New rapid identification test for *Clostridium difficile*

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Abstract

**Aims:** A set of five tests were developed and tested for their ability to confirm the identity of *C difficile* colonies within 30 minutes.

**Methods:** The relevant substrates were incorporated into four filter paper squares attached to a plastic carrier (Diffstrip), five enzymes/products (prolyl aminopeptidase, galactosidase, leucine aminopeptidase, acid phosphatase and indole). The strips were inoculated, incubated for 20 minutes, and reagents added.

**Results:** 96-4% (212 of 220) strains of *C difficile* were immediately differentiated from 51 other *Clostridium* spp tested. The remaining 3-6% (eight of 220) *C difficile* isolates produced a reaction pattern similar to some of the *Clostridium sporogenes* tested and required additional tests. None of the other *Clostridium* spp tested produced reaction patterns similar to *C difficile*.

**Conclusion:** The Diffstrip allowed colonies of *C difficile* to be confirmed within 30 minutes for 96-4% of isolates, with less than 4% requiring any additional tests. No strains of *C difficile* were misidentified and no strains of other *Clostridium* spp tested were misidentified as *C difficile*.

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The pathogenic role of *Clostridium difficile* in the aetiology of intestinal disease has been well documented, causing almost all cases of pseudomembranous colitis (PMC) and about 25% of cases of antibiotic associated diarrhoea (AAD). Laboratory diagnosis of such diarrhoea predominantly consists of culture of the organism from faeces or detection of either toxin A by enzyme immunoassay or toxin B by tissue culture. Although toxin detection correlates best with the clinical infection, culture is still regarded as a highly sensitive method and is required for epidemiological purposes.

Presumptive identification can easily be made by both smell (horse-dung smell) and Chartreuse-green fluorescence under long wave (360 nm) ultraviolet light. Confirmation is then often made by one of three techniques. First, gas liquid chromatography (GLC) has been widely reported as the standard method of identification, but is relatively time consuming and requires both expensive equipment and expertise. A second method requires commercially obtained biochemical identification strips, such as Minitrek anaerobe II (Becton Dickinson, Oxford) or API 20A (API bioMérieux, Basingstoke) but these may take up to 72 hours to perform and may not be reproducible. A third and more recent method is the use of enzyme based identification strips, such as RapID ANA II (Mercia Diagnostics Ltd, Guildford), ATB 32A (API bioMérieux) API ZYM (API bioMérieux), or An-Ident (API bioMérieux) which offer reproducible results, but as *C difficile* is relatively unreactive in such systems, supplementary tests may be required and misidentification can occur. This paper describes the development of a rapid (less than 30 minutes), accurate, and highly reproducible method for confirmation of *C difficile* isolates.

**Methods**

**TEST STRIPS**

All test strips were supplied by Lab M. They consisted of a plastic carrier (65 mm × 12 mm) with attached squares of filter paper which were impregnated with dried test substrates. Numerous identification tests were investigated for their potential use (unpublished data), with five tests finally being chosen. These were incorporated on to the same strip (Diffstrip) which carried four filter paper squares (two tests are combined into one square). They detected the following enzymes (substrates indicated in parentheses):

1. Acid phosphatase (a naphthol phosphate (Lab M) and sodium nitrite buffered to pH 5-0 using phosphate/citrate buffer).
2. Indole (p-dimethylaminocinnamaldehyde (DMCA) (Sigma Chemical Co Ltd, Poole) and toluene sulphonic acid (Sigma Ltd)).
3. Leucine aminopeptidase (leucine-β-naphthylamide) (Lab M).
4. β-galactosidase and prolyl aminopeptidase (5 bromo, 4-chloro, 3-indolyl β-galactosidase and prolyl-β-naphthylamide) (Bachem Ltd, Saffron Walden).

**ORGANISMS TESTED**

Strains (n = 220) of *C difficile* (including NCTC 11204 and NCTC 11209), eight strains of *C sporogenes* (including NCTC 6929, NCTC 8594, NCTC 276 and NCTC 532), nine strains of *C sordellii* (including NCTC 8780), seven strains of *C bifidens* (including NCTC 6801, NCTC 6927, and NCTC...
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Isolate (No. of strains tested)  PA  BG  LA  I  AP
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Clostridium difficile (212/220) + - - - -
Clostridium difficile (9/220)  + - - - -
Clostridium sp (4/8)  + - - - +
Clostridium sporogenes (4/8)  + - - - +
Clostridium sordellii (9/9)  + - - - +
Clostridium bifurcans (3/7)  + - + - +
Clostridium bifurcans (2/7)  + - + - +
Clostridium bifurcans (1/7)  + - + - +
Clostridium bifurcans (1/7)  + - + - +
Clostridium septicum (3/9)  + - - - -
Clostridium septicum (2/5) - - - + -
Clostridium perfringens (8/8) - - - + -
Clostridium glycolicum (7/7)  + - + - +
Clostridium histolyticum (1/1)  + - + - +
Clostridium acetobutylicum (1/1)  + - + - +
Clostridium tetani (1/1)  + - + - +
Clostridium caerulescens (1/1) - - - - -
Clostridium tertium (1/1)  + - + - -
Clostridium beijerinckii (1/1) - - - - -
Clostridium butyricum (1/1) - - - - -

*PA = Prolyl aminopeptidase; BG = β-Galactosidase; LA = Leucine aminopeptidase; I = Indole production; AP = Acid phosphatase.

Results

The table shows the results obtained from the five Diffstrip tests (acid phosphatase, indole, leucine aminopeptidase, β-galactosidase and prolyl aminopeptidase) for the 271 strains of Clostridium spp investigated. All strains of C difficile produced identical test results with the exception of eight isolates (3-6%) which yielded negative leucine aminopeptidase reactions. All other isolates of C difficile produced positive prolyl aminopeptidase and leucine aminopeptidase tests, with negative β-galactosidase, indole, and acid phosphatase results.

All strains of C sordellii could easily be differentiated from C difficile by their positive acid phosphatase and indole reactions. All strains of C bifurcans yielded positive tests for indole, together with three of seven of the isolates also yielding a positive acid phosphatase reaction. Fifty per cent of the strains of C sporogenes could be differentiated from C difficile by their positive acid phosphatase reactions. The remaining 50% (four of eight) resulted in only a positive prolyl aminopeptidase reaction, which is similar to the 3-6% of leucine aminopeptidase negative C difficile strains and would therefore require a supplementary test. All other strains of Clostridium spp tested could also be differentiated from C difficile from the configuration of the positive reactions (table).

There was no difference in results obtained whether cultures were grown on blood agar, CCFA, or CDMN, after either 24 or 48 hours of anaerobic incubation.

Discussion

The new generation of anaerobic identification systems are now predominantly chromogenic enzyme detection tests. These offer results after four hours of incubation, are highly reproducible, and require no additional facilities, such as gas liquid chromatographs. Unfortunately, due to the unreactive nature of many Clostridium spp in terms of the substrates available within these commercial systems, identification may only be to genus level and misidentification can occur.

Investigation of patients with antibiotic associated diarrhoea often entails faecal culture and identification of suspect colonies as C difficile. The identification of Clostridium spp other than C difficile under these circumstances is unnecessary; therefore, a system which rapidly and accurately confirms C difficile from other clostridia would save time spent in fully identifying isolates. From the results of investigating numerous rapid tests for differentiation of various Clostridium spp (unpublished
data) five adequately identified *C. difficile* from other clostridia. Five tests were incorporated on to the Diffstrip, which required a maximum of 20 minutes’ incubation and therefore fulfills these criteria. The prolyl aminopeptidase test, although not helping to differentiate the *C. sporogenes/C. sordellii/C. bifermentans/C. glycolicum* group from *C. difficile*, not only acted as a positive control test for those clostridia which remained unreactive with all other tests, but aided separation of *C. difficile* from the other *Clostridium* spp tested.

The β-galactosidase test was similar in that all the aforementioned group of clostridia did not produce β-galactosidase, but the test allowed most other *Clostridium* sp tested to be differentiated rapidly (no reagents need to be added).

The indole test permitted differentiation of *C. sordellii* and *C. bifermentans* from *C. difficile*, and the incorporation of the leucine aminopeptidase test into the strip allowed 96-4% (212 of 220) of *C. difficile* isolates tested to be differentiated from all other *Clostridium* spp tested.

The acid phosphatase test separated *C. sordellii* and *C. glycolicum* from *C. difficile*, together with 50% of *C. sporogenes* isolates tested. The remaining 50% of *C. sporogenes* produced a similar reaction pattern to 3-6% of the *C. difficile* strains, therefore, on the rare occasions that such a reaction profile occurs, an additional test may be required for final differentiation, such as lipase production. *C. sporogenes* should rarely cause problems as its growth should be inhibited if faeces are cultured on selective media.20

As very little growth of suspect colonies is required to perform the test (unlike the commercially available enzyme based systems), identification can be carried out directly from isolated colonies on the primary culture plate, thereby further reducing the time needed for confirmation. The Diffstrips allowed rapid confirmation of 96-4% of isolates of *C. difficile* and clearly indicated those that required additional testing.

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