Detection and characterization of *Clostridium difficile* from patients with antibiotic-associated diarrhoea in a tertiary care hospital in North India

Clostridium difficile is recognized as a major cause of nosocomial antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis (Riley, 1998) and is a significant financial burden on modern health-care resources. Numerous *C. difficile* reservoirs exist within hospitals including environmental surfaces, ward and surgical staff (Verity *et al.*, 2001) and colonized new admissions. This huge potential for infection of patients from diverse sources necessitates methods for typing *C. difficile* to understand the epidemiology of outbreaks and isolated cases, to identify any possible incidence of cross-infection and to set up surveillance programmes to monitor virulent strain emergence and hospital reservoirs (Northey *et al.*, 2005). *C. difficile*-associated diarrhoea (CDAD) was previously assessed in our hospital in a different patient population (Dhawan *et al.*, 1999). The aim of the present study was to define the incidence of CDAD among patients suffering from AAD and to analyse the genetic heterogeneity of the strains.

A total of 214 stool specimens were received between 2001 and 2003 by the anaerobic laboratory of the All India Institute of Medical Sciences, India, from patients suffering from AAD hospitalized in different wards and were analysed for *C. difficile*. All patients were pre-treated with antibiotics and had loose watery stools (>three to five times a day). In the study group, 58 % were males and 88 % of patients were above the age of 12. Clinical information about the cause of diarrhoea, underlying disease and antimicrobial therapy was obtained by review of patient charts. The stool specimens were cultured on selective cefoxitin-cycloserine-fructose agar and brain heart infusion agar after alcohol shock treatment and incubated anaerobically for 48 h at 37 °C. Isolates were identified as *C. difficile* as described previously (Dhawan *et al.*, 1999).

Production of toxin A and B in the patient samples was detected by commercial ELISA (Premier Toxins A and B test kit; Meridian Diagnostics).

C. difficile isolates were analysed phenotypically for their antibiogram patterns, protein profile (SDS-PAGE) and volatile fatty acids (GLC) as described previously (Chaudhry *et al.*, 2008). They were further analysed at the genotypic level by restriction endonuclease analysis (REA) of the whole-cell chromosomal DNA (Bowman *et al.*, 1991) and by PCR for the toxin A, B and C genes (Chaudhry *et al.*, 2008; Gonçalves *et al.*, 2004). The amplified toxin A gene fragment was then digested with the restriction enzyme *AluI* and the digests were subjected to electrophoresis (Chaudhry *et al.*, 2008). The strains were also analysed by PFGE (Fawley *et al.*, 2005) using a CHEF DR II system (Bio-Rad) and were interpreted as per standard guidelines (Tenover *et al.*, 1995).

A total of 26 (12.1 %) patients were positive for *C. difficile* infection by the toxin dependent assay. Eleven of the 26 toxin-positive stool samples were also positive for *C. difficile* by culture. The highest number of *C. difficile* toxin-positive cases was from stool samples of patients hospitalized in haematology/oncology wards (20/26; 77 %). Six of the 26 positive cases expired.

An antibiogram grouped all the 11 isolates as susceptible to erythromycin, chloramphenicol, ciprofloxacin, penicillin, clindamycin, vancomycin, metronidazole and tetracycline. The identical fatty acid producers were divided into two groups, based on the production of isocaproic acid by GLC. SDS-PAGE placed the isolates into three groups, A, B and C, based on the protein profiles observed (nine isolates in group A, one in group B and one in group C). The whole-cell REA did not provide

bands that were suitable for typing and hence it was excluded. All the clinical isolates were positive for toxin A and B by ELISA and PCR (toxin A, 1.2 kb fragment; toxin B, 399 bp; and *tcdC* gene, 345 bp, which is a part of the pathogenicity locus). The isolates clustered into four different groups by PCR-RFLP of the toxin A gene fragment: five (45 %) isolates in group I, four (36 %) in group II, and one each (9 %) in groups III and IV. PFGE gels showed a range of banding patterns (between 6 and 10 bands) with nine pulsotypes labelled PF-a–PF-i (Fig. 1). Pulsotypes c and h shared two identical strains each. However, there was no epidemiological association between them as these strains were not linked in time or place of hospital stay. The results are summarized in Table 1.

Studies on CDAD in developing countries have been limited, probably due to the lack of technology and facilities for culturing anaerobic pathogens. This study demonstrated a prevalence of 12 % CDAD cases among the AAD cases, which showed a persisting infection rate in this hospital (Dhawan *et al.*, 1999) and also highlights the need for typing in order to obtain information about sources and routes of transmission. However, the percentage of culture-positives in this study was less than the percentage of toxin-positives. The fact that 11 of the 15 (73 %) culture-negative patients were on metronidazole or vancomycin at the time of sample analysis might be responsible for the decrease in rate of isolation of the organism compared with the rate of detection by ELISA.

In the absence of more sophisticated typing methods, a simple antibiogram and SDS-PAGE, although limited in their discriminatory capacity, could be within the scope of microbiology laboratories (Brazier, 1993), and in agreement with this, SDS-PAGE was good enough to discriminate the strains analysed in our

Table 1. Characteristics of *C. difficile* isolates

Strain	Ward of isolation	Date of isolation	Antibiogram	GLC group	SDS-PAGE group	Toxin A/B/C	PCR-RFLP group	PFGE pulsotype
1	Oncology	1/2001	1	1	A	+ / + / +	II	a
2	Oncology	4/2001	1	2	A	+ / + / +	I	b
3	Gastroenterology	6/2001	1	2	A	+ / + / +	I	c
4	Oncology	1/2002	1	2	A	+ / + / +	I	c
5	Orthopaedics	12/2001	1	2	A	+ / + / +	I	d
6	Neurology	1/2002	1	2	A	+ / + / +	IV	e
7	Medicine	1/2002	1	2	B	+ / + / +	II	f
8	Oncology	5/2002	1	2	C	+ / + / +	II	g
9	Neurology	6/2002	1	2	A	+ / + / +	III	h
10	Oncology	6/2002	1	2	A	+ / + / +	I	h
11	Oncology	3/2003	1	2	A	+ / + / +	II	i

previous study (Dhawan *et al.*, 1999). However, in the current analysis, both SDS-PAGE and the antibiogram showed poor discrimination between the isolates. PFGE was the most efficient discriminatory tool in the current analysis, though it was time-consuming. The PCR primer set NK9–NK11 was used as a screening tool to detect the prevalence of *tcdA* variant strains among *C. difficile* as reported by Barbut *et al.* (2002); however, we could not identify any such strains.

There was no evidence of clustering which would have pointed to the possibility of cross-infection. The isolates which shared

common pulsotypes were isolated from patients housed at different wards at different times. No nosocomial outbreak of *C. difficile* was reported during the study period. The cause of death of positive patients in this study was not directly related to *C. difficile* diarrhoea, although this may have been a contributory factor. *C. difficile* diversity may be more characteristic of tertiary care hospitals, which have a high number of transfers from other hospitals (Samore *et al.*, 1994). Transfers add to the pool of *C. difficile*-infected patients and may introduce novel strains to the referral institution.

In conclusion, this study demonstrated that many different strains were prevalent in this hospital population. Furthermore, nosocomial *C. difficile* diarrhoea was associated with a variety of strains simultaneously. The failure to show clustering of diarrhoeal cases reflected the wide diversity of strains in this referral institution at any one time, none of which were responsible for a clonal epidemic. This simultaneous spread of multiple strains of the same organism is a pattern that has been described with other pathogens (Meyer *et al.*, 1993) and poses greater challenges for hospital infection control. In the future, this investigation will be extended by collecting isolates from a larger and more widespread region of the country. The monitoring and molecular typing of a large number of isolates could allow the comparison of these data with those obtained from other countries and acquisition of more information about this important nosocomial pathogen.

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Fig. 1. PFGE patterns obtained with *Sma*I-digested DNA of *C. difficile* isolates. PFGE results obtained for 11 clinical *C. difficile* isolates (lanes 1–11) after the inclusion of 200 µM thiourea in the electrophoresis buffer. The marker lanes (M) contain bacteriophage lambda concatemers (approx. 0.05–1.0 Mbp).

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