

Association between production of toxins A and B and types of *Clostridium difficile*

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SUMMARY One hundred and seventy two strains of *Clostridium difficile* isolated from 62 patients with antibiotic associated diarrhoea or pseudomembranous colitis were analysed for the production of toxins A and B and typed using ³⁵S-methionine labelling followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). There was a correlation between production of toxins A and B and the type of *C difficile*. One hundred and forty four of 172 strains were either high or low producers of both toxins. Toxins were not detected in 28 of 172 strains. Types A and Y were consistently non-toxin producers; types B and E were high toxin producers. Type X, the epidemic strain, showed variable toxin production. Symptoms of 49 patients with haematological malignant pathology who were part of a documented outbreak of antibiotic associated diarrhoea, were analysed in relation to toxin production and type of the clinical strains isolated. In general, there was a correlation between symptoms of antibiotic associated diarrhoea, the type of *C difficile*, and its potential for producing toxins.

Over the past decade many studies have implicated *C difficile* as the primary cause of pseudomembranous colitis and antibiotic-associated diarrhoea (AAD).¹ The pathogenicity of this organism is thought, in part, to be related to its potential for producing toxins.^{2,3} At least two toxins are produced: toxin A is an enterotoxin capable of inducing fluid accumulation in the rabbit ileal loop assay; and toxin B is a potent cytotoxin.⁴⁻⁷

Various typing schemes have been developed in an effort to distinguish among different strains of *C difficile*.⁸⁻¹¹ Previous studies have indicated that the development of symptoms in patients may be related to the type of *C difficile*.^{10,12} Any possible correlations between the type of the organism, its pathogenicity, and its relative toxigenicity have not yet been well established.⁷ In this study we applied a recently reported typing scheme, based on the incorporation of ³⁵S-methionine into soluble bacterial proteins, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE.^{10,11} Nine different types have thus far been reported (A-E, W-Z). Clinical strains of *C difficile* were typed using this method and their production of toxin A and B was assayed to study the association between toxigenicity and

organism type. In addition, symptomatic patients, who were part of a well documented outbreak of *C difficile*-associated diarrhoea (CDAD),¹³ were analysed in the light of the type and the toxigenic potential of the clinical strains obtained.

Patients and methods

Clinical strains of *C difficile* obtained from 62 patients were typed and analysed for toxin production. These patients were studied as part of a six month prospective review of CDAD on a mixed oncology and medical unit during an outbreak of the disease.¹³ Forty nine oncology patients were therefore assessed for symptoms in association with an isolate of *C difficile* with respect to diarrhoea, abdominal pain, and fever, as recorded by nursing and medical staff. Further clinical details of these patients will be published at a later date. Diarrhoea was defined as complaints of passing loose stool more than twice in twenty four hours, reported by either the patient (thus implying a change from normal bowel habit) or by the nursing staff. Symptomatic patients were then analysed in terms of the type of *C difficile* and the ability of their strains to produce toxins A and B.

One hundred and seventy two strains of *C difficile* were isolated from faecal specimens of the 62 patients after faecal inoculation on selective media

(cycloserine, cefoxitin, and fructose agar; Oxoid) and anaerobic incubation for 48 hours. The strains were identified by standard techniques¹⁴ and stored in Robertson's cooked meat medium (RCM; Southern Group Laboratories, London) until required.

The typing scheme used in this report was developed at this hospital and has been described in detail elsewhere.¹² Briefly, stored strains were inoculated from RCM onto pre-reduced blood agar plates and incubated anaerobically for 48 hours at 37°C. Two to three colonies from each strain were then inoculated into 100 µl of pre-reduced methionine free modified Eagle's medium with Earle's salts (Flow Laboratories) containing about 10 µCi of ³⁵S-methionine (Amersham International, Amersham, UK). After anaerobic incubation for two hours an equal volume of double strength electrophoresis buffer was added to each tube and subsequently boiled for two minutes. Electrophoresis on 12.5% polyacrylamide gels was then performed and the protein patterns obtained for each strain were ultimately visualised by autoradiography.

Clinical strains of *C. difficile* were stored in pre-reduced RCM and subsequently analysed for toxin B (cytotoxin) production. When required, strains from the RCM were checked for bacteriological purity and then cultured on blood agar for 48 hours under anaerobic conditions. They were subsequently reinoculated into pre-reduced RCM and incubated at 37°C for five days. The broth was then filtered using a 0.45 µm filter membrane (Acrodisc) and 100 µl of the extract was applied to a pre-seeded microtitre tray of Hep2 cells over a dilution range of 1/2–1/256. Each microtitre plate was controlled using a high titre positive control isolate from a case of clinically diagnosed CDADC. Samples were assessed for a cytopathic effect, neutralised by *Clostridium sordellii* antitoxin after overnight incubation at 37°C, and the titre at which neutralisation occurred was taken as the end point of the assay.

Toxin A purification, the raising of purified antitoxin A, and the enzyme linked immunosorbent assay (ELISA) technique for the quantitative detection of toxin A were performed as described by Redmond *et al.*¹⁵

Briefly, toxin A was prepared from a five litre growth of a strain found to be a good toxin producer in vitro, isolated from a patient with pseudomembranous colitis. The enterotoxin was purified from other protein components by preparative electrophoresis using a discontinuous buffer system and ion-exchange chromatography on a DEAE sepharose CL6B (Pharmacia) column.

Monoprecipitin antisera to toxin A were raised in male Californian rabbits by injecting subcutaneously with 10 µg of pure toxin mixed 1/1 with Freund's

complete adjuvant. At weekly intervals three further injections were made using Freund's incomplete adjuvant. Six weeks later one rabbit received an intravenous boost of 20 µg toxin A (0.6 ml) without adjuvant; this rabbit became moribund after four days and was bled out. Its serum contained low titres of antitoxin A and was used to neutralise toxin A before intravenous boosting of other rabbits with 20 µg of toxin A. Three days later the rabbits received 8 µg of neutralised toxin A, were bled from the ear vein 10 days later, and then rested. The globulin fraction of the rabbit serum was purified by precipitation with an equal volume of 32% (w/v) sodium sulphate and thorough dialysis against phosphate buffered saline (PBS) containing 8.0 g/l NaCl; 0.2 g/l KH₂PO₄; 1.1 g/l Na₂PO₄; 0.2 g/l KCl; pH 7.4. Purified IgG was conjugated to horseradish peroxidase using a method based on that of Nakane and Kawaoi.¹⁶

A direct sandwich ELISA technique was used for the quantitative detection of toxin A. Each well of a flat bottomed microtitre plate (Nunc type II) was coated with 100 µl of rabbit antitoxin A (100 µg/ml purified IgG) in carbonate buffer (0.05 M/l carbonate, pH 9.6) at 37°C for two hours or overnight at 4°C. Without emptying the wells 20 µl of 5% (w/v) bovine serum albumin in coating buffer was added to each well and the plate was incubated for a further 30 minutes at 37°C. The plate was then washed four times in PBS containing 0.05% Tween 20 (Sigma) (PBS-T). Sample antigen consisting of 50 µl of RCM broth which had been inoculated with the organism was added and incubated for 90 minutes at 37°C. The wells were washed before the addition of 100 µl of 0.1% normal rabbit serum in PBS-T and incubated for 30 minutes at 37°C. After further washing 100 µl of rabbit antitoxin A conjugated to horseradish peroxidase diluted 1/1000 in PBS-T was added to each well and incubated at 37°C for two hours. After a final washing 100 µl of an appropriate substrate solution was added to each well for 30 minutes at 37°C. The colour reaction was stopped by the addition of 12% sulphuric acid and the assay was quantified spectrophotometrically at 492 nm (Biorad ELISA plate reader) by comparison with known concentrations of standard samples of toxin A.

Results

A total of 172 strains of *C. difficile* obtained from 62 different patients were analysed for toxins A and B (table 1). The strains were divided for purposes of analysis into three categories; non-detectable toxin, low, and high toxin producing strains. High toxin producing strains were defined as those which produced a titre of 1/128 or greater in the toxin B assay, and more than 40 ng/ml of toxin A in the toxin A

Table 1 Types of *Clostridium difficile* analysed for toxins A and B production

Type	Toxin A			Toxin B			
	No of strains studied	Not detected	Low	High	Not detected	Low	High
A	5	5	0	0	5	0	0
B	5	0	1	4	0	1	4
C	5	2	0	3	2	0	3
D	7	4	0	3	4	0	3
E	17	0	0	17	0	1	16
W	2	0	0	2	0	0	2
X	118	5	57	56	5	72	41
Y	12	12	0	0	12	0	0
Z	1	0	1	0	0	1	0
Total	172	28	59	85	28	75	69

assay. Toxins A and B were not detected in some strains using the above methods.

Fig 1 shows the relative amounts of toxin A and toxin B produced according to type. Types A and Y regularly failed to produce detectable titres of either toxin A or B; types B and E produced high titres of both toxins. One strain with an E type produced high titres of toxin A but low of toxin B (table 1). Types C and D produced either high or undetectable titres of both toxins, but each individual strain produced either high or undetectable titres of both toxins; type X (the epidemic strain and hence the one most com-

monly isolated) produced either high or low titres of toxin. Five strains of type X failed to produce any detectable titres of either toxin. Unlike types C and D, however, 34 strains of X type had titres of two toxins that were inversely related with titres of one toxin appearing to be unrelated to the other. Eighty per cent of all strains examined (137 of 172) produced parallel low or high titres of both toxins. Only one strain of type Z and two of W were analysed as they

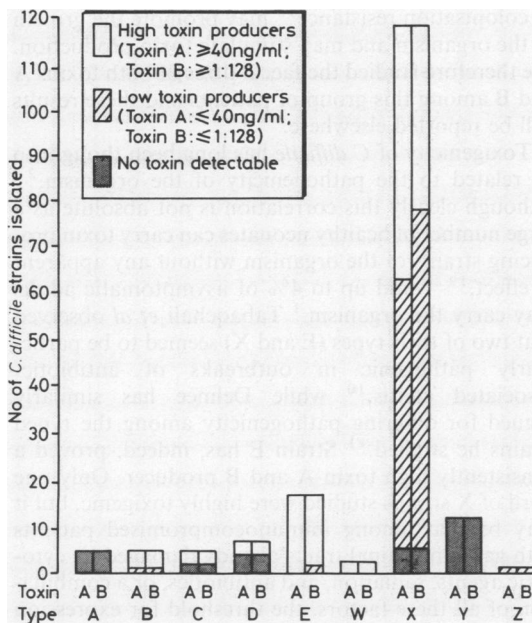


Fig 1 Toxin A and B production by different types of *Clostridium difficile*.

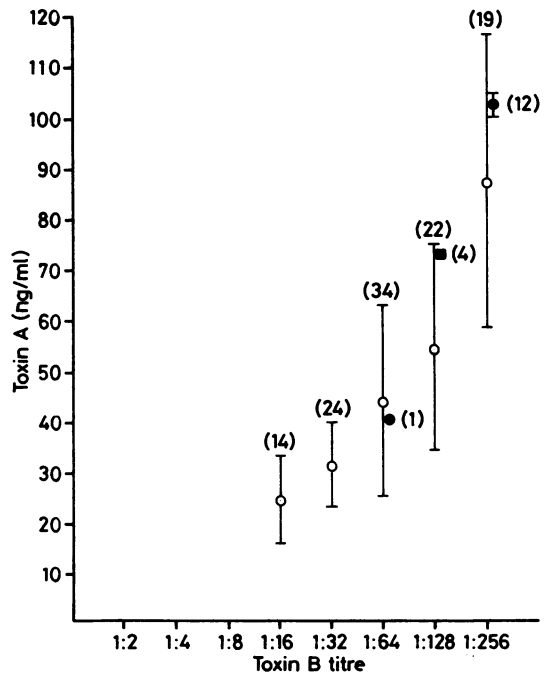


Fig 2 Correlation between toxin A and B production in strains of *Clostridium difficile*; 118 types X and 17 type E (mean SD). ○ type X ● type E.

Table 2 *Toxins A and B from isolates of Clostridium difficile obtained from oncology patients*

Type	High toxin producer (Toxin A > 40 ng/ml toxin B titre \geq 1/128)		Low toxin producer (Toxin A < 40 ng/ml toxin B titre < 1/128)	
	Epidemic	Non-epidemic	Epidemic	Non-epidemic
	Symptomatic patients (n = 33):			
Toxin A	24	3	5	1
Toxin B	21	3	8	1
Asymptomatic patients (n = 16):				
Toxin A	1	5	5	5
Toxin B	1	4	5	6

were only very rarely obtained. The association between toxin production for 118 strains of type X and 17 strains of type E is shown in fig 2. The mean and standard deviations of the various titres of toxins A and B show that the two toxins are inter-related.

Patients were assessed for symptomatic disease as defined earlier. Twenty nine of 33 symptomatic patients carried the epidemic strain (type X), only six of 16 asymptomatic patients carried this type. Other types were carried by the remainder of the asymptomatic patients.

Table 2 gives the results of the toxin analysis of the strains obtained from the oncology patients studied. None of the symptomatic patients carried *C difficile* strains which did not produce toxins. All symptomatic patients had strains isolated which produced toxins A and B. Twenty seven of 33 symptomatic patients had high toxin A producing strains, 24 of which were the epidemic strain type X. Twenty one of those strains were also high toxin B producers, but three strains were low toxin B producers. Only six of the 33 symptomatic patients had strains which produced low titres of toxin A, five of those being the epidemic strain.

Among the 16 asymptomatic patients, there was only one patient from whom a high toxin A and B producing epidemic strain was isolated. The remaining five high toxin producing strains belonged to other types. Ten of the 16 asymptomatic patients had low toxin producing strains.

Discussion

This study shows that an association seems to exist between the type of *C difficile* and the quantities of toxin produced. Types B and E were consistently high producers of both toxins, whereas no toxins were detected from types A and Y (table 1, fig 1). Type X, the epidemic strain in this outbreak, was a high toxin producer among most symptomatic patients, but could also produce undetectable levels in five strains (table 1). In addition, most strains of *C difficile* studied produced parallel levels of the two toxins. In only

three symptomatic patients, however, high toxin A was observed with low yields of toxin B. In this study the toxin B titres were not titrated fully and the titre of 1/128 was chosen as an arbitrary cut off point. Nevertheless, this value can distinguish strains in which toxin B was present in reasonable quantities from those in which it was present in only very low concentration or was undetectable.

A recent report by Haslam *et al* indicated that under limiting growth conditions dependent on the removal of amino acids from the composition of the growth media, the relative production of one or other of the toxins can vary.¹⁷ All the strains that were examined in our study were grown in a well defined medium (RCM), rich in a wide range of amino acids, under the same conditions. This clearly does not simulate in vivo conditions where additional factors such as colonisation resistance¹⁸ may promote the growth of the organism and may stimulate toxin production. We therefore studied the faecal titres of both toxins A and B among this group of patients and these results will be reported elsewhere.

Toxicogenicity of *C difficile* has long been thought to be related to the pathogenicity of the organism,^{2,3} although clearly this correlation is not absolute as a large number of healthy neonates can carry toxin producing strains of the organism without any apparent ill effect,^{19,20} and up to 4% of asymptomatic adults may carry the organism.¹ Tabaqchali *et al* observed that two of their types (E and X) seemed to be particularly pathogenic in outbreaks of antibiotic-associated colitis,¹⁰ while Delmee has similarly argued for differing pathogenicity among the typed strains he studied.¹¹ Strain E has, indeed, proved a consistently high toxin A and B producer. Only one third of X strains studied were highly toxigenic, but it may be that among immunocompromised patients with gastrointestinal tracts already damaged by cytotoxic agents, radiation, and antibiotics, or a combination of all these factors, the threshold for expression of disease by this organism is much reduced. Thus two thirds (21 of 33) of symptomatic patients with a type X isolate had high toxin A and B producing

strains and a further three patients had high toxin A producing strains, while low toxin A and B producing X strains occurred in only five symptomatic patients. The numbers are too small to reach significance, but are consistent with the suggestion that the pathogenicity of the strain, expressed here by symptomatic disease, relates in part to its toxigenic ability which, in turn, may be related to the type of *C. difficile* that is isolated.

A single report by Wust *et al.*⁸ relating the PAGE profile of soluble proteins from *C. difficile* strains to its toxin production potential showed that the single non-toxigenic strain of the 13 studied had, in fact, a unique profile. This result agrees with the observations made in this study, relating the ³⁵S-methionine PAGE profile to the toxin potential of the different types. It has been previously suggested that typing of the organism may be useful in helping to distinguish between pathogenic and non-pathogenic strains.^{10,11} This report provides an explanation as to why this might be useful if studies of strains world wide continue to confirm the view that type, pathogenicity, and toxin production are inter related. Isolations of particular types of *C. difficile*, therefore, may alert clinicians early to their potential for causing disease, especially in patients with additional risk factors such as age, underlying disease, or previous exposure to antibiotics.

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