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Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea

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Clostridium perfringens has been reported as the cause of up to 15% of cases of antibiotic-associated diarrhoea (AAD) and may be diagnosed by detection of enterotoxin (CPent) in faeces. The performance of a commercial ELISA method for CPent, with culture and PCR methods to confirm the presence of enterotoxigenic *C. perfringens*, was evaluated in 200 consecutive specimens from patients with clinical details suggestive of AAD: 8% of the specimens were positive for CPent, 16% were positive for *C. difficile* cytotoxin and 2% gave positive test results for both *C. perfringens* and *C. difficile* toxins. Culture and PCR results confirmed the majority of ELISA results, although 2 (12.5%) reactive specimens were only weakly positive. *C. perfringens* is a potentially important cause of infective AAD and can be detected with the *C. perfringens* enterotoxin ELISA kit, although weak positive results should be considered with caution.

Introduction

Clostridium perfringens was first implicated as a cause of antibiotic-associated diarrhoea (AAD) in 1984 [1], when free faecal *C. perfringens* enterotoxin (CPent) was detected in 11 patients with diarrhoea following antibiotic administration. The evidence for a causal role by *C. perfringens* in AAD has increased and it has been reported to account for between 2% and 15% of all cases [1–4]. CPent is a 35-kDa protein produced constitutively *in vivo* at low levels by bacteria during the vegetative growth phase, but expression is up-regulated during sporulation [5,6]. CPent binds to gut epithelial cells, where it is internalised and alters cell membrane permeability, with resultant epithelial cell death leading to diarrhoea. Unlike *C. difficile* infection, *C. perfringens* AAD does not result in the formation of pseudomembranes [1]. CPent is encoded by the *cpe* gene which has been cloned and sequenced [5], and this has permitted the design of amplification primers [7,8]. A study of *C. perfringens* isolates showed that only 6% of wild-type strains possess the *cpe* gene [8], although the prevalence of such strains in infected symptomatic patients with food poisoning is much higher [9].

CPent can be detected in faecal specimens by the Vero cell cytotoxicity assay or ELISA systems [1,10–12]. Culture of *C. perfringens* is not recommended as a diagnostic method, given the relative ubiquity of the bacterium in human faeces, and because not all isolates will be enterotoxigenic. CPent production from cultured isolates can be difficult to demonstrate because it occurs during sporulation and it is difficult to make this bacterium sporulate *in vitro* [8]. An ELISA method for the detection of CPent has been developed (TechLab, Blacksburg, USA) although the performance characteristics of this kit have not been fully evaluated. We have demonstrated previously that there are problems with the culture of enterotoxigenic *C. perfringens* from faecal specimens found to be CPent ELISA positive [3]. Therefore, this study aimed to determine the relative frequencies of *C. perfringens* and *C. difficile* AAD, and to validate results obtained with the CPent ELISA kit by culture, a cytotoxin assay and a nested PCR method to enhance sensitivity.

Materials and methods

Specimens

Two hundred consecutive faecal specimens from in-patients submitted for *C. difficile* testing at Leeds Teaching Hospitals Trust were studied for evidence of *C. perfringens* infection. All patients had diarrhoeal stools and clinical evidence of AAD. Only specimens

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< 24 h old were eligible for inclusion. All specimens were stored at 4°C before testing.

Cytotoxin detection

C. difficile cytotoxin was detected with Vero cells and *C. difficile* antitoxin (TechLab) and the same Vero cell assay was used to detect *C. perfringens* enterotoxin with *C. perfringens* enterotoxin antitoxin (TechLab) as described previously [1]. Faecal supernate was added undiluted to the test wells (in-well dilution 1 in 10). The assays were repeated with a 1 in 10 dilution of the faecal preparation if evidence of bacterial overgrowth or non-specific cytotoxicity was apparent on reading the wells. The *C. perfringens* Enterotoxin Test kit (TechLab, distributed by BioConnections, Leeds, W. Yorks) was used according to the manufacturer's instructions and results were available within 2.5 h. Results were recorded with a dual-wavelength spectrophotometer (450 nm and 620 nm) as recommended, as this was found to be preferable to visual interpretation of results.

C. perfringens culture

Two Robertson's cooked meat broths (Oxoid) were inoculated with each specimen, one direct and one after alcohol shock treatment [13], and incubated aerobically for 24 h at 37°C. The broths were subcultured on to egg yolk agar (Oxoid) and neomycin blood agar (Oxoid) with a 5-µg metronidazole disc (Oxoid) and incubated anaerobically overnight at 37°C. Identification of *C. perfringens* colonies was by reaction with *C. perfringens* type A antitoxin (TechLab) and Gram's stain. Up to three *C. perfringens* colonies from each culture plate were stored at -20°C in phosphate-buffered saline with glycerol 40%.

PCR detection of the *cpe* gene

DNA was extracted directly from each faecal specimen with the QIAamp® DNA Stool Mini Kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. DNA extracts were stored at -20°C before PCR. After thawing, extracted DNA was added directly to the PCR assay as template. For PCR of *C. perfringens* isolates, stored isolates were cultured on neomycin blood agar. One colony was suspended in 50 µl of sterile distilled water and heated to 99°C for 5 min. This suspension was used as template in the PCR.

A nested PCR technique was used with faecal DNA extracts, but the second round primers were used alone with extracts prepared from isolates. The first round primers were 5'-TGTTAATACTTTAAGGATATGTATCC-3' and 5'-TCCATCACCTAAGGACTG-3' as described by Kokai-Kun *et al.* [7]. Each 25-µl PCR reaction contained 10 pmol of each primer, buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 1 U of *Taq* polymerase (Promega UK, Southampton) and 2.5 µl of template

DNA. The reaction comprised 5 min at 94°C followed by 34 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. There was a final extension step of 10 min at 72°C. The PCR product was used as template for the second round PCR. Second round primers were 5'-ATGTAATAGATAAAGGAGATGGTT-3' and 5'-ATAAATTCAGAAGTAAATCCAACT-3' as described by Van Damme-Jongsten *et al.* [8,14]. Each 25-µl PCR reaction contained 10 pmol of each primer, buffer, 3 mM MgCl₂, 0.2 mM dNTPs, 1 U of *Taq* polymerase and 2.5 µl of template DNA. The reaction comprised 5 min at 95°C followed by 45 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 70°C. The PCR product was 163 bp. The enterotoxigenic *C. perfringens* strain NCTC 8239 was used as the positive control for the PCR assays.

Results

The prevalence of *C. difficile* infection in the study group was 16%.

Fig. 1 summarises the results of the CPent ELISA, *cpe* PCR testing and *C. perfringens* culture. Sixteen (8%) of faecal specimens gave positive test results for CPent with the ELISA kit. Four of these 16 specimens were also positive in the Vero cell assay. Of the ELISA-positive specimens, 14 were *cpe* positive by nested PCR of faecal DNA extracts; 12 of these specimens yielded *cpe*-positive *C. perfringens* on culture. Of the two ELISA-positive PCR-negative specimens, only one specimen yielded *C. perfringens* on culture and none of the colonies tested was *cpe* positive. Four of the ELISA-positive specimens were also positive for *C. difficile* infection.

Of the 200 specimens, 184 were both ELISA and Vero cell assay negative; 177 (96%) of these specimens were *cpe*-negative by nested PCR of faecal DNA extracts. A total of 267 *C. perfringens* isolates from these specimens were stored, and 20% of isolates were randomly selected for further PCR analysis. Of these randomly selected isolates 7 (13%) were *cpe* positive. Seven (4%) of the ELISA-negative specimens were *cpe*-positive by nested PCR of faecal DNA extracts. Five of these seven specimens yielded *C. perfringens* on culture, but only two contained *cpe*-positive isolates.

Discussion

Although *C. difficile* is the most commonly identified pathogen in hospital-acquired infective diarrhoea, the cause(s) of the majority of the cases, in some series up to 80%, currently remain undiagnosed. *Staphylococcus aureus* and *C. perfringens* are the most frequently cited alternative causes of AAD [15]. In the present study, 8% of the study population were found to be CPent positive according to ELISA results. This compares with a 16% prevalence rate of *C. difficile* infection in

Fig. 1. Summary of results of CPent ELISA, *cpe* PCR testing, and *C. perfringens* culture.

the same group. One quarter of the CPent-positive specimens (2% overall), were also positive for *C. difficile* cytotoxin. In 14 of the 16 ELISA-positive specimens there was further evidence of enterotoxigenic *C. perfringens* from a cytotoxin assay, nested PCR on faeces for *cpe*, and culture with direct PCR for *cpe*. It is important to note that nearly half of those specimens containing *cpe*-positive isolates also contained *cpe*-negative isolates. This suggests that multiple colonies need to be tested from each sample. Investigations on two of the ELISA-positive specimens failed to provide any further supporting evidence for infection. It is of interest that these two specimens produced the lowest OD results obtained in the ELISA-positive group, and this was confirmed by repeat testing. Because the ELISA kit has a single cut-off point for results, there is no grey zone for equivocal results. We believe that these two specimens were false-positive results in the absence of further evidence of infection. As such, CPent-positive results that are only slightly higher than the ELISA kit cut-off should be treated with caution. Furthermore, only those specimens that produced a positive ELISA reading greater than that obtained for the positive control were detectable in the Vero cell assay. Therefore, we conclude that the ELISA kit is much more sensitive than the Vero cell assay for the detection of CPent, and indeed that use of the latter assay alone will fail to identify the majority of CPent-positive infections.

The ELISA-negative PCR-positive samples may represent a group of patients who are carrying enterotoxigenic *C. perfringens* but in whom CPent is not produced. Thus, the *cpe* gene may be present but not expressed. Alternatively, CPent may not be captured by the ELISA system, for example, if it was a modified toxin or neutralised by host factors [16]. The 13% *cpe*-positive *C. perfringens* rate seen with isolates from the ELISA-negative faecal DNA PCR-negative specimens

may reflect the prevalence of *cpe*-positive isolates in this group of patients. Although it is higher than the 6% rate previously reported [8], this may be expected in a symptomatic patient cohort. Some of these patients may have had earlier CPent-mediated diarrhoea that has since resolved. Such faecal specimens probably failed to yield *cpe*-positive nested PCR results because of low organism load, which was aided by enrichment of enterotoxigenic *C. perfringens*.

A previous study in this hospital with the CPent ELISA kit found that 15% of patients with AAD were CPent positive [3]. While this appears considerably higher than the 8% prevalence rate found in the current study, it was consistent with the similarly higher prevalence of *C. difficile* cytotoxin (32%) in the tested specimens. The prevalence of the *cpe* gene in *C. perfringens* isolates from ELISA-negative faeces in the previous study was 10%, a figure similar to that identified in the current study. The preliminary study highlighted discrepancies between ELISA results and the isolation of enterotoxigenic *C. perfringens*, with 68% of isolates from ELISA-positive samples lacking the *cpe* gene. As in the current study, testing of multiple colonies demonstrated that specimens could contain a mixture of *cpe*-positive and *cpe*-negative strains. To overcome these problems in the current study, PCR was used to demonstrate the presence of the *cpe* gene directly from the faecal specimen.

Evaluation of the PCR method with quantitative techniques to spike faeces with *C. perfringens* broth indicated that the use of a nested PCR protocol could increase the test sensitivity from 8×10^8 cfu/g faeces to <80 cfu/g of faeces (unpublished data). This was essential for the detection of the *cpe* gene in faecal DNA extracts that may contain low numbers of enterotoxigenic *C. perfringens*. Several methods have been described for the detection of CPent in faeces,

including tissue culture assays, ELISA and RPLA [4]. Vero cell tissue-culture assay has been reported to lack sensitivity (40 ng of enterotoxin/g of faeces) and reproducibility for the detection of CPent [16] and this was observed during the present study. Some workers have suggested that the gold standard test for CPent in faeces should be an in-house ELISA developed by the PHLS Central Food Laboratory, because of its high sensitivity (5 ng of enterotoxin/g of faeces), specificity and reproducibility [16]. However, the development of a similar system in most diagnostic laboratories is not feasible. An RPLA kit is commercially available (Oxoid) and, although sensitive and reproducible, non-specific reaction with faecal matter has been recorded [17].

Because *C. difficile* AAD may markedly prolong hospital stay, it is plausible that *C. perfringens* AAD may also result in extended hospitalisation, particularly in frail elderly patients. There is also some evidence that treatment of *C. perfringens* AAD with metronidazole can produce symptomatic benefit [18]. Therefore, there may be justification to support the routine detection by laboratories of CPent in faecal specimens. Resources released from abandoning the routine examination of faecal specimens for (non-*C. difficile*) conventional bacterial enteropathogens in patients who develop diarrhoea after day three of admission could be redirected to CPent testing [19]. Alternatively, CPent testing could be reserved for those faecal specimens found to be *C. difficile* toxin-negative. We conclude that *C. perfringens* is a potentially important cause of infective AAD and can be reliably detected with the TechLab *C. perfringens* enterotoxin ELISA system, although weakly positive results should be considered with caution.

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