Genetic characterization of clinical isolates of *Clostridium difficile* using an optimized RAPD protocol and PCR ribotyping reveals strain diversity between two tertiary referral Trusts in the West Midlands, UK

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Epidemiological investigations of *Clostridium difficile* often focus on differences between separate geographical areas. In this investigation, two populations of *C. difficile* recovered from separate tertiary referral Trusts within the West Midlands, UK, were characterized using both PCR ribotyping and an optimized RAPD (random amplification of polymorphic DNA) protocol. The PCR ribotyping and RAPD methodologies identified differences between the two *C. difficile* populations, in both the prevalence and the diversity of types identified. The use of PCR ribotyping in conjunction with RAPD further categorized different types within defined PCR ribotypes, identifying different types within the same PCR ribotype and therefore providing a greater discriminatory power than either of the methods when used alone. The differences observed in this study between the two Trusts in the distribution of both RAPD 'type' and PCR ribotype demonstrate the diversity that is present amongst isolates of *C. difficile* within a relatively small geographical area and warrants a need for further investigation into the local epidemiology of *C. difficile*.

INTRODUCTION

Clostridium difficile is still a major cause of hospital-associated infection across the UK despite a continual decline in infection rates since 2008 (Health Protection Agency, 2010). The emergence of a *C. difficile* strain in Canada in 2003 which was associated with greater rates of mortality and increased resistance to fluoroquinolones (Loo *et al.*, 2005; McDonald *et al.*, 2005) was later identified as the hypervirulent PCR ribotype 027/PFGE type NAP1 strain that has since been responsible for large outbreaks of *C. difficile* infection (CDI) across North America and Europe (Warny *et al.*, 2005).

C. difficile PCR ribotype 027 is currently the most prevalent PCR ribotype isolated in England and Northern Ireland, followed by PCR ribotypes 106 and 001, respectively (Health Protection Agency, 2009). Similar patterns of distribution are evident throughout several countries in Europe, although the distribution and predominant PCR ribotypes differ between countries (Kuijper *et al.*, 2008). There is little information, however, on the distribution

and prevalence of PCR ribotypes of *C. difficile* within smaller geographical areas and therefore the localized distribution of PCR ribotypes is relatively unknown.

Currently within England and Northern Ireland, only three PCR ribotypes are responsible for over 50 % of CDI cases (Health Protection Agency, 2009); this has led to PCR ribotyping no longer being discriminative enough to monitor the epidemiology of CDI (Tanner *et al.*, 2010). Methods such as multi-locus variable number tandem repeat analysis (MLVA) are now becoming increasingly employed to type strains of *C. difficile* as they have the capacity to identify greater genotypic variability between isolates of *C. difficile* and identify 'subtypes' within PCR ribotypes (Killgore *et al.*, 2008; Marsh *et al.*, 2006; van den Berg *et al.*, 2007). Subtyping within PCR ribotypes has also previously been demonstrated using other methods such as PFGE, REA and REP-PCR (Rahmati *et al.*, 2005; Northey *et al.*, 2005; Killgore *et al.*, 2008; Fawley *et al.*, 2008; Tanner *et al.*, 2010). Despite this, accessibility, in addition to time and cost restraints, means that MLVA is still not widely utilized. Random amplification of polymorphic DNA (RAPD) PCR is a quick, cost-effective method which has previously been used to characterize *C. difficile* isolates (Barbut *et al.*, 1994; Chachaty *et al.*, 1994; van Dijk *et al.*,

Abbreviations: CDI, *C. difficile* infection; MLVA, multi-locus variable number tandem repeat analysis; RAPD, random amplification of polymorphic DNA.

1996). The method is often criticized, however, due to lack of reproducibility (Brazier, 1998). Optimization of the protocol has been shown to vastly improve reproducibility and therefore an optimized protocol was designed to determine if RAPD could be used to effectively discriminate between strains of *C. difficile*.

The aim of this study was to investigate the prevalence and distribution of different *C. difficile* PCR ribotypes between two tertiary referral Trusts within the West Midlands, UK, and also determine if RAPD could be utilized in the characterization and discrimination of isolates.

METHODS

Isolates. Sixty-two clinical toxigenic isolates of *C. difficile* were recovered from two tertiary referral Trusts within the West Midlands. Isolates were recovered from the Trusts between 2004 and 2005, with 30 of the isolates recovered from Trust A and 32 from Trust B. Eleven PCR ribotype reference isolates (001, 002, 005, 014, 015, 017, 023, 027, 064, 078 and 106) were kindly provided by HPA, Newcastle Laboratory, UK, and were used as comparisons for PCR ribotype profiles.

Isolation of *C. difficile* from faecal samples. Isolates were recovered from faecal samples using alcohol shock methodology, and following isolation were stored at -70°C using a Microbank Bacterial Preservation System until required.

PCR ribotyping. The PCR ribotyping technique used in this investigation was as described by Stubbs *et al.* (1999).

RAPD. The RAPD protocol used in this investigation had been optimized in previous studies to ensure the reproducibility and discriminatory power of the method (data not shown). Two 10 bp primers, previously published by other authors, were used in reactions that were separate from and independent of each other. The primers used were AP3 (5'-TCA CGA TGC A-3') (Martirosian *et al.*, 1995) and AP4 (5'-TCA CGC TGC A 3') (Barbut *et al.*, 1993). Isolates were cultured onto Wilkins-Chalgren anaerobe agar and incubated for 24 h at 37°C in an anaerobic cabinet. Colonies were then suspended in a 0.5% (w/v) Chelex 100 resin suspension, which was briefly vortexed before being incubated in a water bath at 94°C for 12 min; a negative control containing only a suspension of Chelex 100 resin was also used for each experiment. Samples were then centrifuged for 10 min at 15 000 g and the supernatant containing the crude DNA extract was used in the RAPD reaction; samples were stored at 4°C until required. A total reaction volume of 25 μl was used; concentrations for each of the components in the reactions were as follows: 100 μM primer, 2.5 mM each dNTP, 35 mM MgCl_2 , 25 mM KCl, 10 mM Tris/HCl (pH 8.8), 1.25 U *Taq* polymerase and 2 μl DNA sample. Amplification was carried out using cycles as follows: an initial cycle of 94°C for 4.5 min, 5 cycles of 94°C for 30 s, 24°C for 2 min and 72°C for 1 min followed by 35 cycles of 94°C for 30 s, 30°C for 30 s and 72°C for 1 min, and a final extension of 5 min at 72°C . Following amplification, 5 μl loading buffer was added to the PCR samples and gel electrophoresis was carried out using a 2% (w/v) agarose gel and performed in $1 \times$ TAE; 5 μl DNA ladder was added to the centre and end wells and 15 μl PCR product was loaded into the remaining wells. Gels were run at 80 V for 1 h and 24 min until samples reached the end of the gel. To enable gels to be visualized, they were stained by soaking in 0.005% (w/v) ethidium bromide solution for 20 min and then rinsed in sterile distilled water. Gels were visualized under UV light using the

GBOX-EF Gel Documentation System and images captured using Genesnap software.

Profile analysis. Amplicon profiles produced by both RAPD and PCR ribotyping were analysed using GelCompar II software.

Typeability and discriminatory power of PCR ribotyping and RAPD. The typeability and discriminatory power of PCR ribotyping and both primers used in the optimized RAPD protocol were calculated using Simpson's index of diversity (D) using the equation $D = [1 - \sum n(n-1)]/N(N-1)$, where n is the total number of profiles characterized by an individual method, and N is the total number of profiles for both methods.

RESULTS

PCR ribotyping

From the 62 isolates of *C. difficile* characterized, 12 distinct PCR ribotype profiles were identified. Six of these profiles could be matched to reference PCR ribotype profiles and therefore identified a distinct PCR ribotype. The frequency with which each of the PCR ribotypes occurred is presented in Table 1. The remaining six profiles, which represented 23% of the total isolates, could not be matched to any of the 11 reference profiles available and therefore the PCR ribotype of these isolates was designated 'undefined'. The PCR ribotypes most frequently identified amongst the group of isolates used in this investigation were 027 and 106, with rates of 27% and 23% respectively; other PCR ribotypes identified were (rates indicated in parentheses) 001 (8%), 002 (6%), 014 (8%) and 064 (5%).

The distribution and number of PCR ribotypes amongst the isolates varied between the two tertiary referral trusts. Amongst the 32 isolates recovered from Trust A, 10 distinct PCR ribotype profiles were identified, but only six PCR ribotype profiles were identified amongst the 30 isolates recovered from Trust B. Isolates belonging to PCR ribotypes 027, 064, 106 and one undefined type were

Table 1. Frequency with which PCR ribotypes occurred throughout the isolate population (percentage frequency indicated in parentheses)

PCR ribotype	Trust A	Trust B	Total
001	5 (16%)	0	5 (8%)
002	4 (13%)	0	4 (6%)
014	5 (16%)	0	5 (8%)
027	3 (9%)	14 (47%)	17 (27%)
064	2 (6%)	1 (3%)	3 (5%)
106	2 (6%)	12 (40%)	14 (23%)
Undefined (a)	0	1 (3%)	1 (2%)
Undefined (b)	1 (3%)	0	1 (2%)
Undefined (c)	0	1 (3%)	1 (2%)
Undefined (d)	2 (6%)	0	2 (3%)
Undefined (e)	4 (13%)	1 (3%)	5 (8%)
Undefined (f)	4 (13%)	0	4 (6%)

found to be present in both Trusts, with the remaining types and profiles identified being exclusive to only one. Almost 80 % of the isolates recovered from Trust A were identified as belonging to only five of the PCR ribotypes identified. Thirty-two per cent of isolates were found to belong to PCR ribotypes 001 and 014, with each being found at equal frequency; PCR ribotype 002 and two undefined types were also each found in 13 % of isolates recovered from this trust. Amongst the isolates recovered from Trust B, 87 % belonged to PCR ribotypes 027 and 106; the four remaining PCR ribotypes that were identified were each represented by a single isolate.

RAPD

When the 62 isolates of *C. difficile* were characterized using primer AP3, 19 different RAPD types were identified. Thirteen different RAPD types were identified amongst isolates recovered from Trust A (Fig. 1), with six different types identified among isolates from Trust B (not shown). The frequency with which RAPD types (using primer AP3) were observed, and the Trusts from which they were recovered, are presented in Table 2(a). The RAPD profiles produced were exclusive to the location from which they were recovered, with no one type observed in isolates from both of the Trusts. As for PCR ribotyping, 87 % of the isolates recovered from Trust B belonged to two RAPD types consisting of 11 and 15 isolates; each of the remaining four types identified were represented by a single isolate.

When both populations of *C. difficile* isolates were characterized using primer AP4, 10 different RAPD types were defined and in contrast to the results obtained when isolates were characterized using primer AP3, some isolates from both locations shared the same profile. Six different RAPD types were identified amongst isolates recovered from Trust A (Fig. 2), with two of these types also identified in isolates recovered from Trust B. The frequency

Fig. 1. RAPD profiles of isolates recovered from Trust A and characterized using primer AP3.

Fig. 2. RAPD profiles of isolates recovered from Trust A and characterized using primer AP4.

with which RAPD types (using primer AP4) were observed, and the Trusts from which they were recovered, are presented in Table 2(b). Ninety per cent of the 62 isolates

Table 2. Frequency with which RAPD types, characterized using primer AP3 (a) and primer AP4 (b), occurred throughout the isolate population

RAPD type	Trust A	Trust B
(a) AP3 RAPD type		
A	3	0
B	7	0
C	1	0
D	3	0
E	0	1
F	2	0
G	3	0
H	5	0
I	2	0
J	2	0
K	1	0
L	1	0
M	1	0
N	1	0
O	0	11
P	0	1
Q	0	15
R	0	1
S	0	1
(b) AP4 RAPD type		
a	1	0
b	1	0
c	5	0
d	9	1
e	0	11
f	15	15
g	0	1
h	0	1
i	0	1
j	1	0

were identified as belonging to four RAPD types, with the remaining six types each being represented by a single isolate.

Characterization of isolates using both PCR ribotyping and RAPD

Following characterization using both PCR ribotyping and RAPD, the data produced were then used in combination to further discriminate within PCR ribotypes (Table 3). When used to discriminate within PCR ribotypes, primer AP4 generally categorized to an equal or lesser number than primer AP3, with the exception of PCR ribotype 001. Isolates identified as belonging to the most common PCR ribotypes within the UK (001, 027 and 106)

were further categorized into three RAPD types amongst those belonging to PCR ribotype 001, and into four RAPD types amongst those belonging to PCR ribotypes 027 and 106.

Typeability and discriminatory power of PCR ribotyping and RAPD

All isolates were typable by both PCR ribotyping and RAPD. Characterization of isolates using RAPD and primer AP3 demonstrated the greatest discriminatory power (0.89) followed by PCR ribotyping (0.86) and then RAPD using primer AP4 (0.58).

DISCUSSION

Both PCR ribotyping and RAPD produced similar patterns of profile distribution between the two locations, with both methods identifying greater variability throughout the population of isolates recovered from Trust A. Although there were similarities in the discriminatory power of the two methods, the defined types did not map directly on to each other; therefore RAPD type could not be used to predict PCR ribotype. There was also an overlap in the types observed; a single RAPD type was not exclusive to one PCR ribotype, possibly due to the targeting of different genomic targets by each of the methods. This similar pattern of distribution has also previously been observed in earlier phenotypic work which investigated the antibiogram profiles of 12 antibiotics in the same population of isolates (Green *et al.*, 2010). The similar patterns of distribution observed between the two Trusts using PCR ribotyping, RAPD and antibiogram profiling is suggestive of an association between these methods; however, no association could be established.

This investigation highlighted differences in PCR ribotype epidemiology between the two Trusts despite their being relatively close geographically. Almost 90 % of the isolates from Trust B belonged to PCR ribotypes 027 and 106; in contrast, the two predominant PCR ribotypes (001 and 014) recovered from Trust A represented only 32 % of isolates. The reasons for such epidemiology are unknown and there are likely to be several contributing factors such as differences in infection control procedures and the demographics of the patients admitted. The predominance of only two PCR ribotypes (027 and 106) amongst isolates from Trust B may be indicative of hospital-acquired transmission, whereby the same strains of *C. difficile* continually circulate throughout a hospital environment. This observation is speculative, however, if based on PCR ribotyping information alone, due to limitations in the discriminatory power of PCR ribotyping. When RAPD profiling data were also applied to these predominant PCR ribotypes from Trust B, 11 (37 %) and 8 (27 %) of isolates identified as belonging to PCR ribotypes 027 and 106 respectively were also the same RAPD type. Although such characterization is not as discriminative as MLVA, the

Table 3. Distribution of defined RAPD types within PCR ribotypes

PCR ribotype	AP3 RAPD type	No. of isolates	AP4 RAPD type	No. of isolates
001	B	1	a	1
	D	1	d	1
	I	2	e	2
	J	1	j	1
002	C	1	d	3
	F	1	e	1
	H	1		
	K	1		
014	A	2	d	1
	B	1	e	4
	D	1		
	M	1		
027	B	1	c	1
	H	2	e	3
	O	3	f	13
	Q	11		
064	A	1	d	1
	E	1	e	1
	H	1	i	1
106	G	1	f	13
	H	1	h	1
	O	8		
	Q	3		
	R	1		
U(a)	Q	1	f	1
U(b)	F	1	b	1
U(c)	S	1	g	1
U(d)	B	1	d	1
	J	1	f	1
	B	2	c	1
U(e)	L	1	d	2
	N	1	f	2
	P	1		
U(f)	B	1	c	3
	D	1	d	1
	G	2		

identification of these polymorphisms in isolates of *C. difficile* demonstrates how useful such methodologies can be in epidemiological studies, and especially outbreak situations. The PCR ribotype of over a third of isolates (34 %) recovered from Trust A and 10 % of isolates from Trust B could not be identified due to the profiles not matching reference profiles of the 10 most common PCR ribotypes in England and Wales.

To conclude, the differences between the two Trusts in the distribution and prevalence of both RAPD 'type' and PCR ribotype demonstrate the diversity that is present in isolates of *C. difficile* within a relatively small geographical area. The use of PCR ribotyping in conjunction with RAPD has enabled greater discrimination of isolates than could be achieved using either of the methods alone; this is important for studying the epidemiology of CDI as it allows discrimination within PCR ribotypes.

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