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Comparison of alcohol shock enrichment and selective enrichment for the isolation of *Clostridium difficile*

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SUMMARY

Two enrichment methods were compared for their ability to recover *Clostridium difficile* from stool samples. One method used selective enrichment in an antibiotic-containing broth followed by detection with a latex particle agglutination (LPA) reagent. The other used enrichment in a non-selective broth following treatment of the specimen with alcohol. With clinical specimens enrichment culture was significantly more successful at detecting *C. difficile* than direct plating. Alcohol shock enrichment was twice as effective as direct culture, while selective broth enrichment was three times more effective. The use of LPA for screening selective enrichment broths for *C. difficile* should prove a cost-effective measure as only positive broths (about 20%) require subculture for confirmation.

INTRODUCTION

Clostridium difficile is recognized as the causative agent of pseudomembranous colitis and many cases of antibiotic-associated diarrhoea (Bartlett, 1979). Previously, the detection of faecal cytotoxin (toxin B) and the isolation of *C. difficile* was regarded as the essential minima for the microbiological diagnosis of these syndromes, although the finding of faecal cytotoxin is not always consistent (Riley, Bowman & Carroll, 1983). In addition, it was shown recently that *C. difficile* enterotoxin (toxin A) was primarily responsible for the diarrhoea associated with *C. difficile* infection (Stephen, 1986). A commercially available latex reagent for toxin A does not detect this toxin (Lyerley & Wilkins, 1986) and enzyme-linked immunosorbent assay for toxin A (Laughon *et al.* 1984) is not routinely performed in diagnostic microbiology laboratories. Moreover, the production of both toxins A and B depends on the composition of the growth medium *in vitro* and, presumably, on the nutritional status of the gut *in vivo* (Haslam *et al.* 1986). In view of these considerations it seems likely that the isolation of *C. difficile* may be of more value than toxin detection in the diagnosis

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of disease associated with *C. difficile*, and that enrichment media should consequently be used to maximize isolation rates (Carroll, Bowman & Riley, 1983; Levett, 1984).

A variety of enrichment broths have been used to detect *C. difficile* (Carroll, Bowman & Riley, 1983; Buchanan, 1984), although not all workers agree about their value (O'Farrell *et al.* 1984). The assessment of enrichment techniques is difficult because most are compared against direct culture. The purpose of our investigation was to compare the method of Levett (1984), using alcohol shock followed by enrichment in cooked meat medium, with that of Carroll, Bowman & Riley (1983), using selective enrichment in a broth containing antibiotics.

MATERIALS AND METHODS

Samples

Stool samples were obtained from two sources; stools sent to the PHLS Anaerobe Reference Unit with a request to culture *C. difficile*: and diarrhoeal stools sent for routine microbiological examination to the Luton Public Health Laboratory from hospital and general practice patients. Specimens were usually cultured within 24 h of collection; if this was not possible they were stored at −70 °C until processed. With some referred specimens longer delays sometimes occurred during transit to the laboratory but it has been shown that *C. difficile* remains viable in stool samples for up to 4 days (Bowman & Riley, 1986).

Cultural procedures

The isolation method of Levett (1984) used ethanol treatment of faeces to select for clostridial spores followed by enrichment in cooked meat medium. The selective enrichment method of Carroll, Bowman & Riley (1983) was modified by the omission of gentamicin and used brain heart infusion broth containing cycloserine (250 mg/l) and cefoxitin (8 mg/l). For the presumptive detection of *C. difficile* in the selective broth a latex particle agglutination (LPA) test, manufactured by Disposable Products Pty Ltd., South Australia, and marketed in the United Kingdom by Mercia Diagnostics, Guildford, Surrey, was employed (Bowman, Arrow & Riley, 1986). All specimens were processed by both methods, and for comparison were also cultured directly on CCFA (George *et al.* 1979) containing 8 mg/l cefoxitin and 250 mg/l cycloserine. Faecal cytotoxin detection was not performed unless specifically requested.

RESULTS

A total of 218 stool samples was examined for *C. difficile* by the three methods and Table 1 summarizes the findings. Twelve specimens were positive by selective enrichment but negative by alcohol shock enrichment, while only four specimens were positive by alcohol shock enrichment but negative by selective enrichment. There were 14 apparent false-positive results by LPA, 3 of which were due to cross-reactions with *C. sordellii* and *C. bifermentans*. In a further three samples for which LPA was positive but *C. difficile* was not isolated from the selective broth, the organism was recovered by the alcohol shock method. Both enrichment

Table 1. *Comparison of direct and enrichment culture for the isolation of* C. difficile

	Positive	(%)	Negative
Direct culture	12	5·5	206
Alcohol shock enrichment	21	9·6	197
Selective enrichment	31	14·2	187

Table 2. *Percentage sensitivity, specificity and predictability of positive or negative results*

	DC-SE*	AS-SE†	DC−AS‡
Sensitivity	62	75·6	70
Specificity	100	100	100
Predictability			
Positive	100	100	100
Negative	90·8	94·9	95·6

* Direct culture compared with selective broth enrichment.
† Alcohol shock enrichment compared with selective broth enrichment.
‡ Direct culture compared with alcohol shock enrichment.

following alcohol shock and selective broth enrichment were significantly better than direct culture for the recovery of *C. difficile* ($\chi^2 = 2{\cdot}06$, $P < 0{\cdot}25$ and $\chi^2 = 9{\cdot}3$, $P < 0{\cdot}005$, respectively), while selective enrichment was slightly better than alcohol shock enrichment ($\chi^2 = 1{\cdot}08$, $P < 0{\cdot}5$). Table 2 shows the sensitivity, specificity and predictability of the three methods.

DISCUSSION

This study has confirmed the value of enrichment for the isolation of *C. difficile*. With both methods there was a statistically significant improvement over direct culture, which may be important in both clinical and epidemiological studies. *C. difficile* may persist in stool samples for some time after cytotoxin has disappeared (Teasley, Olson & Gebhard, 1983) and in other cases the organism may be present in low numbers or only in the form of spores, particularly after treatment (Onderdonk, Cisneros & Bartlett, 1980). Enrichment media may greatly facilitate its recovery in studies of the carriage and environmental distribution of *C. difficile* where similarly low numbers of organisms may be present in specimens (Levett, 1984).

There are conflicting reports on the value of enrichment culture for the isolation of small numbers of *C. difficile*. One reason for this has been the use of inappropriate media at other stages of the isolation procedure; for example, many studies have used the original concentrations of cycloserine and cefoxitin in CCFA (500 mg/l and 16 mg/l respectively) although it has been clearly demonstrated that by using half these concentrations the isolation rate for *C. difficile* may be increased by 30–50% (Levett, 1984; Bowman, 1985). Other workers (Holst, Helin & Mardh, 1981) subcultured their enrichment broths on to non-selective blood agar, which would permit the growth of a number of inhibitory faecal bacteria

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(Rolfe, Helebian & Finegold, 1981); unless alcohol treatment is used enrichment broths should be subcultured on to CCFA.

When a selective enrichment broth containing antibiotics is used it is possible to introduce too much inoculum; media containing cefoxitin are particularly susceptible to this 'inoculum effect'. Early studies by Bowman (1985) on a selective enrichment broth showed that an inoculum of one drop (0·02 ml) of fluid stool, or two drops of a 50% suspension, was sufficient; if a larger inoculum was used recovery of *C. difficile* was markedly reduced.

In the present study enrichment in an antibiotic-containing medium was superior to enrichment following alcohol treatment. This was a not unexpected finding as alcohol treatment reduces the number of all vegetative cells, those of both *C. difficile* and of contaminating bacteria, thus resulting in a smaller inoculum. The ratio of vegetative cells to spores is of some importance in determining the success or otherwise of the enrichment method. Thus in situations where mainly spores are present, such as following treatment, alcohol shock enrichment should be as successful as selective enrichment. On a number of occasions selective enrichment was positive but alcohol shock enrichment was negative; the reverse occurred less frequently. Although some of these were no doubt due to sampling errors, as the distribution of bacteria in faecal samples is not uniform, it may be concluded that selective enrichment with antibiotics is more effective than enrichment following alcohol shock for the recovery of *C. difficile*.

The commercially available LPA reagent has been useful for the rapid identification of presumptive colonies of *C. difficile* on CCFA, while the reliability of negative results given by LPA on selective broth cultures increases its value as a screening test for detection of *C. difficile* (Bowman, Arrow & Riley, 1986); these findings were confirmed in the present investigation. As previously, there was a number of false-positive results with LPA, some of which were attributed to cross-reactions with either *C. sordellii* or *C. bifermentans*. It is thus necessary to subculture LPA positive selective broth cultures to confirm the presence of *C. difficile*. On three occasions LPA was positive, subculture was negative, but alcohol shock enrichment detected *C. difficile*. These were regarded as false-positive results although, clearly, *C. difficile* was present in the specimen. This may reflect enhanced sensitivity of the LPA reagent in detecting low numbers of *C. difficile* present in the selective broth but not cultivatable. On several occasions some organisms were cultured, notably lactobacilli, which inhibited the growth of *C. difficile* on CCFA. Although *C. difficile* could still be detected in areas of light growth, there remains the possibility that low numbers could have been completely suppressed. The use of LPA to screen selective broths, however, is still a cost-effective measure as approximately 80% of broth cultures do not require subculture. The LPA reagent is inexpensive, and the test simple to perform, thus making it appropriate for use in small laboratories.

Because *C. difficile* may be the most important infectious cause of diarrhoea in hospital patients (Riley, Bowman & Carroll, 1983) all hospital laboratories should be able to detect the organism. Our efforts have been directed towards improving and simplifying techniques for the isolation of *C. difficile*. Enrichment cultures increase isolation rates and the methodology is within the scope of most

laboratories. Although the isolation of *C. difficile*, either on direct culture or using enrichment media, does not constitute a diagnosis of pseudomembranous colitis or antibiotic-associated diarrhoea (Bowman, Arrow & Riley, 1986), the ability of any isolate to produce cytotoxin (and, by implication enterotoxin) may be tested *in vitro* and its pathogenic potential assessed with consideration of the patient's clinical condition.

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