

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) of *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,¹⁻³ causing almost all cases of pseudomembranous colitis (PMC) and about 25% of cases of antibiotic associated diarrhoea (AAD).^{4,5} Laboratory diagnosis of such diarrhoea predominantly consists of culture of the organism from faeces or detection of either toxin A by enzyme immunoassay⁶ or toxin B by tissue culture.⁷ Although toxin detection correlates best with the clinical infection,⁸ culture is still regarded as a highly sensitive method⁹ 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.¹⁰ 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,^{11,12} but is relatively time consuming and requires both expensive equipment and expertise. A second method requires commercially obtained bio-

chemical identification strips, such as Minitex anaerobe II (Becton Dickinson, Oxford) or API 20A (API bioMérieux, Basingstoke),^{13,14} 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)¹⁵⁻¹⁷ 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 bifermentans* (including NCTC 6801, NCTC 6927, and NCTC

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506), five strains of *C. septicum*, eight strains of *C. perfringens*, seven strains of *C. glycolicum*, one strain of *C. histolyticum* (ATCC 4259), one strain of *C. tetani*, one strain of *C. cadaveris*, one strain of *C. tertium*, one strain of *C. beijerinckii* and one strain of *C. butyricum*. NCTC strains were derived from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, and ATCC 4259 was supplied by the American Type Culture Collection, Centers for Disease Control, Atlanta, USA.

TEST PROTOCOL

The test organisms were derived from national collections or from the culture collection at Preston Public Health Laboratory. They were identified using standard methods¹⁸ and grown on Columbia agar (Unipath Ltd, Basingstoke) with 7% v/v whole horse blood, cycloserine cefoxitin fructose agar (CCFA) (Unipath Ltd), and moxalactam/norfloxacin selective agar (CDMN),¹⁹ for either 24 or 48 hours at 37°C in an anaerobic cabinet. After incubation the acid phosphatase, leucine aminopeptidase, and β -galactosidase/prolyl aminopeptidase filter paper test squares were inoculated with a colony of the test organism using a bacteriological loop (sufficient to observe a visible inoculum on the strip). The indole test was supplied with a lighter but still visible inoculum. Each test square was then moistened with a loopful of sterile distilled water and the result of the indole test recorded after 30 seconds (a positive result taken as a blue-purple colour around the edge of the inoculum). The strip was then incubated aerobically at 37°C for 20 minutes in a humidified chamber after which time the result of the β -galactosidase test was recorded (presence of β -galactosidase results in the formation of an azure blue colour) before adding a drop of reagent A (10 mg/ml of sulphanic acid (Aldrich Chemical Co Ltd, Gillingham) in 5N acetic acid) to the acid phosphatase test, and a drop of reagent B (1 mg/ml of DMCA in 1N hydrochloric acid) to the β -galactosidase/prolyl aminopeptidase test and the leucine aminopeptidase test. The

results of these were then recorded after five minutes of incubation at room temperature. Phosphatase activity was detected by the formation of an orange colour while the presence of prolyl aminopeptidase activity was indicated by a red-pink colour and that of leucine aminopeptidase by a pink colour at the site of the inocula.

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. bifermentans* tested produced 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

Diffstrip results for 271 strains of *Clostridium* spp

Isolate (No of strains tested)	Enzyme activity tested*				
	PA	BG	LA	I	AP
<i>Clostridium difficile</i> (212/220)	+	-	+	-	-
<i>Clostridium difficile</i> (8/220)	+	-	-	-	-
<i>Clostridium sporogenes</i> (4/8)	+	-	-	-	+
<i>Clostridium sporogenes</i> (4/8)	+	-	-	-	-
<i>Clostridium sordellii</i> (9/9)	+	-	-	+	+
<i>Clostridium bifermentans</i> (3/7)	+	-	+	+	-
<i>Clostridium bifermentans</i> (2/7)	+	-	+	+	+
<i>Clostridium bifermentans</i> (1/7)	+	-	-	+	+
<i>Clostridium bifermentans</i> (1/7)	+	-	-	+	-
<i>Clostridium septicum</i> (3/5)	-	+	-	-	+
<i>Clostridium septicum</i> (2/5)	-	+	-	-	-
<i>Clostridium perfringens</i> (8/8)	-	+	-	-	+
<i>Clostridium glycolicum</i> (7/7)	+	-	+	-	+
<i>Clostridium histolyticum</i> (1/1)	-	+	-	-	+
<i>Clostridium acetobutylicum</i> (1/1)	-	+	-	-	+
<i>Clostridium tetani</i> (1/1)	-	-	-	-	+
<i>Clostridium cadaveris</i> (1/1)	-	-	-	+	-
<i>Clostridium tertium</i> (1/1)	-	+	-	-	-
<i>Clostridium beijerinckii</i> (1/1)	-	+	-	-	-
<i>Clostridium butyricum</i> (1/1)	-	+	-	-	-

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

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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