IMMUNOCHEMICAL FINGERPRINTING OF *CLOSTRIDIUM DIFFICILE* STRAINS ISOLATED FROM AN OUTBREAK OF ANTIBIOTIC-ASSOCIATED COLITIS AND DIARRHOEA

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SUMMARY. Twenty eight strains of *Clostridium difficile*, isolated from an outbreak of antibiotic-associated colitis and diarrhoea in an orthopaedic ward and from sporadic cases throughout Sweden, were sent to Edinburgh for immunochemical fingerprinting without information about their origin. EDTA extracts of the organisms were examined by crossed immunoelectrophoresis (CIE), polyacrylamide gel electrophoresis (PAGE) and electroblot transfer. Two patterns were revealed by CIE: group A (18 strains) and group B (10 strains). PAGE and electroblot transfer revealed one major group of 10 strains (group 1), six small groups of two or three strains and six strains which were unlike any other strain. The CIE group B and PAGE-electroblot group 1 were identical. Nine of the 10 strains in this group were from patients in the outbreak. These findings indicate that a single strain spread in the orthopaedic ward as a nosocomial infection and that this strain differed from most other strains investigated. The PAGE-electroblot technique should, therefore, greatly aid investigations into the epidemiology of *C. difficile* infections.

INTRODUCTION

Clostridium difficile is now well recognised as the major cause of antibiotic-associated pseudomembranous colitis (Larson *et al.*, 1978; Bartlett, 1979; Möllby, Nord and Aronsson, 1980) and appears also to be responsible in part for a spectrum of related bowel conditions, such as antibiotic-associated diarrhoea, post-operative diarrhoea, exacerbations of chronic inflammatory bowel disease and sporadic non-antibiotic-associated diarrhoea (British Medical Journal, 1981; Brettle *et al.*, 1982). Carriage of *C. difficile* by healthy adults is extremely uncommon but high isolation rates of up to 40% have been reported for infants (George, Sutter and Finegold, 1977).

The epidemiology of *C. difficile* is still poorly understood. Animal studies (Larson, Price and Borriello, 1980) and the clustering of cases (Howard, Sullivan and Troster,

1980; Greenfield *et al.*, 1981; Rogers *et al.*, 1981) suggest that the organism may be transmitted from person to person or from the environment (Mulligan *et al.*, 1980; Kim *et al.*, 1981) rather than being a simple overgrowth of a minor component of the normal flora in a compromised bowel. Even healthy carriage by infants appears to be associated with case-clustering (Larson *et al.*, 1982). More thorough investigations into the modes of transmission of *C. difficile* have been hampered by the lack of a reliable typing system for this species. The aim of the present study was to show by the use of immunochemical fingerprinting that isolates of *C. difficile* obtained from a nosocomial outbreak of antibiotic-associated colitis and antibiotic-associated diarrhoea in an orthopaedic ward were identical. These isolates were also different from 15 isolates from other sources. Crossed immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis and electroblot transfer were used to identify the various strains of *C. difficile*.

MATERIALS AND METHODS

Patients. During a period of 14 days, stool samples from patients on antibiotic therapy in two different wards were analysed for the presence of *C. difficile* cytotoxin and organisms. In an orthopaedic ward at Huddinge Hospital, Stockholm, where there was an outbreak of *C. difficile*-associated colitis and diarrhoea during this period, 34 patients (20 females, 14 males; mean age 74 years) were investigated, and during the same period 29 patients (17 females, 12 males; mean age 59 years) in the Department of Infectious Diseases, Karolinska Institute, Roslagstulls Hospital, Stockholm, with only sporadic incidents of this disease, were investigated. Patients were regarded as having antibiotic-associated colitis (AAC) on the basis of endoscopic examination, or as having antibiotic-associated diarrhoea (AAD) if they had more than five loose stools daily and endoscopy was negative or, as was the case for the majority of these cases, if endoscopy was not performed. The antimicrobial agents used in the two wards were clindamycin, cephalosporins and β -lactamase-stable penicillins, and the patterns of use were roughly comparable between the wards.

Bacterial isolates. Isolation and identification of *C. difficile* and testing for toxin production were done as previously reported (Aronsson, Möllby and Nord, 1981). Fifteen isolates of *C. difficile* from 13 patients in the orthopaedic and infectious diseases wards were fingerprinted immunochemically along with two isolates from another two patients in the orthopaedic ward, one sampled 4 months before and the other 2 months after the outbreak in the orthopaedic ward. Another 11 isolates from 10 patients with AAC or AAD in four other hospital wards in Sweden were also included. All strains were toxigenic and they were stored as cooked-meat-broth cultures. They were sent to Edinburgh for fingerprinting without information about their origin.

Environmental sampling. Stool samples from 37 members of the staff at the orthopaedic ward at Huddinge Hospital were collected and assayed for *C. difficile* and its toxin. Plastic Rodac plates (Falcon, Oxnard, CA) were used to culture environmental samples from beds, toilets, washing rooms, treatment rooms and floors.

Culture of organisms and antigen preparation. Stationary-phase cooked-meat-broth cultures (0.1 ml) were inoculated into 100-ml volumes of PPY medium (Holbrook, Duerden and Deacon, 1977), which contained sodium carbonate 0.04% w/v and cysteine hydrochloride 0.075% w/v, and incubated anaerobically with CO₂ 10% for 16 h at 37°C. Spore formation was negligible as judged by phase-contrast microscopy. Antigens were extracted with EDTA by a method simplified from that of Poxton and Byrne (1981). Bacteria were harvested by centrifugation (20 000 *g* for 8 min), washed twice in phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl). The drained pellet was resuspended in 1 ml of PBS containing 10 mM EDTA and incubated for 30 min at 45°C. The extracted cells were removed by centrifugation (20 000 *g* for 10 min) and the supernatant fluid was used undialysed as antigen. Protein content of the preparation was measured by the method of Lowry *et al.* (1951).

Antiserum preparation. Antisera against u.v.-killed cells of *C. difficile* NCTC11223 and two clinical isolates not from the present study were raised in New Zealand White rabbits by the method of Poxton and Byrne (1981).

Crossed immunoelectrophoresis. This was performed by the procedure of Weeke (1973) as described by Poxton and Byrne (1981). EDTA antigens were prepared in duplicate from all the strains. Protein concentrations in the preparations were *c.* 1–3 mg/ml.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The buffer system of Laemmli (1970) was used with 10% slab gels and the method was that of Poxton and Brown (1979). A 20-track gel was used to run two sets of nine EDTA antigens at the same time. One set was stained with Coomassie Blue and the other was used for transfer to a nitrocellulose membrane.

Electroblot transfer. The method of Towbin, Staehelin and Gordon (1979) was followed. Briefly, the EDTA extract that had been separated on SDS-PAGE was transferred to nitrocellulose membrane (Transblot™ Transfer Medium, BioRad) in a Tris, glycine, methanol buffer, pH 8.3 (Towbin *et al.*, 1979) at 12 V and 40 mA for 18 h. The antigens were probed with the antiserum raised against *C. difficile* NCTC11223 by the Immun-blot™ immunoassay (BioRad): after washing for 10 min in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5), the unbound sites on the membrane were blocked by treatment with gelatin 3% in TBS for 45 min. The membrane was transferred to the *C. difficile* antiserum diluted 1 in 200 in gelatin 1% in TBS and incubated at room temperature for 3 h. After two 10-min washes in Tween 20 0.025% in TBS, the membrane was placed into goat anti-rabbit IgG-horseradish peroxidase conjugate (BioRad) diluted 1 in 3000 in gelatin 1% in TBS and incubated at room temperature for 1 h. After two more washes as above, the membrane was placed into HRP colour development solution (BioRad) which contains 4-chloro-1-naphthol. The colour developed between 5 and 15 min. Several changes of distilled water were used to stop the reaction. All of the above steps were performed with gentle agitation.

RESULTS

In the infectious diseases ward with only sporadic cases of AAC or AAD, no patient on antibiotic treatment developed serious diarrhoea during the time of investigation, and only two of 29 patients were found to be colonised. In the orthopaedic ward, six (four females, two males; mean age 75 years) out of 34 patients developed serious colitis that needed vancomycin therapy. Another seven patients (five females, two males; mean age 72 years) colonised with *C. difficile* showed no or minor intestinal symptoms (table I). In total, 15 strains from these two wards were submitted for immunochemical fingerprinting without information about their origins.

Initially, a sample of each antigen was run in CIE against three different antisera—one raised against the reference strain *C. difficile* NCTC11223, the others

TABLE I
Acquisition of C. difficile by patients on antibiotic therapy in two different wards

Ward	Number of patients	Number of patients (%)	
		who developed colitis	who were colonised but asymptomatic
Orthopaedic ward with <i>C. difficile</i> outbreak	34	6 (18)	7 (20)
Infectious diseases ward with sporadic cases	29	0 (0)	2 (7)

TABLE II
Groups of C. difficile identified by CIE and SDS-PAGE-electroblot analysis

CIE group	SDS-PAGE-electroblot group	Number of strains	Source
B	1	9*	O.W.
B	1	1	S
A	2	3(2)†	S
A	3	2	S
A	4	3	S
A	5	2(1)†	O.W.
A	6	2	S
A	—	2(1)†	O.W.
A	—	2	I.D.
A	—	2	S

O.W. = orthopaedic ward; I.D. = infectious disease ward; S = from hospital wards throughout Sweden.

* Four patients with AAC and five carriers.

† Two strains were isolated from the same patient.

FIG. 1.—Examples of CIE patterns of EDTA extracts of *C. difficile* run against antiserum to whole cells of *C. difficile* NCTC11223. Approximately 15 µg of protein in 10 µl in the first dimension was run into 250 µl of antiserum in the second dimension; (a) and (b) are examples of group-B patterns and (c) and (d) are examples of group-A patterns.

against two clinical isolates. Only the patterns of precipitin lines produced by the NCTC11223 antiserum were considered suitable for further investigation. The patterns produced by the antisera to the clinical isolates were ill-defined. Only line 4 (see below) was at all clear; they were difficult to compare and were not used further.

After all the antigens had been tested with the reference antiserum, it was seen that the duplicate antigen preparations gave identical patterns, but that two distinct

FIG. 2.—SDS-PAGE of EDTA extracts of 15 strains of *C. difficile* on 10% gels, and their corresponding electroblot transfers probed with NCTC11223 antiserum. Approximately 45 μ g of protein in 50 μ l of sample buffer was run on each track. (a) Coomassie Blue-stained SDS-polyacrylamide gel: track 1, group-3 pattern; track 2, group-5 pattern; tracks 3-6, group-1 patterns; tracks 7 and 8, group-2 patterns. (b) Electroblot transfer of fig. 2a. (c) Coomassie Blue-stained polyacrylamide gel: tracks 1 and 2, group-2 patterns; tracks 3-7, ungroupable patterns. (d) Electroblot transfers of fig. 2c.

patterns had been produced overall. One pattern was produced by 18 strains (group A). The rest of the strains (group B) gave a different pattern (table II). Examples of the two patterns are shown in fig. 1. Several of the precipitin lines were shared by both groups, but the group-B pattern had a characteristic, strongly staining, slow-moving line (no. 1). The precipitin lines are numbered as in our previous publications (Poxton and Byrne, 1981; Poxton, 1982). It should be noted, however, that in this study some extra, weakly staining lines have been seen for the first time. This is a consequence of using undialysed EDTA antigens. Extensive freezing and thawing also resulted in the destruction of these antigens. The best results were obtained when antigens were used immediately after preparation, without freezing and thawing.

In the Coomassie Blue-stained polyacrylamide gels, many common bands were present, with identical patterns in some tracks. Duplicate antigen preparations gave identical patterns. Groups of similar patterns could be assembled with some difficulty, but the electroblot transfers immediately revealed groups of similar patterns. Fig. 2 shows two sets of SDS-PAGE patterns and their corresponding electroblots. When analysed in detail, the groups obtained with both techniques were identical (table II). Although some of the patterns produced by other strains were similar to those in groups 1–5, none were identical (group A6, table II). Others were considerably different and unlike any other (group A–, table II).

Comparison of the groups identified by CIE with those based on SDS-PAGE or electroblot analysis shows that group B and group 1 are identical. The isolates belonging to this group were, with one exception, obtained from the orthopaedic ward at Huddinge Hospital during the time of the outbreak of colitis and diarrhoea. Isolates obtained from other sources in Sweden were different from the isolates in the B1 group (table II). These data justify the assumption that the B1 isolates belonged to the same strain of *C. difficile* which spread within this ward, colonising five patients and causing severe colitis in another four patients. However, isolates from two patients with diarrhoea in the ward during the 14-day period did not belong to the B1 group. Thus, more than one strain of *C. difficile* was present in the ward during the period. Isolates sampled after and before the outbreak in this ward were found to be different from the B1-group strain of *C. difficile*.

None of the stool samples obtained from staff members or from the environment in the orthopaedic ward yielded *C. difficile* or its toxin.

DISCUSSION

Studies with a selective medium have shown that there is a very low carrier rate for *C. difficile* in a healthy adult population (Aronsson *et al.*, unpublished observations). It has been suggested that such carriage is transient (Larson *et al.*, 1978). However, the immediate environment of infected patients has been found to be contaminated (Mulligan *et al.*, 1980; Fekety *et al.*, 1981), and spread of the organism from the environment and from person to person has been suggested (Kim *et al.*, 1981; Kim, Dupont and Pickering, 1983). Isolation of *C. difficile* has also been reported from household pets (Borriello *et al.*, 1983), seals, donkeys, camels, sewage, mud and sand (Hafiz and Oakley, 1976) and recently from a Kodiak bear with pseudomembranous colitis (Orchard, Fekety and Smith, 1983). Thus, exogenous sources of *C. difficile* are being increasingly recognised.

Our investigation is the first time that strains of *C. difficile* isolated from an outbreak of *C. difficile* colitis have been fingerprinted immunochemically and shown to be identical, and also different from other isolates in a control group (Wüst *et al.*, 1982). With only one exception, the strains isolated from this 14-day outbreak were identical with each other and different from other strains, as determined by CIE or SDS-PAGE-electroblot methods. Strains isolated before and after the outbreak from the same ward were also different.

In our experience, strains from diverse sources can have similar CIE profiles and it is only when striking differences occur, as in this study, that this method proves useful for typing. SDS-PAGE can be used alone; the patterns produced, however, are complex and are difficult to analyse without the use of specialised equipment, especially if tracks from different gels have to be compared. Electroblot transfer immunoblotting has proved extremely satisfactory. The technique is simple to perform in apparatus that can be made easily in the laboratory. The simplicity of the patterns allows straightforward visual analysis, and we conclude that investigations into the epidemiology of *C. difficile* should be considerably aided by this technique.

Our data strongly suggest that the same strain of *C. difficile* spread within a hospital ward, infecting 38% of the patients and causing disease in susceptible patients. It is not likely that this finding represents an overgrowth of endogenous *C. difficile* in these patients, but we have not been able to determine the means of transmission in this outbreak.

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