029, 046, 053, 056, 070, 075, 078, 081, 087, 095, 106, 126
R 3b	Human ECDC	$A^-B^+CDT^-$	017
R 14 (630)	Human United Kingdom	$A^+B^+CDT^-$	012
R 23	Human United Kingdom	$A^-B^+CDT^-$	110
R 5, R 21, R 22	Human Belgium	$A^+B^+CDT^+$	080, 063, 066
R 17 (ATCC 43593)	Human Belgium	$A^-B^-CDT^-$	060
Sw 2, Sw 4, Sw 15	Human Sweden	$A^+B^+CDT^-$	AU087, 282, 026
Sw 5	Human Sweden	$A^-B^-CDT^-$	010
Si 22	Human Singapore	$A^+B^+CDT^-$	AU100
Si 25	Human Singapore	$A^-B^-CDT^-$	AU126
Ni 4, Ni 8	Human Nigeria	$A^-B^-CDT^-$	AU199, AU091

Strains not identified as UK ribotypes have been designated as Australian (AU) ribotypes. ECDC, European Centre for Disease Control; NSW, New South Wales, Australia; Qld, Queensland, Australia; Vic, Victoria, Australia; WA, Western Australia, Australia.

media.[25] Here we have used pure cultures of *C. difficile* to evaluate the final commercial ChromID C. difficile agar (CDIF) formula and compare it to a formulation of CCFA that contained 0.1% sodium taurocholate (TCCFA) as a germinant using culture conditions stated in the SHEA and IDSA guidelines for the characterisation and recovery of *C. difficile*.

MATERIALS AND METHODS

Media

CDIF plates were supplied by bioMérieux and contained (per L) meat peptone (porcine) 8.0 g, taurocholate (bovine) 1 g, yeast extract 3.5 g, sodium chloride 6.0 g, selective mixture 0.27 g, chromogenic mixture 0.3 g, agar 13 g and purified water. TCCFA plates were supplied by PathWest Media (Australia) and followed the original formulation of George *et al.*[21] apart from the antimicrobial concentrations (cycloserine 250 μg/mL and cefoxitin 8 μg/mL) and egg yolk at 60 mL/L, and the addition of 0.1% synthetic sodium taurocholate (Sigma cat no. T4009), *p*-hydroxyphenylactic acid at 1 g/L and agar at 13 g/L.

Bacterial strains

Table 1 shows the 90 different PCR ribotypes[26–28] used in preliminary testing and Table 2 shows *C. difficile* strains used in standard suspension, alcohol and heat shock experiments. They included human, animal and environmental

Table 2 *Clostridium difficile* strains used for the comparison of CDIF to TCCFA

Strain	Source	Toxin	Ribotype
AI 35	Porcine WA	$A^-B^+CDT^+$	237
ATCC 43598	Human Belgium	$A^-B^+CDT^-$	017
ES 214	Human NSW	$A^+B^+CDT^-$	001
ES 231	Human Vic	$A^+B^+CDT^+$	027
R 10725	United Kingdom	$A^+B^+CDT^+$	078
R 11446	United Kingdom	$A^+B^+CDT^-$	014
SSCC 28297	Quebec Canada	$A^+B^+CDT^+$	027
VPI 10463	Virginia USA	$A^+B^+CDT^-$	
630	Human Switzerland	$A^+B^+CDT^-$	012
WA 3	Human WA	$A^+B^+CDT^-$	002
WA 9	Human WA*	$A^+B^+CDT^-$	001
WA 34	Human WA*	$A^+B^+CDT^-$	014
WA 76	Human WA*	$A^+B^+CDT^-$	001
WA 94	Human WA*	$A^+B^+CDT^+$	078
WA 122	Human WA*	$A^+B^+CDT^-$	002
WA 131	Human WA*	$A^+B^+CDT^-$	014
WA 176	Human WA*	$A^+B^+CDT^+$	027
WA 231	Human WA*	$A^-B^-CDT^-$	
WA 240	Human WA*	$A^+B^+CDT^-$	001

NSW, New South Wales, Australia; Vic, Victoria, Australia; WA, Western Australia, Australia.

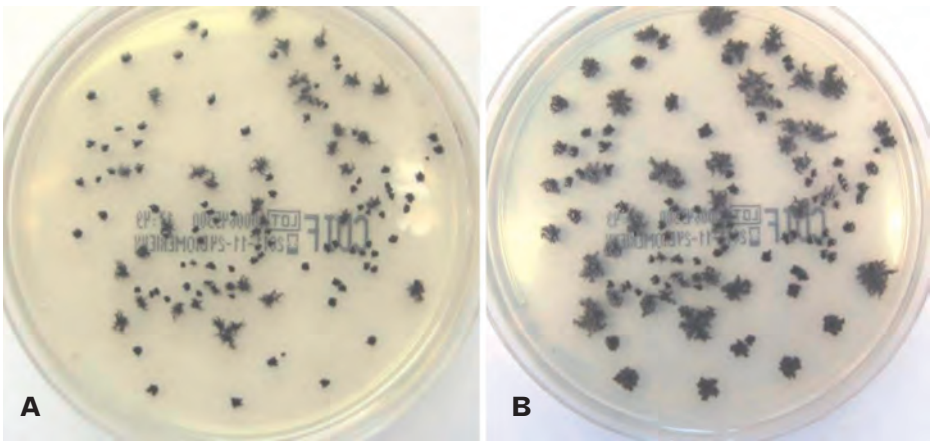

Fig. 1 Colonies of *C. difficile* AI 35 (ribotype 237) on CDIF after (A) 24 h and (B) 48 h of growth.

strains that had been identified with a species specific PCR.[29] PCR was also used to detect toxin A, toxin B and binary toxin genes.[29–31] Frozen stocks were maintained at −80°C in brain heart infusion broth (BHIB) containing 15% glycerol.

Growth of *C. difficile* on CDIF

The 90 different PCR ribotypes were plated onto CDIF for single colonies. Five strains (representing PCR ribotypes 001, 002, 014, 017 and 078) were also tested on CDIF that had been pre-reduced (≥2 h). Plates were incubated in an anaerobic chamber (Don Whitley, UK) at 35°C with 80% N_2, 10% CO_2 and 10% H_2, and 75% relative humidity, and colonies were examined for colour and morphology at 24 and 48 h. No plate spent more than 15 min outside the anaerobic chamber during examination and/or manipulation.

Comparison of *C. difficile* recovery on CDIF and TCCFA

Two different strains from five distinct ribotypes (001, 002, 014, 078 and 027) were used to compare recovery on CDIF to pre-reduced TCCFA. Standard 2.0 McFarland suspensions were prepared in 0.85% saline from 48 h blood agar cultures and serially diluted for total viable counts on each medium. The plates were incubated anaerobically and colonies were counted after 24 and 48 h. The experiment was performed in duplicate on separate occasions and mean data were used in all analyses.

Recovery of *C. difficile* after alcohol shock

To determine the concentration of spores, an aliquot of 2.0 McFarland suspension of 10 *C. difficile* isolates was prepared as above and mixed with an equal volume of ethanol for 1 h before dilutions and viable counts were performed. Mean data were used in all analyses.

Recovery of *C. difficile* before and after temperature shock

Temperature shock was also used to compare CDIF to pre-reduced TCCFA for recovery of spores and vegetative cells, as described previously.[32] Briefly, the total number of culturable cells from 18 h broth cultures of eight strains of *C. difficile* was determined by viable counts on the two media. The proportion of total culturable cells that was spores was determined by heat shock at 56°C for 10 min, and the remaining proportion of cells was considered vegetative *C. difficile*.[32]

Statistical methods

The data from all experiments were analysed using Graphpad Prism 3.03. The Kolmogorov–Smirnov (KS) test was used for normality testing. Matched analyses of non-parametric data were performed using the Wilcoxon matched pairs test and of parametric data by the paired *t* test.

RESULTS

Growth of 90 different ribotypes of *C. difficile* on CDIF

All 90 different ribotypes of *C. difficile* grew well on CDIF after 24 h incubation and typical colonies, coloured or not, were small and irregular with a raised umbonate profile. Pre-reduction of CDIF did not enhance growth in the five strains that were initially tested and so no further pre-reduction of plates was carried out. At 24 h, single colonies of ribotypes 023, 289 and AU090 were colourless, ribotypes 110 and 026 were grey and the remaining 85 ribotypes were black. After 48 h incubation, only ribotypes 023 and AU090 (3%) remained colourless while all other ribotypes were black.

Characterisation and growth of *C. difficile* from standard suspensions

All ribotypes used for standard suspension testing grew well on CDIF within 24 h and most gave characteristic small irregular black colonies with a raised umbonate profile. Strains such as AI 35 (ribotype 237) that gave some large, diffuse colonies on TCCFA formed flat filamentous colonies on CDIF which were more apparent at 48 h (Fig. 1). Strains WA 176 (ribotype 027) and SSCC 28297 (ribotype 027) had various colony morphologies; some characteristic irregular black colonies and a few larger filamentous colonies, while others were small and round and took 48 h to assume characteristic morphology. After 48 h the larger filamentous colonies had taken on a swarming appearance. With all strains, more small grey/black colonies of *C. difficile* appeared between 24 and 48 h of growth.

The colony count of *C. difficile* from standard suspensions varied enormously and was dependent upon the strain and the medium on which it was grown. The highest count was with WA 9 (ribotype 001) on CDIF and the lowest with WA 94 (ribotype 078) on TCCFA where no colonies were seen and the count was below the limit of detection of 100 CFU/mL (Table 3). As a result the data from WA 94 were not included in any further analysis.

At both 24 h ($p < 0.005$) and 48 h ($p = 0.01$) the median counts on CDIF agar were significantly higher than that on TCCFA (Table 3). The median CDIF count at 24 h was 1.5 times that on TCCFA and reduced to only 1.2 times at 48 h even though the TCCFA had been pre-reduced for a minimum of 2 h (Fig. 2). The median counts on both CDIF and TCCFA increased significantly between 24 h and 48 h, by an average of 18% ($p < 0.005$) and 25% ($p < 0.005$), respectively (Fig. 2). The counts on CDIF at 24 h were lower than those on TCCFA at 48 h, but the difference was not significant.

Recovery of *C. difficile* after alcohol shock

The mean colony count on CDIF was 1.5 times that of TCCFA at 24 h and 1.4 times at 48 h (Fig. 3). Counts on CDIF at both

Table 3 Mean colony counts from standard suspensions of *C. difficile*, from separate experiments without alcohol shock and with alcohol shock, plated onto CDIF and TCCFA and incubated for 24 h and 48 h

		Mean colony count $\times 10^6$ (CFU/mL)							
		24 h suspension*		48 h suspension†		Alcohol shock 24 h‡		Alcohol shock 48 h‡	
Strain	Ribotype	CDIF	TCCFA	CDIF	TCCFA	CDIF	TCCFA	CDIF	TCCFA
WA 76	001	8.70	5.60	13.4	8.90	12.2	4.20	12.65	4.75
WA 9	001	87.0	36.0	95.0	40.5	3.65	1.40	3.85	1.65
ES 214	001	ND	ND	ND	ND	6.65	3.90	9.30	6.20
WA 3	002	0.83	0.69	0.89	0.78	1.29	0.92	1.37	0.99
WA 122	002	4.35	2.80	6.20	5.15	16.6	8.05	18.35	8.95
WA 131	014	3.70	2.34	4.40	2.51	1.01	0.34	1.04	0.36
WA 34	014	3.31	2.32	5.20	3.80	5.50	3.55	5.80	4.30
ES 231	027	5.20	3.40	8.90	7.80	13.65	9.20	14.4	10.55
WA 176	027	1.07	0.70	2.03	2.70	6.80	4.90	9.15	8.00
SSCC 28297	027	ND	ND	ND	ND	3.25	3.10	7.70	6.15
WA 94	078	0.31	<0.0001	0.36	<0.0001	0.03	<0.0001	0.03	<0.0001
WA 240	078	2.99	2.43	6.75	5.55	6.80	4.90	11.05	7.05
R 10725	078	ND	ND	ND	ND$^-$	6.00	8.50	8.80	11.75
AI 35	237	ND	ND	ND	ND	1.90	4.45	4.85	5.65

CDIF, ChromID C. difficile agar; ND, not done; TCCFA, cycloserine-cefoxitin-fructose-egg-yolk agar containing 0.1% sodium taurocholate.
* Wilcoxon matched pairs ($p \leq 0.01$) (WA 94 NOT included in analysis).
† Wilcoxon matched pairs ($p \leq 0.05$) (WA 94 NOT included in analysis).
‡ Paired *t* test ($p \leq 0.05$) (WA 94 NOT included in analysis).

24 h ($p = 0.04$) and 48 h ($p = 0.02$) were significantly higher than those on TCCFA (Fig. 3). The increase in the mean colony count on the CDIF and TCCFA plates between 24 h and 48 h was significant with a 27% increase for CDIF ($p = 0.001$) and 33% for TCCFA ($p = 0.001$). When the colony count on CDIF at 24 h was compared to that on TCCFA at 48 h there was no significant difference.

Total counts, vegetative cell counts and spore counts after temperature shock

The recovery of spores and vegetative cells of *C. difficile* on both CDIF and TCCFA was examined at both 24 h and 48 h. At both time points the total number of culturable cells on CDIF, which included both spores and vegetative cells, was significantly higher than that on TCCFA at 24 h ($p = 0.003$) and 48 h ($p = 0.002$) (Table 4). The culturable spore count did not differ significantly while the calculated number of vegetative cells on CDIF was significantly higher than on TCCFA at 24 h ($p = 0.0003$) and 48 h ($p = 0.0002$) (Table 4).

The increases in spore counts between 24 h and 48 h were not significant. The increases in the total and vegetative cell counts on TCCFA at these time points were also not significant. However, on CDIF, both the total count and the vegetative cell count showed a significant increase ($p = 0.03$) between 24 h and 48 h, in line with previous results (Table 4).

DISCUSSION

bioMérieux has recently introduced a new chromogenic medium (CDIF) for the isolation and identification of *C. difficile* after 24 h incubation anaerobically. Colonies of *C. difficile* are grey/black and readily visible against the clear agar. The chromogenic mixture in the agar contains 3,4-cyclohexenoesculetin-β-D-glucoside (CHE-β-glu) and, from 24 h, *C. difficile* is able to free CHE which then combines with iron to form a black insoluble product that results in grey/black colonies.[26] The colonies, though small at 24 h, have the typical irregular morphology seen on CCFA plates but are not as flat. Strains that display variable morphologies on CCFA show the same characteristics on CDIF.

A prototype of the CDIF agar was evaluated by Perry *et al.*[25] who saw <1% increase in the colony count of *C. difficile* between 24 h and 48 h of growth in contrast to the 'optimised' formulation tested here where there was average increase of 18% for the pure culture suspensions and 27% for the spore counts. Whether this means that the prototype agar

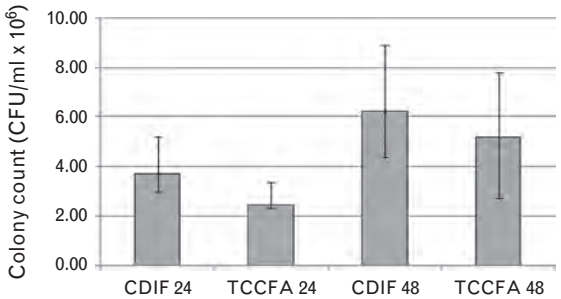

Fig. 2 Median counts of standard suspensions on CDIF and TCCFA at 24 h and 48 h in CFU/mL with 0.25 and 0.75 percentiles marked.

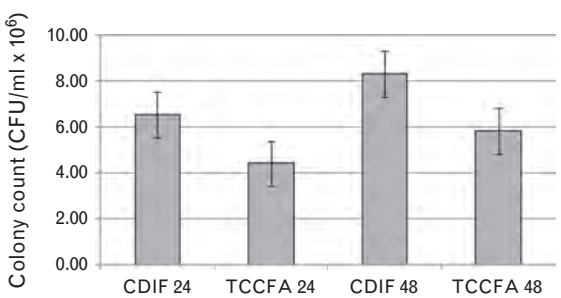

Fig. 3 Mean counts of standard suspensions after alcohol shock on CDIF and TCCFA at 24 h and 48 h in CFU/mL ± 1 SD.

Table 4 Mean cell count of total number of cells, the spore cell count from heat shock and the presumptive vegetative cell count for eight strains of *C. difficile* at 24 h (in parentheses) and 48 h

		Mean colony count × 10^5 (CFU/mL)					
		Total count*		Spore count		Vegetative cell count*	
Strain	Ribotype	CDIF†	TCCFA	CDIF	TCCFA	CDIF†	TCCFA
ES 214	001	122.25 (88.75)	13.88 (9.85)	1.52 (1.51)	1.60 (1.60)	120.73 (87.24)	12.28 (8.23)
R 11446	014	102.75 (98.00)	15.30 (15.25)	0.46 (0.46)	0.44 (0.43)	102.29 (97.54)	14.86 (14.82)
ATCC 43598	017	88.00 (69.00)	32.25 (2.03)	2.30 (2.25)	2.38 (2.03)	85.70 (66.75)	29.88 (4.23)
SSCC 28297	027	72.50 (59.75)	9.03 (6.73)	0.94 (0.58)	1.04 (0.76)	71.56 (59.17)	9.03 (5.97)
R 10725	078	94.50 (92.50)	47.75 (47.00)	2.48 (2.38)	3.30 (3.20)	92.03 (90.13)	44.45 (43.80)
VPI 10463		230.00 (215.00)	73.50 (54.75)	0.40 (0.40)	0.33 (0.33)	229.61 (214.61)	73.18 (54.43)
AI 35	237	184.00 (183.00)	55.25 (55.25)	0.69 (0.60)	0.74 (0.70)	183.32 (182.40)	54.51 (54.55)
630	012	112.75 (111.75)	2.43 (2.18)	0.03 (0.02)	0.03 (0.02)	112.72 (111.73)	2.40 (2.16)

* Paired *t* test at 24 h and 48 h ($p \leq 0.001$).
† Paired *t* test at 24 h and 48 h ($p \leq 0.005$).

allowed all vegetative cells and spores to grow within 24 h, or if the formulation changes produced an increase in colony count, presumably due to further spore germination between 24 h and 48 h, is unknown. The exact nature of any formulation changes that occurred is also unknown.

When compared to TCCFA which had been pre-reduced to give optimal recovery of *C. difficile*, there was approximately 1.5 times greater recovery of *C. difficile*. Colony counts pre- and post-heat shock suggested this was due to enhanced recovery of vegetative *C. difficile*. CDIF was an excellent medium for the recovery of vegetative *C. difficile* and as good as pre-reduced TCCFA for spore recovery.

When alcohol shock was used to select spores, recovery was significantly higher than that achieved on pre-reduced TCCFA, but when heat shock was used there was no significant difference seen. This may be explained by the different methodologies used and the characteristics of the spores. A spore population of *Bacillus* spores may have germination lag times that vary from hours to many days and spores with very long lag times have been termed 'superdormant spores'.[27–29] Such superdormant spores may be activated by heat.[29] Recently, it was shown that temperatures of 63°C and 71°C enhanced germination in aged spores of *C. difficile*,[30] and the heat shock temperature used in experiments here was 56°C. This temperature was probably sufficiently high to induce germination in aged spores and lead to most spores present germinating on both CDIF and TCCFA. When alcohol shock was used, such aged spores would not have germinated on TCCFA but might have been induced to germinate on CDIF and so account for the significant difference seen between the two media.

Another explanation may lie in the characteristics of the spore suspensions, as one was from a 48 h blood agar culture and the other was an 18 h brain heart infusion broth culture. The 18 h culture may not have contained aged spores but only those that germinated more readily. This would explain the lack of significance between the spore counts at 18 h. Again, the CDIF agar would have to have been able to induce more germination of aged spores than TCCFA to explain the significant difference that was seen with the alcohol-shock counts.

Both the saline suspension and the alcohol shock counts on CDIF at 24 h did not differ significantly from that of TCCFA at 48 h, but did increase significantly when incubated for 48 h. This also indicates that CDIF may induce the germination of otherwise dormant spores and implies that if culture on a CDIF plate of a PCR positive stool sample is negative at 24 h it might be worthwhile incubating for a further 24 h. The lack of growth of PCR positive stool samples has been attributed to non-culturable cells of *C. difficile*[31] but aged and superdormant spores may also play a role. Low recovery rates as seen with WA 94 where CDIF had counts from 100-fold to 1000-fold higher than those seen with TCCFA, dependant on the presence of spores alone or a mix of spores and vegetative cells, may also be partially responsible. In a recent study, prolonged incubation (48 h) of stool specimens on CDIF enhanced recovery and increased sensitivity and was recommended by the authors.[33] An environmental study comparing CDIF to cefoxitin-cycloserine-egg-yolk agar with added lysozyme also used a 48 h incubation time.[34]

At present, laboratory culture of *C. difficile* is essential for molecular tracking by those laboratories with such capability. Culture can be a time-consuming process and CDIF appears to facilitate this by lowering the isolation time of *C. difficile* by 24 h and by removing the necessary step of pre-reducing the isolation medium. CDIF is also superior to pre-reduced TCCFA in the recovery of both vegetative cells and spores produced by alcohol shock. It may even aid in the initial isolation of the organism before the patient is placed on antibiotic therapy when there are substantial quantities of vegetative cells present in faecal material.[35] Perry *et al.*[25] who evaluated the prototype both on pure cultures and fecal specimens concluded that the prototype medium 'offers effective isolation of *C. difficile* within only 24 h with or without alcohol-shock treatment' and the data presented here would support this claim.

Culture is not only required for surveillance purposes. If we are to understand fully the rapid dissemination of epidemic strains of *C. difficile* we must look beyond surveillance to all factors that may play a role. These could include asymptomatic carriage of *C. difficile* and the part that this may play in contamination of both the hospital and community environment; the rise in community acquired CDI and the concurrent possible increase in community environmental contamination; animal carriage and contamination of the immediate environment; animal husbandry and abattoir practices and the implications for food contamination; and the risk that all these carry for

the spread of CDI. Isolation and culture of *C. difficile* is required to investigate all of these and CDIF agar would appear to make this task quicker and easier.

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