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An Investigation of the Subtype Diversity of Clinical Isolates of Irish Clostridium Difficile Ribotypes 027 and 078 by Repetitive-Extragenic Palindromic PCR

Denise Drudy
Technological University Dublin, denise.drudy@tudublin.ie

K. Solomon
University College Dublin

S. Murray
University College Dublin

See next page for additional authors

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Authors

Denise Drudy; K. Solomon; S. Murray; L. Scott; S. McDermott,; A. Martin; C. O’Donoghue; M. Skally; K. Burns; L. Fenelon; F. Fitzpatrick,; L. Kyne; and S. Fanning

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An investigation of the subtype diversity of clinical isolates of Irish *Clostridium difficile* ribotypes 027 and 078 by repetitive-extragenic palindromic PCR

K. Solomon,[1] S. Murray,[1] L. Scott,[2] S. McDermott,[2] D. Drudy,[1] A. Martin,[1,3] C. O'Donoghue,[1,3] M. Skally,[2,4] K. Burns,[5] L. Fenelon,[2] F. Fitzpatrick,[4,5] L. Kyne[1,3] and S. Fanning[1]

Correspondence
K. Solomon
katie.solomon@ucd.ie

[1]UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland

[2]Department of Clinical Microbiology, St Vincent's University Hospital, Elm Park, Dublin 4, Ireland

[3]Department of Medicine for the Older Person, Mater Misericordiae University Hospital, Eccles Street, Dublin 7, Ireland

[4]Health Protection and Surveillance Centre, Gardiner Street, Dublin 1, Ireland

[5]Department of Clinical Microbiology, Beaumont Hospital, Beaumont Road, Dublin 9, Ireland

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A repetitive-extragenic palindromic PCR (rep-PCR) subtyping method (DiversiLab) in conjunction with ribotyping, toxinotyping and antimicrobial-susceptibility testing was used to detect subtypes within *Clostridium difficile* ribotypes 027 and 078. Clinical isolates of ribotypes 027 (toxinotype III) (n=30) and 078 (toxinotype V) (n=23) were provided by health-care facilities across the Republic of Ireland over 2 months in 2006 and 1 month in 2009. Ribotype 027 isolates were significantly more related to each other (9 different subtype profiles) when compared to ribotype 078 isolates (14 different profiles) (P=0.001; cut-off >90 % similarity). Almost half of ribotype 078 isolates (45.5 %) showed no relationship to each other. The clonality of ribotype 027 isolates suggests effective adaptation to the human niche, whereas the considerable genetic diversity within ribotype 078 isolates suggests that they may have originated from a variety of sources. Subtyping correlated well with antimicrobial susceptibility, in particular clindamycin susceptibility for ribotype 027, but diverse antimicrobial-susceptibility profiles were seen in ribotype 078 isolates, even within a single health-care facility. Between 2006 and 2009, a change in the predominant subtype of ribotype 027 was seen, with the recent clone representing half of all ribotype 027 isolates studied. This strain exhibited 89 % similarity to a rep-PCR profile of the North American NAP-1 strain.

INTRODUCTION

Clostridium difficile infection (CDI) is recognized as a major cause of health-care-acquired antibiotic-associated diarrhoea and pseudomembranous colitis. Several molecular typing methods have been employed to study *C. difficile*, e.g. PCR ribotyping, PFGE, multilocus variable number tandem repeat analysis (MLVA) and repetitive-extragenic palindromic PCR (rep-PCR), each with various advantages and disadvantages (Killgore *et al.*, 2008; Kuijper *et al.*, 2009). rep-PCR profiling relies on variations in the copy number and location of repetitive elements dispersed throughout the genome. PCR amplification between these elements results in DNA amplicons of various sizes and frequencies giving rise to individual rep-PCR profiles. Discrimination of subtypes within ribotypes by rep-PCR (Northey *et al.*, 2005; Rahmati *et al.*, 2005; Spigaglia & Mastrantonio, 2003), randomly amplified polymorphic DNA techniques (Fawley *et al.*, 2003) and a commercially available semi-automated rep-PCR system (DiversiLab, Bacterial Barcodes; bioMérieux) has been useful for further studying *C. difficile* epidemiology and these methods have shown good reproducibility for this organism (Healy *et al.*, 2005; Pasanen *et al.*, 2011; Spigaglia & Mastrantonio, 2003).

Reports of CDI have been increasingly associated with a hypervirulent strain, PCR ribotype 027 [PFGE type NAP-1 (North American pulsotype-1)] (Kuijper *et al.*, 2006, 2007; McDonald *et al.*, 2005). Studies of ribotype 027/NAP-1 epidemiology using different molecular subtyping methods

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Abbreviations: CDI, *Clostridium difficile* infection; HPSC–HSE, Health Protection Surveillance Centre and Health Service Executive; MLS_B, macrolide–lincosamide–streptogramin B; MLVA, multilocus variable number tandem repeat analysis; rep-PCR, repetitive-extragenic palindromic PCR.

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have identified some clonal strains (Killgore *et al.*, 2008), whereas others have distinguished hospital-related clusters (Fawley *et al.*, 2008). The spread of ribotype 027/NAP-1 mainly correlates with resistance to fluoroquinolones, although outbreaks caused by less common clindamycin-resistant strains have occurred (Johnson *et al.*, 1999; Kuijper *et al.*, 2008), with the first European cases reported in Ireland (Drudy *et al.*, 2008). MLVA of these isolates differentiated distinct clindamycin-resistant and -susceptible clusters, despite the geographical proximity of the sources (Drudy *et al.*, 2008).

A recently emerging PCR ribotype, 078 (PFGE type NAP-7 or -8), has been identified in food and environmental sources, and is associated with community-acquired infections (Jhung *et al.*, 2008; Keel *et al.*, 2007). Prevalence of this strain is increasing in Europe (Brazier *et al.*, 2008; Debast *et al.*, 2009; Goorhuis *et al.*, 2008a, b; Keel *et al.*, 2007; Rupnik *et al.*, 2008). The increase in the prevalence of ribotype 078 appears to have mirrored a reduction in the prevalence of ribotype 027 in The Netherlands (Hensgens *et al.*, 2009). In a recent national study in Ireland, ribotype 078 accounted for 9.4 % of isolates, whereas ribotype 027 accounted for 18.7 % (*n*=107) (Burns *et al.*, 2010b). Antimicrobial-susceptibility patterns of ribotype 078 isolates are less well documented. In a recent study in The Netherlands it was reported that isolates cultured from pigs exhibited identical susceptibility patterns and a genetic relationship to human isolates suggesting possible animal-to-human transmission (zoonosis) (Debast *et al.*, 2009). Human-to-human transmission of ribotype 078 strains is less well characterized, although it has been suggested during a recent outbreak of CDI due to ribotype 078 in the Republic of Ireland (Burns *et al.*, 2010a).

The distinctive differences in genetic diversity, infection origin and antimicrobial susceptibility between *C. difficile* ribotypes 027 and 078 highlight the importance of an epidemiological study of circulating ribotype 027 and 078 strains. One approach, stratifying antimicrobial-susceptibility profiles with enhanced genotyping, may identify subtypes specific to health-care settings, facilitate monitoring of their potential for spread and track the emergence of multi-resistant, increasingly virulent strains of ribotype 078 in the human population.

METHODS

Bacterial isolates and patient clinical history. A total of 53 well-characterized isolates of *C. difficile* ribotypes 027 (*n*=30) and 078 (*n*=23) were selected from isolates previously collected from patients with CDI in 14 hospitals across the Republic of Ireland (Fig. 1) during 2 months in 2006 (*n*=10) and 1 month in 2009 (*n*=43). Ribotypes 027 and 078 comprised 37.7 and 1.9 %, respectively, of all *C. difficile* strains isolated in 2006, and 18.7 and 9.4 %, respectively, of all 2009 isolates (Burns *et al.*, 2010b). Clinical details were collected from patients, including origin of infection as defined by Kuijper *et al.* (2006).

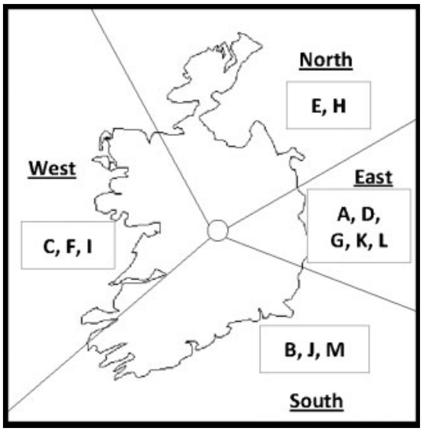


Fig. 1. A schematic map of the Republic of Ireland indicating health-care facilities included in this study. Clinical *C. difficile* isolates in this study were collected from health-care facilities from a variety of geographical locations. The facilities have been coded (A–M) and are separated into their geographical regions for illustration of proximity.

Antimicrobial-susceptibility testing. Antimicrobial susceptibility to MLS_B (macrolide–lincosamide–streptogramin B) antibiotics (clindamycin and erythromycin), fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin), metronidazole and vancomycin were determined by the Etest method (bioMérieux). Isolates of standardized McFarland inoculum were streaked onto pre-reduced Brucella agar plates supplemented with vitamin K1, haemin and 5 % (v/v) sheep blood (BD Biosciences). Etest strips were laid directly on the agar surface and incubated at 37 °C for 48 h. The MIC ($\mu g\ ml^{-1}$) for all antibiotics was measured at the ellipse of inhibited growth. Breakpoints of susceptibility for moxifloxacin, clindamycin and erythromycin were defined by reference to the Clinical and Laboratory Standards Institute (CLSI, 2007) and those for ciprofloxacin and levofloxacin were defined by reference to the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org – 2010, v. 2.6).

DNA purification. Genomic DNA was purified from bacterial pellets following overnight anaerobic growth in Schaedler anaerobe broth (Oxoid) at 37 °C. After enzymic lysis of cell walls, DNA was extracted using the Wizard genomic DNA purification kit (Promega) and assessed for concentration and purity with a NanoDrop spectrophotometer (Thermo Scientific) before use in PCR.

PCR ribotyping. Isolates were characterized as ribotype 027 or 078 by 16–23S intergenic-spacer PCR performed using the method outlined by Stubbs *et al.* (1999). Gel electrophoresis fingerprints were analysed using BioNumerics software (version 6.01; Applied Maths) and ribotypes were assigned by comparison to reference strains from the Leiden University Medical Centre library, Leiden, The Netherlands.

Toxinotyping. Isolates were toxinotyped as described by Rupnik *et al.* (1998). DNA amplicons generated by PCR of the first 3 kb of the toxin B gene (B1) and 3 kb of the C-terminal region of the toxin A gene (A3) were digested by restriction enzymes *Hin*cII, *Acc*I and *Eco*RI producing characteristic banding patterns.

rep-PCR DNA fingerprinting. Genomic DNA was amplified using the DiversiLab *Clostridium* kit (Bacterial Barcodes; bioMérieux) according to the manufacturer's instructions and as described by Pasanen *et al.* (2011). Positive and negative controls (supplied in the kit) were included and assessed in parallel to validate the reaction.

DNA amplicons were separated on microfluidics chips (DNA LabChip device; Caliper Technologies) using a 2100 Bioanalyser (Agilent Technologies) (Healy *et al.*, 2005). DNA standard markers were used to normalize sample runs and a molecular ladder (200–4000 bp marker provided with kit) was included. Data interpretation and analysis were carried out within the DiversiLab software (version 3.4) utilizing dendrograms, similarity matrices and virtual gel images generated from fluorescence intensity graphs after normalization. Percentage similarities between virtual gels generated from isolates were calculated using the Kullback–Liebler band-based method, weighted on the presence or absence of bands.

The basis for discrimination between strains was defined by the DiversiLab strain typing analysis guide (provided online with the software) parameters as follows: similarity greater than 97 % was defined as indistinguishable; similarity greater than 95 % was defined as similar; similarity less than 95 % was considered different but related; and similarity less than 90 % was considered unrelated. Subtypes were assigned where two or more isolates exhibited greater than 90 % similarity to each other. Statistical significance was calculated using χ^2 and Fisher's exact tests.

RESULTS AND DISCUSSION

Characterization of isolates

Isolates were characterized by PCR ribotyping as either ribotype 027 or 078. Toxinotyping confirmed that all isolates of ribotype 078 were toxinotype V. Ribotype 027 isolates were confirmed as toxinotype III.

All isolates were found to be susceptible to both metronidazole and vancomycin (MIC ranges of 0.25–1.5 and 0.0064–0.38 $\mu g\ ml^{-1}$, respectively) and resistant to ciprofloxacin (MIC range 8 to >32 $\mu g\ ml^{-1}$). Antimicrobial-resistance profiles were assigned to isolates based on patterns of susceptibility in response to fluoroquinolones, moxifloxacin, levofloxacin and the MLS_B antibiotics (erythromycin and clindamycin). Susceptibility profiles ranged from resistance to all fluoroquinolone and MLS_B antimicrobials (denoted as profile 1) to susceptibility to erythromycin only (denoted as profile 9).

Diversity of isolates as defined by rep-PCR subtyping

rep-PCR fingerprints for isolates characterized as ribotypes 027 and 078 were generated, and subsequent dendrograms were created and analysed. At a cut-off point of >90 % similarity, four main subtypes within ribotype 027 could be identified (Fig. 2), one of which (denoted subtype 1) contained half of all ribotype 027 isolates analysed. Isolates within this subtype originated from health-care facilities in all geographically distinct regions, thus confirming clonal spread within our study population and that this subtype may therefore exhibit a competitive genetic fitness over others in the current health-care environment nationwide. A comparison between the virtual gel fingerprint profiles of study isolates and those in a commercially provided 'DiversiLab *C. difficile* library' confirmed that isolates of rep-PCR subtype 1 were 89 % similar to a North American pulsotype-1 (NAP-1) isolate (McDonald *et al.*, 2005) (data not shown). Similarities between European ribotype 027 isolates and North American strains have been shown by PFGE (Fawley *et al.*, 2008; Smith, 2005). The predominant subclone of ribotype 027 in Ireland is related to the hypervirulent NAP-1 epidemic strain; however, other subtypes exist that are unrelated.

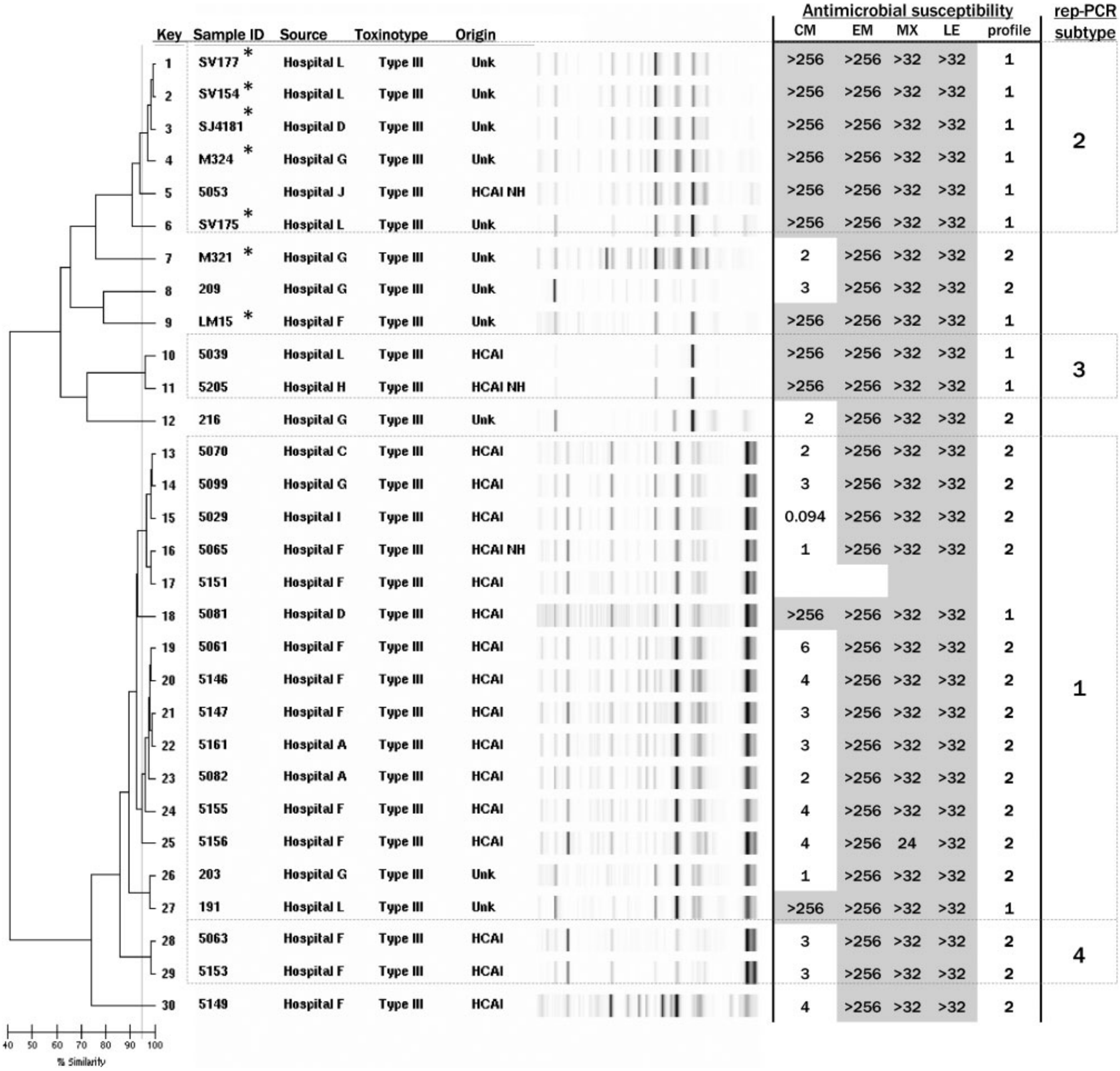
The remaining ribotype 027 isolates clustered within three different rep-PCR subtypes unrelated to the predominant clone. These lesser subtypes may not have been able to dominate due to certain characteristics, such as a slower growth rate, reduced sporulation/environmental persistence or reduced toxin production/virulence, and were either from the same hospital (subtype 4) or neighbouring regions (subtype 3) suggesting a reduced ability to spread. Five isolates (16 %) were unrelated to either of these subtypes or to each other (<90 % similarity).

Heterogeneity within ribotype 027 has been reported by others in European isolates (Fawley *et al.*, 2008) and we postulate that ribotype 027 isolates may not have equal virulence potential, and the emphasis on diagnostic laboratories to identify isolates as markers of potential outbreaks by ribotyping alone may be of limited use.

Ribotype 027 isolates were significantly more likely to be related to each other (similarity ⩾95 %) than ribotype 078 isolates (P=0.001), which exhibited greater genetic diversity; 63 % of ribotype 027 isolates were considered to be indistinguishable from at least one other (>97 % similarity), compared to only 17 % of ribotype 078 isolates. At a cut-off point of >90 % similarity, five main subtypes within ribotype 078 could be identified (Fig. 3), but 45.5 % of ribotype 078 isolates were considered to be unrelated to each other.

The closest related ribotype 078 isolates originated from either the same health-care source (hospitals G and E) or health-care sources within the same region (hospitals G and D). Isolates with a community-associated origin and from nursing homes were closely related to isolates from health-care facilities in the locality, suggesting common sources of infection. Related isolates in subtype 2, however, originated from geographically distinct facilities (>400 km away). Subtype diversity was seen between isolates from health-care facilities of close geographical proximity (within a 15 km radius; hospitals G, A and L). Variation between human ribotype 078/toxinotype V isolates has also been confirmed by PFGE analysis (*Sma*I digestion) with isolates grouped into approximately seven distinct clusters of <90 % similarity including PFGE types NAP-7 and NAP-8 (Jhung *et al.*, 2008).

Other studies using rep-PCR as well as other typing methods have identified that ribotype 078/toxinotype V isolates show a degree of relatedness, in contradiction to the findings presented here (Lin *et al.*, 2011; Pasanen *et al.*, 2011). However, these studies involved either a small

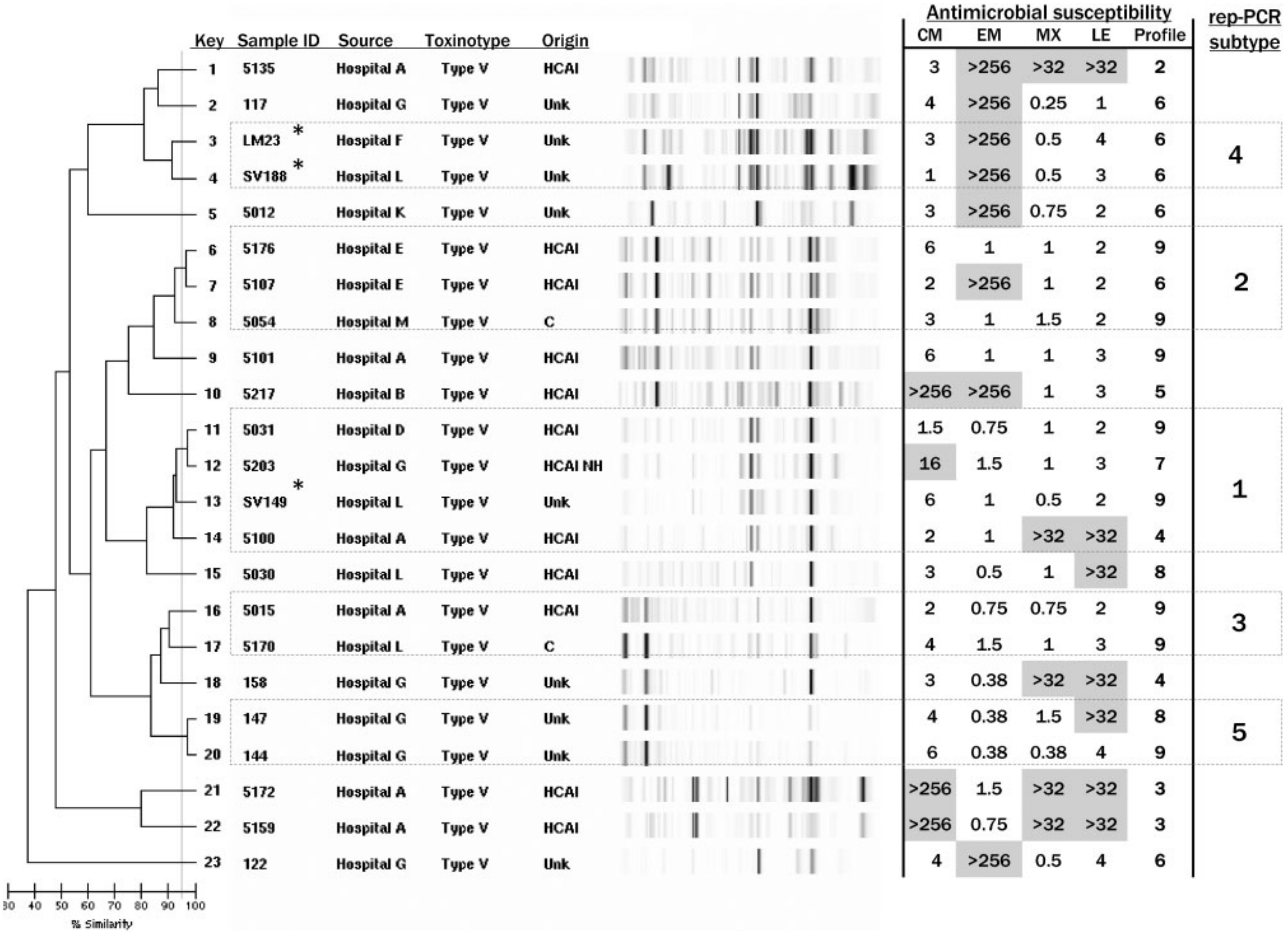


Key	Sample ID	Source	Toxinotype	Origin	CM	EM	MX	LE	profile	rep-PCR subtype
					Antimicrobial susceptibility					
1	SV177 *	Hospital L	Type III	Unk	>256	>256	>32	>32	1	2
2	SV154 *	Hospital L	Type III	Unk	>256	>256	>32	>32	1	
3	SJ4181 *	Hospital D	Type III	Unk	>256	>256	>32	>32	1	
4	M324 *	Hospital G	Type III	Unk	>256	>256	>32	>32	1	
5	5053	Hospital J	Type III	HCAI NH	>256	>256	>32	>32	1	
6	SV175 *	Hospital L	Type III	Unk	>256	>256	>32	>32	1	
7	M321 *	Hospital G	Type III	Unk	2	>256	>32	>32	2	
8	209	Hospital G	Type III	Unk	3	>256	>32	>32	2	
9	LM15 *	Hospital F	Type III	Unk	>256	>256	>32	>32	1	
10	5039	Hospital L	Type III	HCAI	>256	>256	>32	>32	1	3
11	5205	Hospital H	Type III	HCAI NH	>256	>256	>32	>32	1	
12	216	Hospital G	Type III	Unk	2	>256	>32	>32	2	
13	5070	Hospital C	Type III	HCAI	2	>256	>32	>32	2	1
14	5099	Hospital G	Type III	HCAI	3	>256	>32	>32	2	
15	5029	Hospital I	Type III	HCAI	0.094	>256	>32	>32	2	
16	5065	Hospital F	Type III	HCAI NH	1	>256	>32	>32	2	
17	5151	Hospital F	Type III	HCAI						
18	5081	Hospital D	Type III	HCAI	>256	>256	>32	>32	1	
19	5061	Hospital F	Type III	HCAI	6	>256	>32	>32	2	
20	5146	Hospital F	Type III	HCAI	4	>256	>32	>32	2	
21	5147	Hospital F	Type III	HCAI	3	>256	>32	>32	2	
22	5161	Hospital A	Type III	HCAI	3	>256	>32	>32	2	
23	5082	Hospital A	Type III	HCAI	2	>256	>32	>32	2	
24	5155	Hospital F	Type III	HCAI	4	>256	>32	>32	2	
25	5156	Hospital F	Type III	HCAI	4	>256	24	>32	2	
26	203	Hospital G	Type III	Unk	1	>256	>32	>32	2	
27	191	Hospital L	Type III	Unk	>256	>256	>32	>32	1	
28	5063	Hospital F	Type III	HCAI	3	>256	>32	>32	2	4
29	5153	Hospital F	Type III	HCAI	3	>256	>32	>32	2	
30	5149	Hospital F	Type III	HCAI	4	>256	>32	>32	2	

Fig. 2. rep-PCR dendrogram including electropherograms of all isolates of ribotype 027 from 2006 (marked with asterisks) and 2009. rep-PCR subtypes with >90 % similarity are identified (by the dashed boxes). Major subtypes (1 and 2) were assigned where three or more isolates clustered together; minor subtypes (3 and 4) were assigned where pairs of isolates were similar. Isolates are identified by hospital source, toxinotype and origin of infection (HCAI, health-care-associated infection; HCAI NH, health-care-associated infection from a nursing home; Unk, unknown origin). Antimicrobial-susceptibility profiles were assigned on the basis of resistance (shaded) to clindamycin (CM), erythromycin (EM), moxifloxacin (MX) and levofloxacin (LE).

number of ribotype 078 isolates or were not supported directly by alternative typing methods. Bakker *et al.* (2010) determined that out of three ribotype 078 isolates from Ireland, two were identical and one was genetically unrelated based on a single locus variation (summed tandem repeat differences >10) after MLVA analysis. Two of the Irish isolates in this study were isolated in 2008, when an outbreak of ribotype 078 was reported (Burns *et al.*, 2010a), and therefore may be more likely to be clonal. In addition, all three Irish isolates reported by Bakker *et al.* (2010) were found to be tetracycline susceptible and, as shown here, isolates with similar antimicrobial-susceptibility profiles are more likely to be related by rep-PCR.

The diversity identified in ribotype 078 rep-PCR subtypes could be explained by reports that ribotype 078 is more



Key	Sample ID	Source	Toxinotype	Origin	CM	EM	MX	LE	Profile	rep-PCR subtype
1	5135	Hospital A	Type V	HCAI	3	>256	>32	>32	2	
2	117	Hospital G	Type V	Unk	4	>256	0.25	1	6	
3	LM23 *	Hospital F	Type V	Unk	3	>256	0.5	4	6	4
4	SV188 *	Hospital L	Type V	Unk	1	>256	0.5	3	6	
5	5012	Hospital K	Type V	Unk	3	>256	0.75	2	6	
6	5176	Hospital E	Type V	HCAI	6	1	1	2	9	2
7	5107	Hospital E	Type V	HCAI	2	>256	1	2	6	
8	5054	Hospital M	Type V	C	3	1	1.5	2	9	
9	5101	Hospital A	Type V	HCAI	6	1	1	3	9	
10	5217	Hospital B	Type V	HCAI	>256	>256	1	3	5	
11	5031	Hospital D	Type V	HCAI	1.5	0.75	1	2	9	1
12	5203	Hospital G	Type V	HCAI NH	16	1.5	1	3	7	
13	SV149 *	Hospital L	Type V	Unk	6	1	0.5	2	9	
14	5100	Hospital A	Type V	HCAI	2	1	>32	>32	4	
15	5030	Hospital L	Type V	HCAI	3	0.5	1	>32	8	
16	5015	Hospital A	Type V	HCAI	2	0.75	0.75	2	9	3
17	5170	Hospital L	Type V	C	4	1.5	1	3	9	
18	158	Hospital G	Type V	Unk	3	0.38	>32	>32	4	
19	147	Hospital G	Type V	Unk	4	0.38	1.5	>32	8	5
20	144	Hospital G	Type V	Unk	6	0.38	0.38	4	9	
21	5172	Hospital A	Type V	HCAI	>256	1.5	>32	>32	3	
22	5159	Hospital A	Type V	HCAI	>256	0.75	>32	>32	3	
23	122	Hospital G	Type V	Unk	4	>256	0.5	4	6	

Fig. 3. rep-PCR dendrogram including electropherograms of all isolates of ribotype 078 from 2006 (marked with asterisks) and 2009. rep-PCR subtypes with >90 % similarity are identified (by the dashed boxes). Major (1 and 2) and minor subtypes (3, 4 and 5) were assigned as detailed in Fig. 2. Isolates are identified by hospital source, toxinotype and origin of infection (HCAI, health-care-associated infection; HCAI NH, health-care-associated infection from a nursing home; C, community-acquired; Unk, unknown origin). Antimicrobial-susceptibility profiles were assigned on the basis of resistance (shaded) to clindamycin (CM), erythromycin (EM), moxifloxacin (MX) and levofloxacin (LE).

commonly associated with community onset of disease and that this ribotype is more commonly found outside the health-care environment (Notermans *et al.*, 2008). Transmission from environment-to-person may mean that ribotype 078 isolates have not yet fully adapted to the human niche for clonal expansion, although reports of CDI due to ribotype 078 are becoming more frequent (Burns *et al.*, 2010a; Goorhuis *et al.*, 2008a; Rupnik *et al.*, 2008). It could therefore be suggested that ribotype 027 infections are more likely than those of ribotype 078 to be transmitted by person-to-person routes, ensuring a clonal genetic identity within the population of the former.

We postulate that the degree of rep-PCR type variability within PCR ribotypes means that it is not yet possible to directly apply the results of rep-PCR to ribotyping in every case without conducting the two typing methods simultaneously. However, by increasing the numbers of isolates analysed by both methods and uploading the profiles into a central online DiversiLab library, future users of this technique may be able to reliably define subtypes and ribotypes by comparing their profiles to a comprehensive library.

Correlation with antimicrobial-susceptibility profiles

Analysis of the antimicrobial-susceptibility profiles for ribotype 027 identified two different profiles. Isolates of a similar susceptibility profile generally clustered together and isolates of subtype 1 were significantly more likely to be clindamycin sensitive ($P<0.05$) than those in subtype 2, suggesting a strong correlation between subtypes and clindamycin resistance. Of the four isolates from 2009 that were resistant to clindamycin, two were cultured from patients for whom the origin of infection was a nursing home.

Ribotype 078 isolates were categorized into eight very diverse susceptibility profiles that correlated with subtyping in only two instances (subtypes 3 and 4). This diversity within ribotype 078 subtypes suggests that isolates may have acquired novel mechanisms of resistance, possibly due to selection pressures that are encountered in a variety of environmental reservoirs and not normally seen in human reservoirs. Of particular note is the presence of clindamycin resistance in the absence of erythromycin resistance in two isolates of ribotype 078. This unusual phenotype has been reported in *Streptococcus pneumoniae* and in *Enterococcus faecium* associated with the *linB* gene, although it has yet to be confirmed in *C. difficile* (Bozdogan *et al.*, 1999; Montagnani *et al.*, 2007).

Resistance to clindamycin is most commonly associated with the insertion of one or more genes such as *ermB* located on transposons into the *C. difficile* genome, which may have altered the rep-PCR fingerprint profiles of these isolates. Correlation between molecular typing and clindamycin resistance has also been seen in ribotype 027 isolates by MLVA (Drudy *et al.*, 2008) and ribotype 001 by randomly amplified polymorphic DNA (Fawley *et al.*, 2003) with distinct resistant and susceptible clones identified.

Comparison within a hospital

Comparisons between ribotype 027 subtypes within hospital F confirmed the genetic and antimicrobial-susceptibility profile similarity (Fig. 4a). Isolates clustered into two subtypes on the basis of a 90 % similarity threshold, with two outlying isolates, one obtained in 2009 and another from 2006, unrelated to the clusters.

Comparisons between ribotype 078 subtypes within hospital A confirmed the genetic and antimicrobial-susceptibility profile diversity (Fig. 4b). The closest

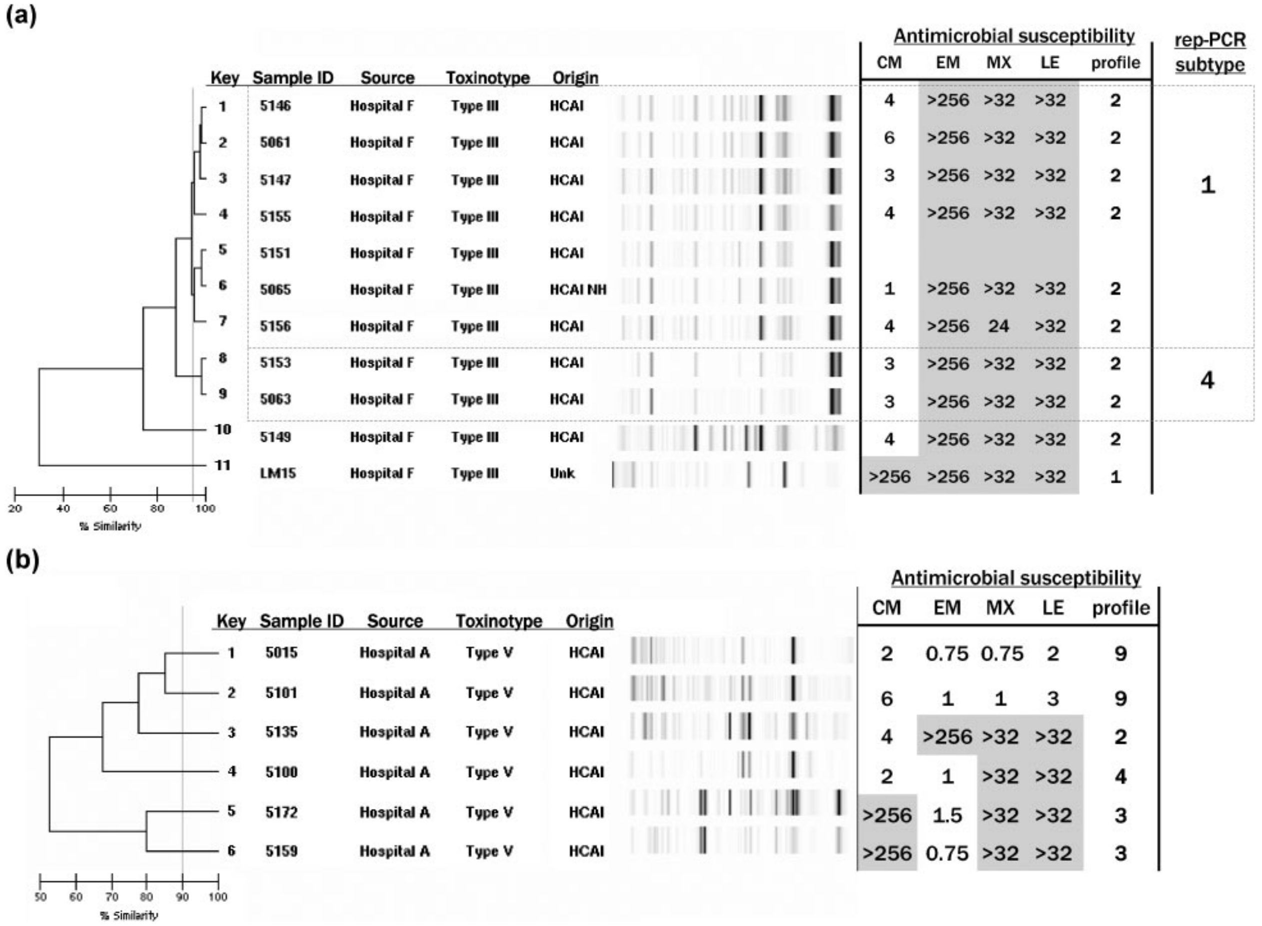


Fig. 4. rep-PCR dendrograms of isolates of ribotype 027 from hospital F (a) and isolates of ribotype 078 from hospital A (b). rep-PCR subtypes with >90 % similarity are identified (by the dashed boxes). Major (1) and minor (4) subtypes were assigned as detailed in Fig. 2. Isolates are identified by hospital source, toxinotype and origin of infection (HCAI, health-care-associated infection; HCAI NH, health-care-associated infection from a nursing home; Unk, unknown origin). Antimicrobial-susceptibility profiles were assigned on the basis of resistance (shaded) to clindamycin (CM), erythromycin (EM), moxifloxacin (MX) and levofloxacin (LE).

similarity between isolates was 85 %. Two susceptibility profiles were unique to this hospital, characterized by the presence of both fluoroquinolone and $\mathrm{MLS_B}$ resistance, which is unusual in ribotype 078 isolates.

Comparison between ribotyped isolates from 2006 and 2009

Members of the major subtype of ribotype 027 isolates analysed from 2006 clustered closely together (subtype 2; Fig. 2), but were unrelated to the predominant subtype circulating in 2009 (all 027 isolates from 2006 marked with an asterisk in Fig. 2). Clindamycin resistance was significantly more common in ribotype 027 isolates from 2006 than in the prevalent subtype in 2009 ($P<0.01$), which were predominantly clindamycin susceptible. A shift in the predominant ribotype 027 subtype has occurred, characterized by the loss of clindamycin resistance, and the new susceptible subtype has spread nationwide. Restriction and pre-authorization for use of clindamycin during nosocomial outbreaks of CDI has proven to be an effective measure to promptly limit outbreaks (Climo *et al.*, 1998; Pear *et al.*, 1994). Implementation of these policies was unlikely, however, to have selected for the recent dominance of the clindamycin-sensitive subtype as clindamycin usage in Ireland is relatively low [0.46 defined daily dose per 100 bed days in 2006 and 0.49 defined daily dose per 100 bed days in 2008 (HPSC–HSE, 2008)]. The most likely cause therefore is the introduction of a clindamycin-resistant strain in 2006 that was initially successful amongst other Irish strains, but was gradually replaced by a sensitive strain without the selection pressure of antibiotics. The prescribing policy in nursing homes, however, may be less restrictive, favouring the emergence of clindamycin-resistant isolates from recent infections that originated in these facilities.

Comparisons between ribotype 078 isolates from 2006 (marked with an asterisk in Fig. 3) and 2009 identified close relationships between isolates (subtype 1; Fig. 3) suggesting the persistence of certain subtypes with very little genetic change. Many new subtypes have, however, been introduced since 2006 that are radically diverse both genetically and in their antimicrobial-susceptibility profiles.

Conclusion

We investigated the heterogeneity of clinical *C. difficile* isolates of ribotypes 027 and 078 by a semi-automated DiversiLab rep-PCR method to investigate the epidemiology of these clinically important strains within health-care facilities. Ribotype 027 isolates were significantly more likely to be closely related to each other than ribotype 078 isolates. Ribotype 078 isolates also exhibited a wider range of antimicrobial-susceptibility profiles, suggesting that ribotype 078 infections may have originated from a variety of non-health-care and environmental sources. A shift in the predominant circulating subtype of ribotype 027 was identified between 2006 and 2009 from a clindamycin-resistant subtype to clindamycin-susceptible subtype similar to PFGE type NAP-1 that is now widespread within Ireland. Preventing the emergence of novel antimicrobial-resistant subtypes, mainly from community and environmental sources, should also be a priority and implemented by employing strict antimicrobial-usage policies in general practice and food production methods.

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