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Relapse versus reinfection with *Clostridium difficile*

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SUMMARY

Relapse of *Clostridium difficile*-associated diarrhoea occurs in 15–20% of patients; however, whether relapse is due to an endogenous source of the organism or reinfection from the environment remains unclear. Restriction enzyme analysis (REA) of chromosomal DNA was used to type multiple isolates from ten patients who had experienced apparent relapses. More than half the relapses were due to infection with a new strain of *C. difficile*. The remaining patients were infected with the same strain, but whether this strain was acquired from the environment or from endogenous sources could not be determined. Relapses with a different strain of *C. difficile* could occur if an individual harboured more than one strain in their gastrointestinal tract. To investigate this possibility ten other patients were assessed for carriage of multiple strains. Ten colonies from a primary culture plate from each patient were typed by REA and tested for their ability to produce cytotoxin. All isolates from the same patient were identical by both methods, indicating that multiple carriage of strains may be a rare event.

INTRODUCTION

Clostridium difficile is the causative agent of pseudomembranous colitis and the major aetiological agent of antibiotic-associated diarrhoea [1]. Previously we demonstrated that *C. difficile* was the most common laboratory-proved cause of diarrhoea among hospital patients [2] and a significant cause of diarrhoea in general practice and community health [3]. Debate continues as to whether infection with *C. difficile* is due to endogenous or exogenous sources of the organism. A growing body of evidence, however, such as case clustering during outbreaks of *C. difficile*-associated disease and isolation of *C. difficile* from the environment during such outbreaks [4–8], points to acquisition of the organism from the environment as the major route of infection.

Relapses of diarrhoea associated with *C. difficile* occur in 15–20% of patients, and it has been assumed that these are due to the persistence of spores in the gut after vegetative cells have been destroyed by antimicrobial therapy [9]. It is also possible that reinfection of the patient occurs with a different strain of *C. difficile* from the environment. This paper describes the investigation of this possibility using restriction enzyme analysis (REA) of chromosomal DNA from isolates of *C.*

difficile obtained from patients who had relapsed. Representatives case histories of relapsing patients are also presented. We also used REA to investigate the report of Borriello and Honour [10], that more than one strain of *C. difficile* could be carried concomitantly by some patients.

MATERIALS AND METHODS

Patients and isolates

Inpatients or outpatients of Sir Charles Gairdner Hospital (SCGH) and Royal Perth Hospital (RPH), from whom multiple isolates of *C. difficile* had been recovered, were investigated. Clinical information was available on eight patients and was obtained from medical records retrospectively. Details of patients and isolates are given in Table 1.

Demonstration of C. difficile *and other enteric pathogens*

Our criteria for culturing for *C. difficile* [11] and the methods employed for the isolation of *C. difficile* and other enteric pathogens and the detection of *C. difficile* cytotoxin by screening sterile filtrates of 50% faecal suspensions in VERO cells [2, 12] have been described previously. They included the use of a selective enrichment broth for *C. difficile* containing gentamicin 5 mg/l, cycloserine 250 mg/l and cefoxitin 8 mg/l (GCC broth) [12]. Screening of GCC broths and identification of *C. difficile* was performed using a commercially available latex particle agglutination test (Mercia Diagnostics Ltd, Guildford, Surrey) [13]. Cross-reacting species were identified according to the methods and criteria of Brazier [14].

Analysis of multiple carriage of strains

Simultaneous carriage of different strains of *C. difficile* within one patient was investigated in the following manner. Stool samples known to contain *C. difficile* were plated on to cycloserine cefoxitin fructose agar plates and incubated in an anaerobic chamber (Don Whitley Scientific Ltd) for 48 h at 37 °C. After incubation ten separate colonies of *C. difficile* from each plate were subcultured into pre-reduced supplemented brain heart infusion broth (BHIB-S) and incubated for 48 h at 37 °C. Sterile filtrates of the BHIB-S cultures were examined for cytotoxin production as described previously [12]. The REA patterns of all ten cultures from each specimen were also determined.

Culture of C. difficile *for REA*

Isolates were inoculated on to blood agar plates and incubated anaerobically for 48 h at 37 °C. They were then inoculated into 5 ml of BHIB-S and incubated at 37 °C for 16–20 h.

Extraction of chromosomal DNA

The method used was a modification of that described by Peerbooms and colleagues [15]. Cells were harvested by centrifugation and resuspended in 250 μl of TES buffer (150 mM-NaCl, 10 mM Tris, 10 mM-EDTA, pH 8·0). Lysozyme was added to a final concentration of 5 mg/ml and the cell suspension was incubated

Table 1. *Details of patients and isolates*

Patient	Sex	Age	Hospital*	Date of isolation	Faecal cytotoxin	Isolate cytotoxigenic	REA pattern
1	M	84	I/SCGH	28.x.88	+	−	A
				15.xi.88	+	+	B
				17.i.89	+	+	B
				7.ii.89	+	+	B
				11.iii.89	+	+	B
				9.v.89	+	+	B
2	M	73	I/SCGH	25.vii.88	−	−	C
				31.viii.88	−	+	D
3	F	65	I/SCGH	15.ix.88	−	+	E
				12.x.88	−	+	E
4	M	53	I/SCGH	25.xi.88	+	+	E
				9.i.89	+	+	E
5	M	76	I/SCGH	18.xi.88	−	−	F
				19.i.89	+	+	G
6	F	25	O/SCGH	20.ii.89	−	+	H
				12.iv.89	−	+	J
7	M	19	O/SCGH	9.ix.88	−	−	K
				30.ix.88	−	−	L
8	M	77	I/RPH	12.ix.88	+	+	M
				3.x.88	+	+	N
9	F	76	I/RPH	16.ix.88	−	−	P
				8.x.88	−	−	P
				16.x.88	−	−	P
10	M	67	I/RPH	7.viii.88	−	−	P
				22.viii.88	−	−	P
				29.viii.88	−	−	P
				13.ix.88	−	−	P

* I, inpatient; O, outpatient; SCGH, Sir Charles Gairdner Hospital; RPH, Royal Perth Hospital.

at 37 °C for 1 h. Cells were lysed by the addition of SDS to a concentration of 1% and incubation at 60 °C for 10 min. The lysate was incubated at 55 °C for 2 h in the presence of 500 μg/ml proteinase K. NaCl was added to a final concentration of 1 M and the DNA was purified by two extractions with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) followed by two extractions with an equal volume of chloroform : isoamyl alcohol (24 : 1). The DNA was precipitated by the addition of two volumes of cold ethanol, pelleted by centrifugation and dried under vacuum for 5 min. It was resuspended in TE buffer (10 mM Tris HCl, 1 mM-EDTA, pH 8·0) to a concentration of approximately 1 mg/ml.

Restriction enzyme digestion and agarose gel electrophoresis

Approximately 5 μg of DNA was digested with 10 U of *Hin*d III (Toyobo) in buffer (10 mM Tris HCl (pH 8), 7 mM-$MgCl_2$, 60 mM-NaCl, 100 μg/ml bovine serum albumin) for 6 h at 37 °C. Digests were electrophoresed for 15 h in 0·7% agarose gels in TAE buffer (40 mM Tris, 20 mM-Na acetate, 2 mM-EDTA, pH 8·0) at 55 V. The gel was stained in 0·5 mg/l ethidium bromide before being photographed under UV illumination with Polaroid 667 film. Different REA patterns were designated with a different letter of the alphabet.

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CASE HISTORIES

Patient 1. An 84-year-old man, with recurrent cholecystitis treated with intravenous cefotaxime, developed diarrhoea. *C. difficile* was isolated from stools and faecal cytotoxin was detected. He was treated with oral metronidazole 200 mg four times a day with resolution in his symptoms; however, at the end of 6 days' therapy *C. difficile* was still cultured from his stool and faecal cytotoxin was still detected. Ten months later, after a further episode of cholecystitis when he was treated with cefotaxime and Augmentin, his diarrhoea due to *C. difficile* recurred. After 16 days' therapy with oral vancomycin, his symptoms abated and a stool specimen was negative for *C. difficile*. Over the next 6 months he had six recurrences of *C. difficile*-associated diarrhoea, which rapidly settled with re-institution of vancomycin therapy. Two years after the initial episode *C. difficile* was cultured from a stool specimen in the absence of significant symptoms.

Patient 2. A 73-year-old man, with a previous carcinoma of the colon requiring colostomy, was admitted with an ischaemic right foot that had not responded to oral therapy with penicillin and flucloxacillin. He was treated with intravenous cloxacillin and metronidazole and, after 11 days, developed diarrhoea associated with *C. difficile*. Faecal cytotoxin was not detected. No specific therapy was given and his symptoms settled. Seventeen days later diarrhoea recurred, *C. difficile* was isolated and, on this occasion, faecal cytotoxin was detected. Once again his symptoms settled spontaneously, only to recur after transfer to the extended care ward before finally resolving without specific therapy.

Patient 4. A 53-year-old man was admitted to the intensive care unit following a subarachnoid haemorrhage requiring placement of a cerebrospinal fluid shunt. A pneumonia was treated with intravenous cefotaxime. Prior to transfer to the neurosurgical ward, diarrhoea due to *C. difficile* with faecal cytotoxin present commenced. He was treated with 12 days of oral vancomycin with slow resolution of his symptoms. Following treatment of a wound infection with flucloxacillin, diarrhoea recurred, but rapidly settled with the re-institution of vancomycin, which was continued for 10 days. He returned to the intensive care unit following clipping of an anterior cerebral artery aneurysm. After return to the ward, *C. difficile*-associated diarrhoea resumed and finally settled after a further 12 days of vancomycin.

Patient 5. A 76-year-old man, with a past history of chronic renal failure, was admitted to hospital with a fractured left neck of femur which was treated with a Richards pin and plate. His postoperative course was complicated by an *Escherichia coli* urinary tract infection for which he received intravenous cephamandole followed by Augmentin. He subsequently developed diarrhoea associated with *C. difficile* in the absence of faecal cytotoxin. Vancomycin, 125 mg four times a day, was administered orally for 16 days and a repeat stool examination on therapy was negative, despite persistent symptoms. He was transferred to an extended care ward and, 6 days after cessation of vancomycin, his symptoms worsened and *C. difficile* and faecal cytotoxin were detected. Following reintroduction of vancomycin his diarrhoea abated and therapy was continued for 9 days. Seven days later he was readmitted for fine-needle aspiration of a squamous cell lung tumour, followed by radiotherapy. After 3 weeks his *C.*

difficile-associated diarrhoea returned, but settled rapidly after re-institution of oral vancomycin.

Patient 7. A 19-year-old man presented following 4 days of diarrhoea, associated with vomiting and abdominal cramps, which had not responded to antispasmodics or tinidazole. He had not previously taken antibiotics and there was no significant past medical history. After transient settling, diarrhoea recurred 2 weeks later. *C. difficile* was isolated from a stool sample in the absence of faecal cytotoxin. Fibreoptic sigmoidoscopy was performed 1 month after the onset of diarrhoea, revealing a nodular rectal mucosa without pseudomembranes. Rectal biopsy demonstrated focal acute inflammation without granulomata. Oral vancomycin (250 mg four times a day) was commenced, and resulted in slow improvement with a slight reduction in stool frequency. A repeat stool specimen at 6 weeks yielded *C. difficile* again. The patient was lost to follow-up.

Patient 8. A 77-year-old man, with a 6-month history of prostatic obstruction with bilateral hydromephrosis and chronic renal failure, was treated with a transurethral resection of prostate and indwelling urethral catheter. Two weeks after admission, and despite receiving no antibiotics, he developed diarrhoea, and *C. difficile* cytotoxin only was detected in a faecal sample. After 10 days of oral vancomycin (250 mg four times a day), his symptoms settled and stools became negative. Three weeks after initial onset his symptoms recurred and both *C. difficile* and cytotoxin were detected in a stool specimen. Metronidazole, 400 mg three times a day, was administered for 10 days and resulted in negative stools without symptoms; however, every time treatment (with vancomycin alone for 10 days, then vancomycin plus cholestryamine for 10 days followed by cholestyramine alone for 2 weeks) ceased his diarrhoea would recur. Currently, he is asymptomatic, receiving cholestyramine one sachet twice daily; however, *C. difficile* and cytotoxin are still detectable in stool specimens.

Patient 9. A 76-year-old woman was admitted to hospital following a cerebrovascular accident. Her inpatient course was complicated by a *Klebsiella* spp. urinary tract infection for which she received 10 days' therapy with oral amoxycillin. Towards the end of treatment she developed diarrhoea; however, faecal samples failed to yield *C. difficile* or cytotoxin. Cotrimoxazole was introduced, as her symptoms of urinary tract infection had persisted. Her diarrhoea continued, so she was given 1 week's therapy with oral vancomycin without effect on her symptoms. She was initially discharged to a nursing home; however, a week later she was readmitted to hospital for a haematemesis, and disseminated ovarian adenocarcinoma was eventually diagnosed. Her diarrhoea continued and, 2 months after the initial onset of diarrhoea, *C. difficile* was isolated in the absence of faecal cytotoxin. Her stools remained persistently positive until her death 1 month later from a presumed pulmonary embolus.

Patient 10. A 67-year-old man was admitted to the intensive care unit following the insertion of a ventricular drain after he had suffered an intraventricular haemorrhage. His subsequent course was complicated by persistent *Enterobacter cloacae* infection of his ventricular drain, pneumonia and purulent maxillary sinusitis, for which he sequentially received cloxacillin, cefotaxime, aztreonam, imipenem and gentamicin. *C. difficile*-associated diarrhoea commenced 12 days after admission and continued intermittently for 9 days after commencement of

vancomycin, 250 mg four times a day orally. A repeat stool culture was negative on treatment but, after a week, diarrhoea associated with *C. difficile* recurred and continued for another 5 days. Despite continuation of vancomycin and the lack of significant bowel symptoms, his stools continued to yield *C. difficile* for another 2 weeks, at which time he suddenly died of cardio-respiratory arrest.

RESULTS

A total of 27 isolates of *Clostridium difficile* was obtained from the ten patients in this study. No other enteric pathogens were isolated. There were 5 inpatients and 2 outpatients from SCGH and 3 inpatients from RPH (Table 1). Six isolates were obtained from patient 1, the last 5 of which were identical. Of the remaining 4 SCGH inpatients, 2 showed maintenance of the same strain (patients 3 and 4) and 2 showed acquisition of a new strain (patients 2 and 5). The isolates from patient 3 and 4 were identical, and were very similar to the last 4 isolates from patient 1.

The two outpatients from SCGH, patients 6 and 7, acquired new strains between episodes of disease. Patient 8 from RPH was reinfected with a new strain, while patients 9 and 10 maintained the same strain through the course of their disease.

Another 100 strains, 10 isolates from each of 10 other patients, were tested for cytotoxin production and examined by REA. All of the isolates from any one patient were identical by both methods, although there were differences in REA pattern and cytotoxicity between groups of isolates from different patients.

In total, 22 different REA patterns were obtained from 20 patients investigated for variation in strain(s) carried either over time or at any point in time.

DISCUSSION

REA of chromosomal DNA has been shown to be a useful and highly discriminating tool in epidemiological studies of *C. difficile*-associated diarrhoea [15–19], but in only one of these studies [16] was REA used to investigate *C. difficile* isolates from patients with multiple episodes of *C. difficile*-associated diarrhoea. We investigated ten patients from two hospitals using this method, and less than half these patients were shown to have relapsed with the same organism. Excluding patients 9 and 10, whose infections never resolved during the period of study, 6 of the 8 remaining patients appeared to have undergone reinfection with a new strain. Thus 75% of apparent relapses were due to reinfection, supporting the findings of Johnson and colleagues [16], who found that nearly half their patients had acquired a different strain of *C. difficile* during the course of their study.

A variety of other techniques have been used to study the epidemiology of *C. difficile* including bacteriocin and bacteriophage typing [20], protein profiles [21], serotyping [22] and plasmid analysis [23]. While each has proved useful for certain aspects of *C. difficile* epidemiology, none has been applied to study patients who have apparently relapsed. McKay and co-workers [24] found sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting the most sensitive and discriminating of various typing strategies, but did not include

REA. They did, however, report relapse in two patients due to acquisition of a new strain.

Patient 1 was unusual in our study in that he appears to have experienced both reinfection and relapse. The first strain of *C. difficile* from this patient was isolated only once, while he was in the extended care ward. This strain did not produce cytotoxin *in vitro*, although faecal cytotoxin was detected, suggesting that he may have carried more than one strain of *C. difficile* concomitantly. The second strain was isolated five times; initially, while he was in the extended care ward, 1 month after the first strain was isolated, and subsequently when he presented to the Accident and Emergency Department with diarrhoea (four times). It would appear that he acquired the second strain while in hospital and suffered a relapse after being discharged. At least the first episode of disease after he had been discharged can be attributed to a true relapse as the strain was apparently hospital-acquired, he had not been on antibiotic therapy and it is unlikely that this strain was present in his home environment. The final three episodes of disease could have been reinfection through contamination of his home environment with this strain during his initial symptomatic period before he presented to the Accident and Emergency Department.

Our findings support the possibility of nosocomial spread of *C. difficile* strains within the hospital environment. Patients 3 and 4 from SCGH were infected with the same strain. Patients 9 and 10, from RPH, were also infected by identical strains. Although none of the isolates of *C. difficile* from RPH was the same as an isolate from SCGH, the potential exists for spread of strains between the two hospitals. Transfer of patients between RPH and SCGH occurs frequently as all neurosurgical procedures on public patients in Perth are performed at SCGH. Clusters of cases of *C. difficile*-associated diarrhoea have been a constant problem in the neurosurgical ward at SCGH and have been reported in other wards elsewhere [25].

Those patients from whom identical isolates were recovered for various episodes of disease cannot conclusively be said to have undergone relapse due to an endogenous source of the organism. It is possible that these patients has been reinfected with an environmental source of the original infecting strain. It is a well documented fact that the environment of patients with *C. difficile*-associated diarrhoea is often heavily contaminated with the infecting organism [3].

As it had been previously reported that both cytotoxic and non-cytotoxic strains of *C. difficile* could be isolated from the same stool specimen of patients with AAD [10] (and our results for patient 1 supported this hypothesis) we determined the frequency of simultaneous carriage of different strains using REA. The apparent reinfections that all our patients had undergone could have been due to the simultaneous carriage of a number of strains, with only one strain being isolated each time a specimen was examined. None of ten patients studied harboured more than one strain concomitantly (as determined by REA and cytotoxin profiles), suggesting that the carriage of multiple strains occurs rarely. Similar findings were reported by Devlin and co-workers [18], who could find no variation in multiple isolates of *C. difficile* from two stool specimens. Sell and colleagues [20] reported that *C. difficile* isolates from 16 patients taken 1–14 days apart were similar in their phage sensitivity pattern. Conflicting results were

reported by Sharp and Poxton [26], who found that 2 of 3 faecal samples contained 2 different strains of *C. difficile* at one time using immunochemical fingerprinting.

In conclusion, we found that a large proportion of apparent relapses with *C. difficile* were due to infection with a new strain. The percentage may be larger than the figure quoted, because cases of apparent maintenance of the same strain could be due to environmental sources of the organism. Relapse or reinfection are particularly common in extended-care facilities, probably due to the length of stay and the environmental reservoir provided by these facilities, factors supporting the suggestion by Bender and colleagues [27] that *C. difficile* may be endemic in chronic care facilities.

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