

Simplified procedure for the routine isolation of *Clostridium difficile* from faeces

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SUMMARY The use of alcohol, at a final concentration of 50%, as a selective procedure for the isolation of *Clostridium difficile* was compared to a selective medium containing 250 µg/ml of cycloserine and 10 µg/ml of cefoxitin. Of 266 faecal samples 82 were shown to be positive by one or other method. Seventy-seven (94%) of these were detected by the selective agar (SA) and 72 (88%) by the alcohol procedure (AP). Ten samples (12%) were positive only by SA and five samples (6%) by AP only. The AP was further modified so that all manipulations prior to incubation were performed on the open bench. Of 18 positive samples, 18 (100%) were detected by SA and 16 (89%) by AP.

There has been an increasing body of evidence generated during the last few years which firmly implicates *Clostridium difficile* as the aetiological agent of both antibiotic and non-antibiotic associated pseudomembranous colitis (PMC).¹⁻⁴ There is also recent evidence to implicate this micro-organism in diarrhoea unrelated to PMC,⁴ and as a possible factor in some exacerbations of inflammatory bowel disease.^{5,6} As a result of these findings, an increasing number of laboratories are receiving requests to analyse stools for the presence of *C difficile*. These investigative procedures were greatly facilitated by the development of a sensitive selective agar incorporating cycloserine and cefoxitin as selective agents.^{7,8} The purpose of this study was to evaluate a simplified procedure for the isolation of *C difficile* from stools that was independent of a selective agar, and to further modify this procedure so that it was also independent of sophisticated anaerobic facilities.

This study was divided into two parts. The first part was concerned with the evaluation of the use of alcohol to select for clostridial spores as a selective procedure (alcohol shock), compared to a selective antibiotic-containing medium, for the recovery of *C difficile* from faeces. The second part of the study evaluated a modification of this procedure that employed the minimum of anaerobe culture facilities (bench procedure).

Material and methods

MATERIAL

Source of specimens

The 266 specimens used in the study were forwarded to this laboratory from a number of hospitals throughout the United Kingdom to be investigated for the presence of *C difficile* or its associated cytotoxin, or both. All samples were processed immediately on receipt.

Brain-heart infusion agar (BHIA)

All constituents are expressed as grams or millilitres per litre of distilled water. Difco brain heart infusion 37 g; Difco yeast extract 5 g; haemin solution 1 ml; vitamin K₁ solution 1 ml; L-cysteine HCL 0.5 g; sodium formaldehyde sulphoxylate 0.3 g; New Zealand agar 20 g; defibrinated horse blood 100 ml; and Tween 80 1 ml.

Cycloserine-cefoxitin agar (CCA)

Cycloserine (Sigma) and cefoxitin (Merck Sharp and Dohme) at concentrations of 250 µg and 10 µg per ml respectively were added as selective agents⁸ to the BHIA.

Haemin solution

One gram of haemin was dissolved in 5 ml of 1 N NaOH and then made up to 100 ml with distilled water.

Vitamin K₁ solution

One gram of Vitamin K₁ was dissolved in 99 ml of absolute ethanol.

ISOLATION PROCEDURES

Three different methods were employed to isolate *C. difficile*. Samples of stool were diluted in glycerol transport broth⁹ to yield a 1/10 dilution. This dilution was passed into an anaerobic chamber. Serial tenfold dilutions over four steps were prepared in the chamber with prereduced brain-heart infusion broth (BHIB).¹⁰

(i) *Selective agar* A sample of 0.1 ml of the serial dilutions was plated on to CCA medium which had been stored at room temperature under an anaerobic environment for at least two days before use.

(ii) *Alcohol shock* Of the first stool dilution, 0.5 ml was added to an equal volume of absolute ethanol which had been sterilised by tyndallisation¹¹ and mixed on a vortex mixer. After being allowed to stand at room temperature for 1 h serial tenfold dilutions over four steps were prepared in pre-reduced BHIB, and samples of 0.1 ml of the serial dilutions were plated on to BHIA medium which had been stored at room temperature under an anaerobic environment for at least two days before use. All procedures were performed in the anaerobic cabinet.

(iii) *Bench alcohol shock procedure* This procedure was as described for the alcohol shock procedure described above with the exceptions that all procedures were performed on the open bench and none of the reagents used were prereduced. Samples of 0.1 ml of the serial dilutions were plated on to routine aerobic 5% blood Columbia agar plates, and no attempt was made to prereduce the medium in any way.

INCUBATION PROCEDURES

Both CCA and BHIA plates were incubated at 37°C under an atmosphere of 10% CO₂, 10% H₂ and 80% N₂ for at least 48 h, in an anaerobic incubator housed in the anaerobic cabinet. Inoculated blood plates from the bench alcohol shock procedure were placed in a conventional anaerobe jar. Anaerobiosis was achieved by the use of a "gas generating kit" system (Oxoid). The anaerobe jar was placed in an incubator at 37°C, and the plates incubated for at least 48 h.

IDENTIFICATION CRITERIA

After incubation all plates were examined under long wave (360 nm) ultraviolet light (UV L-25 Mineralight Lamp. Ultra-violet products Ltd, Cambridge), for the low green/chartreuse fluorescence characteristic of *C. difficile* colonies.⁷ A count of all presumptive *C. difficile* colonies was made and at least three of these colonies when present from each of the plates

were subcultured into Robertsons Cooked Meat (Southern Group Laboratories). After three days incubation the cultures were checked for purity and identified by use of the volatile fatty acid profile of carbohydrate metabolism generated by gas-liquid chromatography and biochemical reaction patterns.¹² All isolates were screened for their ability to produce cytotoxin.

CYTOTOXIN ASSAY

Isolates were analysed for the ability to produce a cytotoxin that was specifically neutralised by *C. sordellii* antitoxin (Wellcome Research Laboratories) by the "conventional tube method" described in a previous publication.¹³ All stool samples were also analysed for the presence of this toxin.

Results

Comparison of CCA selective agar and alcohol shock procedure

The results are presented in the Table. Of 266 faecal samples analysed, 82 were shown to have *C. difficile* present by a combination of the two methods described. Of these 82 positive specimens, 77 (94%) were detected by CCA and 72 (88%) by the alcohol shock procedure. Ten samples (12%) yielded *C. difficile* on CCA only, and five specimens (6%) only by the alcohol shock procedure.

Comparison of methods for recovery of C. Difficile from stools

Method	No tested	Total no of <i>C. Difficile</i> positive stools*	No of stools yielding <i>C. Difficile</i> (%)
CCA selective agar	266	82	77 94†
Alcohol shock	266	82	72 88
Bench alcohol shock	55	18	16 89

*No of stools shown to harbour *C. difficile* by a combination of all the methods described.

†Expressed as a percentage of total *C. difficile* positive stools.

CCA positive alcohol shock negative

Seven of the 10 faecal samples that yielded *C. difficile* only on CCA had no demonstrable cytotoxin present, and one of these yielded only non cytotoxic *C. difficile* strains. In addition, one of these seven samples came from a patient from whom previous samples were positive for stool cytotoxin and for *C. difficile* by both methods employed. Of the remaining three samples from this set two were stool cytotoxin-positive and one had a cytotoxin that was not neutralised by the cross-reacting *C. sordellii* antitoxin. One of these cytotoxin-positive stools

came from a patient from whom previous samples yielded *C difficile* by both methods employed.

In two cases the numbers of *C difficile* present as determined by CCA (100 organisms per gram of stool) were below the limit of detection for the alcohol shock procedure which is 200 organisms per gram due to the additional 1 in 2 dilution that this procedure requires.

Alcohol shock procedure positive, CCA negative

Two of the five specimens in this category were negative for stool cytotoxin. However, the other three cases were stool cytotoxin-positive.

QUANTITATIVE RECOVERY

In all, 44 specimens were analysed for total counts of *C difficile*. The CCA selective agar procedure yielded higher counts of *C difficile* than the alcohol shock procedure in 31 cases (70%), but lower counts in 11 cases (25%). In two instances, these two procedures yielded the same number of organisms (5%). The results demonstrate that more organisms can be recovered by the CCA selective agar method than by the alcohol shock procedure. Analysis of the data by a two-tailed *t* test showed that this difference was highly significant ($p < 0.001$) with a *t* value for 43 degrees of freedom of 3.477.

COMPARISON OF CCA SELECTIVE AGAR AND BENCH ALCOHOL SHOCK PROCEDURE

The results are presented in the Table. Of 55 faecal samples analysed, 18 were shown to have *C difficile* present by a combination of the two methods described. Of these 18 positive specimens, 18 (100%) were detected by CCA and 16 (89%) by the alcohol shock procedure. Two samples (11%) yielded *C difficile* on CCA only. In both of these cases the numbers of *C difficile* detected were small with values of $10^{3.4}$ and $10^{2.0}$.

CONCORDANCE OF STOOL CYTOTOXIN AND *C DIFFICILE* ISOLATION

Cytotoxin that was neutralised by *C sordellii* antitoxin was detected in 61 of the 266 samples analysed. Sixty of these 61 cytotoxin-positive stools (98%) were shown to have *C difficile* present by a combination of the methods being evaluated. Of these, two samples yielded *C difficile* only by the CCA selective agar and three samples only by the alcohol shock procedure. All of the cytotoxin-positive stools of the 55 used to evaluate the bench alcohol shock procedure yielded *C difficile* by both the alcohol and selective agar methods employed. Overall, the CCA selective agar detected *C difficile* in 57 of the 61 cytotoxin-positive stools (93%) and the alcohol shock procedure in 58 of these stools (95%).

Discussion

It is now widely accepted that *C difficile* is the aetiological agent of antibiotic-associated pseudo-membranous colitis¹⁻⁴ and also of pseudo-membranous colitis unrelated to known chemotherapy.^{4,14} Information is now also available to implicate the organism and its toxin in antibiotic-associated diarrhoea.^{4,15} There have also been two recent reports implicating this organism in exacerbation of inflammatory bowel disease;^{5,6} however, these latter associations have not been noted in other studies.^{1,15} This increasing awareness of the potential pathogenicity of *C difficile*, especially in the compromised host, has led to a rapid increase in the numbers of laboratories receiving requests to screen for this organism. Although there is a good correlation between the presence of *C difficile* and stool cytotoxin⁴ this assay requires tissue cultures which are not readily available to many clinical laboratories. The development of a sensitive selective agar^{7,8} has greatly facilitated the isolation of *C difficile*. However, not all microbiology laboratories have sophisticated anaerobic, or media preparation facilities. The data presented here indicate that the use of alcohol as a selective procedure is an efficient method for the detection and isolation of *C difficile* from stools and in particular those containing cytotoxin. In addition, the results indicate that this method can be successfully performed on the bench with the minimum of anaerobic microbiology equipment.

The ability of *C difficile* to fluoresce under long wave UV light noted by George *et al.* on their cycloserine-cefoxitin fructose egg yolk agar is also apparent on a blood base agar.⁷ This characteristic can therefore be utilised to aid in the detection and presumptive identification of this organism when isolated by the alcohol shock procedures, and is especially useful to those who are unfamiliar with the colonial characteristics of *C difficile*. We have not yet isolated any other species of clostridium that exhibits this characteristic. An advantage of the alcohol shock procedure is that there is a normal cellular morphology to the organisms present. The cellular morphology of *C difficile* can be characteristic, with apparent bipolar sporing noted on Gram-stain of 3-day-old cultures, giving a "dumb-bell" appearance, an additional feature which can aid in the detection and presumptive identification of this organism. This contrasts to cells present on media containing cycloserine and cefoxitin as selective agents, where spores are not present. An additional advantage of the alcohol shock procedure is that many other clostridial species are concomitantly isolated. This sort of screening procedure

should therefore increase the likelihood of noting associations between carriage of *C. difficile* and other clostridial species that may enhance its pathogenic effect. This sort of enhancement has been noted for *C. tertium* and *C. perfringens* in *C. difficile*-mediated diarrhoea in axenic neonatal hares.¹⁶ The recent finding by Schwartz *et al.*¹⁷ implicating toxigenic *C. perfringens* type C in an ampicillin-induced enterocolitis also highlights the value of an isolation procedure that selects for other clostridial species in addition to *C. difficile*.

In conclusion, we believe that the alcohol shock procedures described are a valid method for the isolation and detection of *C. difficile* from stools and if adopted will greatly increase the number of laboratories that will be able to screen for this pathogen.

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