

Detection of *Clostridium difficile* cytotoxin and *Clostridium perfringens* enterotoxin in cases of diarrhoea in the community

L. J. Forward,¹ D. S. Tompkins¹ and M. M. Brett²

Correspondence

D. S. Tompkins

david.tompkins@hpa.org.uk

¹Health Protection Agency, Yorkshire and the Humber Region, Leeds Laboratory, Bridle Path, Leeds LS15 7TR, UK

²Food Safety Microbiology Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

Faecal specimens from 843 cases of diarrhoea in the community were tested for the presence of *Clostridium difficile* cytotoxin and *Clostridium perfringens* enterotoxin. *C. difficile* cytotoxin was detected in faecal specimens from 0.6 % of cases aged at least 2 years by using a Vero cell assay. Factors associated with detection of *C. difficile* cytotoxin were antibiotic therapy, age over 60 years and living in a home with other elderly people. Three methods were used for the detection of *C. perfringens* enterotoxin: a Vero cell assay, a commercial (TechLab) enzyme immunoassay (EIA) and an in-house EIA. The lower level of detection of pure *C. perfringens* enterotoxin in buffer was 0.01 µg ml⁻¹ by the TechLab EIA and 1.0 µg ml⁻¹ by the Vero cell assay. *C. perfringens* enterotoxin was detected by using the TechLab EIA in faecal specimens from 2.5 % of cases. This commercial EIA was less sensitive than the in-house EIA, detecting only 31 % of positive cases, but was specific and could be used for outbreak investigation by routine diagnostic laboratories. Age over 60 years was a factor associated with *C. perfringens* enterotoxin detection; this age group may be targeted for testing.

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INTRODUCTION

Gastroenteritis is a major cause of ill-health. It has been estimated that 9.4 million individuals suffer from symptoms of infectious intestinal disease every year in England alone, and one-sixth of those attend their general practitioner (GP) for advice (Wheeler *et al.*, 1999). The aetiology of much of this illness remains unknown (Tompkins *et al.*, 1999).

Clostridium difficile is the major cause of antibiotic-associated diarrhoea (AAD) in hospitals (Spencer, 1998). There have been few studies on the incidence of AAD in the community in Britain (Brazier, 1998; Stone, 1999). The Study of Infectious Intestinal Diseases in England (IID Study) identified 1.7 % of cases that presented to GPs with symptoms of gastroenteritis (1.1 % of those aged over 2 years) with stool specimens that were positive for *C. difficile* cytotoxin (CDC) (Food Standards Agency, 2000). *Clostridium perfringens* is recognized as a cause of outbreaks of food poisoning and *C. perfringens* enterotoxin (CPE) has also been detected in cases of antibiotic-associated and sporadic diar-

rhoea (Borriello *et al.*, 1984; Larson & Borriello, 1988; Samuel *et al.*, 1991; Brett *et al.*, 1992; Mpamugo *et al.*, 1995; Wada *et al.*, 1996). In the IID Study, 4 % of patients that presented to GPs with symptoms of gastroenteritis were CPE-positive; however, there were some technical difficulties with the screening test used, a reversed passive latex agglutination (RPLA) test (PET-RPLA; Oxoid). A commercial enzyme immunoassay (EIA) test has been described as a sensitive method for detection of CPE in hospital patients with AAD (Hancock, 1997). Currently, few laboratories routinely examine stool specimens from patients in the community for CPE or CDC unless investigation for AAD is requested specifically.

Current practice at Leeds Public Health Laboratory Service (PHLS; in England, the PHLS became incorporated into the Health Protection Agency on 1 April 2003) is to investigate outbreaks of food poisoning by culture of faecal specimens for *C. perfringens*. Specimens are submitted to the PHLS Food Safety Microbiology Laboratory (FSML), Central Public Health Laboratory, for CPE detection. Cases of sporadic gastroenteritis are not investigated for *C. perfringens* or its enterotoxin. A Vero cell assay is used to detect CDC in faecal specimens from GPs who either request investigation for *C. difficile* or indicate antibiotic use, according to PHLS standard operating procedures.

Abbreviations: AAD, antibiotic-associated diarrhoea; CDC, *C. difficile* cytotoxin; CPE, *C. perfringens* enterotoxin; EIA, enzyme immunoassay; FSML, Food Safety Microbiology Laboratory; GP, general practitioner; PHLS, Public Health Laboratory Service; RPLA, reversed passive latex agglutination.

The aims of this prospective study were: (1) to evaluate the use of the commercial EIA and the Vero cell assay as screening tests for the detection of CPE in faecal samples from sporadic community cases of gastroenteritis by using the PHLS FSML in-house EIA as the gold standard; (2) to determine the frequency of CPE and CDC detection in stool samples from sporadic cases of gastroenteritis that present to GPs in the Leeds area of England and to identify risk factors for diarrhoeal disease caused by CPE or CDC; (3) to use this information to assist in the development of a cost-effective stool-testing strategy.

METHODS

Between November 1999 and April 2000, 843 faecal specimens from patients with gastroenteritis that presented to GPs in Leeds, England, were tested. All specimens were tested for *Salmonella*, *Shigella* and *Campylobacter* spp. and selected specimens were also tested for *Escherichia coli* O157, group A rotavirus, *Cryptosporidium* and other enteric pathogens, according to routine laboratory protocols. Specimen residue was stored at -20°C and tested within a few days, in batches, for the presence of both CDC and CPE by using Vero cell assays and for the presence of CPE by using a commercial EIA (TechLab) at Leeds PHLS. Subsequently, all specimens were sent to FSML for testing for CPE by their in-house EIA, which was performed as described by Bartholomew *et al.* (1985).

All clinically significant findings were reported by telephone to GPs and also to the local Consultant in Communicable Disease Control, following local and national protocols. Questionnaires were issued to investigate the symptoms experienced and to ascertain any association between disease and antibiotic prescribing, food history and hygiene practices. Ethical approval for this study was obtained from both the Leeds and PHLS research ethics committees.

Vero cell assays. A sterile, flat-bottomed, 96-well microtitre tray was seeded with 50–100 μl of a suspension of Vero cells (approx. 200 000 cells ml^{-1}) in Eagle's minimal essential medium (MEM). The plate was incubated overnight in 5% CO_2 in a moist atmosphere at 37°C to produce a monolayer of cells. The medium was removed and replaced with 160 μl maintenance medium (MEM with 2% fetal calf serum).

Faecal extracts were prepared by mixing approximately 0.2 g faeces in 1.0 ml diluent [PBS (Dulbecco A) that contained 0.002% (w/v) phenol red, 0.95 mM MgCl_2 , 0.81 mM CaCl_2 , 0.0009% (w/v) amphotericin, 0.06% (w/v) benzylpenicillin, 0.01% (w/v) streptomycin and 0.01% (w/v) kanamycin] and clarified by centrifugation at 4000 r.p.m. for 10 min. Each extract was tested as described below and the residuum was forwarded to FSML. Six wells were required to test each specimen for CDC and CPE. Faecal extract (20 μl) was added to well 1, mixed with MEM and transferred to well 2, giving an approximately 1 : 10 dilution. This procedure was repeated with wells 3 and 4 and wells 5 and 6, to which 20 μl diluted antiserum had been added. For identification of CDC, antiserum to *Clostridium sordelli* (PHLS Supplies) was used (diluted 1 : 100) in wells 3 and 4 and for identification of CPE, antiserum to CPE (Biogenesis) diluted 1 : 400 in PBS was used in wells 5 and 6. Dilutions for the antisera were pre-determined by using chequerboard titrations. With each batch of specimens that was processed, one row of the microtitre plate was used for controls for the Vero cells, antisera and positive controls (1 μg CPE ml^{-1} supplied by M. Brett, FSML, and a $1 : 10^5$ dilution in Dulbecco A medium of filter-sterilized supernatant of a cytotoxin-producing culture of *C. difficile*, supplied by J. Brazier, Cardiff PHLS). The plate was covered and incubated in a moist atmosphere with 5% CO_2 at 37°C overnight, then the cell cultures were examined for cytotoxicity. A specimen was considered to contain

CPE or CDC if the characteristic cytopathic effect was observed at either concentration of the faecal supernatant in the absence of specific antiserum and was not observed in the presence of homologous antiserum. Specimens that showed verotoxicity at only the highest concentration of supernatant were classed as toxic, with the effect probably being due to the toxic effect of faecal intestinal proteases rather than to low levels of a specific verocytotoxin.

The sensitivity of the assay for CPE was determined by using pure CPE that was diluted with diluent (as described above) to give tenfold dilutions from 1 μg ml^{-1} to 100 pg ml^{-1} . A 20 μl aliquot of each dilution was used to inoculate Vero cells, which were subsequently covered and incubated in a moist atmosphere with 5% CO_2 at 37°C overnight, then examined for cytotoxicity.

TechLab EIA. As the technical information supplied with the TechLab kit did not state the sensitivity and specificity of the assay, a tenfold dilution series of pure CPE from 10 μg ml^{-1} to 100 pg ml^{-1} was prepared in PBS and 100 μl of each was used in the initial step of the EIA to determine sensitivity.

Liquid faeces (50 μl) or an amount of formed/semi-formed faeces that was equal to approximately 3 mm in diameter was added to 200 μl diluent and the manufacturer's instructions were followed for performance of the test. Optical density was read at 450 and 620 nm in an Anthos Labtec 2001 plate-reader. Wells that contained no conjugated antibody or CPE were included in each plate as negative and positive controls, respectively. Specimens that gave an optical density reading > 0.07 were classed as positive if the positive and negative controls fell in their respective ranges. Optical density readings in the range 0.05–0.07 were classed as equivocal and readings < 0.05 were regarded as negative. All specimens that gave equivocal or positive readings were repeated.

Questionnaires. Two questionnaires were issued for each case in which the detection of CPE or CDC was considered to be clinically significant, following tests performed at Leeds PHLS. One brief questionnaire was completed by the GP, giving details on any antibiotics prescribed in the 4 weeks prior to the specimen being taken and any hospital in-patient stays in the preceding 3 months. The GP also forwarded a questionnaire to the patient, asking for details of their symptoms, food hygiene and food consumption in the 2 days before they became ill. Questionnaires not returned after 2 weeks were followed up by telephone enquiry.

RESULTS

Demographic data and questionnaire compliance

Information on age and sex was available for patients who submitted 808 of the 843 specimens, with 43% male, 57% female and 19% aged 60 years or more (Fig. 1). Of the 24 questionnaire sets that were issued, 13 were returned from cases and 19 were returned from their GPs.

Frequency of clostridial toxins

CDC was detected in 10 specimens from 10 cases, of which five were aged < 2 years (Table 1). Presence of CDC is a normal finding in this age-group; therefore, only five cases were clinically significant (0.6% of specimens tested). No other enteric pathogens were detected by routine methods in the 10 CDC-positive samples.

CPE was detected in 21 specimens from 19 cases by the TechLab EIA (frequency in specimens, 2.5%). The Vero cell

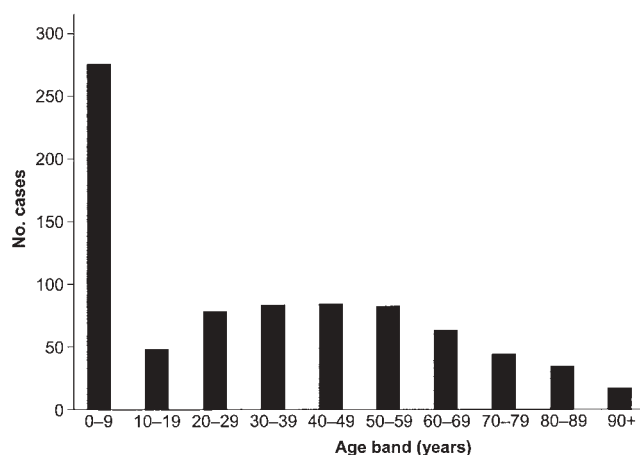


Fig. 1. Age distribution of study participants for whom information was provided.

Table 1. Age distribution of cases that were positive for CDC or CPE by using the TechLab EIA

Age (years)	CDC	CPE
< 2	5	0
30-39	0	1
40-49	0	1
50-59	1	2
60-69	0	1
70-79	0	5
80-89	2	3
90+	2	6

assay detected CPE in 11 of these 21 EIA-positive specimens. No specimens were both EIA-negative and Vero cell assay-positive for CPE. These results reflect the 100-fold difference in sensitivity of the two tests (detection of 0.01 and 1.0 μg purified enterotoxin ml^{-1} in buffer by using the TechLab kit and the Vero cell assay, respectively). One patient with CPE also had a *Campylobacter* infection. No specimens contained both CDC and CPE. The FSML EIA detected CPE in 62 (7.4%) of the 843 specimens, including all 21 detected by the TechLab EIA (Table 2). The age distribution of CPE-positive cases detected by the two EIAs is shown in Fig. 2, with a broad age range of positive cases detected by the FSML EIA, but predominantly elderly cases detected by the less sensitive TechLab EIA.

Demographic and risk factor analysis

Lower numbers of cases with CDC and CPE were identified than had been expected from previous studies, so numbers were too low for meaningful analysis of food history and hygiene. However, living in a residential or nursing home, recent hospital admission and recent exposure to antibiotics were potential risk factors that were recorded in the questionnaires returned by GPs. From Table 3, it can be seen that

Table 2. Comparison of TechLab EIA and FSML EIA for the detection of CPE

FSML EIA	TechLab EIA		
	Positive	Negative	Total
Positive	21*	41	62
Negative	0	781	781
Total	21	822	843

*The Vero cell assay detected CPE in 11 of these specimens.

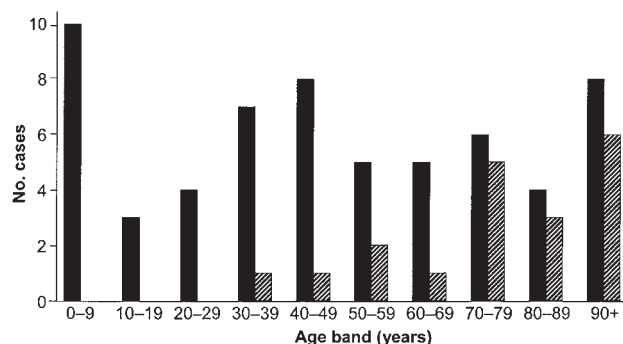


Fig. 2. Age distribution of cases that were positive for CPE by the TechLab EIA (hatched bars) and FSML EIA (filled bars).

age > 60 years, antibiotic therapy and residence in a home with other elderly people were more frequently associated with CDC than CPE in this study, although the numbers are small. Of 19 cases that were positive for CPE with the TechLab EIA test, 15 (80%) were aged > 60 years.

Persistent diarrhoea (median and mean, 9 days) was reported by the nine CPE-positive cases who returned a questionnaire.

DISCUSSION

The purpose of this study was to gather information to enable a strategy to be developed for clostridial toxin testing of routine faecal samples from community patients who attend

Table 3. Putative risk factors for cases of gastroenteritis with CDC or CPE (positive by TechLab EIA) detected in stool samples

	CDC	CPE
Total cases	5	19
Living in nursing or residential home	5/5	7/19
Hospital in-patient in previous 3 months	2/4	3/15
Antibiotics prescribed in previous month	4/4	2/14
Aged > 60 years	4/5	15/19

their GP with gastroenteritis. Although AAD caused by *C. difficile* is a major and increasing problem in hospitals, there has been less interest in defining the extent of this disease in the community setting (Brazier, 1998). Detection of CDC is the accepted diagnostic test for AAD, as 2–3 % of healthy adults are carriers of the organism (Department of Health & Public Health Laboratory Service Joint Working Group, 1994). The IID Study found that 21 of 1866 cases (1.1 %) aged over 2 years who attended GPs were positive for CDC, with a further 1 % that were positive by culture only (Food Standards Agency, 2000). Three of 1616 asymptomatic controls (0.2 %) were CDC-positive. The population incidence for *C. difficile*-associated disease in this GP case-control study was 20 per 100 000 person-years, identical to the findings of a prospective study in Sweden and higher than those of two retrospective studies in the USA (Hirschhorn *et al.*, 1994; Karlström *et al.*, 1998; Levy *et al.*, 2000). Riley *et al.* (1986) tested 1882 community cases of diarrhoeal illness in Western Australia and found 36 (1.9 %) that were positive for CDC. Positivity rates were higher in patients when *C. difficile* investigation was requested by the GP (22.5 %) or when patients were known to have received antibiotics (11.4 %) (Riley *et al.*, 1986). These findings were confirmed in subsequent studies and others have also found strong associations between diarrhoeal disease caused by CDC and antibiotic use in the community setting (Riley *et al.*, 1991; Hirschhorn *et al.*, 1994; Karlström *et al.*, 1998; Levy *et al.*, 2000).

We found 0.6 % of specimens to be positive for CDC, excluding children aged < 2 years, when faecal carriage of *C. difficile* and presence of cytotoxin is a common finding in healthy asymptomatic individuals (Brazier, 1998). All five significant cases that were positive for CDC were living in nursing or residential homes. The problem of *C. difficile*-associated disease and increased death rate in long-term care facilities and nursing homes has been recognized for over a decade (Bentley, 1990; Thomas *et al.*, 1990; Garibaldi, 1999). However, *C. difficile* is still largely considered to be a hospital-based problem (Spencer, 1998). Routine diagnostic laboratories may test all diarrhoeal stools from hospital in-patients for CDC, but specimens submitted by GPs (including specimens from residents of nursing homes) are only tested for CDC when investigation for *C. difficile* is specifically requested or AAD is mentioned in the clinical details.

C. perfringens was first described as a cause of AAD in 1984 and later as a cause of sporadic diarrhoea, particularly in elderly patients in hospital (Borriello *et al.*, 1984; Larson & Borriello, 1988). Detection of CPE is the definitive diagnostic test, as all healthy adults are carriers of the organism (Brett *et al.*, 1992). The RPLA kit has been available for over a decade, but testing for CPE in routine diagnostic laboratories has not been advocated in published guidance documents or in the PHLS standard operating procedures (Pedler & Orr, 1990). The in-house FSML EIA and the RPLA tests are both recognized to be more sensitive than Vero cell assays for detection of CPE (Berry *et al.*, 1988). The Vero cell assay for CPE was included in this study to assess the practical use of

this test in a routine diagnostic setting where a Vero cell assay is in current use for CDC detection. The RPLA kit has been used to investigate cases of sporadic diarrhoea; positivity rates for CPE in faecal specimens ranged from 3.5 to 18.0 % (Samuel *et al.*, 1991; Brett *et al.*, 1992; Mpamugo *et al.*, 1995). However, there have been problems with the specificity of this test (Berry *et al.*, 1988; Food Standards Agency, 2000). Two of the earlier studies found an increased frequency of CPE detection in specimens from elderly patients than those from younger patients and that diarrhoea lasted for longer than in classical food poisoning (Samuel *et al.*, 1991; Brett *et al.*, 1992). There was a significant association with previous antibiotic use in one of these studies (Samuel *et al.*, 1991) but not in the other, although numbers were small. Both studies included cases from the community as well as hospital in-patients. In the IID Study, faecal specimens from 114 of 2871 (4.0 %) of sporadic cases that attended GPs gave a positive result for CPE by using RPLA and were confirmed by the FSML EIA (Tompkins *et al.*, 1999). This combination of tests also detected 15 specimens that were positive for CPE from 2256 asymptomatic controls (0.7 %). The age-range of the positive cases was wide and the median duration of diarrhoea was 4 days (Food Standards Agency, 2000). In the present study, 7.4 % of specimens were positive by the FSML EIA, again with a wide age-range. However, all 19 cases in this present study with CPE detected by the TechLab EIA test were adults and 15 were over 60 years of age, with seven living in nursing or residential homes. All nine cases who completed questionnaires reported diarrhoea, with median and mean durations of 9 days. Only two of 14 cases had received antibiotics in the previous month.

This study confirms that, as in other countries, the incidence of *C. difficile*-associated disease is low in the community in the UK. GPs should be encouraged to request a CDC test in patients with diarrhoea following antibiotics. Although numbers were low, this study has provided some evidence to support the view that *C. difficile*-associated disease is a potential problem in UK nursing homes (Stone *et al.*, 1999). Further study is required but, as the elderly are known to be at greatest risk (Spencer *et al.*, 1998), it would be advisable for laboratories to test all specimens of faeces submitted from nursing home residents for CDC.

The TechLab EIA for CPE was less sensitive than the FSML EIA. From our results, it appears that higher levels of toxin were found in patients aged 60 years or more than in younger patients; however, numbers are small. In our hands, the TechLab EIA was specific and could be used in routine diagnostic laboratories to detect CPE in older patients with diarrhoea of several days' duration and in suspected outbreaks of food poisoning. The Vero cell assay for CPE is too insensitive for routine use, but specimens tested for CDC with an abnormal cytopathic effect that is not neutralized by *C. sordelli* antiserum should be tested locally or referred for specific CPE tests.

The results of this study suggest that testing for CPE would be of value in cases of diarrhoea that are aged 60 years or more,

regardless of recent history of antibiotic treatment. The TechLab EIA kit gives routine diagnostic laboratories a practical test for CPE that should be considered when developing local strategies for testing faecal specimens.

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