Enterotoxigenic *Clostridium perfringens* and sporadic diarrhoea: a study from an Indian tertiary care hospital

Food-borne diseases present a growing health problem worldwide and over 200 different diseases are known to be transmitted by food (Bryan, 1982). Food safety has always been a very significant public health issue, but its global importance is not fully appreciated by many health authorities even now. In the United States, food-borne pathogens have been reported to cause 76 million cases and 5000 deaths each year (Mead *et al.*, 1999). At present, the conventional means for diagnosing food-borne diarrhoea in the microbiology laboratory relies on the culture of bacteria from stool samples. Because there are many food-borne bacterial pathogens, such investigation is usually concentrated only on facultative bacteria. Analyses of food-borne anaerobic bacteria are not routinely performed due to difficulties in their isolation and identification. The role of *Clostridium perfringens* and its enterotoxin in food-borne diarrhoea is well known. More recently, *C. perfringens* enterotoxin has also been implicated as a cause of antibiotic-associated diarrhoea (Borriello *et al.*, 1984), infectious diarrhoea (Larson & Borriello, 1988) and sporadic diarrhoea (Luzzi *et al.*, 1998; Brett *et al.*, 1992). So far no information exists on disease prevalence from our country, even though it is well established in the west. This prompted us to carry out a prospective study to determine the role of enterotoxigenic *C. perfringens* in patients with sporadic, apparently non-food-related, diarrhoea in the Indian population using a duplex PCR and to type the isolates by multiplex PCR.

A total of 200 faecal specimens comprising 100 samples from single isolated cases of acute diarrhoea (both from in-patients and out-patients) and 100 non-diarrhoeal controls received by the Anaerobic Laboratory of the All India Institute of Medical Sciences during the period January 2003–January 2005 were analysed for the presence of *C. perfringens*. All the specimens from which other enteropathogens

(toxigenic *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio*, *Staphylococcus aureus*, *Clostridium difficile*) was isolated were excluded.

Tenfold diluted stool specimens were subcultured onto egg yolk agar (EYA) with neomycin and brain heart infusion agar (BHIA) with a 5 µg metronidazole disc (Hi media). Culture plates were incubated anaerobically at 37 °C for 48 h. Identification of *C. perfringens* colonies was carried out by Gram stain, lecithinase production and other biochemical analyses as described previously (Joshy *et al.*, 2006). Multiple colonies from each culture plate were stored at –20 °C in RCM broth with glycerol (30%) for further analysis.

Detection of *C. perfringens* enterotoxin was performed on the stool specimen by reverse passive latex agglutination (RPLA) (PET RPLA kit; Denka-Sieken) and double sandwich-ELISA (R-Biopharm) using a commercial kit. The assays were performed according to the manufacturer's instructions. The search for enterotoxin production by the isolate was performed after sporulating them *in vitro* in Duncan–Strong sporulation medium.

DNA was extracted from each culture-positive faecal specimen with the QIAamp rDNA stool mini kit (QIAGEN) and 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* stored isolates were cultured on BHI agar. One colony was suspended in 50 µl sterile distilled water and heated to 100 °C for 10 min. Supernatant (10 µl) was used as template in the PCR. For enterotoxin gene detection PCR was done on stool samples as well as on the isolates by duplex PCR (Joshy *et al.*, 2006). Toxinotyping of *C. perfringens* isolates were done using specific primers corresponding to the fragments of toxin genes as described previously (Joshy *et al.*, 2006).

During the two year study period, a total of 200 faecal specimens were examined for *C. perfringens*. Stool specimens from 20 out of 100 (20%) patients were positive for *C. perfringens* by culture. Of these 1% of samples were positive for *C. perfringens* enterotoxin by both RPLA and ELISA. Duplex PCR analysis for enterotoxin gene detection, also showed that 1% of samples were positive for enterotoxin gene.

Of 100 non-diarrhoeal controls 27 (27%) *C. perfringens* strains were isolated. None of the samples were positive for enterotoxin either by RPLA, ELISA or by PCR. Multiplex PCR indicated that all the isolates were of type A, containing only the α toxin gene, β , ϵ and ι genes were absent.

In this study we tried to find an answer to the question whether or not enterotoxin-producing *C. perfringens* strains are casually related to sporadic diarrhoea in our population. Samples known to contain other pathogens were excluded because it would be difficult to interpret the significance of the presence of *C. perfringens* enterotoxin in addition to other enteropathogens such as *Salmonella*. The RPLA kit has been used to investigate cases of sporadic diarrhoea; positivity rates for enterotoxin in faecal specimens ranged from 2.5 to 18% (Samuel *et al.*, 1991; Brett *et al.*, 1992; Mpamugo *et al.*, 1995; Tompkins *et al.*, 1999; Forward *et al.*, 2003). However, there have been problems with the specificity of this test (Food Standards Agency, 2000) and none of those studies involved enterotoxin analysis at the molecular level.

In our study 1 of the 47 isolates gave positive results for both *plc* and *cpe* genes (the presence of 2 bands of phospholipase C gene (283 bp) and enterotoxin gene (426 bp) by duplex PCR. This suggested a prevalence of 2% of *C. perfringens* carrying the *cpe* gene. The observed prevalence of *cpe* gene in *C. perfringens* strains isolated from faecal specimens in this study was close to

those recently reported in molecular epidemiology surveys. Those surveys suggested that only a low percentage (<5%) of all *C. perfringens* isolates from various origins carried the *cpe* gene (Kokai-Kun *et al.*, 1994; Van Damme-Jongsten *et al.*, 1989) whereas the percentage is higher among *C. perfringens* strains isolated from confirmed outbreaks of food poisoning.

None of the *C. perfringens* isolated from healthy controls were carrying the *cpe* gene or were positive for enterotoxin, ruling out the possibility of a carrier state in the analysed group. Toxinotyping showed that all our isolates belonged to type A, which is similar to the other studies reported. Since the numbers of strains analysed is less in the current study, a greater number of strains should be studied before reaching to a conclusion regarding the prevalent geographical types in the area.

Many investigators have described a relation between enterotoxin production and sporulation of *C. perfringens* (Carman, 1997; Duncan, 1973; Zhao & Malville, 1998). Of the isolates 81% (38/47) were producing spores; however, only 2% were producing enterotoxin *in vitro*.

The tests that have been used by previous investigators for diagnosis of *C. perfringens*-associated diarrhoea involved the direct detection of faecal enterotoxin, quantitative stool cultures for *C. perfringens* and the absence of other enteric pathogens in stool specimens. In the present study, we were not able to determine the association between *cpe* gene positive *C. perfringens* in the patient group and other factors, due to the low prevalence of *cpe* gene detected in *C. perfringens* strains isolated from these patients. From this study the *cpe* gene positive case was detected in a patient >15 years, who had fever and abdominal pain. Diarrhoea was of a duration >3 days. Diarrhoea lasting longer than 2 days has been reported in other studies of non-food-borne human gastrointestinal diseases, including antibiotic-associated diarrhoea and sporadic diarrhoea cases (Borriello *et al.*, 1984; Mpamugo *et al.*, 1995). It was also reported that infection was common in the elderly, and symptoms are more severe and longer in duration than

those of *C. perfringens* type A food poisoning (Mpamugo *et al.*, 1995).

To conclude, the results presented here support the fact that sporadic diarrhoea can be caused by enterotoxigenic *C. perfringens*. However, considering the low frequency of enterotoxin in faecal specimens in this study, *C. perfringens* seems to have a limited role in the analysed sporadic diarrhoeal population. The application of these molecular techniques to detect *C. perfringens*, as well as its enterotoxins and their typing, will help not only in diagnosis, but also in epidemiology of infection in various *C. perfringens* outbreaks. This duplex and multiplex PCR will also help us to implicate the aetiology or associating role of *C. perfringens* in diverse clinical syndromes. In the current situation of increasing numbers of immunocompromised patients this relatively mild organism might lead to serious/fatal diseases, if left unrecognized.

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