Malaysian J Pathol 1991; 13(1):29–31

DETECTION OF *CLOSTRIDIUM DIFFICILE* FROM AN ENHANCEMENT BROTH BY GAS-LIQUID CHROMATOGRAPHY

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Summary

Gas-Liquid chromatography (GLC) was used to detect the presence of isocaproic acid produced by *Clostridium difficile* from 54 stool samples grown in cycloserine-cefoxitin brotli. Isocaproic acid was detected in 12 samples of which 5 were confirmed to be *Clostridium difficile* by culture and biochemical tests. The detection of isocaproic acid by GLC together with the presence of presumptive colonies on primary selective culture media provides a more rapid laboratory diagnosis for *Clostridium difficile.*

INTRODUCTION

The role of *Clostridium difficile* in pseudomembranous colitis and antibiotic-associated diarrhoea had been well established. Epidemiological studies of diarrlioea caused by *C. difficile* have shown that it is associated with tlie use of antibiotics,[1] increases in frequency in the elderly[2] and is potentially infectious as shown by several nosocomial outbreaks.[3,4,5]

The isolation of *C. difficile* from stool has been greatly improved with tlie development of new and better selective media and procedures. These media however are not absolutely selective thus allowing the growth of other enteric organisms. Therefore, presumptive isolates of *C. difficile* on these media need definitive identification which may take as long as 5 days. This involves getting pure isolate for biochemical tests and GLC detection of volatile fatty acids. A more rapid and sensitive culture based method is necessary for the detection of tlie organism in stool samples.

To overcome the delay faced by the laboratory to provide a definitive identification within 48 hours, we used GLC to detect isocaproic acid produced by C. *difficile* from stool inoculated in an encliancement brotli. This is to complement the presence of presumptive colonies on primary selective culture plates.

MATERIALS AND METHODS

Specimen

Fifty-four stool samples received from adult patients in University Hospital, Kuala Lumpur were processed for *C. difficile.* All the stools examined were from patients with unspecified diarrhoea.

Media

Taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA) was prepared which contained the following. Columbia Agar Base (BBL 1124) 38 g/l, fructose 6 g/l, neutral red (2%) 1.4 ml/l, egg yolk emulsion 36 ml/l, sodium taurocholate 0.1% (w/v), cycloserine and cefoxitin (Oxoid SR96) 250 mg/l and 8 mg/l respectively.

The encliancement brotli, cycloserine-cefoxitin brotli (CCB), was prepared containing cycloserine 250 mg/l, cefoxitin 8 mg/l and supplemented brain heart infusion base 37 g/l.

Culture

The stools were directly inoculated onto TCCFA and CCB. All plates were incubated anaerobically in Gaspak jars at 37°C and examined for presumptive *C. difficile* colonies after 48 hours. Tlie CCB were incubated at 37°C aerobically for 48 hours before detection of isocaproic acid by GLC. All isocaproic acid positive CCB were subcultured on TCCFA for identification of isolates. A known *C. difficile* (obtained from the Anaerobic Reference Centre in Luton and Dunstable Hospital, England) was used as control throughout the study.

Gas-liquid chromatography

An ether extract for volatile fatty acids was made from the 48 hour CCB. A 1 ml aliquot of the incubated CCB was pipetted out and added to 0.2 ml of 50% H_2SO_4. 1 ml of diethyl ether was added and the mixture was whirlmixed for 15 seconds and then centrifuged. 1 ul sample of the extract from tlie ether layer was injected into the chromatograph.

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The chromatograph used was a PYE Unicam 204 with a flame ionizing detector. The column used was packed with 10% free fatty acid phase (FFAP) on diatomite CLQ, 100/120 mesh (JJ's Chromatography Ltd.). The temperature of tlie column was kept at 200°C and a carrier gas flow rate of 50 ml/min was maintained.

Identification

All colonies with morphology typical of *C. difficile* were subcultured onto blood agar plates and identified using tlie criteria of Holdeman et at[6] and the method of Phillips.[7] Typical colonies of *C. difficile* on TCCFA were yellow with irregular and undefined margins.

Cytotoxin assays

Faecal cytotoxins were detected using monolayer Vero cells. Neutralization test using equal volumes of *Clostridium sordellii* antitoxin were performed concurrently.

RESULTS

Isocaproic acid was detected by GLC in 12 of tlie 54 stool samples inoculated into the CCB. Of these, five were confirmed by tlie criteria of Holdeman[6] as C. *difficile*. The 7 false positive CCB did not grow any *C. difficile* upon subculture. 1 of the 7 false positive CCB was subsequently identified as *Clostridium sporogenes*. No other anaerobe was isolated in the 6 remaining false positive CCB. There wast no faise negative among the 54 stool samples. The 42 samples which were negative for isocaproic acid were all negative for C. *difficile* subculture of tlie CCB. Cytotoxin was not detected in any of the 54 samples.[8]

DISCUSSION

Many authors had advocated tlie use of GLC to detect *C. difficile* from stool samples. Levett et al[8] used GLC to detect tlie presence of caproic acid and p-cresol produced by *C. difficile* grown on modified CCFA. Potvliege et al[9] detected isocaproic acid produced by the organism and reported a sensitivity of 61% and a specificity of 95%. Johnson et al[10] obtained high sensitivity (99 6%) and specificity (99.0%) in their study using GLC to detect four fatty acids produced by *C. difficile* grown in a culture enhanced media.

In this study, we detected isocaproic acid in 12 stool samples grown in the CCB. Of tlie 12 positive CCB, 5 were confirmed to be C. *difficile* and the remaining 7 were false positive CCB. Except for 1 sample, in which *C. sporogenes* was isolated, the remaining samples failed to grow anaerobes upon subculture. Other anaerobes which are know to produce isocaproic acid include *C. bifermentans*, C. *sporogenes* and *C. sordellii*.[8] Tlie positive stool for *C. difficile* had high peaks of isocaproic acid and tlie stools which did not grow C. *difficile* but showed presence of isocaproic acid on GLC had low isocaproic acid peaks. Tlie low specificity in this study could be due to the fact that we only detected one fatty acid instead of the several others produced by C. *difficile* and also because of the small number of specimens sampled. However, further studies carried out on stools of patients with suspected pseudomembranous colitis or antibiotic associated diarrhoea have produced encouraging results. This screening method does seem suitable for such patients as the GLC results correlate well with tlie cultures.

From this study, we maintain that reliance on tlie detection of isocaproic acid using GLC as the sole criterion for the identification of *C. difficile* is unsatisfactory. Tlie GLC results have to be interpreted together with the presence of *C. difficile* colonies on the primary or tlie subculture plates. The 5 positive CCB had corresponding typical colonies on the primary plates and thus a provisional report of the presence of tlie organism can be made with confidence. The 7 false positive CCB however did not show any colonies which were morphologically typical of *C. difficile* on either the primary or tlie secondary plates.

The use of selective enhancement broth is also advocated as this would increase the frequency of isolation of *C, difficile* from the milieu of other enteric organisms. This was shown in a previous study.[11] In this present study we also found that almost 78% of the selective enhancement broth did not require subculture due to tlie absence of isocaproic acid. Therefore, GLC, where available, may be a suitable method to use for screening tlie selective enhancement broth. **Those enhancement broths with no isocaproic** acid need not be subcultured onto TCCFA and this will reduce the cost of making tlie TCCFA plates.

We chose to use TCCFA instead of the much used CCFA because the taurocholate incorporated into the media has been shown to enhance the germination of *C. difficile* spores and therefore would increase the chance of isolating the organism should they be found in small numbers in the stool.[12] The detection of cytotoxin alone for the presence of *C. difficile* is also unsatisfactory as this method would miss the cytotoxin negative but culture positive specimens.

Presently, we found that GLC detection of isocaproic acid from samples grown in enhancement broth together with the isolation of the organism on primary selective media to be of great value in providing clinicians with a more rapid report on the presence or absence of the organism in their patients' stools. GLC detection of isocaproic acid should not be taken as diagnostic of the presence of *C. difficile* as our study shows that the number of false positives can be high and thus relying on this method alone is not justified.

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