CLINICAL MICROBIOLOGY

Enterotoxigenic *Clostridium perfringens* as a cause of sporadic cases of diarrhoea

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Summary. The purpose of this study was to investigate the incidence of cases of sporadic diarrhoea associated with enterotoxigenic *Clostridium perfringens*. Cases were identified by detection of *C. perfringens* enterotoxin with the Oxoid RPLA kit, with confirmation by ELISA, in faecal specimens from isolated incidents of diarrhoea and from which no other enteropathogen had been isolated. In a 2-month study, 65 (18%) of 370 specimens were enterotoxin positive. There was no predominant age group or sex in the enterotoxin-positive group, but a higher proportion (79%) was resident in the community than were enterotoxin-negative cases (34%). Only four of the 65 enterotoxin-positive patients had received antibiotic therapy. Spore counts in most enterotoxin-positive patients were $< 10^5$ /g, indicating that detection of high numbers of *C. perfringens* is not useful in determining the aetiology of sporadic diarrhoea. Diagnosis should be confirmed by the detection of enterotoxin, but further work is required to assess whether an acceptable accuracy is obtained with the RPLA kit or whether ELISA is needed in all cases.

Introduction

It is well recognised that outbreaks of foodborne diarrhoea are caused by enterotoxigenic *Clostridium perfringens*.¹ More recently, the organism has been implicated as a cause of antibiotic-associated diarrhoea,² infectious diarrhoea³ and sporadic diarrhoea.^{4,5} The aim of this study was to investigate the prevalence of sporadic cases of diarrhoea associated with *C. perfringens* enterotoxin.

Patients and methods

Patients

All faecal specimens from single isolated cases of acute diarrhoea received by Ashford Public Health Laboratory in July and August 1991 were examined routinely for salmonellae, shigellae, campylobacters, aeromonas and cryptosporidium. Examination for *C. difficile*, *Escherichia coli* (ETEC and VTEC), *Staphylococcus aureus* and *Bacillus cereus* and their toxins, vibrios, yersiniae, parasites and viruses was done if indicated by clinical details—age, recent foreign travel, antibiotic treatment or the possibility of food

poisoning. All specimens from which no recognised enteropathogen was isolated were sent to the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London for study.

Enterotoxin detection

One volume of faeces was mixed with up to four volumes of phosphate-buffered saline, pH 7.3, centrifuged at 20000 *g* for 20 min at 4°C, filtered through a 0.2- μ m membrane filter, then tested for *C. perfringens* enterotoxin with the Oxoid RPLA kit (Unipath).^{5–7} Positive and negative controls were included on each plate. All samples with a one-well or greater difference between agglutination with sensitised and control latex particles were tested by ELISA.^{5,8} Duplicate samples of each extract were incubated in triplicate for 75 min at room temperature with and without neutralising antibody before incubation for 70 min in plates that had been coated with rabbit IgG against enterotoxin and blocked with bovine serum albumin 1%.^{5,8} After washing, rabbit anti-enterotoxin conjugated to horse-radish peroxidase was added, incubated for 2 h, and removed by washing; then orthophenyldiamine in citrate phosphate buffer was added. The reaction was stopped with 100 μ l of 4 M H₂SO₄ and the absorbance was read at 492 nm in a microtitration plate reader (Titertek, Multiscan MCC 341; ICN-Flow Labora-

tories Ltd, Herts). Wells containing no absorbed antibody or no test extract were included in each plate and gave an absorbance of < 0.1 . A set of dilutions of an enterotoxin standard (0.001 – $0.1 \mu\text{g/ml}$) on each plate was used to create a standard curve from which the enterotoxin concentration was calculated.

Culture of *C. perfringens*

C. perfringens spore counts were determined following alcohol shock.⁹ Serial 10-fold dilutions of faecal suspensions after treatment with an equal volume of ethanol 95% v/v in distilled water were made in Maximum Recovery Diluent (Unipath) and plated on to Columbia Blood Agar plates according to a modified Miles and Misra method.¹⁰ Isolates were identified as *C. perfringens* by their reaction on Egg Yolk Nagler plates with anti-*C. perfringens* toxin A (Wellcome, Dartford).¹¹

Results

During the 2-month study period, Ashford PHL received 370 faecal specimens from isolated, sporadic cases of diarrhoea. Enteropathogens other than *C. perfringens* were isolated from 99 specimens, which were excluded from this study, but not all faecal specimens were screened for all enteropathogens. There was insufficient sample for study in 59 cases and a total of 212 specimens was examined for *C. perfringens* enterotoxin.

The manufacturers make no recommendation as to whether a one-well or a two-well difference is considered positive. With the criterion of a two-well or more difference between agglutination of sensitised and control latex in the RPLA, enterotoxin was detected in 45 specimens. The positive results were confirmed by ELISA in 43 of the 45 specimens. A further 56 specimens gave a one-well difference in the RPLA; enterotoxin was confirmed by ELISA in 22 of these specimens. Thus, enterotoxin was detected by ELISA in a total of 65 of the 212 faecal specimens. A low concentration of enterotoxin was more common in specimens with a one-well difference in the RPLA ($< 0.10 \mu\text{g/g}$ in 17 of the 22 samples) than in specimens

with a two-well difference ($< 0.10 \mu\text{g/g}$ in 17 of the 43 samples).

C. perfringens faecal spore counts were determined in 61 of the 65 enterotoxin-positive samples. Spore counts were $< 10^5/\text{g}$ of faeces in 42 (69%) of the 61 samples. There was no correlation between spore count and enterotoxin concentration.

The ratio of male to female patients in enterotoxin-positive and enterotoxin-negative groups was similar (table). No age group predominated in either group, although one third of cases in both groups were > 60 years old. A higher proportion of the enterotoxin-positive group was resident in the community (71%) than of the enterotoxin-negative group, who were more commonly resident in hospital (66%).

Information on antibiotic treatment was available for 132 patients. In both enterotoxin-positive and enterotoxin-negative groups, most patients had not received antibiotic therapy (table).

The duration of diarrhoea was known in 15 patients with enterotoxin-positive diarrhoea. In 12 cases, diarrhoea was prolonged: 3–6 days in three cases, 1–3 weeks in five cases and 4–6 weeks in four cases. Antibiotic treatment was also known in nine of these patients. One patient receiving antibiotics had diarrhoea which lasted for 2 weeks; eight patients were not treated and the duration of diarrhoea was 8–42 days, with a mean of 23.25 days.

Discussion

Faecal specimens from 370 cases of sporadic diarrhoea were examined but 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 another enteropathogen such as a salmonella.

The Oxoid RPLA kit was used as a preliminary screening test to detect *C. perfringens* enterotoxin. The presence of enterotoxin was confirmed by ELISA in 43 of 45 specimens with a two-well or more difference in the RPLA and in 22 of 56 specimens with a one-well difference; thus, 65 of the 212 specimens were enterotoxin-positive. In the ELISA, extracts were pre-

Table. Comparison of patients with enterotoxin-positive and enterotoxin-negative faeces

Patient characteristics	Enterotoxin-positive faeces (%)	Enterotoxin-negative faeces (%)
Number of patients	65	147
Number resident in		
hospital	19 (29)	97 (66)
community	46 (71)	50 (34)
Antibiotic treatment	4/37 (11)	16/95 (17)
Ratio male/female*	29:35	57:89
<i>C. perfringens</i> spore count $< 10^5/\text{g}$ faeces	42/61 (69)	NT

NT, not tested.

*One unknown in each group.

incubated with and without neutralising antibody against *C. perfringens* enterotoxin, to provide an internal blank for each specimen, so that the lower level of detection was 0.01–0.02 µg/g of faeces.^{5,7,8} Two extracts were positive in the RPLA but this was not confirmed by ELISA. This could be due to errors inherent in the doubling dilutions used in the RPLA or because interference in the ELISA was not removed by the neutralising antibody. Enterotoxin was detected by ELISA in 22 extracts that gave a one-well difference in the RPLA. Non-specific interference was high in the RPLA in 11 of these specimens and the end-point for positive or control latex, or both, was unclear in seven extracts. In the remaining four specimens, the enterotoxin concentration was close to the lower level of detection of the RPLA. In the RPLA, a two-well difference is generally considered to be necessary to keep false-positive results to an acceptably low level, but positive samples with high levels of non-specific interference or with low enterotoxin levels may be missed. Interference by faecal constituents with enterotoxin detection in the RPLA has been reported in some^{12,13} but not other⁷ studies, so that the maximum sensitivity may be only 0.05–0.10 µg/g of faeces^{12,13} rather than 0.002–0.004 µg/g of faeces.⁷ The results presented here indicate that the use of a two-well difference in the RPLA may detect only two-thirds of enterotoxin-positive cases, whereas the use of a single-well difference would result in a false-positive rate of c. 30%.

The prevalence of *C. perfringens* enterotoxin in cases of sporadic diarrhoea in this study (31%) was higher than that of an earlier study (7%).⁵ This may be due in part to the inclusion in this study of specimens with a one-well difference in the RPLA. Faecal specimens in the present study were not screened for all entero-

pathogens, so some may have contained pathogens other than *C. perfringens*. Furthermore, single point food sources of enterotoxigenic *C. perfringens* cannot be excluded in some cases. Diarrhoea was prolonged in 12 of 15 patients in whom information was available, which confirms the results of a previous study⁵ and is in contrast to *C. perfringens* food poisoning, in which diarrhoea generally resolves within 12–24 h.

The proportions of males and females were similar in enterotoxin-positive and enterotoxin-negative diarrhoea, which confirms the findings of the earlier study,⁵ but in the present study a lower proportion of the enterotoxin-positive group were resident in hospital than of the enterotoxin-negative group.

C. perfringens spore counts in faeces were within the normal range, < 10⁵/g, in 69% of enterotoxin-positive patients. High spore counts are found in most, but not all, patients associated with outbreaks of food poisoning^{14–17} but have also been reported in the faeces of healthy elderly people.^{18–20} Low numbers were unlikely to be due to loss of viability *in vitro* as spore counts are stable for weeks or months at –20°C, 4°C and room temperature.^{17,19} There was no correlation between spore count and enterotoxin concentration.

This study confirms that enterotoxigenic *C. perfringens* is associated with sporadic diarrhoea in the absence of antibiotic treatment. A screening test to detect high numbers of *C. perfringens* spores is of limited value, and confirmation of diagnosis can be based only on the detection of the enterotoxin, but further work is needed to assess whether an acceptable accuracy of diagnosis is obtained with the RPLA or whether ELISA is necessary in all cases.

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