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Molecular epidemiology of endemic *Clostridium difficile* infection

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SUMMARY

This is the first study to provide a comprehensive insight into the molecular epidemiology of endemic *Clostridium difficile* and particularly that associated with a recently recognized epidemic strain. We DNA fingerprinted all *C. difficile* isolates from the stools of patients with symptomatic antibiotic-associated diarrhoea and from repeated samples of the inanimate ward environment on two elderly medicine hospital wards over a 22-month period. Notably, *C. difficile* was not recoverable from either ward immediately before opening, but was found on both wards within 1–3 weeks of opening, and the level of environmental contamination rose markedly during the first 6 months of the study period. *C. difficile* infection (CDI) incidence data correlated significantly with the prevalence of environmental *C. difficile* on ward B ($r = 0.76$, $P < 0.05$) but not on ward A ($r = 0.26$, $P > 0.05$). We found that RAPD and RS-PCR typing had similar discriminatory power, although, despite fingerprinting over 200 *C. difficile* isolates, we identified only six distinct types. Only two distinct *C. difficile* strains were identified as causing both patient infection and ward contamination. Attempts to determine whether infected patients or contaminated environments are the prime source for cross-infection by *C. difficile* had limited success, as over 90% of *C. difficile* isolates were the UK epidemic clone. However, a non-epidemic strain caused a cluster of six cases of CDI, but was only isolated from the environment after the sixth patient became symptomatic. The initial absence of this strain from the environment implies patient-to-patient and/or staff-to-patient spread. In general, routine cleaning with detergent was unsuccessful at removing *C. difficile* from the environment. Understanding the epidemiology and virulence of prevalent strains is important if CDI is to be successfully controlled.

INTRODUCTION

Clostridium difficile is the prime pathogen causing antibiotic associated diarrhoea and colitis particularly in the hospital setting [1–4]. While it has been established that certain antibiotics, notably second and third generation cephalosporins have a high propensity to cause *C. difficile* infection (CDI) [2, 5], it is important that exposure of hospitalized patients to sources of *C. difficile* is minimized. This has become

increasingly difficult on hospital wards where susceptible patients share the same living space as *C. difficile* infected individuals. Clusters of cases of nosocomial CDI have been reported in a variety of hospital units, including geriatric and surgical wards, and intensive care and transplantation units [6].

Contaminated environmental surfaces and health-care personnel hand carriage are considered as important sources for *C. difficile* transmission in hospitals [6]. Bacterial spores have been found in far greater quantities in the environment around indi-

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viduals with CDI in comparison with asymptotically colonized patients, presumably secondary to diarrhoea, which can often be unexpected and explosive, so increasing shedding of *C. difficile* [7]. *C. difficile* spores are highly resistant to many commonly used disinfectants and may persist for many months in hospital ward environments [8]. Additionally, it has been shown that the frequency of *C. difficile* positive healthcare personnel hand cultures was highly correlated with the intensity of environmental contamination [9]. The true significance of the environment as a potential reservoir for *C. difficile* and its role in subsequent patient infection remains unclear, primarily because it has proven difficult to determine whether environmental contamination is a cause or consequence of diarrhoea. Studies to date have been limited, however, in that they studied environmental contamination either only as a point prevalence rate or over a short period of time (< 6 months).

Molecular epidemiologic analysis of *C. difficile* isolates collected from geographically distinct hospitals throughout the United Kingdom has demonstrated the presence of a single predominant strain, suggesting the possibility that some strains have a greater propensity for nosocomial transmission [10]. We have previously reported that this epidemic strain was responsible for 75–80% of CDIs encountered as part of a prospective ward crossover study examining antibiotic-related *C. difficile* risk [11]. Elderly inpatients are most closely associated with CDI, yet few reports have studied the epidemiology of *C. difficile* amongst hospitalized patients in non-outbreak situations. Therefore, we prospectively studied all *C. difficile* isolates recovered from symptomatic patients and from repeated environmental sampling on two elderly medicine hospital wards over a 22-month period, from their opening to a planned move. We aimed to investigate the molecular epidemiology of endemic *C. difficile* infection, particularly that associated with the UK epidemic strain, and to explore the relationship between environmental contamination and patient infection.

MATERIALS AND METHODS

Study design

We DNA fingerprinted all *C. difficile* isolates from the stools of patients with symptomatic antibiotic associated diarrhoea and from systematically collected samples of the inanimate ward environment on two

elderly medicine hospital wards over a 22-month period, from ward opening to a planned move, during the period October 1995 to July 1997. The study wards were of similar design, each consisting of four six-bedded bays and containing four side rooms, and were situated on the same floor of a 10-year-old building.

C. difficile infection diagnosis, culture and identification

Faecal samples were tested for the presence of *C. difficile* cytotoxin on request in the routine diagnostic laboratory from patients with diarrhoea suspected to be due to *C. difficile*. Cytotoxin was detected by a microtitre tray method using Hep-2 cells with *Clostridium sordellii* protected controls, and a 1 in 50 final dilution of faeces in cell culture medium. Cytotoxin positive faeces were stored at -20°C pending culture for *C. difficile*.

Environmental sampling was performed monthly. Sites were sampled in a systematic manner (10×10 cm areas) with sterile cotton wool swabs moistened with 0.25% Ringer's solution (Oxoid, Basingstoke, UK), and then cultured immediately for *C. difficile*. *C. difficile* isolates were recovered from environmental and frozen faecal samples by culture on cycloserine cefoxitin supplemented agar without egg yolk (modified CCEY; Lab M Bury, UK) for 48 h in an anaerobic cabinet at 37°C . After direct inoculation onto modified CCEY, environmental swabs were incubated anaerobically in Robertson's cooked meat broth for 48 h at 37°C . Resultant broth cultures were then inoculated onto modified CCEY medium as before. All *C. difficile* isolates were recognized by their characteristic colonial morphology and odour, and in cases of doubt, RapID ANA II System (Innovative Diagnostic Systems, GA, USA) was used. All *C. difficile* strains were stored in PBS/glycerol solution at -20°C .

DNA fingerprinting

DNA fingerprinting of *C. difficile* isolates was performed using both random amplified polymorphic DNA (RAPD) and ribospacer polymerase chain reaction (RS-PCR) techniques in order to maximize the chance of discriminating between strains. Target DNA was extracted from each bacterial strain as previously described [12]. To detect any mixed cultures of *C. difficile*, separate typing reactions were per-

formed on DNA samples extracted from both single and multiple colonies.

RAPD primer ARB11 [13] (5'-CTA GGA CCGC-3') and RS-PCR primers [14] L1 (5'-CAA GGC ATC CAC CGT-5') and G1 (5'-GAA GTC GTA ACA AGG-3'), (all obtained from the Oligonucleotide Synthesis Service, Institute of Pathology, University of Leeds, UK) were used in the study. The following were added to each 25 μ l reaction volume: 5 μ l PCR buffer ($\times 10$ concentrate, BioLine), 8 μ l of deoxynucleoside triphosphate premix (1.25 mM each dNTP) (Pharmacia & Upjohn Inc, Herts, UK), 0.5 μ l BioExtract *taq* polymerase (2 units/reaction, BioLine, UK), and 3 μ l DNA extract. For RAPD, 0.5 μ l ARB11 primer (40 pmol) and 2 μ l of 50 mM MgCl₂ (final concentration 4 mM) were added to the reaction mixture. For RS-PCR, 0.25 μ l L1/G1 premix (25 pmol) and 2.25 μ l of 50 mM MgCl₂ (final concentration 5.5 mM) were added. DNA amplification was carried out in an Ericomp Twinblock EasyCycler™ (Lazer Laboratory Systems, Southampton, UK). RAPD reactions were subjected to 35 cycles, each lasting 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, whereas RS-PCR reactions were subjected to 34 cycles, each lasting 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C. Amplified DNA was separated by agarose gel electrophoresis using Tris-borate EDTA (TBE) buffer pH 8.0 and 2% Metaphor™ gels (Flowgen, Staffs, UK) for 4 h at 180 V/180 mA. DNA fingerprints were visualized, after ethidium bromide staining, with an ImageMaster™ VDS camera (Pharmacia & Upjohn Inc, Herts).

RESULTS

C. difficile strains recovered from patients

During the study period there were 125 separate cases of CDI defined by routine diagnostic testing, 55 cases on ward A and 70 cases on ward B. Specimens from patients with recurrent diarrhoea were excluded. This corresponded to CDI incidences of 9.2 and 8.9 cases per 100 patient admissions for wards A and B, respectively. *C. difficile* was successfully cultured from 86.4% of stored faecal samples, providing 108 strains for DNA fingerprinting.

After visual comparison of DNA fingerprints, isolates that differed by three or more DNA fragments from other strains typed by RAPD were assigned to a new typing group. RAPD based on primer ARB11

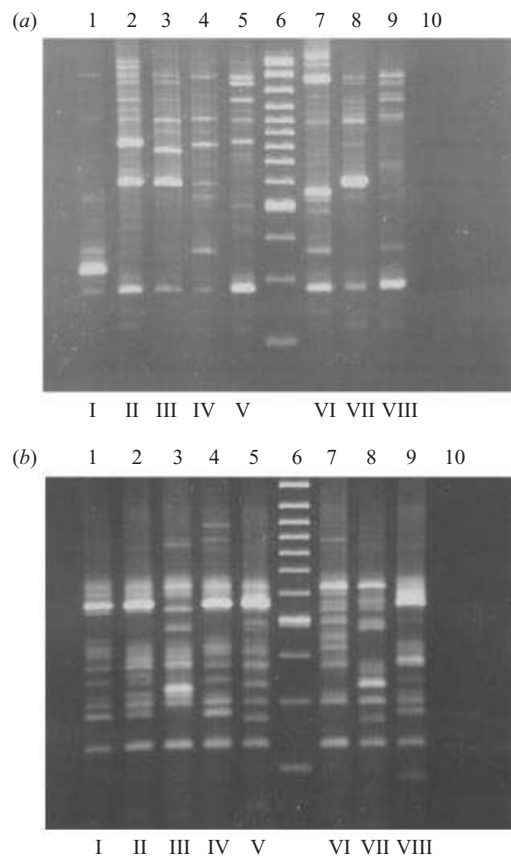


Fig. 1. Summary of DNA fingerprints from *C. difficile* isolates from both patients and the environment on wards A and B using RAPD-PCR technique (a) and RS-PCR technique (b). Prints from ethidium bromide stained 2% Metaphor™ agarose gels are shown. Assigned genotypes are indicated at bottom of lanes. On both gels: lane 6: 100 bp ladder; lane 10: negative PCR control.

successfully separated 108 strains into four distinct types (Fig. 1). Strains typed by RS-PCR were considered distinguishable if > 2 inter-strain band differences were present [15]. This method was found to have an equivalent discriminatory power to RAPD, separating the 108 strains into the same four groups. On ward A, only two genotypes were found (I and II). Apart from one isolate (genotype II), all strains originating from ward A were genotype I (Table 1). On ward B, only three genotypes were discovered (I, III and IV), and genotype I represented 87% of all patient isolates examined.

C. difficile strains recovered from ward environments

Over the 22-month study period, 1122 swabs were taken from pre-defined environmental sites, 572 on ward A and 550 from ward B. The sites sampled

Table 1. *C. difficile* genotypes isolated from patients and their environment on study wards A and B

Ward	Genotype	Clinical isolates (number of isolates)	Environmental isolates	
			Number of isolates	Environmental site
A	I	47/48	40/43	Endemic
	II	1/48	NI	NA†
	V	NI*	1/43	Commodes
	VI	NI	1/43	Radiators
	VII	NI	1/43	Toilet floor
B	I	52/60	55/60	Endemic
	III	1/60	4/60	Toilet floor/air vents
	IV	7/60	NI	NA
	VIII	NI	1/60	Sluice floor

* NI, Not isolated; †NA, not applicable.

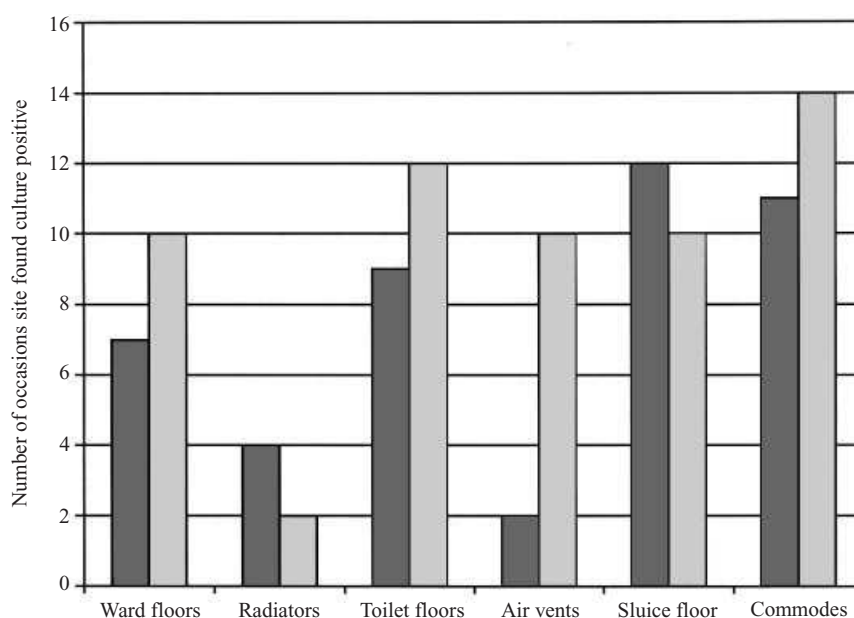


Fig. 2. Frequency of *C. difficile* culture positive environmental sites on study wards A and B. ■, ward A; □, ward B.

comprised radiators (16%), air vents (16%), commodes (16%) and floors (52%) from wards, toilets and sluice rooms. Overall, 34 and 36% of sites were *C. difficile* culture positive on wards A and B, respectively. *C. difficile* was most frequently cultured from commodes and toilet/sluice room floors (Fig. 2). In total, only 21 (6.8%) sites were positive when swabs were cultured on modified CCEY medium alone, the majority (85.7%) of which yielded < 5 bacterial colonies. When swabs were enriched by culture in Robertson's cooked meat broth, total recovery was increased markedly (35.1% of sites were found to be *C. difficile* positive). Five strains were unrecoverable after freezer storage, leaving 103 environmental isolates available for DNA fingerprinting.

Both RAPD and RS-PCR techniques separated 103 strains into six distinct groups (Fig. 1). Ninety-five isolates from both wards were indistinguishable from the patient genotype I strain; 92 and 94% of total isolates from wards A and B, respectively (Table 1). Three other genotypes were found on ward A (genotypes V, VI and VII), and two on ward B (genotypes IV and VIII). Other than genotypes I and IV, environmental strains were isolated only once, and were dissimilar from any patient isolates. Also, two environmental strains were non-toxin producers (genotypes V and VI). Genotype I was the only strain present on both study wards. Apart from this endemic strain, genotype IV was the only strain implicated in both patient infection and ward environmental con-

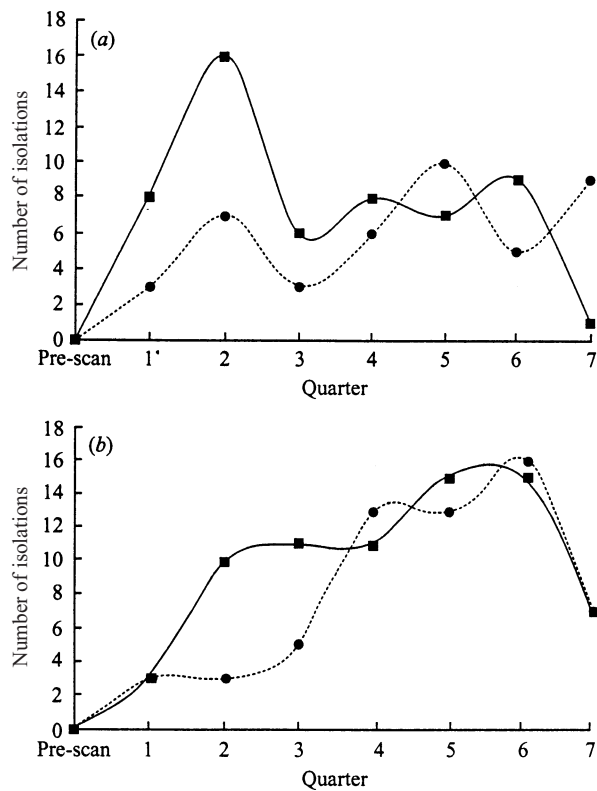


Fig. 3. Quarterly figures: CDI and environmental culture positivity for study wards A (a) and B (b). ■, Patient isolates; ●, environmental isolates.

	6/10	13/10	27/10	5/12	4/1	31/1	9/4
(a)							
Bay floors		+					+
Toilet floors				+	+	+	+
Sluice floors			+			+	+
Commodes					+	+	+
Radiators					+		
Air vents							
(b)							
Bay floors							+
Toilet floors				+	+		
Sluice floors			+			+	
Commodes							+
Radiators							
Air vents				+			

Fig. 4. Recovery of *C. difficile* from environmental sites on wards A (a) and B (b) during months 0–6. + indicates at least one of the samples from site type was *C. difficile* positive.

tamination. Genotype IV was the only example of a *C. difficile* type that was recovered from the environment after, but not before, isolation from six symptomatic patients.

CDI and ward environmental contamination

Quarterly figures for CDI and environmental culture positivity for wards A and B are shown in Figure 3. CDI incidence data correlated significantly with the prevalence of environmental *C. difficile* on ward B ($r = 0.76$, $P < 0.05$) but not on ward A ($r = 0.26$; $P > 0.05$). Figure 4 shows the month-by-month increases in the numbers of environmental site types on the study wards that were *C. difficile* culture positive during the first 6 months of the study. *C. difficile* was not isolated from the environment of either ward before opening, but was found from both wards within 1–3 weeks of opening.

DISCUSSION

We are aware of only one previous study [9] that systematically examined the relationship between environmental contamination and CDI. Samore and colleagues documented marked environmental contamination and transmission to personnel and patient contacts by an endemic *C. difficile* strain over a 6-month period [9]. Several studies have documented the presence of *C. difficile* spores in areas occupied by infected patients, but these have been over short time periods, and evidence of bacterial acquisition from exposure to contaminated environmental sources is scarce. One study concluded that disparate strains responsible for causing disease were more likely to have originated from an environmental source than from cross-infection from patient to patient [16]. Elsewhere, a cluster of CDIs on a surgical unit was associated with an identical strain found in the environment [17]. Conversely, Cohen *et al.* found no evidence to suggest environmental acquisition of *C. difficile* [18].

We found that *C. difficile* was not recoverable from either ward immediately before opening. However, the level of environmental contamination rose markedly during the first 6 months of the study period (Fig. 4). Samore and colleagues found that the frequency of positive personnel hand culture was strongly correlated with intensity of environmental contamination [9]. In our study, the incidence of CDI on ward B, but not on ward A, was strongly correlated with en-

environmental contamination levels over the 22-month testing period ($r = 0.76$, $P < 0.05$). It is interesting to speculate why such a marked difference in strength of correlation was found between wards A and B. We are unaware of marked differences, but several factors not specifically addressed in this study, including antibiotic prescribing practice, patient type and cleaning efficiency, may have influenced either the incidence of CDI or environmental contamination. A comparative trial of CDI risk associated with treatment with cefotaxime or piperacillin-tazobactam occurred on these two wards during part of the present study period [11]. However, this was a ward crossover trial, and thus effects of antibiotic use on CDI or environmental contamination should in theory have been balanced.

Endemic CDI can be controlled by reducing the use of high risk agents [19–22]. However, changes in incidence of CDI following altered antibiotic prescribing practice have generally been seen in non-comparative settings, and/or have not been tested by reintroduction of the suspected antibiotics. Recently, Stone and Kibbler [23] reported that a fall in incidence of *C. difficile* diarrhoea in an elderly medicine unit was associated with a reduction in cephalosporin use. Feedback to clinicians on *C. difficile* rates and antibiotic prescribing levels was relaxed leading to an increase in diarrhoea. Following re-enforcement of an antibiotic policy to limit cephalosporin prescribing, *C. difficile* diarrhoea rates reduced again. The purpose of the present study was not to examine the effects of intervention in environmental cleaning, but rather to determine the baseline relationship between *C. difficile* contamination levels and CDI. Conventional reaction to an outbreak of CDI includes enhanced environmental cleaning, and we have reported the success of this approach [24].

Attempts to determine whether infected patients or contaminated environments are the prime source for cross-infection by *C. difficile* had limited success. Over 90% of *C. difficile* strains isolated in the study were the UK epidemic clone (genotype I). This makes assessment of the interplay of distinct *C. difficile* strains between the patient and the hospital ward difficult. In addition, the undulating frequencies of both infection and ward contamination remained in phase and therefore it was not possible to determine whether patient CDIs preceded a rise in ward contamination or vice versa. We have shown since this study was performed that environmental recovery of *C. difficile* is markedly increased if lysozyme is

incorporated into the selective agar used for sample culture [25]. We believe that use of this improved approach would not have substantially altered the findings of this study, as we have isolated similar limited *C. difficile* types seen here using lysozyme-containing media (unpublished data). The two DNA fingerprinting techniques that we employed had similar discriminatory power. RAPD typing results correlated fully with those of RS-PCR typing in this setting, although despite fingerprinting over 200 *C. difficile* isolates, we identified only six distinct types. As expected, RS-PCR technique was found to be slightly more reproducible than RAPD, given the high susceptibility of the latter even to very small variations in testing conditions. DNA fingerprinting by pulsed-field gel electrophoresis was also examined initially but was abandoned as it was ineffective in producing a pulsotype for the endemic clone. DNA from these strains was repeatedly degraded, presumably by endonucleases (data not shown), as described by several other groups [9, 26, 27].

We identified only two distinct *C. difficile* strains that caused both patient infection and ward contamination (RAPD genotypes I and IV). Interestingly, genotype IV was responsible for a cluster of six cases of CDI, but was only isolated from the environment after the sixth patient became symptomatic. The absence of genotype IV in the environment over this period implies patient-to-patient and/or staff-to-patient spread. Genotype IV was initially isolated from toilet floors on ward B. While contamination was removed from this area by routine cleaning, it persisted on air vents for the remainder of the study. Point prevalence sampling on the study wards 6 months after opening showed that 69 and 40% of high dust sites were *C. difficile* positive on wards A and B, respectively. These observations imply that routine cleaning does not prevent extensive contamination of high-reach sites. Deep cleaning (high-reach site decontamination and wall washing) was performed during the study period on ward B, in response to an outbreak of gastroenteritis (see Fig. 4, in between the 5th and 6th data sets). This resulted in a marked decrease in *C. difficile* culture-positive sites. This observation implies *C. difficile* environmental persistence may in part be due to sub-optimal routine cleaning. Routine environmental cleaning on study wards was carried out using a general-purpose detergent (containing phosphate and ionic and non-ionic surfactants), in line with current advice [28]. However, *C. difficile* genotype I sporulates markedly

more when exposed to non-chlorine based cleaning agents compared with chlorine-containing disinfectants [29]. Therefore, use of some detergents/disinfectants may actually be exacerbating environmental persistence of the UK epidemic *C. difficile* strain. It has been estimated that each CDI case costs more than £4000 [30]. This high figure could be used to justify expenditure on improved standards of hospital cleanliness [31].

The Anaerobic Reference Unit of the Public Health Laboratory Service has confirmed the endemic strain identified here as PCR ribotype 1 (J. Brazier, personal communication). This strain is known to be endemic in 33 of 58 hospitals in England and Wales [32]. It is interesting to speculate on the high prevalence of this strain in hospitalized patients [10]. Results from the present study suggest a relationship may exist between CDI incidence and the level of *C. difficile* spore contamination in the hospital environment. Thus, more cases of endemic infection result in the release of more spores into the environment, creating the potential for more cases of endemic infection. However, although several *C. difficile* strains were found in the study, only genotype I was predominant. This implies that strain-specific characteristics have contributed to persistence. This is the first comprehensive insight into the molecular epidemiology of endemic *C. difficile*, particularly that associated with a recently recognized epidemic strain. Understanding the epidemiology and virulence of prevalent strains is important if CDI is to be successfully controlled.

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