

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 colitis¹ and is frequently linked with many incidents of antibiotic associated diarrhoea.²⁻⁴ 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,⁵ or the subsequent modification containing half the concentration of antibiotics.⁶⁻⁸ 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.⁹ The medium also originally 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 *C 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 *Yersinia 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

Preston Public Health Laboratory, Royal Preston Hospital, PO Box 202, Sharoe Green Lane North, Preston PR2 4HG
S T Aspinall
D N Hutchinson

Correspondence to:
S T Aspinall

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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 10^6 – 10^7 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 (Mercia Diagnostics Ltd, Guildford, England) with all positive isolates being confirmed using API ZYM (API bioMérieux Ltd, Basingstoke, England).^{10 11}

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

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

Medium	Total <i>C. difficile</i> isolations	Growth value	
		<i>C. difficile</i>	Colonies other than <i>C. difficile</i>
CDMN	146	462	2112
CCFA	122	408	3130

of moxalactam against 64 strains of *C. difficile* and 36 other anaerobic organisms. All isolates of *C. difficile* were resistant to 16 mg/l of norfloxacin; this concentration inhibited all strains of faecal streptococci and enterobacteriaceae tested. All *Bacteroides* sp, 50% of anaerobic streptococci, and most strains of *Clostridium* sp (other than *C. difficile*) tested were resistant to 16 mg/l of norfloxacin. The MICs of moxalactam to all 64 isolates of *C. difficile* were ≥ 128 mg/l; 11 of 15 strains of *Bacteroides* sp tested, the four strains of anaerobic streptococci, and all 17 strains of *Clostridium* sp (other than *C. difficile*) had MICs of ≤ 16 mg/l.

COLONIAL MORPHOLOGY

The colonial morphology of the 64 strains of *C. difficile* was identical on the two media, except that beginning with medium containing 16 mg/l of norfloxacin, an increasing proportion of strains showed a slight reduction in the diameter of the colonies.

STUDY OF FAECAL SPECIMENS

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.⁸ 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

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

Isolate	MIC	
	Norfloxacin (No of strains)	Moxalactam (No of strains)
<i>C. difficile</i>	≥ 64 (63)	≥ 128
<i>C. difficile</i>	32 (1)	
Faecal streptococci	≤ 8 (14)	NT
Enterobacteriaceae	≤ 4 (33)	NT
<i>Bacteroides</i> sp	≥ 32 (15)	≤ 16 (11)
<i>Bacteroides</i> sp		≥ 32 (4)
Anaerobic streptococci	≤ 8 (2)	≤ 4 (4)
Anaerobic streptococci	≥ 16 (2)	
<i>Clostridium</i> sp (other than <i>C. difficile</i>)	≥ 16 (12)	≤ 16 (17)
<i>Clostridium</i> sp (other than <i>C. difficile</i>)	≤ 8 (5)	

NT: Not tested.

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 moxalactam 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, moxalactam 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 Chatreuse green fluorescence 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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