Laboratory diagnosis of Clostridium difficile-associated diarrhoea: a plea for culture

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A routine protocol for diagnosing Clostridium difficile-associated diarrhoea (CDAD) based on both faecal-cytotoxin detection and toxigenic culture was adopted by the microbiology laboratory of the St Luc-UCL University Hospital in Brussels in 1997. A toxigenic culture is a faecal culture followed, in the case of positivity, by a direct immunoassay on colonies to detect toxin A production. The results obtained over the past 7 years in the hospital are reviewed here. A total of 10 552 diarrhoeal stools from 7042 patients were analysed, of which 9494 were negative for all tests. A total of 1058 samples (10 %) from 794 patients were culture-positive, of which 460 (4.4 %) were positive for a faecal cytotoxin. The remaining 598 cultures were tested for toxin A on colonies; 355 of them were positive, which is 3.4 % of the total, and the remaining 243 (2.3 %) were negative. The positivity of the faecal-cytotoxin assay was statistically linked to the number of colonies observed on the culture plate. In conclusion, over a 7 year period, toxigenic culture allowed the diagnosis of 355 cases of CDAD that would have been missed by a protocol using a faecal-toxin assay alone. In terms of both patient care, prevention of environmental contamination and prevention of risk of a hospital outbreak, it is proposed that these results justify the recommendation to perform both faecal-toxin assay and culture in routine medical practice.

INTRODUCTION

Clostridium difficile is the main aetiological agent of antibiotic-associated diarrhoea and is considered to be the first cause of nosocomial diarrhoea (Kelly & LaMont, 1998). The virulence is mainly due to the production of two toxins, namely toxins A and B. Some virulent strains may only produce toxin B (Alfa et al., 2000).

The laboratory diagnosis is based on two kinds of tests: faecal culture and toxin detection. Culture on selective medium is very sensitive but lacks specificity because of possible carriage of non-toxigenic isolates. Moreover, it requires a 40–48 h incubation time and is therefore a relatively slow technique. Toxin detection can be performed by inoculation of a faecal filtrate on cultured cells, which will display a typical cytopathic effect neutralized by specific antiserum if the toxin is present. The technique is sensitive and specific but requires facilities for cell cultures and a 6–48 h incubation period before a positive effect is observed. Immunoassays (IAs) directed to toxin A alone or both toxins A and B are available from several companies. Their main advantage is speed, since most kits are made of individual tests, which give a result within only 20–40 min. However, this method is less sensitive than using cultured cells.

Studying guidelines published in the literature highlights the various controversies about the best scheme to follow for an optimal diagnosis. Most of the recommendations issued in the USA and UK limit the diagnosis to a single toxin-detection test on faecal specimens by cultured cells or IA, although in several other European countries both culture and toxin detection are recommended for an optimal diagnosis (Berrington et al., 2004; Bartlett, 2002; Brazier, 1998; Delméé, 2001). A recent survey has clearly shown the large variations in the methods used when comparing laboratories from different European countries (Barbut et al., 2003).

Our laboratory adopted the following scheme for all C. difficile faecal specimens in early 1997. Firstly, both culture and direct faecal-cytotoxin detection on cultured cells have to be performed. Then, if the culture is positive and the faecal toxin negative, colonies grown on selective medium are tested for ‘in vitro’ toxin A production by an IA. This approach, sometimes called toxigenic culture, enables the detection of stools containing toxigenic isolates that would be missed by the single faecal-toxin test.

In this paper we will review the routine laboratory results
obtained in our university hospital over the past 7 years with a view to evaluating the performance and suitability of the diagnostic protocol in use.

**METHODS**

**Faecal specimens.** The bacteriology laboratory operates for an 884 bed university hospital including a 42 bed intensive care unit, a transplantation unit, an oncology unit and a 28 bed geriatric unit. The number of hospitalization days is around 250 000 per year.

Faecal specimens for *C. difficile* diagnosis are accepted under the following conditions: the stools must be liquid or at least unformed and a history of antibiotic therapy or chemotherapy during the last 6 weeks or a previous diagnosis of *C. difficile*. Stools from neonates or children less than 8 years old were sometimes accepted but they will not be considered in this study. Only freshly taken specimens were processed and, in case the tests could not be performed rapidly, they were kept at 4°C until processing. Stools received on Sunday were inoculated for culture on reception and kept at 4°C until Monday morning for toxin detection.

**Culture.** Stool specimens were spread out on home-made cycloserine cefoxaxime fructose agar medium (CCFA) (George et al., 1979) with egg yolk which had been modified to include the selective agents cycloserine at 400 μg ml⁻¹ and cefotaxime at 3-6 μg ml⁻¹ as previously described (Delméé, 1987). They were incubated for 40–48 h in an anaerobic atmosphere created in a jar by the Mart Anoxomat system (Brugman). Colonies of *C. difficile* were identified on the basis of their typical morphology on culture plates when observed with binocular plate microscope and confirmed when necessary by GLC. A semi-quantitative estimation of the numbers of colonies on the plate was made as follows: '+' was recorded when the number of colonies was less than 10, '+' when between 10 and 25, and '++' when greater than 25.

**Faecal-cytotoxin immunoassay.** A suspension of the faecal sample was made in PBS and, after centrifugation, the supernatant was filtered through a 0.2 μm filter. The stool-cytotoxin assay was performed by testing this sterile faecal filtrate on confluent monolayer of HeLa cells. The final dilution of the stools was 1 in 40. The cells were examined both after one night and after 48 h of incubation at 37°C. Positivity was confirmed by neutralization with Techlab *C. difficile* toxin A and B polyclonal goat antiserum (Lucron Bioproducts). If a positive test was not neutralized by the antiserum, the result was reported as 'non-specific' (NSP).

**Detection of toxin A on colonies.** In the case of a positive culture with a negative faecal-toxin test, toxin A detection was performed on colonies using the Oxoid *C. difficile* toxin A IA (Oxoid) as follows. Three to five colonies of *C. difficile* were directly picked from the selective plate with a sterile loop and suspended in 250 μl of the sample diluent. The suspension was shaken with a vortex for 30 s and centrifuged at 9800 g for 10 min. A 125 μl sample of the supernatant was then inoculated on the individual Oxoid panel. A positive result was recorded when a typical blue line appeared in the test window within 30 min.

**RESULTS**

Between 1 March 1997 and 31 March 2004, a total of 15 581 stools from 7042 patients aged over 8 years were received. The number of specimens per patient ranged from 1 to 41 with a mean of 2.2 and a median of 1.

In order to avoid duplicates, the following specimens were discarded from our calculations: all negative samples of a given patient collected within 7 days of a negative result and all the samples of a given patient collected within 10 days of a positive result (regardless of test used), which corresponds to the usual treatment length. So, only 10 552 stool samples were retained for result analysis.

Table 1 summarizes the results obtained for the 10 552 samples. A total of 9494 (90%) cultures were negative. Five of them, however, had a positive cytotoxin result on HeLa cells and were considered as cases of *C. difficile*-associated disease (CDAD). A total of 1058 samples (10%) from 794 patients were culture-positive, of which 460 (44%) had a faecal cytotoxin detected on HeLa cells. The remaining 598 cultures, including nine cases with non-specific results on cultured cells, were tested for toxin A on colonies, and 355 of them were positive, which is 3-4% of the total. The remaining 243 (2.3%) were negative.

A single episode of CDAD was recorded in 623 patients, whereas 107, 45, 9, 6, 3 and 1 patients had, respectively, 2, 3, 4, 5, 6 and 7 different episodes. Among those patients with relapses, two consecutive episodes with both positive faecal-cytotoxin assays were recorded in 55 cases and with both negative tests in 40 cases; a positive test followed by a negative one during the next episode was observed in 40 cases and the contrary in 31 cases.

Table 2 shows the correlation between the results of semi-quantitative cultures and the results of toxin detection in the cases in which a toxigenic isolate was identified (n = 808). There was a statistically significant correlation between the number of colonies on the plate and the probability of having a faecal cytotoxin detected.

**DISCUSSION**

The objective of this study was to evaluate the routine use over a 7 year period of a diagnostic procedure for *C. difficile* slightly modified from that described by Thonnard et al. (1996). Whilst the latter technique had reduced the culture incubation time to 24 h by using sodium taurocholate-enriched CCFA, it could not be applied to routine testing due to the higher price of the plates. Moreover, in that work...
we performed toxin A assays on colonies with the Premier *C. difficile* toxin A IA (Meridian Diagnostics), whereas we used here a much quicker and easier test, the Oxoid *C. difficile* toxin A IA, which is presented on individual panels and yields results within about 40 min. The validity of this test was confirmed by testing reference strains and a set of toxigenic and non-toxigenic strains from our collection (unpublished results).

The procedure combining cytotoxin assay and toxigenic culture (culture plus toxin A assay on colonies) has been applied to every faecal specimen received in our routine laboratory for *C. difficile* diagnosis since 1997. Although it had already been evaluated in several short prospective studies (Barbut et al., 1993; Lozniewski et al., 2001; Fedorko et al., 1999) this is believed to be the first large-scale report including more than 10 000 specimens.

Our results demonstrate that this procedure allows optimal sensitivity and specificity to be reached. We were able to analyse the results of 10 552 stool specimens from adults or children older than 8 years suffering from diarrhoea which was either nosocomial and/or linked to antimicrobial treatment or cancer chemotherapy. Culture was by far the most sensitive method because it enabled the detection of *C. difficile* isolates in 10 580 stool specimens (10 %), a prevalence within the range reported in several other studies. For example, Barbut et al. (1996) reported a prevalence of 10·5 % among hospitalized patients in France whereas Kyne et al. (2002a) observed an 11 % incidence in a prospective cohort study of 252 hospitalized patients receiving antibiotics.

As expected, this high sensitivity of the culture was, however, counterbalanced by a low specificity, which is clearly demonstrated by testing colonies for *in vitro* toxin production. Indeed, 243 specimens (2·3 %) which were positive by culture were then shown to contain non-toxigenic isolates. This negative result implies that any pathogenic role in the diarrhoeal episode can be excluded and unnecessary treatment avoided. One could argue that simultaneous carriage of toxigenic and non-toxigenic isolates is entirely possible. However, the fact that we picked several colonies on the plate in order to perform the IA decreases the probability of missing a toxigenic strain. On the other hand, toxin B-positive/toxin A-negative isolates could also have been missed, a type of strain which has been reported to account for up to 3 % of the strains in some studies (Barbut et al., 2002; Kuijper et al., 2001; Samra et al., 2002). Such strains, however, would have been detected by the cytotoxin assay, which has a sensitivity of about 60 % as discussed below. Moreover, in a recent survey of 280 isolates from our laboratory, included in the present series, we only found one strain that was toxin A-negative/toxin B-positive (unpublished result). However, failure to detect such strains could be prevented by using a different kit allowing the simultaneous detection of both toxins. Several toxin A plus B enzyme-immunoassay kits in microplate format are available and one of them, the Premier Cytoclone A+B (Meridian Diagnostics) was used for toxigenic cultures by Lozniewski et al. (2001). The Meridian Immunocard toxin A and B from the same company was recently introduced. It has the advantage of being presented as individual tests with a very short running time of 10 min without centrifugation, and a first evaluation showed excellent results (unpublished results).

The cost of the toxigenic culture is not very high. By mixing several colonies, a single test is performed per specimen. The cost of the reagent ranges from £5 to £10 (£3.40 to £6.80), and the labour cost is limited to a few minutes. Moreover, in our study the test on colonies was limited to 5·6 % of all specimens. It means that the additional cost of the toxigenic culture per stool specimen is very limited.

By comparison with culture, the direct cytotoxin assay on faecal filtrate showed a rather low sensitivity. Indeed, only 465 stool specimens (4·4 %) were detected as positive. Since an additional 355 culture-positive stool specimens had toxigenic strains detected by IA on colonies, the direct faecal test only detected 56·7 % of a total of 820 toxigenic isolates. This percentage is lower than that reported in several studies. In the study by Thonnard et al. (1996), we reported a sensitivity of 61·7 % and, more recently, Lozniewski et al. (2001) reported a sensitivity of 74 % by comparison to toxigenic culture. The fact that HeLa cells were used may partly explain this rather low sensitivity since they are known to be slightly less sensitive than other cell lines, for example Vero cells (Berrington et al., 2004). It is worth remembering also that this work was done on a routine basis and that stools received during a weekend were only processed for cytotoxin assay the next Monday, a delay which may have a role in the poor sensitivity.

The comparison of the cytotoxin assays and the semi-quantitative measure of positive culture demonstrates that the probability of getting a positive cytotoxin assay is linked to the number of *C. difficile* bacteria contained in the faecal sample. The calculated odds ratio of obtaining a positive

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**Table 2.** Analysis of faecal-cytotoxin assay results according to the semi-quantitative culture results in cases where a toxigenic strain was identified (*n* = 808)

<table>
<thead>
<tr>
<th>Culture</th>
<th>Faecal-cytotoxin assay</th>
<th>Odds ratio (CI 95 %)*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>89</td>
<td>142</td>
<td>1</td>
</tr>
<tr>
<td>++</td>
<td>209</td>
<td>145</td>
<td>2·30 (1·62–3·28)</td>
</tr>
<tr>
<td>+++</td>
<td>162</td>
<td>61</td>
<td>4·24 (2·80–6·43)</td>
</tr>
<tr>
<td>Total</td>
<td>460</td>
<td>348</td>
<td></td>
</tr>
</tbody>
</table>

*Odds ratio of having a positive faecal-cytotoxin test by comparing the results of ++ cultures to those of + or the results of +++ cultures to those of +. CI, confidence interval.
cytotoxin result increased between ‘+’ and ‘+++’ cultures in a statistically significant manner (Table 2). This is logical because stools are never a homogeneous medium and concentrations of bacteria and of toxin may hence vary from one moment to another. Another argument in favour of a random effect in the positivity or negativity of the faecal-cytotoxin assay is the results from the 171 patients with relapses, which shows a random distribution of the results. This allows us to exclude the possibility that the results of the cytotoxin test might be patient- or strain-dependent.

The recent European survey of diagnostic methods and testing protocols by Barbut et al. (2003) clearly shows the wide variations of protocols observed among laboratories from different countries. Although most countries, except Denmark, routinely perform toxin detection on stools, the figure is quite different for culture: more than 90% of the laboratories in Denmark and Belgium perform it but only 28% in Spain and 20% in the UK. Guidelines published in the USA and UK mainly recommend testing for toxin (Barbut et al., 2002; Berrington et al., 2004). Our results show that toxigenic cultures allowed us to recover 355 (3-4%) stool samples with toxigenic C. difficile, that would have been missed by performing only the cytotoxin assay. This has implications not only in terms of patient misdiagnosis but also in terms of environmental contamination and risk of hospital outbreaks. The processed specimens were liquid stools and it has been clearly demonstrated that one such diarrhoeal case can rapidly cause environmental contamination. This could be prevented by the rapid implementation of isolation procedures and room decontamination (Mulligan et al., 1979).

The reasons for which culture is not always recommended in some guidelines are probably linked to cost and technical difficulties. It is true that culture increases the cost of the diagnosis. However, we calculated that the total cost including labour, agar plates and reagents for toxin detection on colonies only takes a few additional minutes and the main goal of culture is to optimize the global result: a 24–48 h delay is surely better than missing the diagnosis.

It is worth emphasizing that culture is also the only way to type strains and to test their antimicrobial susceptibility. Both are of crucial interest in the clinical management of individual cases and hospital outbreaks. Typing allows clonal strains to be traced and recognition of specific virulent groups (Brazier, 2001), whereas susceptibility testing might become mandatory in the future since the observation of the emergence of strains with a decreased susceptibility to vancomycin or metronidazole (Pelaez et al., 2002; Barbut et al., 1999).

This survey clearly shows the advantages of having a protocol including culture for the diagnosis of C. difficile. We recommend toxin testing of every stool specimen suspected of CDAD. This may be done by a cytotoxin assay as described here or by a direct IA on stools. The latter is a bit less sensitive but is easier to perform and much quicker, allowing a result within minutes. Culture should also be performed on every specimen and, in the case of a positive culture result with a negative stool toxin result, colonies should be tested for toxin production with a toxin A IA or, preferably, with a toxin A and B IA, although this has still to be validated.

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REFERENCES


