Typing of *Clostridium difficile* causing diarrhoea in an orthopaedic ward

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SUMMARY In an outbreak of diarrhoeal disease in an orthopaedic ward *Clostridium difficile* was isolated from all six patients with diarrhoea. Attempts were made to type these isolates by means of antibiogram, detection of pre-formed enzymes, analysis of surface proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, and plasmid profile analysis. This showed that a single strain (type E) indistinguishable by the four distinct methods of typing, was isolated from all six patients at some time during their episodes of diarrhoea. Relapse was caused by the acquisition of a new strain in two patients, and by re-emergence or reacquisition of the original strain in two patients. The immunochemical method was the most sensitive and discriminatory of the typing strategies adopted.

Clostridium difficile was first implicated as the cause of pseudomembranous colitis in 1978.¹⁻³ Since then, numerous reports have documented that the organism can cause a broad spectrum of bowel disorders which include antibiotic-associated diarrhoea, chronic inflammatory bowel disease, and non-antibiotic associated diarrhoea.⁴⁻⁸

The role of *C. difficile* in the pathogenesis of these conditions is still the subject of controversy. It has also been difficult to define the epidemiology because of the lack of reliable and readily available typing methods. Several methods have been described. These include antibiograms, analysis of surface proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, plasmid profiles, bacteriocin and bacteriophage typing, autoradiograms of ³⁵S methionine labelled proteins extracted from growing cells, and serogrouping by agglutination.⁴⁻¹¹ In this study we used a combination

of antibiograms, analysis of pre-formed enzymes,¹² SDS-PAGE/immunoblotting and plasmid profiles to investigate an outbreak of diarrhoeal disease due to *C. difficile* in an orthopaedic ward in Glasgow Royal Infirmary.

Patients and methods

The six patients (mean age 79 years; range 72-88) were elderly women who had been admitted to an orthopaedic ward of Glasgow Royal Infirmary. Due to their underlying conditions the patients were all bedbound. Individual details are given in table 1.

Faecal samples were taken from those patients who developed diarrhoea. When the possibility of an outbreak was suspected faecal samples from all the patients in the ward, together with environmental samples from the ward floor, bedpan wagon, "clinimatic", toilets and from ward furniture, were cultured. In addition to the isolates from the "outbreak", five other strains of *C. difficile* from sporadic cases of

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Table 1 Summary of clinical details

Case No	Sex	Age	Reason for admission	Previous use of antibiotics	Outcome
1	F	88	Fractured neck of femur	Yes	Infectious diseases unit
2	F	79	Dislocated Thomson's prosthesis	Yes	Died
3	F	79	Dislocated Thomson's prosthesis	Yes	Infectious diseases unit
4	F	82	Bone graft, right femur	Yes	Infectious diseases unit
5	F	75	Fractured neck of femur	No	Convalesced
6	F	72	Fractured neck of femur	Yes	Infectious diseases unit

Convalesced—patient transferred to another orthopaedic ward.

diarrhoea in the hospital isolated during the previous six months were kept to be used as controls for surface protein analysis.

All samples were inoculated on to cycloserine cefoxitin agar (CCFA)¹³ and incubated anaerobically at 37°C for 48 hours. Isolates were identified as *C. difficile* by colonial morphology, characteristic smell, Gram stain, toxin production, biochemical reactions and by gas-liquid chromatography.¹⁴ Faecal samples were also cultured on MacConkey agar, deoxycholate citrate agar (DCA), campylobacter agar and in selenite F broth for the presence of other enteric pathogens.

TEST FOR TOXIN PRODUCTION

Faecal samples were centrifuged directly at $2000 \times g$ for 20 minutes or, if solid, first suspended in an equal volume of saline before centrifugation. The supernatant was filtered through a $0.45 \mu\text{m}$ membrane filter and then diluted 1 in 50 and 1 in 500 in tissue culture growth medium before inoculation on to Vero cell monolayers: the cells were then incubated for 18 hours at 37°C. The presence of toxin was indicated by the characteristic cytopathic effect. If a sample was found to be positive at the screening dilutions, the concentration of toxin present was established by testing serial doubling dilutions from 1 in 50 to 1 in 6400. The specificity of the toxin was confirmed by neutralisation with *Clostridium sordellii* antitoxin.¹⁴

ISOLATION OF SURFACE PROTEIN ANTIGENS

Organisms were cultured in cooked meat broth (CMB) overnight and 0.1 ml inoculated into 100 ml of protease peptone yeast extract broth (PPY), supplemented with 0.04% (w/v) sodium carbonate and 0.075% (w/v) cysteine hydrochloride as described by Poxton *et al.*⁴ After overnight incubation anaerobically at 37°C the cells were harvested by centrifugation at $20\,000 \times g$ for five minutes at 4°C and the pellet resuspended in phosphate buffered saline, pH 7.4, with 10 mM ethylene diamine tetraacetic acid (EDTA): the suspension was then incubated at 45°C for 30 minutes. The supernatant, which contained the concentrated antigens, was collected after two cycles of centrifugation at $10\,000 \times g$ for 2.5 minutes and used without dialysis as the antigen preparation. The protein content was measured by the method of Lowry *et al.*¹⁵ using bovine serum albumin as standard.

ANALYSIS OF PROTEINS IN SDS-PAGE

The buffer system of Laemmli¹⁶ with 10% slab gels was used as previously described by Poxton and Brown¹⁷. Extracts in EDTA which contained 25 μg of protein in 50 μl of sample buffer were run in duplicate on gels. One set of the separated proteins was stained with

Coomassie blue and the other was used for transfer to nitrocellulose membrane.

IMMUNOBLOT TRANSFER

The method of Towbin *et al.*¹⁸ was used. SDS-PAGE separated EDTA extracts were transferred to nitrocellulose membrane in a Tris, glycine, methanol buffer, pH 8.3, at 12 V and 40 mA for 18 hours. The membrane was then washed and probed with a rabbit antiserum raised against cells of *C. difficile* (NCTC 11223) killed by ultraviolet light followed by application of an antirabbit IgG-horseradish peroxidase (HRP) conjugate and appropriate colour reagent (BioRad).¹⁹

DETERMINATION OF PLASMID PROFILES

Overnight anaerobic cultures (18 hours) in pre-reduced brain heart infusion broth at 37°C were prepared. The cells were harvested by centrifugation at $2000 \times g$ for five minutes, and the pellets resuspended in 200 μl of Tris, EDTA, sodium chloride (TES) dissolved in 50 mM sucrose to which 50 μl of lyso-staphin (1 mg/ml) and 50 μl of lysozyme (40 mg/ml) were added. After incubation for 45 minutes at 37°C 80 μl of 0.25 M EDTA was added before incubation at 56°C for 30 minutes, after which 400 μl of 10% w/v sodium dodecyl sulphate was added to complete lysis. Crude lysates were centrifuged for 15 minutes at $15\,000 \times g$, and the supernatants run on 0.7% agarose gels, as described previously.^{20,21} Plasmids of known size were used as molecular weight standards. A strain of *C. difficile* known to harbour an 80 megadalton plasmid was processed in parallel as a positive control.

ANTIBIOGRAM TYPING

Antibiogram patterns were determined by estimation of minimum inhibitory concentrations (MICs) of erythromycin, tetracycline, chloramphenicol, rifampicin and clindamycin. This was done by an agar dilution method with two-fold dilutions of antibiotic incorporated in Wilkins-Chalgren agar.^{9,22} The antibiotic concentrations tested ranged from: erythromycin 1 to 64 mg/l; tetracycline 0.25 to 32 mg/l; chloramphenicol 8 to 128 mg/l; rifampicin 0.25 to 256 mg/l and clindamycin 8 to 256 mg/l. Plates were inoculated with about 100 000 organisms/ml and incubated anaerobically overnight at 37°C. Sensitive strains were defined as those that did not grow at the lowest antibiotic concentration, and resistant strains as those which were not inhibited by the highest concentration tested.

ENZYME PROFILES

Pre-formed enzymes were detected by the API ZYM system. Overnight broth cultures of each isolate were centrifuged and the pellet suspended in 2 ml sterile

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Table 2 Comparison of antibiogram pattern and immunochemical type

Case No	Date	Strain	Antibiogram					Anti-biotype	Immuno-chemical type
			Erythromycin	Chloramphenicol	Clindamycin	Rifampicin	Tetracycline		
1	28/4	Primary	R	S	S	S	S	2	D
	20/5	Relapse	R	S	R	S	S	1	E
2	22/5	Primary	R	S	R	S	S	1	E
	26/5		R	S	R	S	S	1	E
3	26/5	Primary	R	S	R	S	S	1	H
	30/5		R	S	R	S	S	1	E
	7/6	Relapse	R	S	R	S	S	1	E
	9/6		R	S	R	S	S	1	E
4	26/5	Primary	R	S	R	S	S	1	E
	19/6	Relapse	R	S	S	S	S	2	A
5	4/6	Primary	R	S	R	S	S	1	E
	7/6		R	S	S	S	S	2	D
6	6/6	Primary	R	S	R	S	S	1	E
	10/6		R	S	R	S	S	1	E
	25/6	Relapse	R	S	R	S	S	1	E

R = resistant; S = sensitive.

water to obtain a suspension with a turbidity which measured between McFarland values No 5 and No 6. Volumes (65 µl) of this suspension were then inoculated into the 20 cupules of the test strip (one control + 19 tests) and incubated in darkness at 37°C for four hours.

The test strips were processed, and the results interpreted in accordance with the manufacturer's instructions. Each isolate was tested on two separate occasions.

Results

In late May and early June 1987 six patients in the orthopaedic ward of the Glasgow Royal Infirmary

were involved in an outbreak of diarrhoea (table 2). Three weeks earlier, one patient (case 1) had required treatment for an isolated episode of diarrhoea associated with *C difficile*. This patient was treated with vancomycin for 10 days with good clinical response but five days later she again developed diarrhoea. Over the next 17 days five other patients had episodes of diarrhoea due to *C difficile*.

Five of the six patients in the outbreak had received antibiotics within the previous four weeks. These included flucloxacillin (four patients), ampicillin and amoxycillin (two patients), fusidic acid (one patient), erythromycin (one patient) and cephalixin (one patient).

All patients were treated with oral vancomycin. One

Fig 1 Initial 19 strains typed by immunochemical method (14 from outbreak; 10 patients, four environmental, five from elsewhere in hospital).

Lanes: 1 A; 2 B; 3, 4 C; 5 G; 6 H; 7 D; 8 F; 9-19 E.

Fig 2 Eight different stains A-H. Strains A, D, H, E isolated from patients in "outbreak". Strains B, C, G, F from elsewhere in hospital.

Lanes: 1 A; 2 B; 3 C; 4 G; 5 H; 6 D; 7 F; 8 E.

patient (case 2) died. The cause of death was not directly related to *C. difficile* diarrhoea, although this may have been a contributory factor. Of the other five patients, four had further episodes of diarrhoea from which *C. difficile* was isolated. The outbreak was eventually controlled by closure of the ward to further admissions and transfer of those patients with relapses to the local infectious diseases unit.

C. difficile was isolated from the six patients on the ward (in some cases on more than one occasion) and from the bedside locker, bedpan holder, and surrounding floor of one of the affected patients and from the bedside locker of an asymptomatic patient in the ward. *C. difficile* was not isolated from any of the patients without diarrhoea. No other enteric pathogens were isolated from any patient.

The 15 isolates of *C. difficile* from the six patients and the four environmental isolates produced toxin. All faecal samples from the six patients were also found to contain high titres (> 1 in 6400) of *C. difficile* toxin.

Antibiotic sensitivity tests separated the isolates into two "antibiotypes" on the basis of clindamycin sensitivity. Type 1 was resistant to clindamycin and type 2 was sensitive. All the environmental samples were "antibiotype" 1. A comparison between antibiogram patterns and immunochemical types in the patient samples is shown in table 2.

None of the *C. difficile* isolates studied harboured plasmids.

The following enzymes were present in all isolates from patients and the environment: alkaline phosphatase, esterase (C4), esterase lipase (C80), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase.

Nineteen strains of *C. difficile* from the outbreak and five sporadic isolates from elsewhere in the hospital were submitted with no indication of source for SDS-PAGE/immunoblot fingerprint analysis. Eight immunochemical types, arbitrarily designated A-H, were found (figs 1 and 2). Four of these, A, D, E, and H, were found only in samples from the "outbreak". The other four patterns, B, C, F, and G were produced from the five strains isolated from elsewhere in the hospital. Strains with pattern E were isolated at least once from all the affected patients and from the surrounding environment at some time during their episodes of infection. Types A, D, and H were only found sporadically and were present only in single samples from four different patients (table 2).

Discussion

Results of this study suggest that a single common strain (type E) of *C. difficile*, isolated from all six patients and from the environment, was almost certainly responsible for the outbreak of diarrhoea.

These results support the observations of other workers^{8,9,11,23} that *C. difficile* can be responsible for nosocomial infection in the hospital environment, especially in elderly debilitated patients. It is clear from this study that diarrhoea can relapse after treatment with an appropriate agent (vancomycin) and that the same strain may again be implicated. The reasons for such relapses are not clear but for the first time we have shown that relapse can be caused by acquisition of a new strain (cases 1 and 4) or by re-emergence, or reacquisition of the original strain (cases 3 and 6).

The immunochemical method was shown to be the most discriminatory of the typing strategies evaluated. Antibiograms showed the presence of two types, distinguishable only by their clindamycin sensitivity. Type 1 (clindamycin resistant) was further divided into the "outbreak" type E and another strain, type H, by immunochemical methods. Type 2 (clindamycin sensitive) was further divided into types A and D by immunochemical methods.

The presence of four strains in the outbreak complicated the epidemiological investigation. Although not as complex as a recent outbreak in a renal unit,²⁴ this outbreak shows that multiple strains of *C. difficile* may be involved in a susceptible patient population.

Ideally, 10 colonies should be picked from primary isolation plates for immunochemical analysis²⁵

because more than one strain may be present in primary cultures from individual patients. Unfortunately, this was not done in this study, because it was not at first clear that we were dealing with an outbreak. A similar caveat, however, could equally be applied to many other epidemiological studies of outbreaks which have used this and other typing strategies. Although types A, D, and H were isolated from four of the patients at some time during their diarrhoeal episodes, type E might well have been present on the primary isolation plate.

Levett¹² used the API ZYM system to identify *C difficile* from other clostridial species. He noticed that a few strains had other enzymes present in addition to the common enzymes found in all the *C difficile* strains tested. We found that all our isolates produced the same enzymes and that this result was reproducible. Consequently this method is unlikely to be useful as a typing method for *C difficile*, unless an unusual enzyme pattern is found.

Plasmid profile data have been used to provide useful epidemiological information in outbreaks of infection caused by a variety of different bacterial genera.²⁶ Most strains of *C difficile*, however, do not harbour plasmids and it is well recognised that absence of plasmids or very limited plasmid diversity limits the usefulness of this technique as an epidemiological tool.

Peerbooms *et al* have described the use of restriction endonuclease digest patterns of *C difficile* chromosomal DNA as a means of typing,²⁷ and this method may find wider application in the future.

Immunochemical and plasmid profile analysis can be undertaken in a laboratory with standard electrophoresis equipment, but technical expertise is required to operate these systems successfully, so that these techniques are likely to be restricted to specialist centres. "Antibiotyping" is much less technically demanding and is probably a useful initial step in the investigation of an outbreak but a more discriminating technique should also be used.

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