Molecular Epidemiology of Endemic *Clostridium difficile* Infection and the Significance of Subtypes of the United Kingdom Epidemic Strain (PCR Ribotype 1)

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We previously identified two subtypes of the epidemic strain *Clostridium difficile* PCR ribotype 1, one clindamycin-sensitive strain (arbitrarily primed PCR [AP-PCR] type Ia) and a closely related clindamycin-resistant strain (AP-PCR type Ib) in our institution. We have now carried out prospective epidemiological surveillance for 4 years, immediately following the relocation of two acute medicine wards for elderly patients (wards A and B), to determine the clinical epidemiology of subtypes of the epidemic *C. difficile* PCR ribotype 1 group. To maximize the chance of strain discrimination, we used three DNA fingerprinting methods, AP-PCR, ribospacer PCR (RS-PCR), and pulsed-field gel electrophoresis (PFGE), to analyze *C. difficile* isolates recovered from symptomatic patients and from repeated environmental samplings. On ward B the incidence of *C. difficile* infection correlated significantly with the prevalence of environmental *C. difficile* both in ward areas closely associated with patients and health care personnel ($r = 0.53$; $P < 0.05$) and in high-reach sites ($r = 0.85$; $P < 0.05$). No such relationships were found on ward A. Seventeen distinct *C. difficile* genotypes were identified, 17 by AP-PCR, 12 by PFGE, and 11 by RS-PCR, but only 4 of 17 genotypes caused patient infection. Isolates recovered from the hospital ward environment were much more diverse (14 genotypes). AP-PCR type Ia represented >90% of the *C. difficile* isolates. In addition to this genotype, only two others were isolated from both patient feces and environmental surfaces. AP-PCR type Ib (clindamycin-resistant PCR ribotype 1 clone) was not associated with any cases of *C. difficile* infection and was isolated from the environment on only two occasions, after having been implicated in a cluster of six *C. difficile* infections 5 months before this study. The disappearance of this strain implies that differences in virulence and/or selective pressures may exist for this strain and the closely related, widespread *C. difficile* AP-PCR type Ia strain. Our findings emphasize the need to understand the epidemiology and virulence of clinically significant strains to determine successful control measures for *C. difficile* infections.

Clostridium difficile is a major nosocomial pathogen. It is the most commonly identified pathogen in antibiotic-associated diarrhea and is the etiological agent of pseudomembranous colitis (3, 18). The numbers of reported cases of *C. difficile* infections in England, Wales, and the United States have continued to rise (1, 10, 13). These trends highlight an increasing burden on hospital resources (25, 35). Suboptimal infection control procedures have been implicated in the spread of *C. difficile* infections in hospitals (8, 19), and in some settings the level of *C. difficile* contamination on hospital wards has correlated with transmission to health care personnel or patient contact (27, 34). PCR ribotyping has been used to characterize 116 different *C. difficile* genotypes at the Anaerobic Reference Laboratory (ARL), National Public Health Service of Wales. *C. difficile* PCR ribotype 1 is notably distributed widely (28). This ribotype, which belongs to serogroup G (7), has been referred to as the UK epidemic strain and was reported to be present in 33 of 58 hospitals in England and Wales, which accounts for 55% of all isolates referred from hospital cases of *C. difficile* infection (5). *C. difficile* PCR ribotype 1 has been reported to be responsible for 93% of *C. difficile* infection

outbreaks in the United Kingdom (13), the largest of which involved 175 patients (including 17 deaths) on 34 wards in three hospitals during a 6-month period (8). A recent report from the United States described outbreaks of *C. difficile* infection in New York, Arizona, Florida, and Massachusetts and implicated a PCR ribotype 1 strain that carried the *ermB* gene, which confers resistance to clindamycin (21). PCR ribotype 1 was also identified as the predominant genotype isolated from elderly male patients in a hospital in California and was present among patient isolates from hospitals in Sweden and Japan (6, 23, 36).

Using different DNA fingerprinting techniques, we recently demonstrated that not all *C. difficile* strains belonging to PCR ribotype 1 are clonal and, furthermore, that resistance to clindamycin is not conserved across this ribotype (14, 16). We established the widespread presence of a clindamycin-susceptible PCR ribotype 1 clone at our own institution and also identified a clindamycin-resistant PCR ribotype 1 strain responsible for a cluster of six cases of *C. difficile* infection (15). This investigation ended soon after the cluster was identified due to the relocation of the study wards, and we highlighted the need for long-term study of the distribution of endemic and epidemic *C. difficile* clones. Therefore, we have analyzed all *C. difficile* isolates recovered from symptomatic patients and from repeated environmental samplings in an endemic setting for more than 4 years, immediately after the opening of two med-

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icine wards for elderly patients. We have investigated the molecular epidemiology of *C. difficile*, including subtypes of the epidemic PCR ribotype 1, and aimed to determine their significance in both patient infection and environmental contamination.

MATERIALS AND METHODS

Study design. We fingerprinted the DNA of all *C. difficile* isolates recovered from patients with symptomatic antibiotic-associated diarrhea and from monthly environmental samplings on two hospital medicine wards for elderly patients over a 51-month period (August 1997 to October 2001). The study began immediately following a planned move of the two wards to a different hospital building. The study wards were of similar design, and each consisted of five six-bed bays and at least two patient side rooms. The new ward locations were in the same building (which was approximately 40 years old), with one ward on the floor directly below the other. The new locations of wards A and B were previously gastrointestinally surgery and general medicine wards, respectively.

Diagnosis of *C. difficile* infection, culture, and identification. Fecal samples from patients with diarrhea suspected to be due to *C. difficile* infection were tested for the presence of *C. difficile* cytotoxin in the routine diagnostic laboratory. Cytotoxin was detected by a microtiter tray method with Vero cells with *Clostridium sordellii*-protected controls and a 1-in-50 final dilution of feces in cell culture medium. All cytotoxin-positive feces were stored at -20°C pending culture for *C. difficile*.

C. difficile isolates were recovered from environmental and frozen fecal samples by culture on modified Brazier's cycloserine-cefoxitin-egg yolk agar (Bioconnections, Bardsey, United Kingdom) without egg yolk and supplemented with 5 mg/liter lysozyme (CCEYL) for 48 h at 37°C in a WISE Anaerobic Workstation (Don Whitley Scientific, Shipley, United Kingdom). After direct inoculation onto CCEYL, environmental swabs were incubated anaerobically, as described above, in Robertson's cooked meat broth. Fresh CCEYL plates were then inoculated with the resultant broth culture as before. *C. difficile* isolates were recognized as irregularly edged grey-brown colonies with a characteristic horse manure odor. In cases of doubt, the RapID ANA II system (Biostat, Stockport, United Kingdom) was used to biochemically confirm the identities of the *C. difficile* isolates. All *C. difficile* isolates were stored in nutrient broth supplemented with 0.5% glycerol at -70°C pending DNA fingerprinting studies.

Environmental decontamination. The hospital ward floors and other general surfaces were cleaned daily with a neutral detergent (Hospec; GWP Chemicals, United Kingdom). Sinks, toilets, and commodes were disinfected with a chlorine-release sanitizing agent (Divocare; GWP Chemicals). Isolation rooms housing infected patients were cleaned twice daily with hypochlorite solution (1 in 1,000 ppm chlorine). Mattresses and bed frames were cleaned with a neutral detergent upon patient discharge or transfer. For 2 years (March 1999 to February 2001) within the study period, a ward crossover study was performed; during this time environmental cleaning was carried out with either hypochlorite solution (1 in 1,000 ppm chlorine) or neutral detergent (34). Each ward received the same total duration of cleaning with either agent. The frequency of environmental cleaning was constant throughout.

Environmental sampling. Environmental sites from the hospital wards were sampled for the presence of *C. difficile* spores. Sampling of those sites considered to be commonly exposed to patients and health care staff was performed monthly. In addition, high-reach sites were sampled at 6-month intervals. Sampling was performed in a systematic manner (100-cm² areas) with sterile cotton wool swabs moistened with 0.25% Ringer's solution (Oxoid, Basingstoke, United Kingdom) and then cultured immediately for *C. difficile*.

DNA fingerprinting. Fingerprinting of the DNA of *C. difficile* isolates was performed by the arbitrarily primer PCR (AP-PCR), ribospacer PCR (RS-PCR), and pulsed-field gel electrophoresis (PFGE) techniques in order to maximize the chance of discriminating between strains. For PCR-based typing, target DNA was extracted from each bacterial strain, as described previously (32). To detect any mixed cultures of *C. difficile*, separate typing reactions were performed with DNA samples extracted from both single and multiple colonies. AP-PCR primer ARB11 (5' CTA GGA CCGC 3') (24) and RS-PCR primers L1 (5' CAA GGC ATC CAC CGT 3') and G1 (5' GAA GTC GTA ACA AGG 3') (20) (all from MWG Biotech, Milton Keynes, United Kingdom) were used to fingerprint all *C. difficile* isolates under the conditions described previously (15).

For PFGE analysis, the isolates were cultured in prereduced Schaedler's anaerobic broth (Oxoid) overnight at 37°C in an anaerobic atmosphere. Fresh bacterial growth was harvested from 5 ml broth culture by centrifugation, and the resultant pellets were washed twice in 5 ml sterile phosphate-buffered saline. The

cells were resuspended in 100 μl lysis buffer (10 mM Tris, 0.5 mM EDTA, 0.8% *N*-lauryl sarcosine, 5 mg/ml lysozyme) (J. E. Corkill, personal communication). This suspension was mixed with an equal volume of molten 2% PFGE-grade, low-melting-point agarose (Bio-Rad, Hertfordshire, United Kingdom), dispensed into molds, and allowed to solidify at 4°C . The plugs were incubated for 1 h at 37°C in 1 ml lysis buffer and then transferred to 5- μl glass screw-capped bottles containing 1 ml ESP buffer (0.5 mM EDTA, 1% *N*-lauryl sarcosine, 10 mg/ml proteinase K) and incubated overnight at 50°C . The following morning, the buffer was replaced with fresh solution and the plugs were incubated at 50°C for a further 6 h. The plugs were washed four times in TE buffer (10 mM Tris, 1 mM EDTA). DNA was digested with 20 U of the SmaI restriction enzyme for 5 h at 30°C . The digestion products were separated in a 1% PFGE-grade agarose gel by using a CHEF II PulseMaster PFGE apparatus (Bio-Rad, Hertfordshire, United Kingdom). A bacteriophage lambda DNA concatemer (Bio-Rad) was used as the molecular size marker. If DNA from any of the isolates was suspected to be susceptible to degradation during electrophoresis, 200 μM thiourea (Sigma, Dorset, United Kingdom) was added to the electrophoresis buffer (11). Digestion products were exposed to a field strength of 6 V/cm, with linear ramping from 5 s to 55 s, over 21 h. The PFGE gels were soaked in 0.5 $\mu\text{g/ml}$ ethidium bromide (BDH-Merck, Leicestershire, United Kingdom) and viewed and documented with an ImageMaster VDS camera (Pharmacia, Milton Keynes, United Kingdom).

Analysis of AP-PCR, RS-PCR, and PFGE profiles. The DNA profiles were analyzed with BioNumerics software (Applied Maths, BioSystematica, Devon, United Kingdom). Dendrograms were constructed by the unweighted pair group method with arithmetic mean clustering by using the Dice correlation coefficient (12).

RESULTS

***C. difficile* strains recovered from symptomatic patients.** The diagnostic microbiology laboratory identified 192 new cases of *C. difficile* infection by cell cytotoxicity assay during the study period. Specimens from patients with recurrent diarrhea were excluded from the study. Thus, there were 110 cases on ward A and 82 cases on ward B, which represented incidences of 5.9 and 3.9 per 100 admissions, respectively. Only 45% of reported cytotoxin-positive laboratory investigations could be matched to fecal samples stored at -20°C . The other specimens either had not been stored or an insufficient amount of sample remained after routine laboratory toxin testing. However, the available fecal samples were distributed evenly across the study period, and we failed to recover *C. difficile* from only five cytotoxin-positive fecal specimens after storage at -20°C . Hence, 82 patient *C. difficile* isolates were available for DNA fingerprinting.

On ward A, only three genotypes were identified among isolates from *C. difficile* infection cases: AP-PCR types Ia, IIIa, and IV (Table 1). Apart from single isolates of AP-PCR type IIIa (Fig. 1) and AP-PCR type IV, all *C. difficile* from symptomatic infections on ward A were AP-PCR type Ia. This genotype represented 95.2% of all clinical isolates studied, was clindamycin sensitive, and was confirmed to be PCR ribotype 1 by ARL (Brazier, personal communication). On ward B, only two genotypes were identified among isolates from *C. difficile* infection cases: AP-PCR types Ia and XI (Table 2). Only one AP-PCR type XI isolate was implicated in disease. AP-PCR type Ia represented the other 97.5% of typed patient isolates.

***C. difficile* strains recovered from ward environments.** During the study, 2,550 swab specimens were taken from predefined "frequent-contact" environmental sites: 1,326 on ward A and 1,224 on ward B. The sites sampled comprised bed frames (19%), radiators (19%), commodes (8%), side room curtain rails (8%), and floors (46%) from ward bays, toilets, sluices, domestic storage compartments, and patient side

TABLE 1. *C. difficile* AP-PCR types isolated from symptomatic patients, hospital environments, and the hands of health care workers on ward A

AP-PCR type	No. of clinical isolates/total no. tested	No. of isolates from hand impressions/total no. tested	Environmental isolates	
			No. of isolates/total no. tested	Environmental site(s)
Ia	40/42	14/15	181/201	Multiple
IIb	NI ^a	NI	1/201	Bed frame
IIIa	1/42	1/15	5/201	Bay floor ($n = 2$), bed frame, toilet floor, curtain rail
IIIb	NI	NI	3/201	bay floor ($n = 2$), window frame, bay floor
IV	1/42	NI	1/201	
V	NI	NI	1/201	Bed frame
VIIIb	NI	NI	4/201	Radiator ($n = 2$), bed frame, bay partition
VIIIc	NI	NI	2/201	Isolation room floor, radiator
IX	NI	NI	1/201	Isolation room floor
XIII	NI	NI	1/201	Bed frame

^a NI, not isolated.

rooms. Overall, 29.2% and 32.8% of environmental swab specimens from wards A and B, respectively, were *C. difficile* culture positive. Each environmental site was culture positive for *C. difficile* at least once during the study period. The organism was most frequently cultured from commodes, toilet floors, and bed frames (Fig. 2). In addition to samples taken from frequent-contact sites, an additional 544 swab specimens were taken from predefined high-reach environmental sites: 272 on ward A and 272 on ward B. The sites sampled comprised overbed lamps (15%), bed-bay partitions (15%), window frames (15%), door tops (12%), door frames (12%), fire hoses (5%), smoke detectors (3%), the top surfaces of storage cupboards (3%), and interbed curtain rails (20%). Overall, 15.8% and 12.1% of the high-reach sites on wards A and B, respectively, were *C. difficile* culture positive. Each environmental site except the smoke detector and the storage cupboards was culture positive for *C. difficile* at least once during the study period. The organism was most frequently cultured from overbed lamps, bed-bay partitions, and fire hoses (Fig. 2).

A total of 886 environmental *C. difficile* strains were recovered during the study period. In order to reduce the number of strains for DNA fingerprinting, we selected strains for further study as follows: environmental isolates recovered in the first and last 12 months of the study period and environmental isolates (from the intermediate period) recovered during the 6 weeks before and the 6 weeks after the detection of non-*C. difficile* AP-PCR type Ia isolates from fecal samples. Culture techniques failed to recover 14 *C. difficile* strains after storage at -70°C . Hence, 401 environmental isolates were subjected to DNA fingerprinting, which separated these into 14 types. AP-PCR type Ia represented 90.5% and 87.5% of isolates from wards A and B, respectively. Nine other types were found in the environment of ward A (Table 1), and six other types were found in the environment of ward B (Table 2). Other than AP-PCR type Ia, AP-PCR genotypes IIb and V were the only strains isolated from both study wards. Eight strains were non-toxicogenic (all strains belonged to AP-PCR types IV and V). In addition to AP-PCR type Ia, only AP-PCR type IIIa and IV strains were isolated from both patient feces and the environment (Fig. 1). The clindamycin-resistant PCR ribotype 1 subclone was isolated only once from both a bed frame and a radiator on ward B and was not isolated at all from ward A.

***C. difficile* strains recovered from the hands of health care workers.** A total of 527 hand impressions for culture were taken during a concurrent, 2-year ward-cleaning crossover study (March 1999 to February 2001) (34). Overall, 5.4% and 2.4% of samples on wards A and B, respectively, were *C. difficile* culture positive. All isolates were successfully recovered from frozen storage. Hence, 21 strains isolated from the hands of health care workers during the period of present study were also subjected to DNA fingerprinting analyses. AP-PCR type Ia represented 93% and 83% of such strains on wards A and B, respectively. The only exceptions were a single AP-PCR type IIIa strain on ward A and a single AP-PCR type IIa strain on ward B (Tables 1 and 2).

Evaluation of RS-PCR, AP-PCR, and PFGE *C. difficile* typing techniques. AP-PCR technique successfully classified a total of 483 *C. difficile* strains into 17 distinct genotypes (Fig. 3), whereas PFGE produced 12 genotypes (Fig. 4) and RS-PCR produced only 11 genotypes (Fig. 5). Figure 1 illustrates the different interpretations of strain epidemiology (for genotype III) that resulted from the use of the three fingerprinting methods. The AP-PCR and PFGE techniques successfully divided the predominant genotype in the study (confirmed to be PCR ribotype 1 by ARL) into two subtypes, AP-PCR types Ia and Ib. The AP-PCR technique produced consistent, visually distinguishable profiles for types Ia and Ib of 3 and 11 bands, respectively (Fig. 3). Type Ia represented 90.3% of strains DNA fingerprinted in the study, while type Ib accounted for only 0.4% of the total. The PFGE DNA profiles for the *C. difficile* isolates belonging to both AP-PCR type Ia and type Ib were initially consistently degraded. Successful PFGE analysis of these strains was achieved by adding thiourea to the electrophoresis buffer, as described earlier (11).

***C. difficile* infection and ward environmental contamination.** The *C. difficile* infection frequency and environmental culture positivity for wards A and B are shown in Fig. 6. These data are presented as crude numbers, as the denominators are stable; the patient admission data did not vary significantly during the study period, and the number of environmental sites sampled on each occasion was fixed. The *C. difficile* infection incidence data correlated significantly with the prevalence of environmental *C. difficile* isolates from sites closely associated with patients and health care workers on ward B ($r = 0.53$; $P <$

Strain	PCR ribotype	PFGE pulsotype	AP-PCR genotype	Sample origin	Sample site	Sample date
e38	III	IIIa	IIIa	Environment	Bay floor	12/97
45	III	IIIa	IIIa	Environment	Bay floor	12/97
46	III	IIIa	IIIa	Environment	Bed frame	12/97
P1020	III	IIIa	IIIa	Patient	Feces	05/98
152	III	IIIa	IIIa	Environment	Curtain rail	06/98
690	III	IIIa	IIIa	Staff	Hand impression plate	06/00
943	III	IIIb	IIIb	Environment	Bay floor	09/01
944	III	IIIb	IIIb	Environment	Bay floor	09/01
959	III	IIIb	IIIb	Environment	Curtain rail	09/01
144	III	VII	IIIc	Environment	Toilet floors	06/98

FIG. 1. Distribution of AP-PCR genotype III isolates on ward A. Open circles, AP-PCR genotype IIIa isolates; stippled circles, AP-PCR genotype IIIb isolates; closed circles, AP-PCR genotype IIIc isolates; closed ovals, environmental sites commonly associated with patients and health care workers (sampled monthly), including floors (FL), radiators (R), bed frames (BF), curtain rails (CR), and commodes (C); closed diamonds, high-reach environmental sites (samples every 6 months), including overbed lamps (OL), window frames (WF), curtain rails (CR), bay partitions (BP), door tops (DT), door frames (DF), storage cupboards (SC), fire hoses (FH), and smoke detectors (SD).

0.05) but not on ward A ($r = 0.14$; $P > 0.05$). Similarly, there was a significant correlation between *C. difficile* infection incidence and the prevalence of environmental *C. difficile* isolates from high-reach sites on ward B ($r = 0.85$; $P < 0.05$) but not on

ward A ($r = 0.30$; $P > 0.05$). Table 3 shows the increases in the numbers of environmental sites (in frequent contact with patients and staff) on the study wards that were *C. difficile* culture positive during the first 6 months of the study. *C. difficile* was

TABLE 2. *C. difficile* AP-PCR types isolated from symptomatic patients, hospital environments, and the hands of health care workers on ward B

AP-PCR type	No. of clinical isolates/total no. tested	No. of isolates from hand impressions/total no. tested	Environmental isolates	
			No. of isolates/total no. tested	Environmental site(s)
Ia	39/40	5/6	175/200	Multiple
Ib	NI ^a	NI	2/200	Bed frame, radiator
IIa	NI	1/6	NI	NA ^b
IIb	NI	NI	5/200	Overbed lamp, window frame, bay floor, radiator, bed frame
V	NI	NI	6/200	Curtain rails (<i>n</i> = 3), toilet floors (<i>n</i> = 2), radiator
VII	NI	NI	7/200	Bay floor (<i>n</i> = 2), bed frame (<i>n</i> = 2), overbed lamp, bay partition, floor
VIIIa	NI	NI	4/200	Commodes, overbed lamp, bed frame (<i>n</i> = 2)
XI	1/40	NI	NI	NA
XIV	NI	NI	1/200	Radiator

^a NI, not isolated.^b NA, not applicable.

not isolated from the environment of ward A before it was opened and was not detected until 8 to 10 weeks after it was opened (from 4 of 24 sites). In contrast, *C. difficile* AP-PCR type V (nontoxigenic) was isolated from 2 of 24 sites tested (a toilet floor and a curtain rail in a patient side room), and AP-PCR type Ia was isolated from a single site (a bed-bay partition) on ward B before it was opened. Table 4 shows the *C. difficile* culture-positive high-reach environmental sites at 6-month intervals throughout the study. *C. difficile* was not isolated from high-reach environmental sites on ward A before it was opened but was isolated from 5 of 34 high-reach sites tested approximately 9 months later. In contrast, *C. difficile* was isolated from 3 of 34 sites tested on ward B before it was opened. AP-PCR type V isolates (nontoxigenic) were recovered from interbed curtain rails and a toilet curtain rail, and a single AP-PCR type Ia isolate was recovered from the top surface of a bed-bay partition. The prevalence of culture-positive high-reach sites on ward A increased from 0 to 33.3% during the study period. In contrast, on ward B this figure decreased from 13.9% (at its highest) to 0.

DISCUSSION

This study has highlighted the endemic distribution of *C. difficile* AP-PCR type Ia in elderly medical patients in our institution. Notably, we found marked differences in the epidemiology of this and its closely related subtype strain. *C. difficile* AP-PCR type Ia was indistinguishable from PCR ribotype 1, an established UK epidemic strain that accounts nationally for 57% of all patient isolates (5). This ribotype accounted for 33% of *C. difficile* patient isolates in a U.S. East Coast tertiary referral hospital (27). Only 2 years earlier, a similar study in the same hospital failed to identify a predominant strain (26). Such observations may be indicative of the early epidemic spread of *C. difficile* PCR ribotype 1 in the United States. However, the high prevalence of *C. difficile* PCR ribotype 1 (serotype G) in the United Kingdom is in sharp contrast to those indicated in reports from other countries in Europe, including Belgium and France, where serotypes C and H, respectively, are most common (2, 30). This geographical

diversity suggests that different *C. difficile* strains have the propensity to flourish in different clinical settings and may be selected by environmental or antibiotic pressure within certain health care institutions.

We have established here and previously (14, 16) that *C. difficile* PCR ribotype 1 isolates can be subtyped by both randomly amplified polymorphic DNA and PFGE fingerprinting techniques and by determination of their susceptibilities to clindamycin. In a previous study we designated a strain causing a cluster of six cases of *C. difficile* infection on a unit for the care of elderly individuals as *C. difficile* genotype IV (15). We since recognized this strain as a clindamycin-resistant subtype of *C. difficile* PCR ribotype 1. We have therefore modified our nomenclature to distinguish clindamycin-sensitive *C. difficile* PCR ribotype 1 strains (AP-PCR type Ia) and clindamycin-resistant *C. difficile* PCR ribotype 1 strains (now designated AP-PCR type Ib). The clinical significance of *C. difficile* PCR ribotype 1 subtypes had not been elucidated. In our institution, *C. difficile* AP-PCR type Ib was not implicated in patient infection for 4 years after the original cluster of six cases on the same ward. Similarly, this strain was isolated from the ward environment on only two occasions during this same period. The environments of both wards were sampled before they were opened, and *C. difficile* AP-PCR type Ib was not isolated. Such isolates were also not recovered from symptomatic patients. Thus, the source of *C. difficile* AP-PCR Ib isolates found exclusively in the environment remains unclear. These may have been introduced by an asymptomatic carrier, via the hands of health care workers or visitors, or possibly, from an infected patient whose fecal isolate was not available for analysis. We note that increased resistance to clindamycin does not appear to have afforded this strain a clinical advantage over the closely related, clindamycin-susceptible subtype (*C. difficile* AP-PCR type Ia). This result is not in accord with those in reports from other health care institutions, where type clindamycin-resistant PCR ribotype 1 strains have predominated (21).

Johnson et al. (21) reported that all epidemic *C. difficile* isolates from four U.S. hospitals (later confirmed to be PCR ribotype 1 by ARL) were highly resistant to clindamycin and

FIG. 2. Frequency of *C. difficile* culture-positive environmental sites commonly associated with patients and health care workers (A) and high-reach sites (B) on study wards A (grey bars) and B (white bars).

carried the *ermB* gene. They concluded that clindamycin use was a risk factor for diarrhea due to this strain. Clindamycin is a restricted antibiotic in our institution and as such is very rarely used. The consequent lack of clindamycin selective pressure may explain why the clindamycin-resistant *C. difficile* AP-PCR type Ib strain has not become dominant, but it does not account for the endemic spread of the clindamycin-susceptible strain (AP-PCR type Ia). Kato et al. (23) recently described a non-PCR ribotype 1 *C. difficile* strain (designated ribotype

smz) that was predominant in three Japanese hospitals and that also displayed various levels of clindamycin susceptibility. They reported that the isolation rate of high-level clindamycin-resistant strains among type smz was similar to that among non-type smz isolates and concluded that clindamycin resistance did not affect the epidemic potential of ribotype smz. Our own data also show that some sporadic *C. difficile* clinical isolates are clindamycin resistant, and yet, they have not become endemic (data not shown). Interestingly, we previously

FIG. 3. Analysis of AP-PCR profiles of *C. difficile* strains isolated from the stools of patients with *C. difficile* infection, the hands of health care workers, and the environments of two medicine hospital wards for elderly patients. The dendrogram includes a small representative type of the predominant hospital genotype (type Ia) and all other genotypes found in the study. Strains isolated from the hands of health care workers are marked with an asterisk. DNA profiles were analyzed by using BioNumerics software (Applied Maths, BioSystematica). Dendrograms were constructed by the unweighted pair group method with arithmetic mean clustering by using the Dice correlation coefficient. The percentage level of similarity chosen for type assignments (roman numerals) is indicated by the thick black line and was based on the guidelines recommended by Tenover et al. (29).

highlighted that *C. difficile* ribotype 1 is highly resistant to fluoroquinolones and cephalosporins, antibiotic classes that are used widely at our institution (16, 33). Endemic *C. difficile* PCR ribotype 1 isolates had markedly reduced susceptibilities to six fluoroquinolones compared with those of genotypically distinct, sporadic strains. A recent outbreak of *C. difficile* infection was associated with clindamycin administration and particularly with the formulary replacement of levofloxacin by gatifloxacin in a medical unit for elderly patients (17). The predominant strain was fluoroquinolone resistant, and the out-

break ended after the antibiotic switch was reversed. Antibiotic exposure may be not only a prerequisite for *C. difficile* infection but also an important determinant of which *C. difficile* strains are likely to cause infection. However, the effects of antibiotic exposure on the gut flora and the confounding presence of multiple variables in the clinical setting mean that it is extremely difficult to determine the relative contribution of such potential infection determinants. For this reason, in the present study we did not attempt to correlate antibiotic consumption data with strain epidemiology.

FIG. 4. Analysis of PFGE profiles of *C. difficile* strains isolated from the stools of patients with *C. difficile* infection, the hands of health care workers, and the environment of two medicine hospital wards for elderly patients. The dendrogram includes a small representative type of the predominant hospital genotype (subtype 1a) and all other genotypes found in the study. Strains isolated from the hands of health care workers are marked with an asterisk. The DNA profiles were analyzed by using BioNumerics software (Applied Maths, BioSystematica). Dendrograms were constructed by the unweighted pair group method with arithmetic mean clustering by using the Dice correlation coefficient. The percentage level of similarity chosen for group assignments (roman numerals) is indicated by the thick black line and was based on the guidelines recommended by Tenover et al. (29).

Whether infected patients or contaminated environments are the prime source for cross-infection by *C. difficile* remains largely unresolved. During the present study, it became increasingly difficult to trace distinct *C. difficile* isolates between symptomatic patients and the hospital ward environment in the setting of endemic *C. difficile* AP-PCR type Ia. In addition, we fingerprinted isolates from only 43% of the cases of *C. difficile* infection due to the unavailability of stored fecal specimens or, in a minority of cases, poor recovery from stored fecal mate-

rial. The available fecal specimens and, thus, clinical isolates were distributed evenly throughout the study period, therefore minimizing the risk of sampling bias. We have shown that the sporulation capacity of *C. difficile* PCR ribotype 1 strains is superior to those of other randomly selected *C. difficile* genotypes (31). This may result in better adaptation to environmental survival and recovery from fecal material over other genotypes and may thus explain the higher prevalence of *C. difficile* AP-PCR type Ia.

FIG. 5. Analysis of RS-PCR profiles of *C. difficile* strains isolated from the stools of patients with *C. difficile* infection, the hands of health care workers, and the environment of two medicine hospital wards for elderly patients. The dendrogram includes a small representative group of the predominant hospital genotype (type I) and all other genotypes found in the study. Strains isolated from the hands of health care workers are marked with an asterisk. The DNA profiles were analyzed by using BioNumerics software (Applied Maths, BioSystematica). Dendrograms were constructed by unweighted pair group method with arithmetic mean clustering by using the Dice correlation coefficient. The percentage level of similarity chosen for group assignments (roman numerals) is indicated by the thick black line and was based on the guidelines recommended by Tenover et al. (29).

Use of the three DNA fingerprinting techniques applied in this study represents a robust approach to the molecular epidemiological study of *C. difficile*. There are technique-specific advantages and disadvantages associated with all three methods, and there remains a lack of consensus about the optimal approach to *C. difficile* typing. The discriminatory powers of the typing methods were AP-PCR > PFGE > RS-PCR. The level of discrimination was increased by 43% when all three methods were used in combination compared with that from

the use of RS-PCR alone. Studies have reported problems associated with the use of the RS-PCR technique, notably, poor discrimination of *C. difficile* isolates belonging to serogroups C and D (4, 30). This may be due to the conserved nature of the rRNA spacer regions within these serotypes. In the present study the AP-PCR and PFGE techniques were more discriminatory for RS-PCR ribotypes II, III, and VIII. RS-PCR was the only method that failed to detect subtypes within the UK epidemic *C. difficile* strain. These data suggest

FIG. 6. *C. difficile* infection and environmental culture positivity for study wards A and B; ■, patient isolates; ●, environmental isolates from sites regularly in contact with patients and ward staff; ◇, environmental isolates from high-reach sites.

that suboptimal discrimination by RS-PCR might be extended to serogroup G *C. difficile* isolates. DNA from serogroup G isolates is repeatedly degraded during the PFGE protocol, making this technique unsuitable for the typing of such *C. difficile* strains (10, 30, 22). Recently, the inclusion of thiourea in the agarose gel and electrophoresis buffer has minimized the

amount of DNA degradation, thus permitting successful PFGE fingerprinting of *C. difficile* PCR ribotype 1 (11, 14). In the present study, the PFGE and AP-PCR profiles were fully concordant in their discrimination of subtypes within *C. difficile* PCR ribotype 1. Hence, PFGE may still represent a useful technique for identifying subtypes of this epidemic strain. As

TABLE 3. Recovery of *C. difficile* from environmental sites regularly in contact with patients and ward staff on wards A and B during months 0 to 6

Ward and location	Culture positivity on the following sample date (mo/yr) ^a :					
	08/97	09/97	10/97	11/97	12/97	01/98
Ward A						
Bay floors					+	+
Radiators					+	+
Bed frames			+	+	+	+
Toilet floor					+	
Sluice floor					+	+
Commodes			+	+	+	+
Side room floors			+	+	+	+
Side room curtain rails						
Ward B						
Bay floors			+		+	
Radiators			+	+		
Bed frames			+	+	+	+
Toilet floor	+			+	+	
Sluice floor				+		+
Commodes			+	+	+	+
Side room floors			+	+	+	
Side room curtain rails	+		+	+	+	+

^a +, at least one of the samples from site type was culture positive for *C. difficile*.

expected, RS-PCR fingerprinting was slightly more reproducible than AP-PCR, given the high degree of susceptibility of the latter procedure to variations in testing conditions (9). Nevertheless, the reproducibility of AP-PCR was adequate, and this relatively straightforward technique had a high degree of discrimination. Wullt et al. (36) recently reported on repro-

ducibility problems with AP-PCR during reexamination of *C. difficile* isolates associated with symptomatic recurrences and concluded that PCR ribotyping offered superior experimental robustness. However, 140 distinct AP-PCR genotypes were identified, whereas only 43 RS-PCR genotypes were identified. These observations and our results highlight the importance of selecting the appropriate fingerprinting technique when designing studies to optimize strain discrimination; otherwise, very different conclusions about strain epidemiology (Fig. 1) may result.

We observed a marked increase in the frequency of *C. difficile* culture-positive environmental sites on both wards within 3 months of their opening. This implies that *C. difficile* was repeatedly introduced into the ward environment and that hospital cleaning regimens were largely ineffective at removing *C. difficile* from the populated ward environment. We observed a decrease in the environmental prevalence of *C. difficile* on ward B but not on ward A in high-reach sites during the 4-year study period. We cannot be certain why this difference occurred, but it is possible that the cleaning personnel were more assiduous on the former ward. Wards A and B were temporarily closed to further patient admissions on six and three occasions, respectively, due to clusters of cases of viral gastroenteritis during the study period. Following such unit closures, routine environmental cleaning is enhanced to reduce the risk of nosocomial virus transmission. Thus, we would have expected that microbial contamination on ward A would be less than that on ward B. The timing of ward closure due to viral gastroenteritis did not correlate with the reduced environmental prevalence of *C. difficile* in high, dusty sites. Notably, the *C. difficile* infection incidence on ward B correlated significantly with the prevalence of environmental *C. difficile* contamination in both sites that were frequently and sites that were rarely

TABLE 4. Recovery of *C. difficile* from high-reach environmental sites on wards A and B at 6-month intervals throughout the study

Ward and location	Culture positivity on the following sample date (mo/yr) ^a :							
	08/97	06/98	05/99	11/99	06/00	11/00	05/01	10/01
Ward A								
Bay curtain rails		+	+				+	+
Bay overbed lamps		+	+			+	+	+
Bay partitions				+		+	+	+
Bay window frames						+	+	+
Door tops		+		+		+	+	
Door frames							+	+
Fire hoses				+	+		+	+
Ward smoke detector								
Toilet curtain rails							+	+
Treatment room supplies caddy								
Ward B								
Bay curtain rails	+	+			+			
Bay overbed lamps		+	+	+	+		+	
Bay partitions	+	+		+	+			
Bay window frames			+			+		
Door tops		+	+					
Door frames			+	+				
Fire hoses		+			+			
Ward smoke detector								
Toilet curtain rails	+		+					
Treatment room supplies caddy								

^a +, at least one of the samples from site type was culture positive for *C. difficile*.

associated with patient or health care worker contact. Thus, although contact with high-reach sites is rare, the potential remains for these areas to act as reservoirs for *C. difficile* spores, presumably via spore transfer during periods of air disturbance, for example, that induced by air-conditioning systems, open windows, or floor-buffing machines. Failure to clean such high-reach areas on the basis of infrequent contact with patients or health care workers may therefore be short-sighted. We did not formally measure compliance with environmental cleaning protocols. It is accepted that on occasion compliance may be suboptimal due to workload pressures, staff turnover, and motivation. We therefore cannot distinguish between the effectiveness of a cleaning regimen per se and the end effect on the environmental *C. difficile* burden. The study results do, however, represent the real-world scenario and highlight the difficulty of achieving *C. difficile* removal from the environment.

In conclusion, we observed high-level patient and environmental endemicity by *C. difficile* AP-PCR type Ia, in contrast to that of the other PCR ribotype 1 subtypes. Why different PCR ribotype 1 subtypes appear to predominate in different health care institutions is unclear but could relate to antibiotic prescription pressures. Discriminatory fingerprinting techniques are required to elucidate the epidemiology of *C. difficile* infection and to aid with determination of the virulence characteristics of endemic and epidemic strains.

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