Prevalent PCR ribotypes of clinical and environmental strains of *Clostridium difficile* isolated from intensive-therapy unit patients in Kuwait

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Ninety-five isolates of *Clostridium difficile* from symptomatic and asymptomatic patients and 18 from their environment in the intensive-therapy units (ITUs) of four teaching hospitals in Kuwait were typed by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). A total of 32 different ribotypes was detected among the clinical isolates. The predominant ribotypes from the clinical isolates were types 097 and 078, which accounted for ~ 40 % of all isolates in the ITUs in Kuwait. Ribotypes 097 (toxigenic), 078 (toxigenic) and 039 (non-toxigenic) were three distinct clones that were circulating in all four hospitals. Ribotypes 097, 078 and 076 (i.e. 50 % of isolates from symptomatic patients) were the predominant isolates associated with *C. difficile*-associated disease (CDAD). The environmental isolates belonged to a diverse range of ribotypes, with no particular types common to all the hospitals. Ribotype 078 was found only in the patient environment in Mubarak hospital, while ribotype 097 was restricted to Amiri hospital. The hospital environment occupied by symptomatic as well as symptom-free patients was contaminated with *C. difficile*. Eight new strains that did not match any in the PCR ribotype library established at the PHLS Anaerobe Reference Unit, Cardiff, UK, were assigned ribotypes 105, 125, 128, 129, 131, 134, 140 and 141. These findings show that the isolates associated with CDAD in Kuwait are different from those found in the UK and some other European countries.

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INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus. It is often associated with a spectrum of diseases, referred as *C. difficile*-associated disease (CDAD), which manifest as self-limiting antibiotic-associated diarrhoea (AAD), antibiotic-associated colitis (AAC) and pseudomembranous colitis (PMC) with toxic megacolon and possible gut perforation. Rarely, it can present as extra-intestinal infections, such as arthritis, osteomyelitis, soft-tissue infection and bacteraemia (Levett, 1986). CDAD is an important clinical problem because it is often acquired by hospitalized patients, and *C. difficile* is associated with outbreaks of diarrhoea and colitis in hospitalized adults receiving antibiotics (Bartlett *et al.*, 1978; Djuretic *et al.*, 1999).

Abbreviations: AAC, antibiotic-associated colitis; AAD, antibiotic-associated diarrhoea; CDAD, *Clostridium difficile*-associated disease; ITU, intensive-therapy unit; PMC, pseudomembranous colitis.

In spite of major efforts to control the spread of CDAD in hospitals and nursing homes, this organism has remained a major problem worldwide, and it continues to be responsible for endemic and epidemic nosocomial diarrhoea (McFarland *et al.*, 1989; Johnson *et al.*, 1990; Barbut *et al.*, 1996). *C. difficile*, or its toxins, has been identified in 8–10 % of cases of nosocomial diarrhoea, while other common bacterial enteric pathogens, *Salmonella*, *Shigella* and *Campylobacter* spp., are rarely isolated (Fan *et al.*, 1993; Rohner *et al.*, 1997).

It appears that the most important sources of *C. difficile* in a hospital setting are symptomatic patients and asymptomatic carriers who are the main reservoirs of *C. difficile* in the hospital. The environment of these patients is also an important source. There is evidence that environmental contamination in rooms of patients with diarrhoea is substantially greater than in rooms with asymptomatic patients (49 vs 29 %) (McFarland *et al.*, 1989). In addition, rooms currently occupied by *C. difficile*-negative patients

can, in spite of routine cleaning, be contaminated with *C. difficile* spores that can survive for several months in the hospital environment. Person-to-person transmission on hospital wards, especially geriatric wards, as well as environmental contamination and carriage on the hands of hospital workers have been documented (Kim *et al.*, 1981; Malamou-Ladas *et al.*, 1983; Savage & Alford, 1983; McFarland *et al.*, 1989). Transmission occurs mainly via the faecal oral route and direct contact with contaminated surfaces (Barbut & Petit, 2001).

Epidemiological studies of *C. difficile* strains isolated from different environmental and clinical sources involve detailed comparison of the different isolates from all sites. Several typing schemes have been developed to determine the relatedness of strains of *C. difficile* associated with infections. However, of these, PCR ribotyping offers several advantages over the other methods because it is highly discriminative, reproducible and relatively rapid and easy to perform (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999).

Our main aim was to study the epidemiology of *C. difficile* isolates from patients admitted into the intensive-therapy units (ITUs) of the four main teaching hospitals in Kuwait by PCR ribotyping.

METHODS

Bacterial isolates from patients. Stool samples were collected from all patients admitted to the ITUs of Mubarak Al-Kabeer hospital (ITU-1), Ibn Sina Burn Unit (ITU-2), haematology wards of Kuwait Cancer Control Centre (ITU-3) and Amiri hospital (ITU-4) who had stayed in the units for a minimum period of 3 days. The study was carried out over a period of 1 year (February 2001–January 2002). Freshly passed stool (rectal swabs, in Amies transport medium, if collection of stool was not feasible) was taken on the day of admission and weekly thereafter until the patient was discharged or developed diarrhoea secondary to *C. difficile* infection/colonization.

Environmental and other samples. A sterile swab, pre-moistened with sterile normal saline, was wiped over selected surfaces in the units, e.g. bed sheets, mattress, bed edges, bed ledges, surfaces of the side table next to the patients, suction regulator, oxygen regulator, ventilator surfaces, IV stand and the floor under the bed. Hands of doctors, nurses and physiotherapists who had had contact with the positive cases were cultured, by contact agar plates, for evidence of *C. difficile* contamination.

Inoculation, isolation and identification. The stool and environmental samples were inoculated into Robertson cooked meat (RCM) medium containing 25 ml fastidious anaerobe broth (FAB; Lab M) and incubated anaerobically for 48 h at 37 °C. Five-hundred microlitres of the FAB from the RCM was heated for 10 min at 80 °C. Next, cycloserine-cefoxitin egg yolk agar (CCEYA; Oxoid) and cycloserine-cefoxitin fructose agar (CCFA; Oxoid) plates were inoculated with one loop of the heated broth and incubated anaerobically for 48 h in an anaerobic chamber (H₂ 10%, CO₂ 10%, N₂ 80%). Isolates that were Gram-positive bacilli with characteristic horse-dung smell and fluoresced yellowish-green under long-wave UV light (365 nm) were selected and their identity was confirmed as *C. difficile* by API 20A (bioMérieux).

Toxin detection. Single colonies were subcultured on pre-reduced Columbia agar base (Oxoid) supplemented with 5% horse blood,

vitamin K and haemin and incubated at 37 °C under anaerobic conditions for toxin A detection. Toxin A was detected by ELISA (TOX-A) kits (Tech Lab); the procedure was carried out according to the manufacturer's instructions. Toxin B was detected by cytotoxicity assay on Vero cells. Production of cytopathic effects by filtered supernate of *C. difficile* RCM broth culture on Vero cells indicated toxin B production. Toxins A and B were detected in the stool by the TOX-A/B kits (Tech Lab).

PCR ribotyping. All isolates were typed by the PCR ribotyping method described by O'Neill *et al.* (1996). Briefly, after obtaining a pure culture, a single colony was subcultured on fastidious anaerobe agar (FAA; Lab M) supplemented with 6% horse blood and incubated for 24 h at 37 °C. DNA was extracted from a suspension of 10 colonies in 100 µl 5% Chelex 100 (Bio-Rad) by heating at 100 °C for 10 min. Cell debris was removed from the suspension by centrifugation for 10 min at 17 000 g. The supernate obtained was then used as the DNA template. Primers P3 (5'-CTGGGGTGAAGTCGTAACAAGG) and P4 (5'-GCGCCCTTTGTAGCTTTGACC) were used for the PCR amplification. The reaction mixture (final volume, 100 µl) contained 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 2.5 U *Taq* polymerase, 200 mM of each dNTP, 50 pmol of each primer and 10 µl DNA template. The PCR programme was 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 2 min. A negative control was included in each run. Amplification products were concentrated to a final volume of 25 µl by heating at 75 °C for 90 min before electrophoresis at 200 V, 100 A in Metaphore agarose gel (FMC) for 3 h at room temperature. DNA fragments were then visualized by staining the gel for 20 min in 0.5% ethidium bromide. Gel images were analysed with GelCompar image analysis software (version 4.0; Applied Maths). Our results were then compared with the library of PCR ribotypes already established at the PHLS Anaerobe Reference Unit, Cardiff, UK.

RESULTS

C. difficile was isolated from 95 of 922 patients (430 in ITU-1, 63 in ITU-2, 88 in ITU-3 and 341 in ITU-4) screened during this period. Thirty (31.6%) of these 95 patients were symptomatic with CDAD; two patients had PMC, four had AAC and 24 had AAD. The overall incidence of *C. difficile*-positive patients was 10.3%, with incidence in each hospital being 10.5% for ITU-1, 6.3% in ITU-2, 18% in ITU-3 and 9.6% in ITU-4. The organism was isolated from 18 of 380 environmental samples; six from ITU-1, three from ITU-3 and nine from ITU-4.

The distribution of PCR ribotypes among the clinical and environmental isolates is shown in Tables 1 and 2, and the banding of representative ribotypes is demonstrated in Fig. 1. Of the 95 isolates, 72 (75.8%) were toxin-producers, while 23 (24.2%) were non-toxigenic. A total of 28 (38.9%) of the 72 toxigenic and two (8.7%) of the 23 non-toxigenic isolates were associated with diarrhoea. As shown in Table 1, 32 distinct, genotypically different ribotypes were identified among the 95 clinical isolates. Of these, three distinct clones were detected in all four hospitals, ribotypes 097 (toxigenic), 078 (toxigenic) and 039 (non-toxigenic). The remaining 58 isolates belonged to a set of diverse ribotypes. In ITU-1, the ribotypes associated with diarrhoea in the 18 symptomatic patients were ribotypes 078 (4, 22%), 076 (3, 17%), 012 (2, 11%), 056 (2, 11%), 094 (2, 11%), 097 (2, 11%), 105 (2, 11%) and 046 (1, 6%). The five isolates associated with

Table 1. Distribution of PCR ribotypes and toxigenic strains of *C. difficile* in four teaching hospital ITUs

Ribotype	Toxin (A/B)	ITU-1	ITU-2	ITU-3	ITU-4	Total
002	+	0	1	1	0	2
010	—	3	0	0	0	3
012	+	2	0	0	0	2
013	+	1	0	0	0	1
014	+	1	0	1	1	3
017	—/+	0	0	0	1	1
020	+	1	0	0	0	1
026	—	1	0	0	0	1
029	+	1	0	1	0	2
035	—	1	0	0	0	1
039	—	6	1	1	2	10
045	+	0	0	0	2	2
046	+	1	0	0	0	1
051	+	0	0	3	0	3
054	+	1	0	0	0	1
056	+	2	0	0	3	5
064	+	1	0	0	0	1
070	+	0	0	1	0	1
076	+	3	0	0	3	6
077	+	0	0	3	0	3
078	+	4	1	2	2	9
081	+	1	0	0	0	1
094	+	2	0	1	0	3
097	+	3	1	1	13	18
098	+	0	0	0	2	2
105	+	2	0	0	0	2
113	—	0	0	1	0	1
128	—	5	0	0	0	5
129	+	1	0	0	0	1
131	+	1	0	0	0	1
140	—	0	0	0	1	1
141	—	0	0	0	1	1
Total		44	4	16	31	95

diarrhoea in ITU-3 represented ribotypes 077 (2, 40 %), 014 (1, 20 %), 097 (1, 20 %) and 113 (1, 20 %), while the seven associated with diarrhoea in ITU-4 represented ribotypes 017 (1, 14.3 %), 039 (1, 14.3 %) and 097 (5, 71.4 %).

All symptomatic patients with toxigenic ribotypes had detectable toxins in their stools; two patients with non-toxigenic ribotypes 039 and 113 had no detectable level of toxin in their stools. Two PMC cases in ITU-1 were infected with ribotype 076. Single AAC cases in ITU-1 and ITU-3 were infected with ribotypes 046 and 097. Two AAC cases in ITU-4 were infected with ribotype 097.

As demonstrated in Table 2, the 18 environmental isolates were assigned to nine genotypically distinct ribotypes (six toxigenic and three non-toxigenic); the predominant types were 010 (3), 078 (3) and 097 (4). Ribotypes 078 and 097 were isolated primarily from the environment of sympto-

Table 2. Distribution of PCR ribotypes and toxigenic strains of *C. difficile* in the environment of four teaching hospital ITUs

Ribotype	Toxin (A/B)	ITU-1	ITU-2	ITU-3	ITU-4	Total
001	+	0	0	0	1	1
010	—	3	0	0	0	3
056	+	0	0	0	2	2
078	+	3	0	0	0	3
097	+	0	0	0	4	4
105	+	0	0	1	1	2
120	+	0	0	1	0	1
125	—	0	0	1	0	1
144	—	0	0	0	1	1
Total		6	0	3	9	18

Fig. 1. Representative PCR ribotype profiles of *C. difficile* isolated from patients in Kuwait. Lanes: 1, 6, 11 and 14, 100 bp ladder; 2, ribotype 029; 3, 039; 4, 097; 5, 039; 7, 039; 8, 039; 9, 078; 10, 078; 12, 097; 13, 051.

matic patients infected with the same ribotypes in ITU-1 and ITU-4, respectively. Ribotype 010 was isolated from the environment of asymptomatic patients in ITU-1. Ribotype 105, associated with diarrhoea in ITU-1, was absent in its environment but present in the environment of ITU-3 and ITU-4, where it did not contribute to CDAD. Ribotypes 001 and 144 were present in the environment of ITU-4, but were not isolated from any patient in the unit. Similarly, ribotypes 120 and 125 were isolated from the floor of ITU-3, but were not isolated from the patients in the unit.

No *C. difficile* was isolated from the hands of healthcare providers.

DISCUSSION

In spite of the growing number of studies devoted to CDAD in Western countries, studies on CDAD in the Middle East

are lacking, especially in Kuwait, where information on the incidence of *C. difficile* carriage and CDAD is almost non-existent. This is partly as a result of inertia in anaerobic bacteriology prompted, until recently, by lack of expertise, technology and facilities for culturing anaerobic pathogens. We have, in the recent past, encountered a relatively high proportion of asymptomatic carriers of *C. difficile* in our hospitals, and the interpretation of positive cultures was equivocal. *C. difficile* or its toxin has been reported in one study from nearby Saudi Arabia (Akhter *et al.*, 1994), in Turkey (Soyletir *et al.*, 1996) and in a few reports from Israel (Rudensky *et al.*, 1993; Rivlin *et al.*, 1998; Boaz *et al.*, 2000). However, this is the first report on PCR ribotyping of *C. difficile* strains isolated from symptomatic and asymptomatic patients in the Middle East.

The data generated from the present study showed that all 95 and 18 *C. difficile* isolates respectively originating from patients and their environment were typable by the PCR ribotyping method, and they revealed some interesting epidemiological findings. Secondly, none of the patients admitted to the ITUs carried *C. difficile* into the units on admission. Thus, an incidence rate of hospital-acquired *C. difficile* infection/colonization of 10.3 % was established for the four hospital ITUs in Kuwait, ranging from 6.3 to 18 % in different hospitals. Interestingly, the 95 *C. difficile* culture-positive patients harboured 32 different, highly diverse PCR ribotypes. Three different DNA clones (PCR ribotypes 097, 078 and 039) were detected among patient isolates in all hospitals. The other isolates were assigned to 29 distinct and diverse types. Ribotype 097 was the single most prevalent type and was responsible for about 27 % of CDAD, while ribotype 078 was responsible for about 13 % of CDAD. Thus, ribotypes 097 and 078 were responsible for over one-third of the cases of CDAD seen. From an epidemiological point of view, this is an interesting finding, in that the dominant ribotypes causing diarrhoea in Kuwait are completely different from those seen in Europe, particularly the UK (Stubbs *et al.*, 1999), Hungary (Urban *et al.*, 2001) and Poland (Martirosian *et al.*, 1995). Fifty-five per cent of *C. difficile* infections seen in UK hospitals are caused by ribotype 001 (Stubbs *et al.*, 1999), while ribotype 087 accounted for 39 % of all isolates in Hungary (Urban *et al.*, 2001). In the Polish study, all the environmental isolates and 11 of 31 neonatal isolates belonged to ribotype 001.

It is noteworthy that one of our patients in ITU-4 who had AAD was infected with ribotype 017, which is toxin-variable (toxin A-negative, B-positive). He gave a history of travel to Thailand and he was hospitalized there for unrelated illness. An outbreak of toxin A-negative, B-positive *C. difficile* associated with diarrhoea has been reported in a Canadian tertiary-care hospital (Al-Barrak *et al.*, 1999). This is not our experience in Kuwait so far, as the single isolate was restricted to only one hospital and one patient. Why some patients have more marked symptoms than others and some strains are more associated with outbreaks of CDAD in different countries is unclear. Systemic symptoms are caused mainly by toxin-mediated inflammatory mediators released in the

colon (Dallal *et al.*, 2002). Thus, it is conceivable that the ability of the host to mount an effective antibody-mediated response to the *C. difficile* toxin plays a major role in this regard. However, the bacterial factors involved in the geographical differences seen in the two locations are worthy of further investigation.

The environmental strains were heterogeneous in each hospital. The 18 isolates were assigned to nine different ribotypes. The environment of two patients each in ITU-1 and ITU-4 was contaminated by the same ribotypes as found in the patients' clinical samples, confirming that the patient's environment is a potential source of *C. difficile* and that cross-contamination may play an important role in the acquisition of nosocomial CDAD (Cohen *et al.*, 2000). In some cases, there was no correlation between the environmental and patient isolates. For instance, the environmental isolates in ITU-3 and some in ITU-4 differed from those found in the patients. Our finding is not an isolated one. Earlier, Cohen *et al.* (1997) found none of the environmental *C. difficile* genotypes among isolates from patients, and Simor *et al.* (1993) found no cross-transmission in their institution in a survey of *C. difficile* infection in a long-term care facility.

In conclusion, the prevalent PCR ribotypes of *C. difficile* strains circulating in Kuwait hospitals are different from those found in many hospitals in Europe and are also different from those associated with CDAD in many patients. Further work is needed to elucidate the factors responsible for the geographical differences and the ability of one strain to cause more systemic symptoms than another.

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