New selective medium for isolating *Clostridium difficile* from faeces

S T Aspinall, D N Hutchinson

**Abstract**

**Aims:** To compare CCFA (cycloserine, cefoxitin fructose agar) with a new selective medium CDMN (containing cysteine hydrochloride, norfloxacin, and moxalactam) for the isolation of *Clostridium difficile* after direct faecal culture.

**Methods:** The minimum inhibitory concentration (MIC) of norfloxacin was determined for 64 strains of *C. difficile*, 17 strains of other *Clostridium* sp, and 66 various isolates of faecal origin, together with MIC determinations of moxalactam against the 81 strains of *Clostridium* sp and 15 isolates of *Bacteroides* sp. Using C *difficile* agar base with 0.5 g/l of cysteine hydrochloride, norfloxacin and moxalactam were incorporated into the medium and compared with CCFA for the isolation of *C. difficile* after direct faecal culture.

**Results:** Norfloxacin (12 mg/l) inhibited the growth of enterobacteriaceae and faecal streptococci; moxalactam (32 mg/l) inhibited the growth of most strains of *Bacteroides* sp tested, together with *Clostridium* sp other than *C. difficile*. Using the antibiotics in combination (CDMN), the growth and colonial morphology of 64 strains of *C. difficile* were unaffected. When CDMN medium was compared with CCFA for the isolation of *C. difficile* from 832 faeces from inpatients with diarrhoea, the CDMN agar isolated 20% more strains and reduced the number of contaminating colonies by 30%.

**Conclusions:** CDMN both improves the isolation rate of *C. difficile* from faecal specimens and reduces the growth of other organisms compared with CCFA.

*Clostridium difficile* is the causal agent of most cases of pseudomembranous colitis and is frequently linked with many incidents of antibiotic associated diarrhoea. In these instances confirmation that *C. difficile* is the infecting agent is made by detection of the specific cytotoxin or enterotoxin and isolation of the organism. Most laboratories use cycloserine (500 mg/l), cefoxitin (16 mg/l) fructose agar (CCFA) for culture, or the subsequent modification containing half the concentration of antibiotics. Irrespective of antibiotic concentration, the selectivity of the medium is relatively poor and, the use of alcohol shock on the sample before it is plated out is recommended. The medium alsooriginally included egg yolk which has more recently been replaced by 7% (v/v) whole horse blood. This report presents the results of a comparison of CCFA with a new selective medium (CDMN) that uses the same basal agar as CCFA, but incorporates cysteine hydrochloride as a growth supplement, and norfloxacin and moxalactam as selective agents, in the direct culture of faeces for the isolation of *C. difficile*.

**Methods**

**DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC)**

(i) **Antibiotics**

Norfloxacin (Merck, Sharp and Dohme Ltd, Hoddeston, England) and moxalactam (Eli Lilly and Co Ltd, Basingstoke, England) were supplied as powders by the manufacturers.

(ii) **Micro-organisms**

The following organisms were tested: 64 strains of *C. difficile* (including NCTC 11207 and NCTC 11209), three strains of *C. sordellii* (including NCTC 8780), three strains of *B. bifermentans* (including NCTC 6801 and NCTC 6927), three strains of *C. sporogenes* (including NCTC 6929), three strains of *C. perfringens*, one strain of *C. butyricum*, two strains of *C. septicum*, one strain of *C. tertium*, one strain of *C. novyi*, 15 strains of *Bacteroides* sp, four strains of anaerobic streptococci, 14 strains of faecal streptococci, four strains of *Salmonella* sp, one strain of *Shigella* sp, four strains of *Versinia enterocolitica* and 24 strains of other enterobacteriaceae.

Isolates (other than NCTC strains) were obtained from clinical specimens. The MIC for each strain was determined against norfloxacin, but against moxalactam, only isolates of *Clostridium* sp and *Bacteroides* sp were examined.

(iii) **Media**

MICs were performed using two basal media. (a) *Clostridium difficile* agar base (Unipath Ltd, Basingstoke, England) with 0.5 g/l of cysteine hydrochloride (BDH Lab Supplies, Poole, England) and 7% (v/v) defibrinated whole horse blood (Lab M Bury, England).

(b) Diagnostic sensitivity test (DST) agar (Unipath Ltd) with 7% (v/v) defibrinated whole horse blood (Lab M).

(iv) **Method**

Freshly prepared *C. difficile* and DST agar containing doubling dilutions of norfloxacin (4 mg/l–512 mg/l) and moxalactam (0.0625 mg/l–256 mg/l) were used for MIC determinations, together with control plates containing no antibiotics. All isolates for testing were incubated overnight aerobically in
20 ml volumes of fastidious anaerobe broth (FAB) (Lab M) at 37°C before diluting the cultures with fresh FAB until a turbidity was reached roughly equivalent to 10⁶–10⁷ organisms/ml (previously assessed). Diluted broths (20 µl) were then inoculated on to each of the test media using a Ledwell multipoint applicator (LEEC Ltd, Nottingham, England).

All plates were incubated in an anaerobic atmosphere at 37°C for 48 hours.

The MICs of the strains were recorded as the lowest dilution of antibiotic required to inhibit fully growth of the organism. Variation in colonial morphology was also recorded.

STUDY OF FAECAL SPECIMENS

Using a sterile swab, 832 fresh faecal specimens from patients with diarrhoea were directly inoculated on to:

(i) Clostridium difficile base (with 0.5% g/l of cysteine hydrochloride and 7% blood) containing 12 mg/l of norfloxacin and 32 mg/l of moxalactam (CDMN).

(ii) Clostridium difficile base with 500 mg/l of cycloserine and 16 mg/l of cefoxitin (C difficile selective supplement, Unipath Ltd) (CCFA).

The plates were then spread four times using a sterile loop and incubated anaerobically at 37°C for 48 hours.

All suspect colonies of C difficile were presumptively identified using the C difficile microscreen latex test (Merca Diagnostics Ltd, Guildford, England) with all positive isolates being confirmed using API ZYM (API bioMérieux Ltd, Basingstoke, England).

Growth was assessed semiquantitatively for the isolation of (i) C difficile and (ii) all colonies other than C difficile—contaminants. This was achieved by designating a growth value of between 1 and 5 according to the amount of growth obtained. Growth in the primary inoculation only being given a value of 1, growth extending into the primary streaks, 2, growth into the secondary streaks, 3, and so on up to a growth value of 5.

Results

MIC DETERMINATIONS

The MICs of norfloxacin and moxalactam against all isolates tested were similar on C difficile agar base and DST agar base. Table 1 shows the MICs for norfloxacin against 64 strains of C difficile and 83 other bacteria, and of moxalactam against 64 strains of C difficile and 36 other anaerobic organisms.

Table 1. MICs of norfloxacin against 64 strains of C difficile and 83 other bacteria, and of moxalactam against 64 strains of C difficile and 36 other anaerobic organisms

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Norfloxacin (No of strains)</th>
<th>Moxalactam (No of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C difficile</td>
<td>≥64 (63)</td>
<td>≥128</td>
</tr>
<tr>
<td>C difficile</td>
<td>32 (1)</td>
<td></td>
</tr>
<tr>
<td>Faecal streptococci</td>
<td>≤8 (14)</td>
<td>NT</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>≤4 (33)</td>
<td>NT</td>
</tr>
<tr>
<td>Bacteroides sp</td>
<td>≥32 (15)</td>
<td>≤16 (11)</td>
</tr>
<tr>
<td>Bacteroides sp</td>
<td>≤8 (2)</td>
<td>≤4 (4)</td>
</tr>
<tr>
<td>Anaerobic streptococci</td>
<td>≥16 (2)</td>
<td>≤4 (4)</td>
</tr>
<tr>
<td>Anaerobic streptococci</td>
<td>≤8 (2)</td>
<td>NT</td>
</tr>
<tr>
<td>Clostridum sp (other than C difficile)</td>
<td>≥16 (12)</td>
<td>≤16 (17)</td>
</tr>
<tr>
<td>Clostridum sp (other than C difficile)</td>
<td>≤8 (5)</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT: Not tested.

Table 2. Isolation of C difficile and total growth values obtained from 832 faecal specimens

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total C difficile isolations</th>
<th>C difficile</th>
<th>Colonies other than C difficile</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDMN</td>
<td>146</td>
<td>462</td>
<td>2112</td>
</tr>
<tr>
<td>CCFA</td>
<td>122</td>
<td>408</td>
<td>3130</td>
</tr>
</tbody>
</table>

The number of C difficile isolations obtained from 832 faecal specimens, when cultured directly on to both CDMN and CCFA media, together with the total growth values, are shown in table 2. The CDMN agar gave a 20% higher isolation rate of C difficile than the commercially available CCFA, with the CDMN agar recovering 146 isolates and CCFA recovering 122. The CDMN agar was also more selective than the commercial CCFA in that it reduced the total growth value of contaminating colonies from 3130 to 2112 (a 33% reduction), whilst increasing the total growth value of C difficile isolates from 408 to 462 (13% increase).

Discussion

Since George and coworkers developed their selective medium in 1979 using cycloserine and cefoxitin (CCFA) no suitable alternative selective agents for the isolation of C difficile from faeces have been commercially available. Using the cycloserine and cefoxitin supplement at the recommended concentration (500 mg/l and 16 mg/l, respectively) reports have suggested that it may inhibit some strains of C difficile. To overcome this the supplement has been used at half the concentration but as this reduces the selectivity of CCFA, a form of pretreatment such as alcohol shock has been recommended. Though reducing the number of contaminants, this also reduces the number of vegetative C difficile organisms, and moreover some laboratories do not wish to add an
additional stage to their technique. None the less if faeces are cultured directly on to CCFA the selectivity is relatively poor, often allowing a heavy growth of other faecal isolates. Due to the complex nature of faecal bacterial flora and the necessity to select C difficile from other Clostridium sp present, the choice of which antibiotics to use as potential selective agents is a difficult one.

The MICs of numerous antibiotics were determined against a broad range of faecal isolates and strains of C difficile (unpublished data). From these results, norfloxacin and moxalacatam were chosen as potential agents. Norfloxacin inhibited all the strains of enterobacteriaceae and faecal streptococci tested at a concentration of 16 mg/l while maintaining the growth of all C difficile isolates. The norfloxacin was not effective at this concentration against either Bacteroides sp or strains of Clostridium sp (other than C difficile). For this reason, moxalacatam was combined with the norfloxacin at a concentration of 32 mg/l as this inhibited the growth of all strains of Clostridium sp (other than C difficile) and 11 of 15 strains of Bacteroides sp, without affecting the growth of any of the 64 strains of C difficile tested.

To ensure that neither antibiotic, either alone or in combination, affected the colonial morphology of C difficile, 64 strains were studied. Six isolates were slightly affected at 16 mg/l of norfloxacin, yet remained unaffected at 12 mg/l. The colonial morphology then remained similar to that on control plates (no antibiotics) when 12 mg/l of norfloxacin was combined with 32 mg/l of moxalactam (CDMN) agar. Cysteine hydrochloride (0·5 g/l) was also included in the medium as this was not only shown to increase greatly the growth rate of C difficile in brain heart infusion broth (Unipath Ltd), but also significantly to improve the Chatreux green fluorecence of the colonies under long wave ultraviolet (366 nm) light (unpublished data).

The CDMN medium was 20% more effective in its isolation rate than in CCFA. The possibility that the 24 additional isolates obtained using the CDMN medium were sensitive to the antibiotics present in the CCFA was investigated by subculturing all 24 strains on to CCFA. All strains grew after 48 hours of anaerobic incubation. One explanation for this is that on primary isolation, the antibiotics present in CCFA were too inhibitory for potentially stressed organisms. On subculture, these were then able to tolerate the antibiotics. The more likely explanation is that the large (33%) reduction in the growth of contaminating organisms on the CDMN medium facilitated recognition of lower numbers of C difficile colonies within the faecal culture. This view was supported by the increase in total growth values for isolates of C difficile from 408 to 462 on CDMN medium.

The study therefore concludes that C difficile base with 0·5 g/l of cysteine hydrochloride, 7% whole horse blood, 12 mg/l of norfloxacin and 32 mg/l of moxalactam is a more sensitive medium supplement for direct culture and isolation of C difficile from faeces than the present commercially available cycloserine cefoxitin fructose agar.

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doi: 10.1136/jcp.45.9.812

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