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The *Clostridium difficile* PCR ribotype 027 lineage: a pathogen on the move

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Abstract

Clostridium difficile is a Gram-positive, spore-forming, human and animal pathogen that is the major cause of antibiotic-associated diarrhoea worldwide. The past decade has seen the rapid emergence of the hypervirulent PCR ribotype (RT) 027 complex, which has been associated with increases in the incidence and severity of disease and mortality. In this review, we describe the potential virulence factors that have been reported in strains from the RT 027 complex. We review the emergence, population structure, dissemination and evolution of this lineage.

Keywords: *Clostridium difficile*, dissemination, evolution, genome analysis, pathogen, PCR RT 027
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Clostridium difficile: the Pathogen and Disease

C. difficile is a Gram-positive spore-forming anaerobic bacillus that is the leading cause of healthcare-associated diarrhoea in developed countries [1–4]. Infection is caused by the ingestion of spores from infected patients or the environment. After antibiotic treatment, the protective gut microbiota is disrupted, allowing *C. difficile* to flourish and heavily colonize the gastrointestinal tract; this can lead to asymptomatic carriage or infection. *C. difficile* infection (CDI) ranges in severity from mild and self-limiting, watery or bloody diarrhoea to severe disease, with ileus, toxic megacolon, and pseudomembranous colitis, that can be fatal [5]. Typical features of CDI include watery diarrhoea, abdominal pain and cramps, lower-quadrant tenderness, fever, leukocytosis, and hypoalbuminaemia [6]. Re-occurrences are common, with rates of up to 20% after the first episode of CDI and 50% after the second episode, even after treatment [7].

Typing, Epidemiology, and Phylogenomics—the Emergence of PCR Ribotype (RT) 027

A variety of molecular typing approaches have been developed to investigate CDI, including amplified fragment length polymorphism, pulsed-field gel electrophoresis, restriction endonuclease analysis, and PCR ribotyping [8]. The preferred method in North America is pulsed-field gel electrophoresis, whereas PCR ribotyping is most widely accepted in Europe [9].

In addition, multilocus sequence typing (MLST) has become a standard technique for CDI epidemiological studies [10]. MLST studies based on diverse collections of *C. difficile* strains have suggested that there are five distinct lineages, which can generally be divided according to the most important PCR ribotype (RT): 027, 017, 023, 078, and a large group including the other PCR RTs [11,12] (Fig. 1). The RT 078 complex is an outlying group that is highly divergent and distantly related to the other lineages (Fig. 1) [13]. These findings have also been

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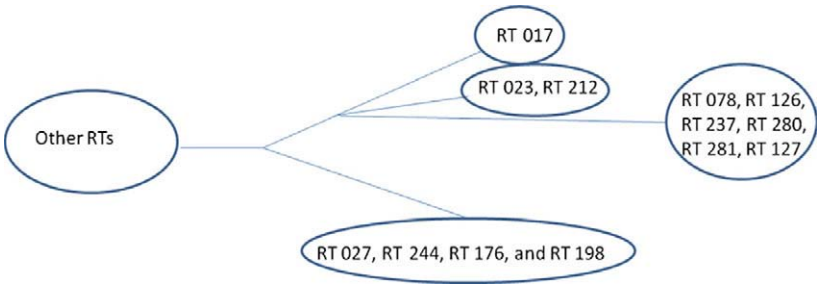

FIG. 1. Diagram showing the five *Clostridium difficile* lineages and their relationship with common ribotypes (RTs). Each lineage is formed by a specific RT complex, apart from clade/lineage 1, which is composed of a pool of different RTs.

confirmed with other genome-based techniques, including a comparative microarray-based study [14] and a whole genome sequencing (WGS) study based on single-nucleotide polymorphisms (SNPs) [15].

The earliest recorded RT 027 isolate was strain CD196, which is a non-epidemic strain isolated from a single patient with CDI in a Parisian hospital in 1985 [16]. The next recorded RT 027 isolate was a non-epidemic strain designated BI-1, which was from a patient with CDI in a Minneapolis hospital in 1988 [17] (Fig. 2). In 2003–2004, Canada experienced outbreaks involving >30 hospitals [18], with notably severe CDIs. Between August 2004 and July 2007, >20 000 nosocomial cases were reported to the Quebec provincial surveillance program (R. Gilca, INSPQ, personal communication). Retrospective analysis showed that a similar strain had also been isolated in several regions of the USA between 2000 and 2002. From 2003 to 2006, two CDI outbreaks at Stoke Mandeville Hospital, UK, caused a total of 127 deaths (Fig. 2). The annual mortality attributable to CDI rose by 72% in the UK to 6500 cases in 2006 [19]. By 2005, RT 027 had been detected in an outbreak in The Netherlands [20], and by 2007 it had been isolated in 16 European countries [21,22]. The application of genotyping methods confirmed that RT 027 strains are genetically highly uniform.

In 2008, RT 027 had reached Korea, Hong Kong, and Singapore, and by 2009 it had been reported in Australia and Costa Rica [23–27]. This was followed by RT 027 being isolated in New Zealand in 2010 [28]; in Chile in 2011 [29], in Panama in 2013 [30], and in China in 2014 [31] (Fig. 2).

RT 027 is associated with an intensifying worldwide epidemic of nosocomial disease, with high mortality and recurrence [19,32–34]. Since 2005, US studies have continued to report increased CDI rates [35], and RT 027 has persisted as the dominant clone [36]. In 2007, CDI surveillance in Canada showed decreases in RT 027 infections and CDI deaths in Quebec. However, there were increases in the incidence rates of RT 027 infection in British Columbia, Ontario, and the Atlantic provinces, accompanied by an increase in CDI-related mortality (Annual Meeting of the Society for Healthcare Epidemiology of America, 2009, Abstract 350). Nevertheless, between 2008 and 2011, England saw a 61% reduction in the number of reports of CDI, which was reflected in a decline in the number of cases of CDI caused by RT 027. The large reduction in the number of cases of CDI caused by RT 027 in England also parallels the decrease in CDI-related mortality (Office for National Statistics 2012). Alongside this decline, an increase in the prevalence of other RTs has been observed, most notably RT 015, RT 002, RT 078, RT 005, RT 023, and RT 106 (*Clostridium difficile* Ribotyping Network for England www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317133396963). In The Netherlands, a 3% decrease in the prevalence of RT 027 has also been detected [37]. All of these data demonstrate that RT 027 spreads rapidly, and, although it is in decline in some geographical areas, its transcontinental dissemination continues [38].

C. difficile PCR RT 027: Virulence Factors

C. difficile has traditionally proven difficult to genetically manipulate, especially RT 027 strains. Despite this, some virulence determinants of *C. difficile* have been studied in detail.

C. difficile pathogenesis is thought to be largely based on the production of the UDP-glycosylating enterotoxins, encoded by *tcdA* and *tcdB* situated within a pathogenicity locus [39]. The 19.6-kb pathogenicity locus of *C. difficile* also contains an accessory gene, *tcdE* [40], along with regulatory components. These toxins cause extensive tissue damage, and are considered to be major virulence factors in CDI [41–45]. It has been reported that some RT 027 strains can produce more toxin *in vitro* [46], and this has been attributed to deletions in the negative regulator gene *tcdC*. Further studies have revealed that the 18-bp in-frame deletion has no effect on toxin production [47]. It has also been hypothesized that RT 027 isolates have differences in the sequence of TcdB in comparison

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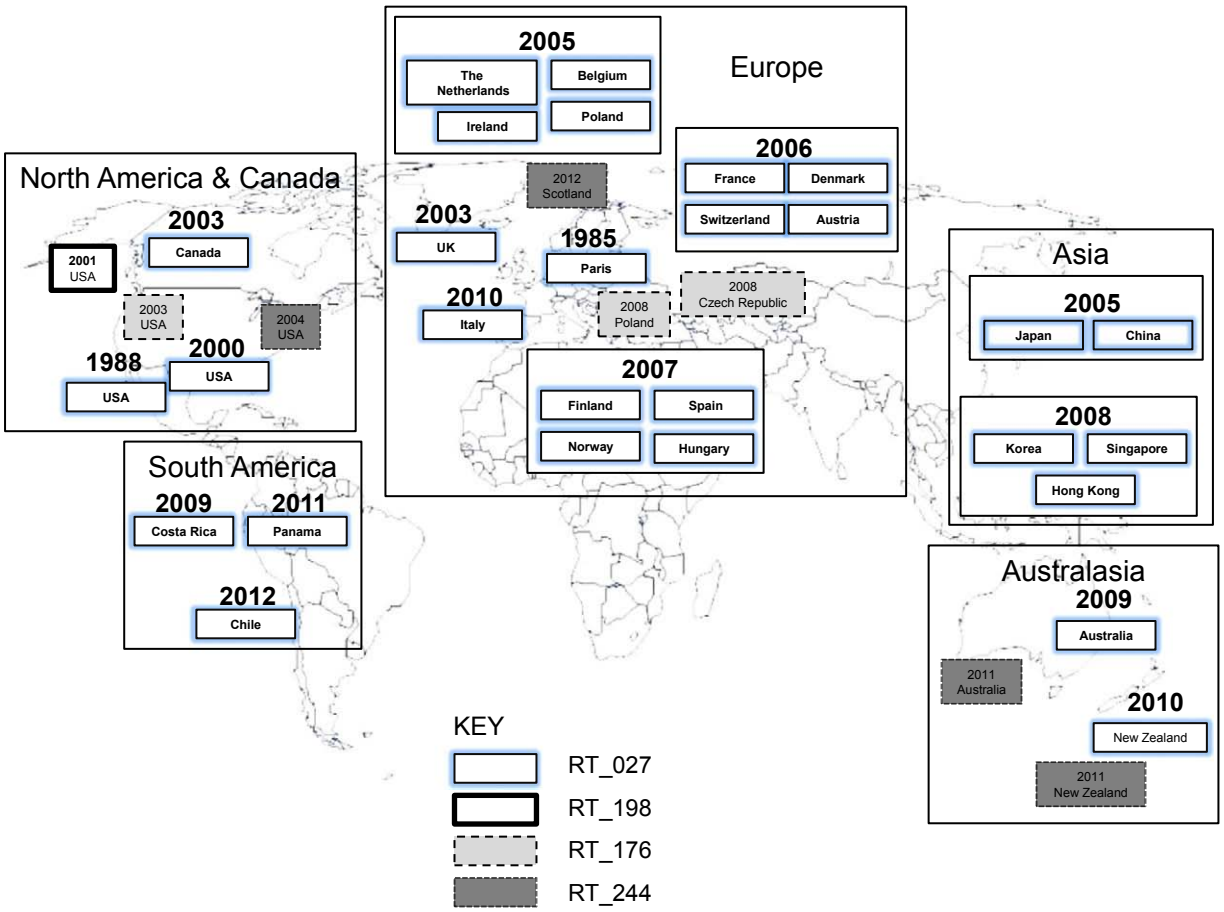

FIG. 2. Map of isolation dates and locations of *Clostridium difficile* ribotype (RT) 027 globally.

with RT 012 and RT 003 [48]. This variation could explain the differences in activity found by Stabler *et al.* [49] in 2009, whereby TcdB from RT 027 isolates was more potent on cultured cells than TcdB from the historical RT 012 strain. Variations between TcdBs from previously predominant RTs and RT 027 strains could be a major contributory factor in the increased virulence of the RT 027 strains [50].

In addition to TcdA and TcdB, RT 027 strains have also been found to produce a binary toxin [51,52], encoded by two genes, *cdtA* and *cdtB* [53], and this has been linked with increased severity of disease [54,55].

C. difficile also produces spores that are highly resistance to adverse conditions. Spores play a key role in the transmission of CDI, as they are resistant to a wide range of chemical and physical stressors, and can persist in a contaminated environment for several weeks to months [56,57]. Spore germination in the gut enables *C. difficile* vegetative cell growth, metabolism, and production of toxins A and B, which mediate intestinal epithelium injury, inflammation, and colitis [58,59]. In a healthy gut, indigenous microorganisms form a protective barrier against *C. difficile* colonization, but this protective function can be weakened by antibiotic therapy [60]. It has been reported that the proportion of RT 027 spores that germinate is much higher than the proportion of strain 630 spores that germinate [61]. In addition, RT 027 isolates showed higher germination efficiencies in the presence of 0.1% (w/v) sodium taurocholate than RT 106 and RT 078 [62]. More recent studies have found that *C. difficile* spore germination in response to sodium taurocholate varies significantly, even within the RT 027 group [63]. A link between high germination rate and CDI recurrence has previously been reported [64].

C. difficile also produces a surface layer [65], composed of the high molecular weight surface layer protein (SLP) and the low molecular weight SLP [66,67]. It has been demonstrated that SlpA is conserved in RT 027 strains. Sequencing data of different RTs (001, 012, 014, 017, 027, and 078) revealed that SlpA is conserved among strains belonging to the same RT. In addition, it has been reported that the antigenic role of the low molecular weight SLP may involve immune evasion [68].

C. difficile also has peritrichous flagella, which are O-linked-glycosylated. Additionally, it has been found that

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flagellum glycosylation plays a role in flagellar assembly and motility [69].

C. difficile can be resistant to many antibiotics, and, through genomics approaches, resistance can be traced over time. The epidemic RT 027 strains that caused outbreaks worldwide were found to be highly resistant to fluoroquinolones as compared with the historic strain 630, owing to point mutations in the DNA gyrase genes [70]. It has been suggested that, after antibiotic treatment, recolonization of the human gut by commensal bacteria is prevented by the production of *p*-cresol by *C. difficile* [71]. The production of *p*-cresol from tyrosine appears to be specific to *C. difficile* and lactobacillus strains [72].

To date, many virulence factors have been proposed to contribute to the difference in virulence between historical RTs and RT 027, such as toxin production and sporulation [50,61]. The relative importance of these factors is still under investigation.

C. difficile PCR RT 027: Comparison with the historical PCR RT 012 Strain 630

A recent study compared the first fully sequenced strain 630 (RT 012), a multidrug-resistant isolate from a patient with pseudomembranous colitis at a hospital in Zurich in 1982 [73], with both a historical (CD196) and modern (R20291) RT 027 strain (Table 1). The study found 234 genes to be unique to both RT 027 strains. Recent genome re-annotation in our laboratory has revealed that RT 027 strains have a lower number of unique genes than in the Stabler *et al.* [49] study (Table 2).

In addition, it has been shown that, in strain 630, flagellum-associated genes are found in two loci, F1 (CD0226–CD0240) and F3 (CD0245–CD0271), which are separated by an inter-flagellum locus F2 (CD0241–CD0244) [49]. Microarray analysis of this region previously showed a loss of, or high divergence in, F1 and F2 in all RT 027 isolates tested [49]. The different genes present in the F2 region of strain 630 and RT 027 may be important in the glycosylation of the flagella. Studies in other enteric bacteria, such as *Campylobacter jejuni*, have shown that both flagellin and post-translational modifications of flagellin are required for autoagglutination, which is linked to virulence [74].

TABLE 1. Strains described in this review

Strains	PCR ribotype	Date isolated	City, state/province
630	012	1982	Zurich, Switzerland
CD196	027	1985	Paris, France
R20291	027	2006	Aylesbury, UK

A large proportion of the RT 027-specific genes are regulatory genes, with eight two-component regulators and 15 other transcriptional regulators having been found [49].

C. difficile PCR RT 027: Emergence and Microevolution

He *et al.* have confirmed that RT 027 isolates are members of a single lineage (Fig. 1) [15]. A Bayesian skyline plot analysis [15] showed that RT 027 isolates underwent a population expansion around the start of the century, which coincided with the reporting of major hospital outbreaks caused by RT 027 strains. The study also revealed that the *C. difficile* genome harbours a significant number of mobile elements, many of them coding for a variety of antibiotic resistance genes, suggesting a significant role for horizontal gene transfer in resistance acquisition [15]. Study of the distribution of SNPs along the conserved *C. difficile* genome revealed an exchange of very large chromosomal regions, within both the deep-branching phylogeny and the RT 027 hypervirulent group [15]. He *et al.* also showed that, between deeply diverging lineages, there is evidence for strong purifying selection [15].

A more recent study showed that the core genome of RT 027 isolates shows a relatively low level of genetic diversity, consistent with its recent emergence, with very few SNPs defining the major branches in the phylogeny [38]. The study also showed the presence of two genetically distinct lineages, which have independently acquired an identical mutation (encoding a p.THr82Ile alteration) in the DNA gyrase subunit A gene *gyrA* by either mutation or recombination, leading to high-level fluoroquinolone resistance (FQR) [75]. This study designated these recently emerged lineages as FQR1 and FQR2 [38].

The FQR1 lineage contains epidemic isolates associated with healthcare-related outbreaks in the USA [76] and isolates associated with sporadic infections in South Korea [77] and Switzerland between 2007 and 2010 [78]. Bayesian phylogeographical analysis indicated a 99% probability that the FQR1 lineage originated in the USA [38]. The earliest isolate in the FQR1 lineage was obtained in Pittsburgh, Pennsylvania in 2001, representing one of the earliest reports of an increase in CDI frequency caused by FQR RT 027 [79,80]. FQR1 seems to represent an epidemic lineage that originally emerged in the north-eastern USA and that was subsequently transmitted to South Korea and Switzerland [38]. The FQR2 lineage represents the majority of epidemic isolates that show a widespread geographical distribution. The most remarkable feature in the FQR2 phylogeny is a star-like topology in the early part of the lineage, which is generally consistent with

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TABLE 2. Unique genes of ribotype 027 strains

R20291	CD196	Function
CDR20291_0223	CD196_0236	DTDP-4-dehydrorhamnose reductase
CDR20291_0224	CD196_0237	Glucose-1-phosphate thymidylyltransferase
CDR20291_0242	CD196_0255	Glycosyl transferase family 2
CDR20291_0243	CD196_0256	Glycosyl transferase, group 2 family
CDR20291_0310	CD196_0323	TetR (putative transcriptional regulator)
CDR20291_0439	CD196_0453	Sensor histidine kinase
CDR20291_0861	CD196_0881	Sensor protein
CDR20291_0862	CD196_0882	Predicted ABC transporter, ATPase component
CDR20291_1278	CD196_1301	Transcriptional regulator, araC family protein
CDR20291_1415	CD196_1438	Prophage lambdaBa04, site-specific recombinase, phage integrase family
CDR20291_1463	CD196_1488	*N*-acetylmuramoyl-L-alanine amidase (cell wall hydrolase)
CDR20291_1464	CD196_1489	Cell surface protein (putative penicillin-binding protein)
CDR20291_1847	CD196_1802	Possible transcriptional regulator
CDR20291_2278	CD196_2230	Putative peptidoglycan-binding/hydrolysing protein
CDR20291_2760	CD196_2713	Two-component system, sensor histidine kinase
CDR20291_2964	CD196_2917	Transcriptional regulator, padR-like family
CDR20291_2981	CD196_2934	Spermidine/putrescine ABC transporter ATP-binding subunit
CDR20291_2994	CD196_2947	CRISPR-associated helicase Cas3
CDR20291_2995	CD196_2948	CRISPR-associated autoregulator, devR family
CDR20291_2997	CD196_2950	CRISPR-associated protein Cas6
CDR20291_2998	CD196_2951	CRISPR-associated protein Cas5 family
CDR20291_3050	CD196_3004	ABC transporter, permease associated with salivaricin lantibiotic
CDR20291_3051	CD196_3005	Possible sensor histidine kinase
CDR20291_3123	CD196_3077	ABC transporter, ATP-binding protein
CDR20291_3124	CD196_3078	Sensor protein
CDR20291_3184	CD196_3138	Putative membrane protein
CDR20291_3185	CD196_3139	ABC transporter, ATP-binding protein
CDR20291_3186	CD196_3140	Membrane protein, putative
CDR20291_3187	CD196_3141	Accessory gene regulator
CDR20291_3188	CD196_3142	Sensor histidine kinase virS
CDR20291_3276	CD196_3230	Transposase, mutator type
CDR20291_3277	CD196_3231	Putative exported protein
CDR20291_3278	CD196_3232	Putative exported protein
CDR20291_3281	CD196_3235	Transposon Tn21 resolvase
CDR20291_3284	CD196_3238	Phage portal protein
CDR20291_3289	CD196_3243	Leucine-rich repeat protein
CDR20291_3461	CD196_3415	Chloramphenicol *O*-acetyltransferase
CDR20291_3469	CD196_3423	Transcriptional regulator, tetR family
CDR20291_3470	CD196_3424	ABC transporter, ATP-binding protein
CDR20291_3472	CD196_3426	Putative ABC transporter, permease protein

rapid population expansion from a common progenitor. Bayesian analysis suggested that the FQR2 lineage also originated in North America [38].

The Bayesian phylogeny contains multiple highly supported sublineages of FQR2 associated with distinct geographical locations, suggesting rapid transcontinental dissemination from North America to continental Europe, the UK, and Australia. The study suggested a single introduction of FQR2 into Australia and at least four separate introductions into continental Europe, including two trans-Atlantic transmission events and two transmissions from the UK, giving rise to actual isolates in Austria and Poland [81] (Fig. 2). Similarly, the descendants of the FQR2 lineage were introduced into the UK on at least four occasions, including three trans-Atlantic transmissions from North America and one from continental Europe, leading to a series of highly publicized outbreaks in UK hospitals from 2004 to 2006 [82]. Despite multiple introductions, the majority of current UK isolates appear to be descendants of one sublineage, which is a 2002 Birmingham isolate. The dominant sublineage was also responsible for the Stoke Mandeville outbreak [82].

It is believed that the acquisition of FQR in two distinct lineages is associated with the emergence of RT 027 strains. In addition, horizontal gene transfer is a pivotal mechanism driving *C. difficile* evolution [15,73]. He *et al.* have shown that the separate acquisitions of FQR and a conjugative transposon in two distinct lineages of RT 027 are the key genetic changes linked to its rapid emergence during the early 2000s [33,76]. This study also demonstrated that the acquisition of resistance to commonly used antibiotics is a major feature of the continued evolution and persistence of RT 027 in healthcare settings.

C. difficile PCR RT 027 in Disguise

A group of 19 strains designated as BI (all presumed to be RT 027) were isolated and characterized by Gerding *et al.* (D. N. Gerding, personal communication). A recent study revealed that some of the BI strains that were presumed to be RT 027 are, in fact, other RTs: BI-6 (RT 176), BI-11 (RT 198), and BI-14 (RT 244). The banding patterns of these RTs are very similar to that of RT 027 (plus or minus one band) (Fig. 3). A close association between RT 027 and RT 176 has also been found in strains isolated in the Czech Republic and Poland [81]. The reported RT 244 isolate obtained in New Zealand also

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shares several features with RT 027 [28]. Additionally, a study that performed comparative genome microarray analyses found these three RTs to be in the same clade as RT 027 [14], and an MLST study found that all three RTs cluster together with RT 027 (Fig. 3) [13]. These results indicate that the different typing methodologies do not correspond as well as previously thought, and suggest that they these RTs have co-evolved and possibly evolved from each other (Fig. 1). Moreover, these findings highlight the importance of monitoring high similarity in RT patterns between RT 027 and other RTs, as strains belonging to these other RTs may be as problematic as RT 027 [83]. In addition, this MLST study showed that RT 126, RT 237, RT 280, RT 281 and RT 127 cluster together with RT 078, and that RT 212 clusters together with RT 023 (Fig. 1). These findings suggest that RTs in disguise are also present in other lineages, and need to be monitored closely.

To date, the data generated with MLST, microarrays and traditional typing methods have shown inadequate resolution for definitive epidemiological conclusions to be drawn. There is a pressing need to understand the micro-evolution of *C. difficile*. The most definitive approach is WGS, and the advent of next-generation sequencing has greatly facilitated the re-annotation of bacterial genomes. The application of WGS comparisons for epidemiological and phylogenetic studies has already been published for several bacterial pathogens [84–86].

We do not yet fully understand the species population structure and evolutionary history of *C. difficile*. WGS promises to provide further insights, and it is envisaged that this approach, alongside strong geographical, patient and epidemiological data, will enable the monitoring of persistence, transmission and evolution within hospitals by spatial-temporal mapping of the genotypes down to the level of hospital floor plans and patient movement within and outside hospitals.

It is anticipated that more WGS data for multiple strains from multiple sources will allow for a detailed description of the population structure of *C. difficile*. Such a global reference map will facilitate the contextualization of additional sequenced strains, and the identification of virulence determinants.

Conclusion and Future Perspectives

C. difficile is the major cause of healthcare-associated diarrhoea worldwide, in part because of the rapid emergence of RT 027, which, in the last decade, has been associated with outbreaks and increased mortality [87]. Although RT 027 is still persistent in North America and Canada, it seems that there has been a decrease in the frequency of CDI caused by RT 027 in Europe, most notably in the UK (Public Health England report, 2013). Recently, RT 027 has also been isolated in South America [27,29] and New Zealand [28]. It is clear that RT 027 has evolved rapidly, and its transcontinental spread is ongoing [38]. Horizontal gene transfer and genetic recombination have played an important role in this sequence diversification [15].

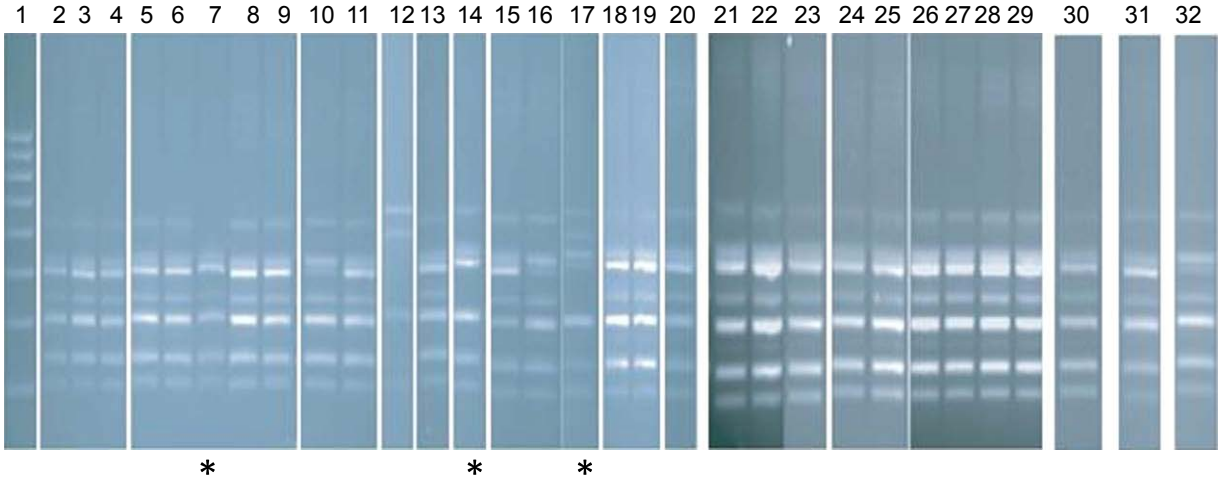

FIG. 3. PCR ribotyping profiles obtained with *Clostridium difficile*. Ribotype (RT) 176, RT 198 and RT 244 are marked with asterisks. Lane 1: 100-bp ladder. Lane 2: BI-1. Lane 3: BI-2. Lane 4: BI-3. Lane 5: BI-4. Lane 6: BI-5. Lane 7: BI-6 (RT 176). Lane 8: BI-6p. Lane 9: BI-6p2. Lane 10: BI-7. Lane 11: BI-8. Lane 12: BI-9. Lane 13: BI-10. Lane 14: BI-11 (RT 198). Lane 15: BI-12. Lane 16: BI-13. Lane 17: BI-14 (RT 244). Lane 18: BI-15. Lane 19: BI-16. Lane 20: BI-17. Lane 21: 027-01. Lane 22: 027-02. Lane 23: 027-03. Lane 24: 027-04. Lane 25: 027-05. Lane 26: 027-06. Lane 27: 027-07. Lane 28: 027-08. Lane 29: 027-09. Lane 30: 027-10. Lane 31: R20291. Lane 32: CD196.

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The RT 027 genome is highly dynamic [38]. The separate acquisitions of FQR and a conjugative transposon in two distinct lineages of RT 027 are the key genetic changes linked to its rapid emergence during early 2000 [38]. Given the current mortality associated with CDI, despite antibiotic therapy, it may be time to take a different approach. Targeting of specific pathways that disable *C. difficile* could be an adjunct to or even a replacement for antibiotic therapy.

In this review, we have presented a timeline that reflects the isolation dates and locations of RT 027. A uniform typing methodology should be implemented in all countries worldwide, to monitor the spread of strains that are as or more hypervirulent than RT 027. In particular, there is a need to understand that there are RTs very similar to the hypervirulent RT 027 (RT 176, RT 244, and RT 198), RT 078 (RT 126, RT 237, RT 280, RT 281, and RT 127), and RT 023 (RT 212).

In the past, typing methods such as PCR ribotyping have been valuable for monitoring outbreaks of *C. difficile*. However, with the increase in the number and variety of PCR RTs over time, the discriminatory power of such methods has diminished, affecting their ability to characterize potential outbreak strains as the same bacterial clone. Additionally, in a query outbreak situation, identifying genetically very similar RTs, such as RT 027, RT 176, RT 198, and RT 244, suggesting that an outbreak has not occurred poses problems. It is possible that the outbreak RT 027 strain has, during an outbreak, undergone changes in the size and copy number of the intergenic spacer regions that are the diagnostic targets for PCR RT, thereby altering the RT identity and misrepresenting an outbreak situation. Sequencing of whole genomes offers optimal discriminatory power, allowing laboratories to detect transmission pathways between patients, hospital wards, hospitals, regions, countries, and continents.

Analyses of RT 027 isolates have demonstrated that the acquisition of resistance to commonly used antibiotics is a major feature of its continued evolution and persistence in healthcare environments. With the decline in RT 027 in the UK, its increased dissemination globally, and the simultaneous increase in other equally virulent RTs, there is a pressing need to understand the evolution of this lineage. The use of next-generation technologies combined with an understanding of the emergence and evolution of the hypervirulent RT 027 strain will allow us to monitor the emergence and spread of other potentially hypervirulent strains in real time. It is envisaged that this real-time genomic data coupled with strategies to control the spread and treatment of CDIs will result in improved infection control and reductions in the morbidity, mortality and costs associated with CDI.

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Transparency Declaration

No conflict of interest is declared.

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