Analysis of latex agglutination test for *Clostridium difficile* toxin A (D–1) and differentiation between *C. difficile* toxins A and B and latex reactive protein

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**Summary** Virulent toxigenic and avirulent non-toxigenic strains of *Clostridium difficile* gave a positive result in the latex agglutination test (LAT) for *C. difficile* toxin A (D–1). Similar concentrations of latex agglutinating antigen were produced by these strains in vivo. Positive reactions were also given by *C. sporogenes*, proteolytic *B. botulinum* Types A, B, and A/F, and *Bacteroides* *assaccharolyticus*. The latex agglutinating antigen was denatured by boiling for 10 minutes, but not by heating at 56°C for 30 minutes. The reaction was abolished by incubation of test material with crude *C. difficile* antitoxin but not with other clostridial antitoxins or specific antitoxin to *C. difficile* toxin A. The latex agglutinating antigen present in *C. difficile* eluted between 0.39% and 0.47% M sodium chloride, and that produced by the other clostridia, between 0.35% and 0.43% M sodium chloride by fast protein liquid chromatography. The latex agglutinating antigen of *C. difficile* was neither cytotoxic nor mouse lethal and was distinct from toxin A and toxin B.

In the analysis of faecal specimens from patients with diarrhoea the latex agglutination test correlated better with the presence of *C. difficile* than with toxin B and detected both toxigenic and non-toxigenic strains. The latex agglutination test should only be used in the laboratory as an alternative to culture for *C. difficile* and not as a method for the detection of *C. difficile* toxins.

*Clostridium difficile* is an important enteric pathogen which causes a spectrum of gastrointestinal disease ranging from mild diarrhoea to fulminant pseudomembranous colitis. Several methods for the diagnosis of *C. difficile* mediated disease are currently available, including detection of the organism directly or indirectly, or detection of toxin B (cytotoxins) by enzyme linked immunosorbant assay (ELISA), counterimmunoelctrophoresis, or, more commonly, tissue culture. There are several problems associated with these approaches, especially the indirect techniques for detecting the organism and toxin B. Detection of toxin B by tissue culture is the most sensitive method, but not all laboratories have access to tissue culture facilities. To overcome this problem a simplified tissue culture system has been described, and latex agglutination systems which concentrate on detection of toxin A, have been developed. One of these latex agglutinating toxin A systems is now commercially available (Marion Laboratories, Kansas City, USA, marketed under licence from Mitsubishi Chemical Industries, Japan). The specificity of the test for detection of toxin A has been questioned, however, and our preliminary findings which showed a positive reaction with a non-toxigenic avirulent strain of *C. difficile*, with other species of clostridia, and with faeces which contained neither *C. difficile* nor cytotoxin, stimulated us to investigate further these unexpected findings.

**Material and methods**

**Source and identification of bacterial strains**

The 23 strains of *Clostridium difficile* consisted of 11 toxigenic and 12 non-toxigenic strains. The toxigenic strains were isolated from the faeces of patients with pseudomembranous colitis (two strains), antibiotic-associated diarrhoea (two strains), asymptomatic carriage in infants (two strains), a geriatric patient (one strain), a cat (one strain), and a dog (one strain), the...
caecal contents of a hamster with ileoaeacitis (one strain), and the environment of a neonatal unit (one strain). The non-toxigenic strains were isolated from the faeces of patients with diarrhoea due to causes other than *C. difficile* (three strains), asymptomatic carriage in infants (three strains), and dogs (two strains), and the environment of a veterinary clinic (four strains). All strains were identified as described in detail elsewhere.\(^{11}\)

The following strains of clostridia were also examined: *C. acetobutylicum* NCIB 8052; *C. beijerinckii* NCIB 9362; *C. bifermentans* NCTC 506 and 2914 and four faecal isolates of *C. bifermentans*; the sterile filtrates of three strains each of the proteolytic *C. botulinum* Type A and Type B, *C. botulinum* Type C, *C. botulinum* Type A/F, and *C. botulinum* Type G (provided by MJ Hudson BMRL CAMR; *C. butyricum* NCIB 9380, and one strain isolated from faeces; two strains of *C. cadaveris*, *C. paraputrificum*, *C. perfringens* Type A (CN 4015, Food Hygiene Laboratory, Colindale, London), and six entero-toxigenic strains of *C. perfringens* from two patients with diarrhoea; *C. septicum* NCTC 547; *C. sordellii* NCTC 8780, and five strains isolated from faeces; *C. sporogenes* NCTC 532 and 533, and 18 faecal isolates of *C. sporogenes*; and two isolates of *C. subterminale*. All of the isolates other than those obtained from recognised collections were isolated from faeces at the Clinical Research Centre, London and the General Hospital, Birmingham, and identified by established criteria.\(^{12}\)

**DETECTION OF LATEX AGGLUTINATING (LA) ANTIGENS**

All bacterial strains were grown anaerobically in brain heart infusion broth (BHIB) (Gibco) with added reducing agents\(^1\) at 37°C for three days (in London) or in tryptic-nitrate broth (TNB-Difco) (in Birmingham). Aliquots of sterile filtrates (50 µl) (0.45 µl filters) or aliquots of supernatant after centrifugation of spent culture media were screened directly for the presence of reacting antigen as instructed by the manufacturers.

**DETECTION OF LA ANTIGEN PRODUCED BY *C. DIFFICILE* IN HAMSTERS**

A toxigenic and a non-toxigenic strain of *C. difficile* were each used to infect two hamsters pretreated with antibiotics, as described in detail elsewhere\(^11\) - \(^13\) but with minor modifications to the number of days on which they received *C. difficile* after pretreatment with clindamycin. In brief, hamsters pretreated with clindamycin and housed individually in sterile isolator

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**Figure** Fast protein liquid chromatography profile of toxigenic strain of *C. difficile*. **LA-1** fractions in which latex agglutinating antigen produced by *C. sporogenes* and proteolytic *C. botulinum*: Types A, B, and A/F eluted. **LA-2** fractions in which latex agglutinating antigen produced by toxigenic and non-toxigenic strains of *C. difficile* eluted.
cages were given 0.5 ml of a washed suspension of C. difficile (p.o) containing 10^7 cfu/ml. The toxigenic strain of C. difficile (strain B-1) was given five days and the non-toxigenic strain (M-1) 10 days after the administration of clindamycin. At sacrifice 24 hours later, caecal material was analysed for the presence of C. difficile, toxin B, and latex agglutinating antigen.

DETECTION OF LATEX AGGLUTINATING ANTIGEN IN FECES

Faeces from 123 patients with diarrhoea were examined at the General Hospital Birmingham. In addition, faeces from 84 patients with diarrhoea received at the Clinical Research Centre were examined within a week of receipt and storage at room temperature after prior screening for C. difficile and toxin B. Two faecal specimens from a patient excreting a non-toxigenic avirulent strain of C. difficile (strain M-1) after oral challenge with the organism as part of a different study, five faecal specimens from five patients with cystic fibrosis asymptptomatically excreting C. difficile, and nine faecal specimens stored at 4°C for up to one month from seven patients with C. perfringens enterotoxin associated diarrhoea were also examined. All clostridia isolated from latex agglutinating antigen positive faecal samples, which were negative for C. difficile and cytotoxin, were inoculated into TNB, and after four days' anaerobic incubation at 37°C the supernatant from the spent culture medium was tested for latex agglutinating antigen. For one such specimen an enrichment was also performed in Robertson's cooked meat medium (Southern Group Laboratories) and all isolates from this latex agglutinating culture were examined individually after subculture into this medium.

One faecal specimen was retested two months, one after four months, and one five months after storage at 4°C.

TITRATION OF LATEX AGGLUTINATING ANTIGEN

Spent culture media or caecal contents were prepared for the latex agglutinating test as described above. This material was diluted in a two-fold dilution series in the buffer provided by the manufacturers. End points were determined as the last dilution at which a positive reaction was seen. For the positive control material supplied by the manufacturers (reagent 3), this was 1:7 corresponding to 71 ng/ml. Conversion of end points to concentrations was based on this apparent limit of detection. A microscopic end point was also determined by examination with oil immersion by light microscopy at a magnification of 100. Using this method a negative test showed individual latex particles moving freely by Brownian movement; a positive test showed clumping of the latex beads into rafts.

CHARACTERISATION OF LATEX AGGLUTINATING REACTIVE PRODUCTS

The spent culture medium of 24 hour cultures of C. difficile strains B-1, M-1, C. sporogenes NCTC 532, C. botulinum Types A, B, and A/F, and a faecal isolate of Bacteroides asaccharolyticus grown in brain heart infusion broth were treated in a variety of ways before screening for latex agglutinating antigen. One ml aliquots were individually heated at 56°C for 30 minutes and boiled at 100°C for 10 minutes: 0.5 ml aliquots were mixed either with equal volumes of C. difficile antitoxin (TD Wilkins, Virginia Polytechnic Institute, Blacksburg, USA); the cross reacting C. sordellii antitoxin (Wellcome Research Laboratories, Beckenham, Kent); specific antitoxin to C. difficile toxin A (Dr J Stephen, Birmingham University); anti-C. perfringens enterotoxin (Food Hygiene Laboratory, Colindale, London); or normal horse serum (Wellcome Research Laboratories) and left at room temperature for one hour. The positive control material (reagent 3) provided by the manufacturers was examined in the same way.

ANION EXCHANGE CHROMATOGRAPHY

Anion exchange chromatography was performed on a mono Q column HR5/5 (5 cm × 5 mm Pharmacia) incorporated into a fast protein liquid chromatography apparatus (Pharmacia). Samples (500 µl) of filter sterilised (0.22 µm) 48 hour cultures of C. difficile strain B-1 (toxigenic), C. difficile strain M-1 (non-toxigenic), C. sporogenes NCTC 532, and C. botulinum Types A, B, and A/F in brain heart infusion broth and a 1/2 dilution of a latex agglutinating positive stool sample in start buffer (20 mM Tris pH 7.5), were individually applied to the column. Uninoculated brain heart infusion broth served as a negative control. In addition, 250 µl samples of C. difficile strain B-1 and C. sporogenes NCTC 532, and of C. difficile strain B-1 and M-1 were applied simultaneously. The material was eluted with a linear gradient of 0 to 1M sodium chloride in 25 ml of 20 mM Tris-hydrochloric acid buffer (pH 7.5) at a flow rate of 2 ml/minute. Eluting proteins were detected by absorbance at 280 nm and collected in 1 ml fractions, which were examined for cytotoxicity and reaction in the latex agglutination test kit. Neutralisation of cytotoxocity was performed with C. difficile antitoxin and C. sordellii antitoxin which contain antibodies to both toxins A and B, and with specific antitoxin to C. difficile toxin A. Fractions corresponding to those which reacted in the latex agglutination test were also collected after anion exchange chromatography of 3 ml of a 48 hour culture of C. difficile strain B-1 (toxigenic). These fractions, which were confirmed as being latex agglutination positive, were screened for mouse lethality.
DETECTION OF TOXINS A AND B

Isolates of *C. difficile* were tested for their ability to produce toxin B in vitro after growth in chopped meat carbohydrate (Southern Group Laboratories) by screening for cytotoxic effects in African green monkey kidney (VERO) cells, cultured in microtitre wells by the micromethod, as described elsewhere. All caecal and faecal material and fractions obtained after anion exchange chromatography were also screened for toxin B by this method. Production of toxin A by isolates was screened for by an ELISA technique. Fractions obtained after anion exchange chromatography were analysed for toxin A by screening for cytotoxic effects in VERO cells and specific neutralisation by antitoxin to *C. difficile* toxin A.

NEUTRALISATION OF TOXIN A WITH LATEX BEADS

An attempt was made to use the antibody coated latex beads provided in the latex agglutination test to neutralise the cytotoxic fractions, obtained after anion exchange chromatography, which neutralised specifically with antitoxin to toxin A. Cytotoxic material of titre 1/4, 1/8, 1/16 and 1/32 was mixed with an equal volume of latex beads and left for one hour at room temperature. In one set of experiments the beads were removed after centrifugation and in another set the beads were left. In both cases the material was screened for cytotoxicity in tissue culture as described above. The control latex beads provided with the kit were used as a negative control.

DETERMINATION OF VIRULENCE OF *C. DIFFICILE* STRAINS

Mouse lethality test

Strains were tested for the production of biologically active toxins by screening cell free filtrates of spent culture media after three days' growth in brain heart infusion broth for lethality to mice (three per strain) after intraperitoneal injection, as described by Banno et al. Various fractions obtained by anion exchange chromatography were also similarly tested after intraperitoneal injection of 0.3 ml aliquots. Negative controls consisted of corresponding concentrations of sodium chloride in Tris buffer collected after a blank run.

Virulence in hamsters

Strains were examined for virulence in hamsters pretreated with clindamycin (three per strain) after oral feeding with viable cultures of *C. difficile*, as described in detail elsewhere.

Results

REACTION OF CLOSTRIDIAL STRAINS WITH THE LATEX AGLUTINATION TEST

Table 1 shows the results for *C. difficile*. Of the 11 toxigenic strains, nine were shown to produce toxin A by ELISA and two were not tested. All 11 strains were lethal to pretreated hamsters, and sterile culture filtrates were lethal to mice on intraperitoneal injection. Of the 12 non-toxigenic strains, four were shown not to produce toxin A by ELISA and eight were not tested; all were avirulent in hamsters pretreated with antibiotics, and none was lethal to mice on intraperitoneal injection. All of the strains of *C. difficile* gave a positive reaction in the latex agglutination test, irrespective of their toxigenic status.

Of the other species of clostridia examined, all 20 strains of *C. sporogenes* and the seven strains of proteolytic *C. botulinum*—that is, Types A, B, and A/F—were positive. All other strains were negative, though the six faecal isolates of enterotoxigenic *C. perfringens* were positive in both the test and the negative control.

Four toxigenic and four non-toxigenic strains of *C. difficile* as two groups produced the same amounts of latex agglutinating antigen, with a mean titre of 1/8 (range 1/4 to 1/16). The titre determined microscopically was 1/24 (range 1/8 to 1/64) for the toxigenic strains and 1/32 (range 1/16 to 1/64) for the non-toxigenic strains. The strains of *C. sporogenes* NCTC 532 and 533 produced titres of 1/16 and 1/4, respectively, and a strain from a patient with latex positive, but *C. difficile* and cytotoxin negative faeces,
produced a titre of 1/16. The titres determined microscopically were 1/128 and 1/4, respectively, for the two NCTC strains.

**Production of latex agglutinating antigen by C difficile in hamsters**

Both toxigenic virulent strains and non-toxigenic avirulent strains of *C difficile* produced similar quantities of latex agglutinating antigens in vitro. The possibility that a non-toxigenic avirulent strain may fail to do so in vivo was therefore investigated in the hamster animal model (table 2). The non-toxigenic avirulent strain of *C difficile* colonised hamsters and produced latex agglutinating antigen to the same extent as the virulent toxigenic strain, but did not cause ill health. Similar results were found in man.

**Presence of latex agglutinating reactive material in patient faeces**

Two faecal specimens from a patient infected with the non-toxigenic avirulent *C difficile* strain M-1 contained $10^4$ and $10^5.5$ cfu of *C difficile* /g, and both specimens were positive in the latex agglutination test. The patient did not have diarrhoea at the time of sampling, however, or at any stage after receiving strain M-1. All five faecal specimens from the patients with cystic fibrosis who were asymptomatically excreting *C difficile* were also positive in the latex agglutination test. None of the stools contained toxin B and the one stool examined for toxin A was negative; two of the patients excreted non-toxigenic strains and three patients excreted toxigenic strains.

Of the nine faecal specimens from patients with *C perfringens* enterotoxin associated diarrhoea, one gave an uninterpretable result—that is, gave a positive reaction with the negative control latex beads—but was shown to be negative for *C difficile* and toxin B; two gave a positive result, both of which were positive for *C difficile* but negative for toxin B; and the other six were negative. One of these negative specimens, however, was positive for *C difficile* though negative for toxin B.

Table 3 shows the results of the correlation between toxin B, *C difficile*, and latex agglutination for the 207 faecal specimens from patients with diarrhoea of unknown cause. Thirty two of 45 (71%) *C difficile* positive specimens were positive by latex agglutination. In two of these cases *C difficile* was only isolated after enrichment. Four specimens were positive by latex agglutination but negative for *C difficile*. Fifteen of 20 (75%) toxin B positive specimens were positive by latex agglutination and 21 were positive by latex agglutination but negative for toxin B. Two specimens gave uninterpretable results and both of these were positive for *C difficile*, though only one was positive for toxin B. Overall, the latex agglutination test correlated better with the presence of *C difficile* than with toxin B.

In total there were 11 false negative and four false-positive results given by the latex agglutination test when comparing with the presence of *C difficile* or toxin B, or both. Four of the specimens that were negative by latex agglutination had both *C difficile* and toxin B present. It was possible to explain the latex agglutination in two of the four false positive specimens; in one case the reaction was due to the presence of *C sporogenes* and in the other to *B asaccharolyticus*, both organisms being shown to produce latex agglutinating antigen in vitro. In a further false positive specimen large amounts of anti-

**Table 3** Correlation between toxin B *C difficile* and latex agglutination test

<table>
<thead>
<tr>
<th>No of specimens</th>
<th>Latex agglutination test</th>
<th><em>C difficile</em></th>
<th>toxin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>158</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>?</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2** Production of latex agglutinating antigen in hamsters infected with *C difficile*

<table>
<thead>
<tr>
<th>Animal and strain of <em>C difficile</em></th>
<th>Log$_10$ No of <em>C difficile</em>/g of faecal content</th>
<th>Reciprocal of toxin B titre</th>
<th>Titre of latex agglutination antigen</th>
<th>Animal health at sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain B-1 (toxigenic, virulent)</td>
<td></td>
<td></td>
<td></td>
<td>Moribund</td>
</tr>
<tr>
<td>A</td>
<td>5·5</td>
<td>$1 \times 10^4$</td>
<td>1/2 (1/4)*</td>
<td>Moribund</td>
</tr>
<tr>
<td>B</td>
<td>6·0</td>
<td>$2 \times 10^6$</td>
<td>1/2 (1/8)</td>
<td>Moribund</td>
</tr>
<tr>
<td>Strain M-1 (non-toxigenic, avirulent)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>4·5</td>
<td>Negative</td>
<td>1/2 (1/4)</td>
<td>Healthy</td>
</tr>
<tr>
<td>E</td>
<td>7·5</td>
<td>Negative</td>
<td>1/4 (1/8)</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

*Titre determined by light microscopy.
biotic (piperacillin, augmentin, and metronidazole) at concentrations that would be inhibitory to both \textit{C. difficile} and \textit{C. sporogenes} were present. \textit{C. perfringens}, \textit{C. butyricum}, and other unidentified clostridia were isolated from the remaining false positive specimen; but cultures of these isolates failed to give a positive latex reaction.

Three of the 25 specimens that were positive for \textit{C. difficile} but negative for faecal toxin B were due to the excretion of non-toxigenic strains; 10 of these patients, however, excreted toxigenic strains. The toxigenic status of isolates from the other 12 specimens was not determined.

The positive faecal material that had been stored at 4°C for two, four, and five months remained positive on retesting.

\textbf{CHARACTERISATION OF LATEX AGGLUTINATING ANTIGEN}

Boiling for 10 minutes denatured the latex agglutinating antigen produced by \textit{C. difficile} strains B-1 and M-1. \textit{C. sporogenes} NCTC 532, \textit{C. botulinum} Types A, B, and A/F, \textit{B. asaccharolyticus}, and those present in reagent 3 (positive control material), resulting in a negative latex agglutination test. Heating to 56°C for 30 minutes had no such effect. In all cases the reaction was abolished by prior incubation of test material with \textit{C. difficile} antitoxin but not by incubation with any of the other antitoxins, including the specific antitoxin to \textit{C. difficile} toxin A and cross reacting \textit{C. sordelli} antitoxin, or normal horse serum.

Reagent 3 was cytotoxic with a titre of 1/4. The cytotoxicity was abolished by preincubation with \textit{C. difficile}, \textit{C. sordelli}, and \textit{C. difficile} toxin A antitoxins, but not with normal horse serum.

The figure shows the results of analysis of different bacteria by anion exchange fast protein liquid chromatography for toxic material and latex agglutinating antigen. The latex agglutinating antigen produced by both toxigenic and non-toxigenic strains of \textit{C. difficile}, and that present in the latex agglutinating positive stool sample which was also \textit{C. difficile} positive and cytotoxic, eluted within a 2 ml volume between 0.39% and 0.47% M sodium chloride; and that produced by \textit{C. sporogenes} and the proteolytic \textit{C. botulinum} eluted over a 2 ml volume between 0.35% and 0.43% M sodium chloride. Simultaneous analysis of the toxigenic and non-toxigenic strains of \textit{C. difficile} gave an identical elution profile for latex agglutinating antigen as either alone, whereas simultaneous analysis of \textit{C. sporogenes} and the toxigenic strain of \textit{C. difficile} gave an elution profile identical with the sum of the two individual species—that is, elution within a 3 ml volume between 0.35% and 0.47% M sodium chloride.

The latex agglutinating antigen produced by either strain of \textit{C. difficile} was not cytotoxic to VERO cells. The latex agglutinating antigen obtained after fractionation of 3 ml of starting material from the toxigenic strain of \textit{C. difficile} had a latex agglutinating titre of 1/32 (2272 ng/ml) and administration of 0.3 ml (757 ng) to mice was not lethal. The non-toxigenic strain of \textit{C. difficile} did not yield any cytotoxic fractions, whereas the toxigenic strain did. Two consecutive one ml fractions eluting between 0.27% and 0.35% M sodium chloride and three consecutive one ml fractions eluting between 0.59% and 0.70% M sodium chloride were cytotoxic. The cytotoxicity of all fractions could be neutralised with \textit{C. difficile} and \textit{C. sordelli} antitoxins (which contain antibodies to both toxins A and B of \textit{C. difficile}). The cytotoxicity of only those fractions eluting between 0.27% and 0.35% M sodium chloride, however, could be neutralised by specific antiserum to \textit{C. difficile} toxin A. This material was not neutralised by the latex beads provided in the latex agglutination kit.

\textbf{Discussion}

This investigation clearly shows that the latex agglutination test for the detection of \textit{C. difficile} toxin A agglutinates with bacterial products that are neither toxin A nor toxigenic. This conclusion is based on the following observations: firstly, all of the non-toxigenic strains of \textit{C. difficile} examined caused agglutination. On the assumption that the test was detecting toxin A, this finding could be explained if the non-toxigenic strains were in fact producing very low concentrations of toxin A. The non-toxigenic strains, however, produced similar amounts of material, causing agglutination of the latex beads both in vitro and in vivo as the toxigenic strains. Furthermore, the non-toxigenic strains examined were all confirmed as completely avirulent in the hamster model of disease. The agglutination caused by the non-toxigenic strains can not be due to the presence of biologically inactive toxin A, because if this were the case, the strains would react positively in an ELISA test, which they do not.

Secondly, it was possible to separate the material that caused agglutination of the latex beads from both toxins A and B by fast protein liquid chromatography. The isolated latex agglutinating material had the same characteristics as that present in crude culture filtrates and was not biologically active in our test systems. In addition, fractions obtained after fast protein liquid chromatography that were identified as toxin A on the basis of cytotoxicity and neutralisation with specific antitoxin, failed to cause agglutination of the latex beads.
Thirdly, the agglutination caused by crude culture filtrates of *C. difficile*, or its isolated latex agglutinating material, could not be neutralised by antitoxin to *C. difficile* toxin A. As crude culture filtrates of *C. sordellii* (a non-cytopathic strain) did not cross react in the latex agglutination test, it was predicted that *C. sordellii* antitoxin, which cross reacts with *C. difficile* toxins A and B, would fail to neutralise agglutination of the latex agglutinating antigen, but that crude *C. difficile* antitoxin, which contains antibodies to other *C. difficile* antigens, as well as toxins A and B, should neutralise agglutination. This was found to be the case.

Fourthly, antigens from other bacteria would also cause agglutination of the latex beads. The agglutinating antigens produced by *C. sporogenes* and proteolytic *C. botulinum* coeluted with fast protein liquid chromatography and were chromatographically distinct from that produced by *C. difficile*. This material had the same neutralisation pattern as that of *C. difficile* and probably reflects cross reactivity. More interestingly, it was found that *B. asaccharolyticus* also caused agglutination. This latex agglutinating antigen coeluted with that of *C. difficile* (data not shown).

Finally, the latex agglutination test gave positive results for faecal material from patients without diarrhoea that contained only non-toxigenic strains of *C. difficile* and in which toxin A was absent as determined by ELISA.

It has been reported that the enterotoxic activity of preparations of toxin A in rabbit ligated ileal loops could be neutralised by preincubation with the latex beads available in the kit (Kono H, personal communication, 1986). The positive loops described, however, had a volume length ratio of less than 1, which most people would find unacceptable: and were non-haemorrhagic, which indicates that the amount of toxin A used was very low. These findings imply that there may be sufficient titres of antibodies to toxin A on the beads to neutralise the biological activity of low concentration of the toxin but, that from the above observations, there is insufficient antibody to cause agglutination. Nevertheless, we were unable to neutralise the cytotoxicity of toxin A by preincubation with the latex beads.

The positive control material (toxin A) provided with the kit (reagent 3) is only partially purified (Wilkins TD and Lyerly DM, personal communication, 1986), and consists of at least five antigens. The major antigen is the one that causes agglutination of the test beads. One of the minor antigens is toxin A. Our study also clearly showed that reagent 3 contains a latex agglutinating antigen, which cannot be neutralised with antitoxin to toxin A, and a cytotoxic antigen which can.

The enterotoxin isolated by Kono has a molecular weight of 47,000 daltons (Kono H, personal communication, 1986), and is therefore different to the one originally described by Banno *et al* and Sullivan *et al* which has a molecular weight of 450,000 to 600,000 daltons, and is more in keeping with the molecular weight of 52,000 reported by Rihn *et al*. This difference is explained on the basis that the higher molecular weight enterotoxin is an aggregate of the 47,000 molecular weight entity; the aggregate being induced by ammonium sulphate precipitation used in their earlier purification procedures (Banno, Kobayashi T, and Kono H, personal communication, 1986). This assumption has been used to explain the lack of agglutillation with purified toxin A described by Lyerly and Wilkins. The cytotoxic fraction that corresponds to toxin A used in our studies had not undergone any ammonium sulphate precipitation step, and therefore this apparent explanation for the Lyerly and Wilkins finding did not apply. The cytotoxic fraction that was neutralised with antibodies to toxin A described in our study and the one we obtained directly from clinical material eluted a little later than purified toxin A (obtained from Dr Haslam, Birmingham University), which has the high molecular weight, in keeping with the data published by others.

Despite the problems outlined the use of the latex test is an option which some diagnostic laboratories may find acceptable. Removal of the cross reactivity with *C. sporogenes* and proteolytic *C. botulinum*, noted recently by others, and *B. asaccharolyticus* would improve the specificity of the test. We found the latex test to be a highly sensitive method for detecting *C. difficile*, and in two cases the organism could only be recovered after enrichment. Some laboratories may find the test more reliable than culture. During a hospital outbreak of pseudomembranous colitis, the rapidity and ease of use of the latex test should prove invaluable in presumptively identifying infected patients and thereby facilitating their prompt isolation. As a routine diagnostic screening method, however, it must be remembered that it will not discriminate between toxigenic virulent strains and non-toxigenic avirulent strains of *C. difficile*. Clinicians will need to understand that the test is not diagnostic of clinically important infection and merely serves to alert them to the possibility of pseudomembranous colitis or antibiotic associated diarrhoea. The same is, of course, true for the cytotoxin (toxin B) test, except that the correlation with significant infection is a closer one. Incorrect diagnosis of pseudomembranous colitis and antibiotic associated diarrhoea, with resultant unnecessary treatment with vancomycin or metronidazole, is a likely consequence of the use of the latex agglutination test and should be watched for.
Recommendations

1. The test should only be used in the laboratory as an alternative to culture for *C. difficile* and not as a method for the detection of *C. difficile* toxin A.

2. In reporting the findings it should be made clear that “the results strongly indicate the presence of *C. difficile*.” It would be unwise to report the findings as *C. difficile* culture positive, or even as latex agglutination positive, which would be accurate but prone to misinterpretation due to previous sales literature indicating its use as a toxin A detection system.

3. Ideally, positive specimens should be screened for toxin B by culture for confirmation, as the test will not differentiate between toxigenic and non-toxigenic strains and there will also be the occasional false positive result due to cross reacting antigens produced by other bacteria.

Addendum

Due to these and other recent findings, Marion Scientific has elected to delete reference to “toxin A” from the CDT kit label and package insert.

The ELISA for toxin A was performed by Dr Haslam, University of Birmingham.

References


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