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ORIGINAL ARTICLE

Asymptomatic carriage of *Clostridium difficile* in an Irish continuing care institution for the elderly: prevalence and characteristics

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Abstract
Introduction *Clostridium difficile* is an increasing cause of nosocomial diarrhoea and colitis. The aim of this study was to identify the prevalence and characteristics of asymptomatic *C. difficile* carriage in a continuing care institution for the elderly.
Methods Stool samples were collected from 100 asymptomatic patients, whose median age was 83 years. Samples were tested for *C. difficile* using traditional culturing methods, 16s rDNA and 16s–23s intergenic spacer (IGS) rDNA sequencing, and analysed for toxin production and toxin genes.
Results The prevalence of *C. difficile* carriage was 10/100 (10%) following culture and 16s and IGS sequencing. An additional seven isolates, initially identified as *C. difficile*, were subsequently identified by IGS rDNA sequencing as *C. sordellii* of the 10% that tested positive for *C. difficile*, seven tested positive for toxin A and B. A significant number of *C. difficile* carriers had recent antibiotic exposure compared with non-carriers, $P = 0.046$.
Conclusion The prevalence of asymptomatic *C. difficile* carriage in this institution was 10%, 7% of which were toxin positive. This study underscores the importance of increased vigilance for *C. difficile* using microbial and molecular methodology and identifies patients at increased risk following antibiotic administration.

Keywords *Clostridium difficile* prevalence · Continuing care institution · Nosocomial diarrhoea · *Clostridium difficile* toxin · *Clostridium difficile* culturing · IGS rDNA sequencing · *Clostridium sordellii*

Introduction

Worldwide, the reported prevalence of *Clostridium difficile* colonisation in long-term care residents, apart from recognised outbreaks, is 4–20% [1–5] and *C. difficile* infection is increasing in hospitals and long-stay institutions. Improved diagnostic techniques and reporting of infection, widespread use of broad-spectrum antibiotics, increased burden on western health services resulting in overcrowding, poorer infection control and the emergence of more virulent pathogenic strains have resulted in an increased prevalence of *C. difficile* [6, 7].

Transmission of *C. difficile* is likely facilitated within a closed environment with a high rate of exposure to antimicrobials, but why it appears endemic in some institutions and not in others is unknown. It is most likely a result of differences in resident morbidity, antimicrobial protocols and regional differences in strain virulence and infection control practices. Various associations have been reported; certain antibiotics, in particular cephalosporins, have been implicated in the increased incidence of *C. difficile* in elderly patients, so also the use of H_2 antagonists [5]. An association between nasogastric and gastrostomy feeding and faecal

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incontinence with higher rates of *C. difficile* infection has also been noted [1].

Clostridium difficile became a notifiable infection in Ireland in 2008; however, data on the extent of *C. difficile*-associated diarrhoea in an institutional setting is, as yet, unclear. The only source of national data is from the third Hospital Infection Society (HIS) prevalence study of healthcare-associated infections in acute hospitals in the UK and Ireland, conducted in 2006 [8]. The number of patients with current *C. difficile* diarrhoea was recorded and 0.5% prevalence was found. Outbreaks of infectious diseases, however, have been notifiable in Ireland since 2004 [9] and eight outbreaks of *C. difficile* infection were reported to the Health Protection Surveillance Centre (HPSC) from 2004 to 2007, five in acute hospital settings and three in residential institutions [10]. However, the importance of the asymptomatic carriers in an at-risk population as a reservoir for this nosocomial infection highlights the need to determine the prevalence of carriage in this group of patients.

The aim of this study was to identify the prevalence of carriage of *C. difficile* in a continuing care institution for the elderly, to determine the toxin production of the predominant strains of bacteria and to identify the patient characteristics associated with carriage.

Methods

Subject recruitment and clinical data collection

The patient cohort consisted of both male and female patients from the rehabilitation and long-term care wards. Of 175 residents in these wards at the time of the study, 100 patients were recruited. Patient recruitment was dependent on each patient's ability to provide a stool sample and give consent. Exclusion criteria included patient's inability to give a stool sample, end of life illness and frailty. Ethical approval was obtained from the University College Cork and Affiliated Hospitals' Board of Ethics to recruit residents of St Finbarr's Continuing Care Hospital for the Elderly, Cork, Ireland. Demographic and clinical data of the study population were collected.

Microbiological screening of faecal samples for the presence of *C. difficile*

One fresh stool sample was collected from each patient, aliquoted on the day of collection and stored at −80°C until analysis. Prior to use, all media were conditioned by boiling and allowing the media to cool in an anaerobic cabinet. *C. difficile* strains were initially isolated from all faecal samples using Braziers cycloserine cefoxitin egg yolk-selective agar (CCEY, LAB M Ltd, Topley House 52 Wash Lane, Bury, Lanc BL9 6AS UK) and incubated anaerobically at 37°C for 48 h. The remainder of each specimen was frozen after processing at −20°C for use in the toxin detection assay.

All faecal samples were inoculated into cooked meat broth (CMB, Oxoid Ltd, Basingstoke, Hampshire, UK), which was supplemented with cefoxitin (16 mg/ml, after autoclaving) and cholic acid (0.1%, prior to autoclaving; Sigma-Aldrich Company Ltd, The Old Brickyard New Road Gillingham, Dorset SP8 4XT UK) and incubated at 37°C for 48 h. Samples were then plated onto Brazier's CCEY-selective agar and incubated anaerobically at 37°C for 48 h. Typical *C. difficile* colonies were identified as 4–6 mm in diameter, irregular, raised opaque, glossy grey colonies with a rough edge (ground glass appearance) and a horse stable odour. Isolated colonies were purified by subculturing onto reinforced clostridia agar (RCA, Oxoid) three times and subsequently Gram stained.

Remel PRO disc kit (Oxoid) was used to confirm the presence of *C. difficile* following the manufacturer's instructions. A positive test resulted in the development of a red to pink colour within 1 min. Two strains, *Bifidobacterium infantis* (Alimentary Health Ltd, NUI Cork, Ireland) and *Clostridium perfringens* (ATCC 110803), were used as negative controls.

Molecular screening of faecal samples for the presence of *C. difficile*

Putative *C. difficle* isolates from the microbiological screening were extracted using the Sigma eExtract-N-Amp Kit (Sigma, XNAT2 Kit). The genomic DNA solution was then quantified using a Nanodrop spectrophotometer and stored at 4°C.

Polymerase chain reaction (PCR) was performed using the intergenic spacer (IGS) primers, IGS L and IGS R [11], and 16s rDNA primers (L and R Table 1) (courtesy of Dr. Christain Reidel, University Ulm, Germany). The cycling conditions were 94°C for 3 min (1 cycle), 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s (28 cycles). The PCR reaction contained 50 ng of DNA, PCR mix (XNAT2 kit) and 10 pmol of IGS L and R primers. The PCR reactions were performed on an Eppendorf thermocycler. The PCR products (10 µl) were run alongside a molecular weight marker (100 bp Ladder, Roche diagnostics Ltd, Charles Avenue, Burgess Hill, RH15 9RY, UK) on a 2% agarose ethidium bromide (EtBr)-stained gel in Tris–acetate–EDTA (TAE) buffer, to determine the IGS profile.

Polymerase chain reaction products (single band) were purified using the Promega Wizard PCR purification kit (Promega, Madison, USA). The purified PCR products were sequenced using the primer sequences (as shown) for the IGS region by Cogenics (Hope End, Essex, UK).

Table 1 PCR primer sequences used to characterise *Clostridium* strains

Gene ID	Primer sequence (5′–3′)	Product size (bp)	References
Housekeeping tpi gene			
TpiF	AAAGAAGCTACTAAGGGTACAAA	230	Lemee et al. [14]
TpiR	CATAATATTGGGTCTATTCCTAC		
Toxin A			
TcdA-F	AGATTCCTATATTTACATGACAATAT	369 (toxin A+ve, B+ve)	Sambol et al. [12]
TcdA-R	GTATCAGGCATAAAGTAATATACTTT	110 (toxin A−ve, B+ve)	
Toxin B			
398CLDF	GAAAGTCCAAGTTTACGCTCAAT	177	Van den Berg et al. [15, 16]
399CLDR	GCTGCACCTAAACTTACACCA		
16srDNA			
16sL	CTGATCTCGAGGGCGGTGTGTACAAGG	1,000	C. Riedel, U Ulm, Germany
16sR	CTGATGAATTCGAGACACGGTCCAGACTCC		
Intergenic spacer 16s–23s rDNA (IGS)			
IGSL	GCTGGATCACCTCCTTTC		Brigidi et al. [11]
IGSR	CTGGTGCCAAGGCATCCA		

PCR was performed, using the intergenic spacer (IGS) primers, IGS L and IGS R, and 16s rDNA primers (L and R), to characterise *Clostridium* isolates. The purified PCR products were sequenced using the primer sequences listed for the IGS region. Sequence data were then searched against the NCBI nucleotide database to determine the identity of the strain by nucleotide homology. The resultant DNA sequence data were then subjected to the NCBI standard nucleotide-to-nucleotide homology BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST/). The nearest match to the sequence was identified and then the sequences were aligned for comparison using DNASTAR MegAlign software (DNASTAR Inc., Madison, USA). The sequence differences were noted and the variable regions identified

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Analysis of *C. difficile* culture filtrates for the presence of *C. difficile* toxin

Clostridium difficile culture filtrates were produced by inoculating the bacterial strains into 10 ml of reduced reinforced clostridial media (RCM, Oxoid) and incubated anaerobically at 37°C for 18–24 h. Cultures were then centrifuged and the supernatant filtered through 0.45-μm syringe filters. The X/pect *C. difficile* toxin A/B kit (R24650, Oxoid) was used to assay *C. difficile* culture filtrates for the presence of toxin A and/or B.

Analysis of isolated *C. difficile* for the presence of *C. difficile* toxin genes by PCR

Oligonucleotide primers were prepared for the entire toxin B gene (*tcdB*) and for the toxin A–C gene region (*tcdA–C*) and designed according to previously validated methods [12]. Template DNA was isolated from *C. difficile* strains, as described previously for restriction endonuclease analysis [13]. DNA extractions were in the range of 50–100 ng/ml. Three sets of primers were used for *C. difficle* typing (Table 1). The *tpi* primer set was used for amplifying an internal fragment of the *C. difficile*-specific tpi gene (housekeeping gene) and generated a 230-bp amplified product [14]. The *tcdA* primer set was designed to flank the smallest of the three deletions in the 3′ region of tcdA characterised in A−B+ variant *C. difficile* strains (generates a 369-bp fragment for A+B+ strains and a 110-bp fragment for A−B+ strain); the *398CLDs/399CLDas* primer set was derived from the non-repeating sequence of toxin B gene [15, 16]. All primers were synthesised by MWG Biotech, Germany.

A toxigenic and a non-toxigenic *C. difficile* strain were used as positive and negative controls, respectively. The toxin gene sequences were amplified using the eLONGase system, according to the manufacturer's directions (Gibco-BRL Life Technologies, Grand Island, NY, USA). In brief, 200 ng of template DNA was amplified in a reaction mixture of 1× eLONGase buffer, $MgSO_4$ at 2.0 mM, 200 μM of each dNTP, 10 pmol of each paired primer and 1 μL of eLONGase enzyme. The cycling parameters consisted of denaturation at 94°C, annealing at the melting point for each primer pair minus 5°C and extension at 68°C for 1 min per kilobase of amplicon. The *tcdA–C* amplicon

was then digested with *Pst*I, while each *tcdB* amplicon was digested with *Hin*cII. The resultant products were then analysed by electrophoresis on a 0.7% agarose gel and were visualised by EtBr staining.

Statistical methods

Statistical analyses were performed using non-parametric two-tailed statistical tests: chi-squared test was used to analyse proportions; Fisher's exact test was used to analyse proportions when the numbers in either group were <5 and Mann–Whitney was used for non-parametric medians. P values ≤ 0.05 were considered to be statistically significant.

Results

Prevalence of *C. difficile* carriage

Of 100 asymptomatic patients recruited and screened for the presence of *C. difficile* by culturing techniques, 17 were *C. difficile*-positive on culturing of stool samples and using Remel PRO disc kit. These 17 samples underwent molecular analysis by PCR using the IGS primers and a BLAST search confirmed 10 as being *C. difficile*. The remaining seven culture/Prodisc-positive isolates were found to be *C. sordellii* strains (Table 2).

Only the 17 samples found to be *C. difficile* positive following culturing were isolated and assayed for toxin production. *C. difficile* toxin A was detected in 10/17 samples and 12/17 were found to be toxin A/B positive. Following amplification and restriction endonuclease analysis of toxin genes, it was revealed that 7/10 of the confirmed *C. difficile* strains had the genetic material for toxin A production and 9/10 were positive for the presence of toxin B genes.

Demographic correlates

The demographics of the study population, including gender, age, length of hospital stay, concomitant medications, co-morbidities, use of proton pump inhibitors (PPI), antibiotic and previous *C. difficile* exposure, are summarised in

Table 2 Molecular (PCR, 16s and intergenic sequencing), microbiology and immunoassays on presumptive *C. difficile* isolates

Strain ID	*C. difficile*	PCR		Microbiology	Immunoassay		16S rDNA	IGS 16s–23s rDNA
		Toxin A	Toxin B	Prodisk	Toxin A	Toxin A/B		
CD001	−	−	−	+			*C. bifermentans*	*C. sordellii*
CD003	−	−	−	+	+	+	*C. bifermentans*	*C. sordellii*
CD005	−	−	−	+			*C. bifermentans*	*C. sordellii*
CD006	+	−	+	+				
CD017	+	−	+	+		+	*C. bifermentans*	*C. sordellii*
CD018	+	−	+	+				
CD025	+	+	+	+	+	+		
CD027	+	+	+	+	+	+		
CD028	+	+	+	+	+	+		
CD037	+	−	+	+	+	+	*C. bifermentans*	*C. sordellii*
CD045	+	+	+	+	+	+		
CD047	+	+	+	+	+	+		
CD056	+	−	−	+	+	+		
CD070	+	+	+	+	+	+		
CD079	−	−	−	+			*C. bifermentans*	*C. sordellii*
CD086	+	−	+	+		+	*C. bifermentans*	*C. sordellii*
CD087	+	+	+	+	+	+		
Bifidobacterium infantis 35624 (negative control)	−	−	−	−	−	−		
Clostridium perfringens ATCC 110803 (negative control)	−	−	−	−	−	−		
C. difficile ribotype 001	+	+	+	+	+	+		

A total of 17 isolates from 100 patients were identified as *C. difficile* using conventional microbiology screening techniques. Following PCR screening, 10 of these 17 were confirmed to be *C. difficile*. Further molecular identification using 16S and IGS sequencing of the other seven strains revealed that they were *C. sordellii*

Table 3 Patient demographics

	C. difficile Carriers	*C. difficile* non-carrier	*P* value
Number	10	90	
Gender % male	40%	31%	0.72^
Age years [median (range)]	82 (76–89)	83 (67–97)	0.80<
Length of stay [median days (range]	526.5 (38–2,694)	573.5 (5–5,591)	0.54<
Concomitant medications (median)	5 (3–10)	5 (1–12)	0.87<
Antibiotic exposure	8	42	0.046*
Proton pump inhibitors	4	30	0.73^
Comorbidities (median)	6.5	5	0.09<
Previous *C. difficile* infection	3	15	0.38^

Demographic and clinical data of the study population were collected. Of 100 patients, 10 were *C. difficile*-positive based on molecular identification; 40% of these were male. There was no association between *C. difficile* carriage and sex, age, length of stay, concomitant medication and use of proton pump inhibitors, previous *C. difficile* infection or number of comorbidities. Eight of ten *C. difficile* carriers had recent antibiotic exposure compared with 42/90 non-carriers, $P = 0.046$, chi-squared test. Statistical analyses included ^Fisher's exact test, <Mann–Whitney test and *chi-squared test

Table 3 and carriers and non-carriers compared. Previous *C. difficile* infections were noted in the carrier and non-carrier groups, but were not significantly different between the groups. There was, however, a significant difference in antibiotic exposure between the carrier and non-carrier groups. Of the ten carriers, eight were on a course of broad-spectrum antibiotics at the time of sample collection compared with only 42/90 of those in the non-carrier group, which was statistically significant ($P = 0.046$ chi-squared test). The most commonly identified antibiotic group in the carriers was broad-spectrum penicillin (six patients), cephalosporins (two patients) and fluoroquinolones (two patients). We also found that 8/10 of patients who tested positive for *C. difficile* toxin in their stool had been exposed to an antibiotic within the preceding 2 months. Non-carriers were less likely to have been exposed to antibiotics with only 43/90 of this group identified as having recent antibiotic exposure; again the difference was found to be significant ($P = 0.0046$ chi-squared test) (Table 3).

Comorbidities were defined as a co-existing, chronic, infectious, inflammatory or neoplastic disease. There was no difference in the number of comorbidities between the groups. The carrier group had a median number of 6.5 (range 4–12) and the non-carrier population had a median of 5 (range 1–9). It was found that 3/10 of the carrier population had a past history of stool positive for *C. difficile* disease. Of 90 of the non-carrier group, 15 had a previous diagnosis of *C. difficile* disease. There was no significant difference between the two groups in this respect ($P = 0.38$, Fisher's exact test).

Discussion

This study determined the asymptomatic carriage rate of *C. difficile* in a cohort of hospitalised elderly patients in an Irish continuing care institution and identified the epidemiological characteristics of this carrier population compared with non-carriers within the same institution. The 10% carriage rate is comparable to reported prevalence rates from other groups [1–5], but considerably less than that found by Riggs et al. [17] who reported a 51% carriage rate in asymptomatic patients of a long-term care facility.

Seventeen isolates were identified as *C. difficile* by conventional microbiology of which ten were confirmed to be *C. difficile* positive by PCR techniques. The seven remaining strains not identified as *C. difficile* were revealed to be *C. sordellii* following further analysis. This pathogen is rarely encountered in clinical specimens (1% of *Clostridium* species), but has been described as a human pathogen with fatal potential [18]. Toxigenic isolates of *C. sordellii* produce two toxins, namely haemorrhagic toxin (HT) and lethal toxin (LT) that are very similar to *C. difficile* toxin A and toxin B and consequently are sensitive to the standard immunoassay [19]. The PRO disc technique for identifying *C. difficile* did not distinguish between *C. difficile* and *C. sordelli*. This and other studies have highlighted the importance of using various techniques in the molecular study of *C. difficile*; however, there remains a lack of consensus about the optimal approach to *C. difficile* typing.

There was no association between *C. difficile* carriage and gender or age in this over-65 years population. Contrary to previous studies, which have shown an association between length of stay and increased risk of developing *C. difficile* infection [20], there was no difference in mean length of stay between the groups. In this study, it was found that a significant association exists between *C. difficile* carriage and the concurrent use of antibiotics or exposure to broad-spectrum antibiotics within the preceding 2 months, similar to previous studies [17, 21, 22]. The most common implicating class of antibiotics in this study, broad-spectrum penicillins, cephalosporins and fluoroquinolones, were also noted to be associated with *C. difficile* carriage. Judicious antibiotic prescribing policies should be considered, as their implementation has previously resulted in a reduction in symptomatic *C. difficile* disease in this population [23, 24]. Previous infection with *C. difficile* has also been implicated as a risk factor for further infection or colonisation [17, 25]; however, this study showed no significant difference between the groups in this regard. There was no difference in

the number of comorbidities between the groups, and there are few studies published on this worldwide. There are many conflicting reports on the association between the use of PPI and *C. difficile* disease [21, 26, 27], but this study did not show an association.

The identification of asymptomatic *C. difficile* carriers within a continuing care institution may represent an important reservoir of patients who may be at risk following subsequent antibiotic exposure. Further investigation of this group may reveal a role in disease transmission. Our data suggest that the use of broad-spectrum antibiotics is associated with *C. difficile* carriage and although this association is widely accepted, these antibiotics continue to be used. More stringent antibiotic prescribing policies need to be adhered to and these classes of antimicrobials avoided where possible in this at-risk population.

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