Isolation of *Clostridium difficile* from faecal specimens – a comparison of chromID *C. difficile* agar and cycloserine-cefoxitin-fructose agar

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The culture of toxigenic *Clostridium difficile* from stool specimens is still seen as the gold standard for the laboratory diagnosis of *C. difficile* infection (CDI). bioMérieux have released ChromID Cdiff chromogenic agar (CDIF) for the isolation and identification of *C. difficile* in 24 h. In this study, we compared CDIF to pre-reduced cycloserine-cefoxitin-fructose agar with sodium taurocholate (TCCFA) in the examination of glutamate dehydrogenase-positive faecal specimens that were either GeneOhm positive or negative, using direct culture or culture following alcohol shock. Direct culture on CDIF had a sensitivity of 100 % and recovery of 94 % while for TCCFA these were 87 % and 82 %, respectively. For GeneOhm-positive alcohol-shocked faecal samples, sensitivity and recovery on CDIF was similar to direct culture while on TCCFA they were about 10 % higher. For direct culture, there was a significant difference between growth on CDIF at 24 h and TCCFA at 48 h ($P < 0.001$) and between the two media at 48 h ($P < 0.001$). A total of 142 strains of *C. difficile* were recovered in pure culture from all GeneOhm-positive samples used in this study and 11 (7.7 %) of these were A’B’CDT+ and may represent mixed infections of toxigenic and non-toxigenic *C. difficile*. The most dominant ribotype was UK 014 (14.7 %) followed by 002 (11.9 %) and 020 (11.9 %), and 36 % of toxigenic isolates, including an A’B’*+CDT+ strain, could not be assigned a UK ribotype. CDIF outperformed pre-reduced TCCFA by negating the need for alcohol shock treatment and by giving a time saving of 24 h in the isolation of *C. difficile*. CDIF plates were also more selective than TCCFA and *C. difficile* colonies were easy to identify and subculture prior to strain typing.

**INTRODUCTION**

The culture of toxigenic *Clostridium difficile* from stool specimens is still seen as the gold standard for the laboratory diagnosis of *C. difficile* infection (CDI) but it is difficult, laborious and time consuming. Although stool culture has been promoted as part of some diagnostic algorithms (Fenner et al., 2008; Schmidt & Gilligan, 2009; Shin et al., 2009), the advent of strains that cause outbreaks of more severe disease (Debast et al., 2009; Goorhuis et al., 2008; Warny et al., 2005) has driven the need for the isolation of *C. difficile* to facilitate molecular tracking and follow the spread of particular strains. A chromogenic media has recently been designed to assist in the isolation and identification of *C. difficile*.

*C. difficile* can be isolated from faecal specimens on a prototype chromogenic medium (bioMérieux, IDCd) within 24 h without the use of alcohol shock treatment (Perry et al., 2010). When culturing Vidas-positive stools, IDCd had a sensitivity of 97 %, at 24 h and 99 % at 48 h compared to CLO (bioMérieux) with sensitivities of 59 % and 76 %, respectively (Perry et al., 2010). Using pure cultures of 10 distinct ribotypes on five different selective media all ribotypes showed a higher count on IDCd than on all other media under any conditions with a mean count 3.7 times higher than on cefsulodin-cycloserine-egg yolk agar (CCEY) and over 30 times higher than on CLO (Perry et al., 2010).

bioMérieux has released and made commercially available the final formulation of their selective chromogenic agar for the isolation of *C. difficile*, ChromID Cdiff (CDIF). In this study, we compared this optimized medium to cycloserine-cefoxitin-fructose agar (CCFA) with sodium taurocholate.
taurocholate (TCCFA) that had been pre-reduced for a minimum of 2 h. We examined glutamate dehydrogenase (GDH)-positive faecal specimens that were either positive or negative for the toxin B gene by GeneOhm (Becton Dickinson), and used direct culture or culture following alcohol shock. In addition, isolates were ribotyped as part of an on-going surveillance program to monitor for the emergence of strains of *C. difficile* with enhanced virulence.

**METHODS**

**Media.** CDIF media were supplied as pre-poured ready to use plates by bioMérieux (Craponne, France); each contained meat peptone (porcine) 8.0 g, taurocholate (bovine) 1.0 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 Laboratory Medicine Excel Media and followed the original formulation of CCFA (George et al., 1979) apart from reducing the antibiotic concentrations by 50% (cycloserine 250 µg ml⁻¹ and cefoxitin 8 µg ml⁻¹) and including egg yolk at 60 ml l⁻¹, plus the addition of 0.1% synthetic sodium taurocholate (Sigma Cat # T4009), p-hydroxyphenylacetic acid at 1 g l⁻¹ and agar at 13 g l⁻¹. All plates were dried at 4 °C until used.

**Faecal samples.** A total of 234 GDH-positive (Wampole C. DIFF) samples that had also been tested by GeneOhm (Becton Dickinson) were obtained from the PathWest Laboratory Medicine Enteric Laboratory and stored at either 4°C or −70°C until used. All of 50 GeneOhm-positive stool specimens was used for direct culture. One hundred GeneOhm-positive stools and 84 GeneOhm-negative stools were cultured following alcohol shock.

**Direct culture.** All plate media were dried prior to use and TCCFA plates were pre-reduced in an anaerobic chamber in 80% N₂, 10% CO₂ and 10% H₂ at 35 °C for a minimum of 2 h. Stools were diluted with an equal volume of 0.85% sterile saline and vortexed vigorously; 50 µl of the suspension was streaked for single colonies on the two media. If the stool was liquid, then a 25 µl inoculum was used. The initial inoculum was streaked out across the agar surface into the second quadrant, and subsequently into the third and fourth quadrants of the plate. All plates were incubated in the anaerobic chamber and growth of *C. difficile* and faecal flora quantified at 24 and 48 h. No plate spent more than 15 min outside the anaerobic chamber during examination and/or manipulation. Growth of both *C. difficile* and faecal flora was assessed semiquantitatively as growth in the initial inoculum=1, the second and third quadrant=2 and the fourth quadrant=3. The number of isolated colonies of *C. difficile* (≤5 or >5) and the colony characteristics of *C. difficile* were also recorded.

**Alcohol shock culture.** Alcohol shock was performed on 100 GeneOhm-positive and 48 GeneOhm-negative stools. One gram of stool was mixed with 1 ml of ethanol and, for liquid stools, 1 ml was mixed with 1 ml of ethanol, left for 60 min and then mixed; 10 µl was streaked for single colonies on CDIF and pre-reduced TCCFA. If there was little stool available, then any remaining faecal material was collected on a swab and the swab left in contact with 1 ml ethanol for 60 min, then mixed and 10 µl streaked for single colonies onto the two media. The CDIF plates and TCCFA plates were examined after incubation at both 24 and 48 h. No plate spent more than 15 min outside the anaerobic chamber during examination and/or manipulation.

**Identification, toxin profiling and ribotyping.** Presumptive *C. difficile* colonies were subcultured onto blood agar (BA) plates for purity and identified by their characteristic morphology on BA and CCFA; natural chartreuse colony fluorescence under UV light on BA; lack of lecitinase (i.e. lack of opalescence surrounding colonies on CCFA containing egg yolk); and their ability to produce t-proline-aminopeptidase. Further confirmation of species identity was via a species-specific PCR for toxin A, toxin B and binary-toxin genes.

All isolates were screened by PCR for the presence of toxin A (tcdA) and toxin B (tcdB) genes (Kato et al., 1991) and binary-toxin (cdtA and cdtB) genes (Stubbs et al., 2000). PCR ribotyping was performed (Stubbs et al., 1999) with the PCR ribotyping reaction products concentrated using the Qiagen MinElute PCR Purification kit (Ambion) and run on the QIAxcel capillary electrophoresis platform (Ambion). The PCR products were visualized on QIAxcel ScreenGel software (v1.0.2.0, Ambion).

PCR ribotyping banding patterns were identified by comparison of banding patterns with a reference library, which consisted of a collection of 50 UK ribotypes that included 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and a collection of the most prevalent PCR ribotypes currently circulating in Australia (B. Elliott, unpublished data).

Interpretation of PCR ribotyping bandpatterns was performed by dendrogram and cluster analysis using the Dice coefficient with the BioNumerics software package v6.3 (Applied Maths).

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

**RESULTS AND DISCUSSION**

**Direct culture from stools**

Both the Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA) recommend culture for *C. difficile* on CCFA to which a geriminant, either lysozyme or taurocholate, has been added, and which has been pre-reduced under anaerobic conditions (Cohen et al., 2010). In this study, we compared CDIF with TCCFA following these guidelines and did not pre-reduce CDIF as in a previous study this was not deemed necessary (Boseiwaqa et al., 2013).

Growth of *C. difficile* and the other faecal flora on the 50 GeneOhm-positive stools was quantified numerically, and statistically analysed by the Wilcoxon matched-pairs test. When growth on CDIF at 24 h was compared to the growth on TCCFA at 48 h there was a significant difference between the medians (P=0.001) as was the difference between the two media at 48 h (P<0.001) (Fig. 1). The difference between the growth on CDIF at 24 and 48 h was also significant (P=0.019) (Fig. 1). Growth of other faecal flora on CDIF at 24 and 48 h was significantly less than that on TCCFA at the same time points (P<0.0001 at 24 h and 48 h) (Fig. 2) and more markedly so at 24 h. There was less contaminating faecal flora on CDIF than on TCCFA, as at 24 h only two plates of CDIF had ≤2 isolated single colonies of *C. difficile* and at 48 h there were five (10%). At 48 h there were 15 TCCFA (30%) plates that had ≤2 isolated single colonies of *C. difficile*. 
C. difficile grew on 47 CDIF plates at 24 h and 41 TCCFA plates at 48 h. All isolates of C. difficile were black on CDIF at 24 h and had the characteristic yellow ground-glass appearance on TCCFA at 48 h. This gave a 12% higher recovery and sensitivity for CDIF at 24 h over TCCFA at 48 h (Table 1). CDIF was more selective than TCCFA, with less other faecal flora present and with more single isolated colonies of C. difficile, and confirms the findings of Eckert et al. (2013). The increased isolation of C. difficile on CDIF may be due in part to increased growth of vegetative cells present in faecal samples (Boseiwaqa et al., 2013) along with the greater selectivity. However, as with all selective media, prolonged incubation times resulted in breakthrough growth. Despite this, a further 24 h incubation may be warranted with plates negative for C. difficile at 24 h as the increase in growth on CDIF between 24 and 48 h was significant in this and an earlier study (Boseiwaqa et al., 2013). A prolonged incubation of 48 h has been recommended as it increased the sensitivity of CDIF from 74% to 87% (Eckert et al., 2013).

Culture from alcohol shock

For the GeneOhm-positive alcohol-shocked faecal samples, growth was seen on 95 CDIF plates at 24 h and on 92 TCCFA plates at 48 h, which gave similar recovery and sensitivities (Table 1). Of the 84 GeneOhm-negative faecal samples, 46 grew C. difficile; 41 on CDIF at 24 h and 36 on TCCFA at 48 h to give sensitivities of 89% and 78%, respectively. No significant association was found between the GeneOhm result and culture medium (Fisher’s exact test, \( P = 0.7868 \)). With all alcohol-shocked faecal samples, there was little or no other faecal flora present. When compared to direct culture, a similar sensitivity and recovery was seen for CDIF, whereas on TCCFA these were about 10% higher (Table 1).

Ribotypes

Forty-six of the 47 isolates of C. difficile from direct culture were available for toxin profiling and ribotyping. Two isolates were \( A^{+}B^{+}CDT^{+} \) (both ribotype 244), one isolate was \( A^{-}B^{-}CDT^{-} \) and all others were \( A^{+}B^{+}CDT^{-} \). The non-toxigenic strain could not be assigned a UK ribotype from our collection of reference strains along with eight of the \( A^{+}B^{+}CDT^{-} \) strains. The most prevalent ribotypes were 014 \( (n = 7) \) and 070 \( (n = 7) \), followed by 054 \( (n = 6) \), 002 \( (n = 4) \) and 081 \( (n = 3) \). There was one ribotype 027 recovered from an elderly patient who had recently returned from the USA.

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**Fig. 1.** Median C. difficile growth on CDIFF and TCCFA for 50 GeneOhm-positive faecal samples (a) and number of samples with growth of 1, 2 or 3 from direct culture on CDIF and pre-reduced TCCFA at 24 h and 48 h, respectively (b). Growth in the initial inoculum is 1, in the second quadrant 2, and in the third and fourth quadrants 3.

**Fig. 2.** Median growth of contaminating faecal flora for 50 GeneOhm-positive faecal samples (a) and number of samples with growth of 1, 2 or 3 from direct culture on CDIF and pre-reduced TCCFA at 24 h and 48 h, respectively (b). Growth in the initial inoculum is 1, in the second quadrant 2, and in the third and fourth quadrants 3.
A total of 96 isolates of *C. difficile* were recovered after alcohol shock from the 100 GeneOhm-positive samples, including 81 that were $A^+B^+CDT^+$, five that were $A^+B^-CDT^+$ and 10 that were non-toxigenic. Four of the non-toxigenic isolates were UK ribotype 010. The toxigenic isolates belonged to several different ribotypes with the predominant types being 014 ($n=24$) and 002 ($n=13$), followed by 020 ($n=7$). All other ribotypes contained two or fewer isolates. A ribotype could not be assigned for 38 isolates, including four of the five $A^+B^-CDT^+$ strains.

Among the 46 *C. difficile* isolates from the GeneOhm-negative samples, 11 were $A^+B^+CDT^-$, one was $A^-B^+CDT^-$ and 34 were non-toxigenic. Four $A^+B^-CDT^-$ isolates were ribotype 020 and one was ribotype 014. For the other six, and the $A^-B^-CDT^-$ strain, a ribotype could not be assigned. Of the 34 non-toxigenic isolates, eight were ribotype 010.

A total of 142 strains were recovered in pure culture from all the GeneOhm-positive samples used in this study. Eleven (7.7%) of these strains were toxin profiled as $A^+B^+CDT^-$. The most dominant ribotype found among all toxigenic isolates was UK 014 (14.7%) followed by 002 and 020 (11.9% each). Approximately one-third of strains (36%), including an $A^-B^-CDT^-$ strain, could not be assigned a ribotype based on comparison with our reference collection of 50 UK ribotypes. Also, 11 were non-toxigenic ($A^-B^+CDT^-$), and it is possible that these may have been recovered from mixed infections of both toxigenic and non-toxigenic *C. difficile*. The proportion of non-toxigenic strains seen here (7.7%) was close to the 7% seen by Eyre et al. (Eyre et al., 2012) in a study from Oxfordshire. Ribotype 014 was the most dominant ribotype (14.7%) found followed by 002 and 020 (11.9% each). Of the non-toxigenic isolates only eight were ribotyped as 010 and the remaining 26 could not be assigned a ribotype. It is interesting to note that 36% of the toxigenic isolates, including the $A^-B^+CDT^-$ strain, also could not be assigned a UK ribotype from our collection of reference strains. There were seven $A^-B^-CDT^+$ isolates that included three ribotype 244 strains, a strain responsible for an outbreak of community-acquired *C. difficile* infection in Australia during 2011/12 (Riley, 2012). In this study, all ribotypes of *C. difficile* produced black colonies on CDIF although strains producing colourless colonies (e.g. ribotype 023) have been reported at low frequency (Boseiwaqa et al., 2013; Perry et al., 2010).

Black colonies of *C. difficile* on CDIF are distinctive and readily distinguished on the light background of the agar but there is a need to train staff to recognize the colony morphology of *C. difficile* on CDIF due to the small proportion of strains that do not turn dark grey or black within 24–48 h. The medium outperforms pre-reduced TCCFA by negating the need for alcohol shock treatment and by giving a time saving of 24 h in the isolation of *C. difficile*. CDIF plates are also more selective than TCCFA and *C. difficile* colonies are easily identified for subculture prior to identification and strain typing.

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### REFERENCES


