

Clostridium difficile and cytotoxin in routine faecal specimens

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SUMMARY Over a five-month period 1239 unselected, routine faecal specimens from 856 patients were examined for *Clostridium difficile*. One hundred specimens representing 69 patients were culture-positive. Toxin was detected in the stool of ten. During the study period, there were 41 Salmonella, 12 Campylobacter and 9 Shigella infections. *C difficile* was isolated together with Salmonella from 12 patients. No patient required specific treatment for *C difficile* infection. The significance of these findings is discussed.

The role played by *Clostridium difficile* in the aetiology of pseudomembranous colitis (PMC)¹⁻⁴ and some cases of antimicrobial-induced diarrhoea⁵⁻⁷ is now well documented. However, this organism with its cytotoxin has also been found in healthy neonates^{4, 8} and in asymptomatic adults on antibiotics.⁹ Clearly, if the detection of *C difficile* cytotoxin in stool specimens is to be a useful diagnostic tool a thorough knowledge of its distribution in stool specimens from all sources is necessary. This study was designed to investigate the occurrence of *C difficile* and its cytotoxin in "routine" specimens of faeces received at a microbiological laboratory and to determine whether estimation of cytotoxin titres in culture-positive stool specimens would help to distinguish between benign as opposed to harmful symptomatic carriage of *C difficile*.

Material and methods

SPECIMENS

Examination was carried out on 1239 (unselected) consecutive stool specimens received at the routine microbiological laboratory at Whipps Cross Hospital. These included specimens from hospital inpatients and outpatients as well as Public Health Laboratory specimens.

CULTURE

All specimens were cultured for *Salmonella*, *Shigella* and *Campylobacter* spp, using recommended laboratory methods. A selective agar medium, cycloserine, cefoxitin, fructose, blood agar (CCFA Oxoid) was

used for the isolation of *C difficile*.^{10, 11} Faecal specimens were diluted tenfold in Ringer's solution and emulsified before inoculation. All cultures were incubated for 48 h in Gaspak jars (Becton Dickinson, UK).

Colonies resembling *C difficile* were inoculated on blood agar medium (Oxoid) and incubated anaerobically for a further 48 h. Suspect *C difficile* strains, recognised by their typical morphological appearance, smell, and green fluorescence under longwave ultraviolet light, were inoculated into Robertson cooked meat medium and incubated for 48 h. The strains were further identified by their typical pattern of volatile short chain fatty acids,¹² and concentrations calculated on a DP 101 computing integrator (Pye Unicam).

CYTOTOXIN DETECTION

Faecal suspensions were stored at 4°C for 48 h (72 h at weekends) to await the preliminary culture result from the reading of the CCFA plates. Supernatants were prepared from all culture-positive suspensions by separation in a refrigerated centrifuge. Decanted supernatants were screened for cytotoxin in Hela cell tissue culture and stored at -20°C for further testing. *C difficile* cytotoxin was said to be present when the development of a cytopathic effect after 24 or 48 h could be neutralised by *Clostridium sordellii* antiserum (Wellcome Research Laboratory).^{1, 2} All cytotoxin containing specimens were titred to an end point in doubling dilutions.

CYTOTOXIN PRODUCTION BY *C DIFFICILE* STRAINS

Supernatants from 48-hour cooked meat broth

cultures of *C. difficile* strains were tested at an initial dilution of 1/8 for cytotoxin in a similar manner to the faecal specimens.

Results

A flow chart (Fig. 1) summarises the findings, 1239 specimens from 856 patients were examined. *C. difficile* was isolated from 69 patients. More than one culture-positive specimen was received from 13 patients, the range was 2-6 specimens over a period of two weeks to three months. The total number of *C. difficile* isolates was 100. Each of these 13 patients consistently carried either a cytotoxin-producing or a non-toxin producing strain.

The age distribution of culture-positive patients is

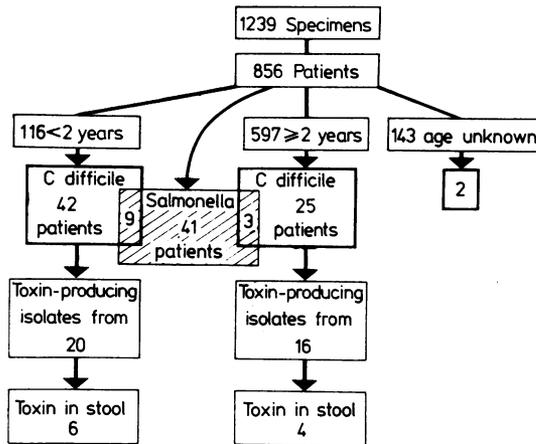


Fig. 1 A flow chart summarising the *C. difficile* isolation rates and results of cytotoxin detection tests. Specimens referred to are routine faecal samples.

shown in Figs 1 and 2. The overall isolation rate of *C. difficile* was 8% (69/856); 36% in those less than two years old and 4% in those more than two years of age. Cytotoxin-producing strains were carried by 36 of the 69 positive patients (52.2%). The percentage of cytotoxin-producing strains was higher in those over two years of age (64%) than in those under two years (47.6%).

Cytotoxin was detected in the stool of 10 patients (Table 1), all were carriers of toxin-producing strains. Six were infants, four were adults on antibiotics, one of whom had sigmoidoscopic evidence of colitis. In the infant group, one acquired the organism and gradually developed a rising cytotoxin titre during the study period (patient 5—Table 1). Another had a diminishing cytotoxin titre after a *Salmonella* sp infection. Among the adult patients, one changed from cytotoxin-negative stool to a cytotoxin titre of 1/160 over a period of five days. Another had a falling cytotoxin titre (patient 10), after withdrawal of antibiotics and resolution of symptoms.

Table 2 illustrates the findings in eight adult patients complaining of diarrhoea. *C. difficile* was isolated from all patients, six of the eight strains were cytotoxin producers in vitro, but none of the patients had detectable cytotoxin in their stools. Five were on antibiotics, one had ulcerative colitis, and one had returned from abroad but no pathogens were isolated.

Details of the 41 *Salmonella* infections appear in Table 3. *Clostridium difficile* was isolated from 9/9 of those below two years of age and 3/20 from those older than 2 yr. There were 12 *Campylobacter* and nine *Shigella* infections. One of each (aged 58 and 7 yr respectively) were culture-positive for *C. difficile*. The overall incidence of infections with other enteric pathogens in association with *C. difficile* was 20%.

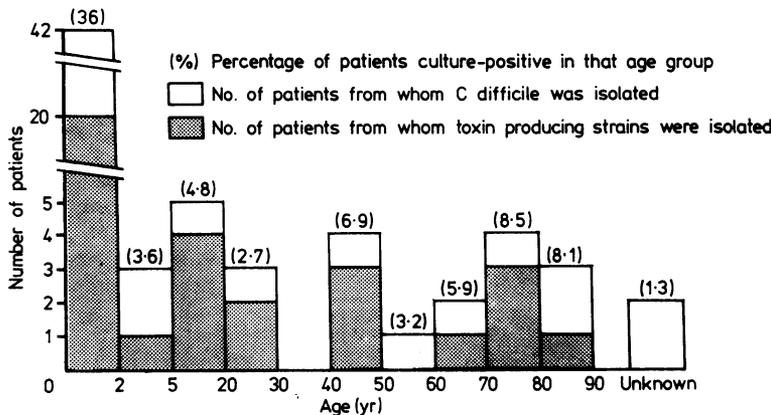


Fig. 2 Histogram showing the detailed distribution of *C. difficile* isolates in the different age groups.

Table 1 Details of patients with *C difficile* toxin in their faeces

Patient No	Sex	Age	<i>C difficile</i> * isolated	Cytotoxin titre (in faeces)	Day (after 1st specimen)	Antimicrobial therapy	Clinical information
1	M	7 wk	+	1/160		Nil	Febrile convulsion, no diarrhoea
2	F	5 months	+	1/160		Nil	"Food Poisoning"
3	M	4 months	+	1/6400		Nil	Shigella contact. No symptoms
4	M	5 months	+	1/1280		Nil	Vomiting, weight loss, failure to thrive
5	F	3 months	-	1/2560 Not tested	60	Nil	Premature baby. <i>E coli</i> 055 carrier, no symptoms
6	F	5 months	+	1/40 320 1/640	10 59	Nil	<i>Salmonella typhimurium</i> infection
7	M	45 yr	+	1/320 1/40 1/800	30 100	Metronidazole	No symptoms, post treatment of amoebic colitis
8	F	40 yr	+	Not detected		Flucloxacillin + gentamicin	Diarrhoea, staphylococcal septicaemia
9	F	82 yr	+	1/160 1/2560	5	Cephazolin Cephalexin	Cholecystitis, antibiotic-associated colitis. Multiple ulcers in rectum. Spontaneous recovery off antibiotics
10	F	76 yr	+	1/2560 1/80	30	Cephazolin + metronidazole	Post gastroenterostomy, diarrhoea, spontaneous recovery off antibiotics

*All strains were cytotoxin producers in vitro. + = present. - = absent.

Table 2 Findings in eight patients presenting with diarrhoea but no *C difficile* cytotoxin in their faeces

Patient No	Age (yr)	<i>C difficile</i> isolation	Toxin production by strain	Cytotoxin in faeces	Antibiotic therapy	Clinical details
11	40	+	+	-	Salazopyrine	Ulcerative colitis
12	76	+	-	-	Amoxycillin	Chest infection
13	65	+	+	-	Ampicillin	Gastrectomy
14	86	+	-	-	Nil	Carcinoma of colon
15	75	+	+	-	Penicillin G	Amputation
16	80	+	+	-	Ampicillin + tobramycin	Oesophageal perforation, carcinoma
17	24	+	+	-	Amoxycillin	Chest infection
18	40	+	+	-	Nil	Holiday abroad

+ = present. - = absent.

Table 3 *Clostridium difficile* and cytotoxin in patients with *Salmonella* infections

Age (yr)	No of patients	<i>C difficile</i> isolated	In vitro toxin production	Cytotoxin in stool
<2	9	9	3	1
>2	20	3	2	0
Unknown	12	0	-	-
All	41	12	6	1

Discussion

The *C difficile* isolation rate of 8% found by this study indicates that the organism is a relatively common bowel inhabitant occurring with greater frequency than *Salmonella* (4.7%) in the routine faecal specimens received at this laboratory. However, its role as a pathogen is less easily defined.

In infants less than two years of age, the carriage rate of 36% was striking and is consistent with previous reports of isolation from healthy neonates.⁴⁸ No patients in this group had symptoms thought to be attributable to *C difficile* infection, nor was there an association with antimicrobial therapy. In direct contrast, *C difficile* carriage in the older age group frequently followed antimicrobial therapy

and was accompanied by symptoms of bowel disturbance in some patients. The agents involved were the penicillins, cephalosporins, aminoglycosides and metronidazole. One patient with ulcerative colitis was also receiving salazopyrine, similar cases have been reported previously^{13 14} in whom *C difficile* and its cytotoxin have been implicated with relapses of chronic inflammatory bowel disease. Two further patients who were culture-positive for *C difficile* but had not received antimicrobials were suffering from diarrhoea associated with carcinoma of the colon and recent travel abroad. The patient with carcinoma of the colon had not received any chemotherapy. Cancer chemotherapy was associated with relapsing pseudomembranous colitis and *C difficile* isolation in one patient with embryonal carcinoma of the testicle.¹⁵ Recent travel abroad has been linked with increased carriage of *C difficile*.¹⁶ Our patient complained of diarrhoea after a holiday abroad and examination of the stools for other pathogens was negative.

An association with infection by other enteric pathogens is suggested by the finding that 20% of *C difficile* carriers were infected with either *Salmonella*, *Shigella* or *Campylobacter* species. This association however, is not as marked as the 36% incidence reported by Falsen.¹⁶

Cytotoxin production tests carried out on cell-free supernatants of Robertson cooked meat broths were positive for strains from 52% (36/69) of the *C difficile* carrier, a lower incidence than previously reported.¹⁷ This may be due to the shorter incubation period (48 h) and a higher initial dilution (1/8) used in this study compared with the five-day incubation and 1/2 dilution tested by Viscidi *et al.*¹⁷ Indeed, cytotoxin from some of their strains was only detectable at the initial 1/2 dilution. In view of the important role ascribed to *C difficile* toxin in the aetiology of colitis associated with this organism,²⁻⁴ those patients with toxin in their stool are of particular interest. Ten of the 36 carriers of cytotoxin-producing strains had detectable toxin in their stool (Table 1), the titres ranging from 1/40 to 1/6400. However, the presence of cytotoxin did not correlate closely with symptoms of diarrhoea or colitis. Six of the toxin-positive patients were infants (Table 1), five of whom were asymptomatic. The sixth was suffering from *Salmonella* gastroenteritis. Two of the asymptomatic infants had titres of toxin which were relatively high (1/2560 and 1/6400) and comparable to those reported in cases of pseudomembranous colitis.^{1 3 11 18 19} Examination of serial specimens from three of the infants (patients 4, 5, and 6) revealed that cytotoxin was still detectable 60, 49, and 100 days respectively, after their first toxin-positive stool specimen.

In the adult group, all four toxin-positive patients (Table 1) were receiving antibiotic therapy. Three had diarrhoea including one with sigmoidoscopically confirmed colitis and a cytotoxin titre of 1/2560. The fourth with a cytotoxin titre of 1/800 and no symptoms had completed a course of metronidazole therapy for amoebic colitis. One of the adults (patient 8, Table 1) developed a positive cytotoxin titre in her stool over the five-day period after her initial culture-positive and toxin-negative specimen thus demonstrating the potential of screening by culture for anticipating toxin-related problems. A similar observation in one patient was noted recently.⁷ A further eight adult patients with diarrhoea (Table 2) from whom *C difficile* was isolated had no cytotoxin present in their stool.

It has been suggested that there is a correlation between pseudomembrane formation in the colon and cytotoxin titres greater than or equal to 1/6400.²⁰ Only one patient in our study, an infant with no symptoms, had a titre of this magnitude.

The absence of any bowel disturbance in five of the patients with *C difficile* cytotoxin in their stool casts doubt upon its importance as an aetiological agent in the pathogenesis of diarrhoea and colitis. However, the recent discovery of a second *C difficile* toxin, an endotoxin with biological and immunological characteristics distinct from that of the cytotoxin²¹ may provide an explanation for the apparent discrepancy.

In conclusion, this study has demonstrated the feasibility of screening routine faecal specimens for *C difficile* and its cytotoxin. Despite the uncertain role of this toxin, its detection in tissue culture remains a convenient and rapid diagnostic procedure. Clearly, other factors, such as changes in the normal bowel flora and bacterial interactions,²² may also have a part to play in the pathogenesis of the *C difficile* syndrome.

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