ORIGINAL ARTICLE

Characteristics of *Clostridium difficile* strains isolated from asymptomatic individuals and from diarrheal patients

S. Wongwanich[1], P. Pongpech[2], C. Dhiraputra[3], S. Huttayananont[1] and P. Sawanpanyalert[1]

[1]National Institute of Health, Nonthaburi, [2]Chulalongkorn University and [3]Mahidol University, Bangkok, Thailand

Objectives To characterise genotypes of *Clostridium difficile* strains isolated from asymptomatic individuals and patients with diarrhea.

Methods Fecal specimens from 235 asymptomatic infants <12 months, 76 asymptomatic children 1–11 years and 132 adult patients with antibiotic-associated and non-antibiotic-associated diarrhea obtained from Siriraj Hospital, Bangkok from October 1998 to April 1999 were examined for *C. difficile* by cycloserine–cefoxitin–fructose agar culture. The presence of the *C. difficile* toxin A gene was determined by specific PCR with the use of primers 5-(CCC AAT AGA AGA TTC AAT ATT AAG CTT)-3 and 5-(GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT)-3. All *C. difficile* isolates were subsequently genotyped by pulsed-field gel electrophoresis (PFGE).

Results The *C. difficile* strains were found in 28 (11.9%) asymptomatic infants, 16 (21.1%) asymptomatic children and 33 (25%) adult patients. In total, 14 PFGE types and eight subtypes designated as types A, B, C, D, E, F, G, H, I, J, K, L, M and N, and A1, A2, A3, A4, B1, B2, B3 and E1, respectively, were identified. Only two isolates from infants and 18 isolates from adult patients were toxin A gene positive by PCR. Both isolates of toxigenic *C. difficile* were from infants in the same ward and were PFGE type B. PFGE type A was the predominant type among all toxigenic isolates (12 of 18 isolates) from adult patients. The other PFGE types of toxigenic *C. difficile* found in adult patients were: type A1, one isolate; type B, four isolates; and type C, one isolate. Types B2 and D were identified in 38.5% and 46.2%, respectively, of the toxin A gene-negative isolates of *C. difficile* from infants.

Conclusions These results revealed the occurrence of three distinct clusters from different wards in Siriraj Hospital. The toxigenic *C. difficile* of PFGE type A and related subtypes was a predominant infective clone in adult patients, whereas non-toxigenic *C. difficile* types B2 and D were encountered in asymptomatic infants. This information can be useful in epidemiologic investigations.

Keywords *C. difficile*, epidemiology, typing, pulsed-field gel electrophoresis, diarrhea

Accepted 2 July 2001

Clin Microbiol Infect 2001; 7: 438–441

INTRODUCTION

Clostridium difficile is a spore-forming anaerobic bacterium. It has been recognised as a major cause of pseudomembranous colitis, antibiotic-associated diarrhea, antibiotic-associated colitis, and non-antibiotic-associated diarrhea [1–4]. This bacterium produces at least two exotoxins: toxin A, an enterotoxin, and toxin B, a potent cytotoxin. Both toxins are involved in the diseases [1,5].

Because of the increasing incidence of nosocomial diarrhea caused by *C. difficile*, there is a need for an effective procedure for typing these bacteria in order to obtain information about sources and routes of transmission. In a previous study, the genotypic data obtained by the use of pulsed-field gel electrophoresis (PFGE) were shown to be particularly reliable in establishing relatedness or identity among 11 *C. difficile* isolates [6]. In the present study, all *C. difficile* strains isolated from fecal specimens collected from asymptomatic infants, asymptomatic children and adult patients with diarrhea were investigated for the presence of the toxin A gene by using polymerase chain reaction, in order to assess their occurrence and clinical significance. We have extended PFGE analysis to a large sample of both toxigenic and non-toxigenic *C. difficile* isolates in order to search for correlations in genome characteristics.

MATERIALS AND METHODS

Patients and fecal specimens

In total, 446 fecal specimens were collected from three different groups of patients at Siriraj Hospital, Bangkok, Thailand, from October 1998 to April 1999. These included 235 asymptomatic infants, 76 asymptomatic children and 132 adult patients who

Corresponding author and reprint requests: Mrs. Siripan Wongwanich, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, 88/7 Tiwanonth Road, Nonthaburi 11000, Thailand
Tel: +66 2 589 9850 8
Fax: +66 2 591 5449
E-mail: siripan@dmsc.moph.go.th

developed diarrhea during their hospital stays. Diarrhea was defined as six or more unformed feces in 36 h. The adult patients with diarrhea in this study could be divided into two subgroups according to the physician's diagnosis. The first subgroup included 84 adult patients who developed diarrhea during their hospital stays. The other subgroup of 48 adult patients comprised the suspected cases of antibiotic-associated diarrhea. There were two adult patients whose feces were collected more than once, because of the development of chronic diarrhea during their long hospital stays.

Culture

All fecal specimens were cultured for *C. difficile* by the use of a selective medium, cycloserine–cefoxitin–fructose agar (CCFA, Oxoid Ltd, Basingstoke, UK), and alcohol shock as previously described [7].

Detection of the toxin A gene

The presence of the toxin A gene in all isolates of *C. difficile* was determined by specific PCR with the use of the published primers: 5-(CCC AAT AGA AGA TTC AAT ATT AAG CTT)-3 and 5-(GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT)-3 [8]. The DNA samples were amplified on a Perkin-Elmer Cetus Model 480 Thermocycler for 35 cycles at 95 °C for 95 s, ramping to 55 °C in 90 s; 55 °C for 30 s, ramping to 60 °C in 30 s; and 60 °C for 120 s, ramping to 95 °C in 75 s. The toxin A-specific PCR product was 252 bp long.

PFGE analysis

DNA extraction and PFGE of all *C. difficile* isolates were performed as described previously [6]. Briefly, *C. difficile* cells grown overnight in 20 mL of brain–heart infusion broth were resuspended in TES (Tris-HCL + EDTA + NaCl) buffer to achieve a McFarland turbidity of 3, and mixed with an equal volume of 1.6% low melting agarose (Bio-Rad Laboratories, Richmond, CA, USA) to make plugs for PFGE. The cells in the plugs were treated with lysis solution and then with protease. The DNA was digested overnight with *Sma*I (New England Biolabs Inc,, Beverly, MA, USA). For PFGE, a CHEF DR II system (Bio-Rad Laboratories, Richmond, CA, USA) was used with switch times of 5 and 30 s for 24 h at 6 V/cm. The gels were stained with ethidium bromide and photographed under UV light. Chromosomal DNA of *Saccharomyces cerevisiae* (Bio-Rad Laboratories) was used for molecular standard markers. The isolates' chromosomal fingerprints were compared by eye and assigned to PFGE types and subtypes, according to the published guideline [9]. Isolates with indistinguishable patterns were considered to belong to the same PFGE type and were designated by the same unique letter (e.g. A). Isolates with patterns closely related (1–2-band difference) to that of an already defined type were designated by the letter of that type followed by a number (e.g. A1, A2).

Statistical analysis

Data were analysed using Epi Info Software version 6. Categorical variables were compared using chi-square test. A *P*-value of <0.05 was considered significant.

RESULTS

C. difficile in three groups of patients

The *C. difficile* strains were found in 28 (11.9%) of 235 asymptomatic infants, 16 (21.1%) of 76 asymptomatic children, and 33 (25%) of 132 adult patients, as shown in Table 1. The proportions positive for *C. difficile* among the three groups of patients were significantly different ($P = 0.004$). Thirteen of 84 (15.5%) adult patients with diarrhea and 20 of 48 (41.7%) adult patients with antibiotic-associated diarrhea had *C. difficile* in their stools ($P < 0.001$). Toxigenic *C. difficile* were found in three (8.8%) of 20–40-year-old, four (9.5%) of 41–60-year-old and 11 (19.3%) of >60-year-old patients. Although the highest numbers of both *C. difficile* culture isolates (31.6%) and toxin A gene-positive isolates (19.3%) were observed in diarrheal patients at >60 years of age, there was no significant difference in detection rates ($P = 0.287$).

Table 1 Recovery of *C. difficile* from asymptomatic individuals and diarrheal patients

Patient group	No. of persons	No. (%) of *C. difficile* positive	No. (%) of toxin A gene positive by PCR
Asymptomatic infants <12 months	235	28 (11.9)	2 (0.9)
Asymptomatic children 1–11 years	76	16 (21.1)	0
Adults	132	33 (25.0)	18 (13.6)
With diarrhea	84	13 (15.5)	8 (9.5)
With antibiotic-associated diarrhea	48	20 (41.7)	10 (20.8)
Total	443	77 (17.4)	20 (4.5)

Detection of the toxin A gene

Only two of 18 isolates from infants and 18 of 33 isolates from adult patients were positive for toxin A gene by PCR. The toxin A gene was found in eight *C. difficile* isolates of patients with diarrhea (9.5%), while ten *C. difficile* isolates of patients with suspected antibiotic-associated diarrhea had the toxin A gene (20.8%) (Table 1).

PFGE analysis

In total, 14 PFGE types and eight subtypes designated as types A, B, C, D, E, F, G, H, I, J, K, L, M and N, and A1, A2, A3, A4, B1, B2, B3 and E1, respectively, were identified. The DNA patterns obtained with PFGE analysis are shown in Figures 1 and 2. Both isolates of toxigenic *C. difficile* from infants in the same ward were PFGE type B. PFGE type A was the predominant type among all toxigenic isolates (12 of 18 isolates) from adult patients. The other PFGE types of toxigenic *C. difficile* found in adult patients were: type A1, one isolate; type B, four isolates; and type C, one isolate. Among 14 non-toxigenic *C. difficile* isolates from the symptomatic adults, nine PFGE types were observed. Of these, types A3, B, B1, G and M were each represented by two non-toxigenic strains, while B3, F, I and N were each represented by only one strain. Types B2 and D were identified in 38.5% and 46.2% of the toxin A gene-negative isolates of *C. difficile* from infants. The greatest genotypic variety of non-toxigenic *C. difficile* was obtained in the asymptomatic children group (13 PFGE types, 16 strains).

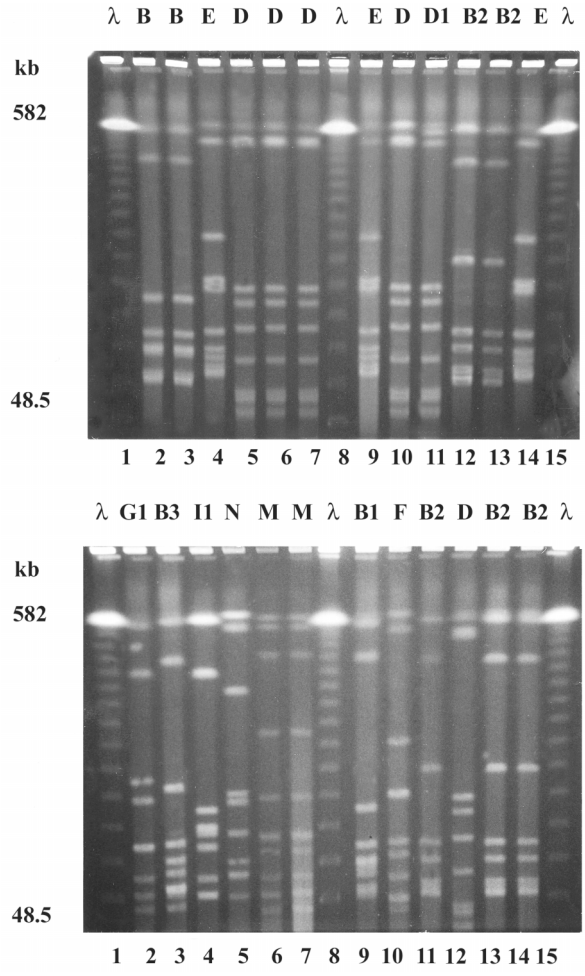

Figure 1 PFGE major patterns and subtypes of *Sma*I digests. The PFGE types are shown in the lanes. Lanes 1, 8 and 15: lambda ladder molecular size markers. Lanes 2–7 and 9–14: clinical isolates.

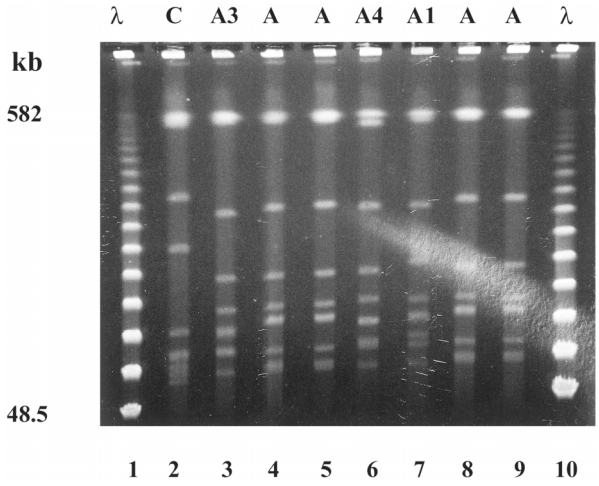

Figure 2 Fragment patterns of the *C. difficile* genome digested with *Sma*I in strains isolated from diarrheal adult patients. The PFGE types are shown above the lanes. Lanes 1 and 10: lambda ladder molecular size marker. Lanes 2–9: clinical isolates.

There were two adult patients who stayed in the hospital longer than 2 months. They both developed chronic diarrhea during that time. The genotype did not change among the isolates obtained from the serial specimens from these patients. Sequential isolates changed in subtype in only one patient after 2 months and 20 days (PFGE types A and A4). In another patient, the PFGE type A strain persisted throughout the time of the hospital stay (4 months and 20 days).

DISCUSSION

Five to 70 per cent of healthy infants have *C. difficile* in their feces, but few of them develop the disease [1,2]. No one knows why infants are protected, but there are a number of hypotheses, including colostrum-containing substances which neutralise toxins A and B [10], the immature nature of the intestinal flora, and the lack of the toxin receptors in the intestine [5]. However, asymptomatic infants can spread this organism to healthcare workers and the hospital environment, which could be the possible mode of nosocomial transmission. In this study,

we found that 11.9% of infants harbored *C. difficile*. Two of these infants carried toxigenic *C. difficile* and showed no signs or symptoms of diarrheal disease.

The review by Bartlett [1] showed that toxigenic *C. difficile* was implicated in 10–25% of cases of antibiotic-associated diarrhea, and in 50–75% of cases of antibiotic-associated colitis. More than 90% of those with pseudomembranous colitis carried toxigenic *C. difficile* in their feces. A trend of increasing prevalence of *C. difficile* has been reported in Europe and the USA during the past 10 years.

In Thailand, there was no routine screening for *C. difficile* infections in the clinical laboratory in the hospitals throughout the country. The importance of *C. difficile* as a cause of diarrhea appears to be under-recognised by our medical community, whereas this study demonstrated an occurrence rate of 13.6% for toxigenic *C. difficile* infections in hospitalised adult patients. However, the difference in positivity rates of *C. difficile* stool culture between adult patients with diarrhea and those with antibiotic-associated diarrhea was statistically significant (13/84 versus 20/48, $P < 0.001$). It is now widely accepted that toxigenic *C. difficile* is the most frequently encountered agent of diarrhea among hospitalised patients. Routine laboratory work-up protocols for fecal specimens should therefore be designed for *C. difficile* culture and/or its toxin(s) detection.

In this study, the polymerase chain reaction was used for the detection of the toxin A gene of *C. difficile*. Only toxin A was determined, because both toxins (A and B) normally act in a synergistic manner to cause *C. difficile*-associated disease. It has been rarely reported that isolation of *C. difficile* from patients could produce only toxin A or toxin B alone [11,12].

C. difficile has become the most common agent of nosocomial diarrhea in adults [13–15]. Typing methods could be important in the epidemiologic investigation of nosocomial infections. In this study, all 77 *C. difficile* strains isolated from study patients were typed, but one isolate could not be typed by PFGE, because of DNA fading. Our results confirmed that the PFGE assay could generate a good discriminative capacity for investigating the relatedness of isolates recovered from different patients. Two isolates from fecal specimens collected at different periods of time from one adult patient were both PFGE type A. The other two *C. difficile* isolates from one adult patient were closely related PFGE types (type A and type A4).

In conclusion, the results showed that the occurrence of *C. difficile* infections in adult patients was comparable to that reported in developed countries. Routine detection of *C. difficile* and/or its toxin(s) is therefore necessary in this region. This study also confirms that the genotypic characteristics of *C. difficile* isolates could be clearly identified by PFGE. It was found that the major clones of both toxigenic and non-toxigenic isolates of *C. difficile* obtained from asymptomatic individuals and diarrheal patients were different. This may be very useful in epidemiologic studies of the clonal distribution of *C. difficile* in other settings.

ACKNOWLEDGMENTS

This work was presented in part at the 11th European Congress of Clinical Microbiology and Infectious Diseases, Istanbul, Turkey, 2001 [16].

REFERENCES

1. Bartlett JG. *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. *Clin Infect Dis* 1994; 18(suppl 4): s265–72.
2. Knoop FC, Owens M, Crocker IC. *Clostridium difficile*: clinical disease and diagnosis. *Clin Microbiol Rev* 1993; 6: 251–6.
3. George WL, Rolfe RD, Finegold SM. *Clostridium difficile* and its cytotoxin in feces of patients with antimicrobial agent-associated diarrhea and miscellaneous conditions. *J Clin Microbiol* 1982; 15: 1049–53.
4. Brettle RP, Poxton IR, McMurdoch J, Brown R, Byrne MD, Collee JG. *Clostridium difficile* in association with sporadic diarrhoea. *Br Med J* 1982; 284: 230–3.
5. Lyerly DM, Krivan HC, Wilkins TD. *Clostridium difficile*: its disease and toxins. *Clin Microbiol Rev* 1988; 1: 1–18.
6. Wongwanich S, Ramsiri S, Kusum M, Warachit P. *Clostridium difficile* infection in HIV-positive patients. *Southeast Asian J Trop Med Public Health* 2000; 31(3): 537–9.
7. Wongwanich S, Ramsiri S, Vanasin B, Khowsaphit P, Tantipatayangkul P, Phan-urai R. *Clostridium difficile* associated disease in Thailand. *Southeast Asian J Trop Med Public Health* 1990; 21(3): 367–72.
8. Kato N, Ou CY, Kato H *et al*. Identification of toxigenic *Clostridium difficile* by polymerase chain reaction. *J Clin Microbiol* 1991; 29: 33–7.
9. Tenover FC, Arbeit RD, Goering RV *et al*. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–9.
10. Kim K, Pickering LK, Dupont HL, Sullivan N, Wilkins T. In vitro and in vivo neutralizing activity of human colostrum and milk against purified toxins A and B of *Clostridium difficile*. *J Infect Dis* 1984; 150: 57–62.
11. Rupnik M, Avesani V, Janc M, Eichel-Streiber C, Delmee M. Novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* 1998; 36(8): 2240–7.
12. Kato H, Kato N, Watanabe K *et al*. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* 1998; 36: 2178–82.
13. Johnson S, Clabots CR, Linn FV, Olson MM, Peterson LR, Gerding DN. Nosocomial *Clostridium difficile* colonization disease. *Lancet* 1990; 336: 97–100.
14. Larson HE, Price BA, Honour P, Borriello PS. *Clostridium difficile* and the aetiology of pseudomembranous colitis. *Lancet* 1978; i: 1063–6.
15. McFarland LV, Mulligan M, Kwok RYY, Stamn WE. Nosocomial acquisition of *Clostridium difficile* infection. *N Engl J Med* 1989; 320: 204–10.
16. Wongwanich S, Pongpech P, Dhiraputra C *et al*. Characteristic of *Clostridium difficile* strains isolated from asymptomatic individualsand from diarrhoeal patients. *Clin Microbiol Infect* 2001; 7(suppl 1): 319.