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Clostridioides difficile infections were predominantly driven by fluoroquinolone-resistant *Clostridioides difficile* ribotypes 176 and 001 in Slovakia in 2018–2019

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ABSTRACT

Aim: To investigate the epidemiology of *Clostridioides difficile* infection (CDI) in Slovakian hospitals after the emergence of ribotype 176 (027-like) in 2016.
Methods: Between 2018 and 2019, European Centre for Disease Control and Prevention CDI surveillance protocol v2.3 was applied to 14 hospitals, with additional data collected on recent antimicrobial use and the characterization of *C. difficile* isolates.
Results: The mean hospital incidence of CDI was 4.1 cases per 10,000 patient bed-days. One hundred and five (27.6%) in-hospital deaths were reported among the 381 cases. Antimicrobial treatment within the previous 4 weeks was recorded in 90.5% (333/368) of cases. Ribotype (RT)176 was detected in 50% (n=185/370, 14 hospitals) and RT001 was detected in 34.6% (n=128/370,13/14 hospitals) of cases with RT data. Overall, 86% (n=318/370) of isolates were resistant to moxifloxacin by Thr82Ile in GyrA (99.7%). Multi-locus variable tandem repeat analysis showed clonal relatedness of predominant RTs within and between hospitals. Seven of 14 sequenced RT176 isolates and five of 13 RT001 isolates showed between zero and three allelic differences by whole-genome multi-locus sequence typing. The majority of sequenced isolates (24/27) carried the *erm*(B) gene and 16/27 also carried the *aac(6′)-aph(2′′)* gene with the corresponding antimicrobial susceptibility phenotypes. Nine RT176 strains carried the *cfr*(E)gene and one RT001 strain carried the *cfr*(C) gene, but without linezolid resistance.
Conclusions: The newly-predominant RT176 and endemic RT001 are driving the epidemiology of CDI in Slovakia. In addition to fluoroquinolones, the use of macrolide–lincosamide–streptogramin B antibiotics can represent another driving force for the spread of these epidemic lineages. In *C. difficile*, linezolid resistance should be confirmed phenotypically in strains with detected *cfr* gene(s).

1. Background

Clostridioides difficile is a Gram-positive, spore-forming anaerobe. When gut microbiota is depleted, *C. difficile* can cause gastrointestinal infection with symptoms ranging from diarrhoea to life-threatening intestinal damage [1]. Two large clostridial glycosylating toxins, toxin A (TcdA) and toxin B (TcdB), are the main virulence factors in the pathogenesis of CDI. Some strains also produce 'binary toxin', a specific ADP-ribosyltransferase [2].

Since 2006, the incidence of *C. difficile* infection (CDI) has increased due to the spread of certain epidemic strains. As such, the implementation of molecular characterization of causative strains has become one of the key components of infection and prevention control. In Europe, polymerase chain reaction (PCR) ribotyping was adopted by reference laboratories, and use of a capillary electrophoresis (CE) modification increased its discriminatory power and enabled the identification of new variants of epidemic lineages [e.g. ribotype (RT)176, the genetic variant of notorious RT027] [3].

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Ribotyping data from Slovak *C. difficile* isolates from 2011 to 2013 showed low diversity of RTs and clear predominance of RT001; *C. difficile* RT176 was not identified at that time [4–6]. Later, in 2016, a large outbreak of 75 RT176 CDI cases was reported in a tertiary care centre in the northern part of Slovakia [7]. In the same year, 36 hospitals in Slovakia participated in a national CDI surveillance and 12 hospitals also submitted *C. difficile* isolates for characterization. In addition to the persistent predominance of RT001, the emergence of RT176 was recognized in six hospitals [8].

The genetic relatedness of RT176 to epidemic RT027 and reports on multi-centre clusters in Eastern Europe [7] stressed the need for enhanced surveillance, including the characterization of causative strains. Thus, in order to obtain current data on CDI epidemiology after the emergence of RT176 in Slovakia, this CDI surveillance study was undertaken to characterize *C. difficile* isolates from all CDI cases.

2. Materials and methods

2.1. Ethics

Ethical approval and informed consent were not required for this study as it was surveillance-based, *C. difficile* isolates were obtained during routine diagnostic testing, and data were anonymized. The results of the study had no impact on the care of the participating patients.

2.2. Epidemiological surveillance

Between May 2018 and May 2019, 14 Slovakian hospitals followed European Centre for Disease Prevention and Control (ECDC) CDI surveillance protocol v 2.3 [9], with no post-discharge follow-up of death or re-admission. Additionally, data on antimicrobial consumption were acquired from the hospital records of each CDI case for the 4 weeks prior to their CDI diagnosis, independent of hospitalization.

2.2.1. CDI case definitions

Healthcare-associated CDI (HA CDI) cases were defined as patients who either had onset of symptoms ≥3 days following admission to a healthcare facility, or had onset of symptoms in the community within 4 weeks of discharge from a healthcare facility.

Community-associated CDI (CA CDI) cases were defined as patients who were not discharged from a healthcare facility in the 12 weeks preceding symptom onset. The onset of symptoms occurred either outside the healthcare facility or on the day of admission or the following day.

Recurrent CDI cases were defined as patients with diarrhoea and a positive laboratory test after completion of CDI treatment, between 2 and 8 weeks after the onset of symptoms from a previous episode of CDI.

A complicated course of CDI was defined as admission due to community-onset CDI; admission to an intensive care unit; surgery (colectomy) for toxic megacolon, perforation or refractory colitis; or death [3,9].

2.3. Statistical analysis

Differences between groups were evaluated using χ^2-test or Fisher's exact test for categorical variables and the Wilcoxon test for continuous variables. A univariable logistic regression model was used for the analyses of associations between individual variables and outcomes of interest. The *P*-values were adjusted for multiple comparisons using the Holm–Bonferroni method, and $P \leq 0.05$ was considered to indicate statistical significance. Analyses were conducted using R Version 3.5.1 [10].

2.4. Identification and confirmation of CDI

At the physician's request, unformed stool samples from patients suspected of having CDI were tested using RIDA QUICK *Clostridium difficile* glutamate dehydrogenase (GDH) and RIDA QUICK *Clostridium difficile* toxin A/B tests (R-Biopharm AG, Hessen, Germany). GDH and toxin A/B positive stool samples were cultured on selective media for *C. difficile* (Brazier agar, Oxoid, Basingstoke, UK) after alcohol shock treatment at 36.9°C under anaerobic conditions for 48 h (Whitley A35 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK).

2.5. Infection prevention and control measures at the time of the study

Any patient with diarrhoea was isolated immediately and a stool sample was taken for CDI testing. A hospital epidemiologist monitored compliance with the barrier isolation regimen, communicated daily with the attending physicians, and the network clinical pharmacologists guided treatment regimens. Metronidazole was used for the treatment of patients with laboratory-confirmed CDI.

2.6. Characterization of C. difficile

The identification of *C. difficile* isolates was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Biotyper v 3.1, Bruker Daltonics, Billerica, MA, USA). *C. difficile* isolates were characterized by CE ribotyping and detection of toxin genes (*tcdA*-toxin A, *tcdB*-toxin B, *ctdA* and *cdtB* – binary toxin) by multiplex PCR [11,12]. One isolate for the two most frequently detected RTs was selected from each hospital, where applicable, for whole-genome sequencing (WGS; Illumina, San Diego, CA, USA; Eurofins Genomics, Louisville, KY, USA).

2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for metronidazole, vancomycin and moxifloxacin was performed for all isolates by agar dilution [Wilkins-Chalgren anaerobe agar containing 0.005 g/L of haemin (Oxoid)] categorized according to the European Committee on Antimicrobial Susceptibility Testing epidemiological cut-off values for metronidazole (>2 mg/L) and vancomycin (>2 mg/L), and the Clinical and Laboratory Standards Institute breakpoint for moxifloxacin (≥8 mg/L) [13,14]. The quinolone-resistance-determining region in the *gyrA* gene was amplified and sequenced in all strains [15], and compared with *C. difficile* BR81, NZ_CP019870.1. In WGS data, acquired resistance genes were detected using ResFinder 4.1 from FASTQ data available at: www.genomicepidemiology.org [16]. Resistant genotypes were tested phenotypically using minimum inhibitory concentration (MIC) strips (Liofilchem, Roseto Degli Abruzzi, Italy): *aac*(6')-*aph*(2'') – amikacin, gentamicin; *erm*(B) – erythromycin, clindamycin; *cfr*(C) and *cfr*(E) – linezolid; and *tet*(M) – tetracycline. Breakpoints for susceptibility categorization were ≥16 mg/L for tetracycline and ≥8 mg/L for clindamycin [14]. No breakpoints are currently available for amikacin, gentamycin, erythromycin and linezolid.

2.8. Genetic relatedness analysis of predominant RTs

Multi-locus variable tandem repeat analysis (MLVA) was performed on strains of predominant RTs using seven previously published regions with short tandem repeats (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd) with a change of reverse primer for G8Cd loci, as described elsewhere [17,18]. For visualization of the genetic relatedness of isolates belonging to the same RT, a minimum spanning tree (MST) was constructed using the Manhattan coefficient

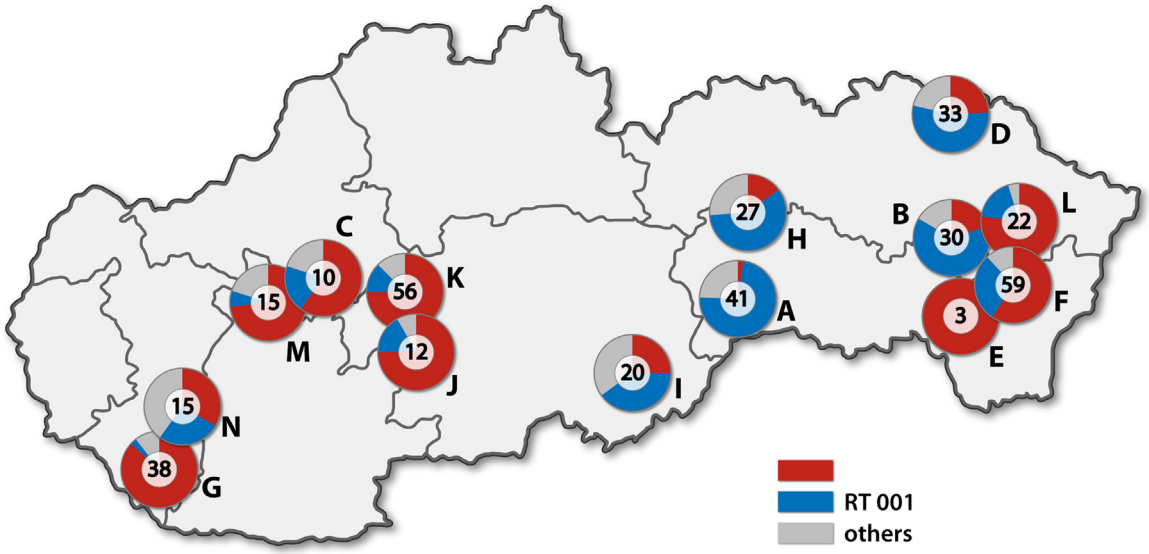

Figure 1. Distribution of 14 Slovak hospitals participating in this study. Pie charts show the representation of *Clostridioides difficile ribotypes* (RT) 001 and 176 identified per hospital. The numbers in the centre represent the number of *C. difficile* isolates cultured for further characterization.

(Bionumerics v5.0, Applied Maths, Sint-Martens-Latem, Belgium). A clonal complex (CC) was defined as the sum of tandem repeat differences (STRD) ≤2, and a genetically related cluster was defined as STRD ≤10 [17].

In sequenced isolates, a multi-locus sequence type (ST) and a core-genome MLST (cgMLST) were determined from FASTQ data using MLSTFinder 2.0 and cgMLSTFinder 1.1 [19,20], available at: www.genomicepidemiology.org. Whole-genome MLST (wgMLST) was performed and an MST was constructed using BioNumerics (v8.1, Applied Maths).

3. Results

3.1. Reported CDI cases

In total, 14 hospitals in Slovakia participated in the study. Three hundred and eighty-one cases of CDI were reported, of which 288 (75.6%) were healthcare-associated (HA), 24 (6.3%) were community-associated (CA), and 20 (5.3%) were of unknown origin. Forty-nine patients (12.9%) had recurrent CDI. The mean hospital incidence of CDI was 4.1 cases/10,000 patient-days [95% confidence interval (CI) 2.5–5.7].

A complicated course of CDI was reported for 47/381 (12.3%) cases. These cases had lower odds of being HA CDI than CA CDI [odds ratio (OR) 0.362, 95% CI 0.134–0.980; P=0.046], and were more likely to have a fatal outcome within 30 days of hospitalization (OR 5.00, 95% CI 2.65–9.435; P<0.001; see online supplementary table). An in-hospital fatal outcome was recorded for 105/381 (27.6%, 95% CI 23.0–32.1) cases. The cases who died during hospitalization within 30 days of CDI diagnosis (n=98, 25.7%) tended to be older than those who did not (median 80 vs 75 years; adjusted P=0.011; see online supplementary table). Death was reported to be 'possibly' or 'definitely' related to CDI for 64.8% of fatal cases.

Antibiotic use in the 4 weeks prior to CDI was recorded for 368/381 cases (96.6%). Of these, 333 (90.5%) were reported to have received an antimicrobial agent in monotherapy or in combination. The most common were cephalosporins (n=139), fluoroquinolones (n=105), co-amoxiclav (n=70), penicillins (n=47), metronidazole (n=31), glycopeptides (n=29), lincosamides (n=27) and aminoglycosides (n=25). The '4C antimicrobials' (i.e. fluoroquinolones, clindamycin, co-amoxiclav and cephalosporins) [21] comprised 80.8% (n=269) of prior antimicrobial therapy (see online supplementary table).

3.2. Characterization of C. difficile isolates

In total, 370/381 (97.1%) cases had *C. difficile* isolate cultured, and 27 RTs were identified. The most prevalent were RT176 (n=185, 50.0%), identified in all 14 participating hospitals, and RT001 (n=128, 34.6%), detected in 13/14 hospitals (Fig. 1; also see online supplementary table). Of the other 25 detected RTs, only RT014 was identified in >2% of isolates (n=8). Genes for all three toxins (A, B, Binary) were detected in 198/370 (53.5%) isolates of the following RTs: 176 (n=185), 027 (n=5), 078 (n=4), 023 (n=3) and 126 (n=1). The remaining 172 isolates carried genes for toxins A and B (see online supplementary table).

Resistance to moxifloxacin (≥8 mg/L) was detected in 86% of isolates (n=318/370), with a Thr82 substitution in GyrA present in 99.7% (n=317) (see online supplementary table). Reduced susceptibility to metronidazole (>2 mg/L) was observed in 16 isolates, of which 13 were RT176 and three were RT001; however, after a subculture and re-testing using MIC test strips (Liofilchem), these isolates were susceptible to metronidazole and were negative for the presence of pCD-metro [22], investigated as described previously [23].

There was no significant evidence of an association between origin of infection (HA/CA), complicated course of infection, recurrent CDI, fatal outcome within 30 days of diagnosis, and age and carriage of binary toxin genes or *C. difficile* strains of RT176 or RT001 compared with other RTs (Tables 1 and 2). In contrast, resistance to moxifloxacin was more common in HA CDI cases than in CA CDI cases [249/261 (95.4%) vs 30/42 (71.4%); P<0.001; see online supplementary table].

3.3. Genetic relatedness of predominant RTs

Analysis of MLVA results from all 128 RT001 and 183/185 RT176 isolates identified 19 CCs (STRD ≤2) for both RTs (Figs 2 and 3; also see online supplementary table). RT001 had 12 of the 21 CCs with less than five isolates, and its five largest CCs had 3.2 isolates per hospital. In contrast, the five largest CCs of RT176 isolates had an average of 7.4 isolates/hospital. RT176 also had the largest CC (CC8, n=28 isolates, n=8 hospitals), the largest single hospital CC (CC1,

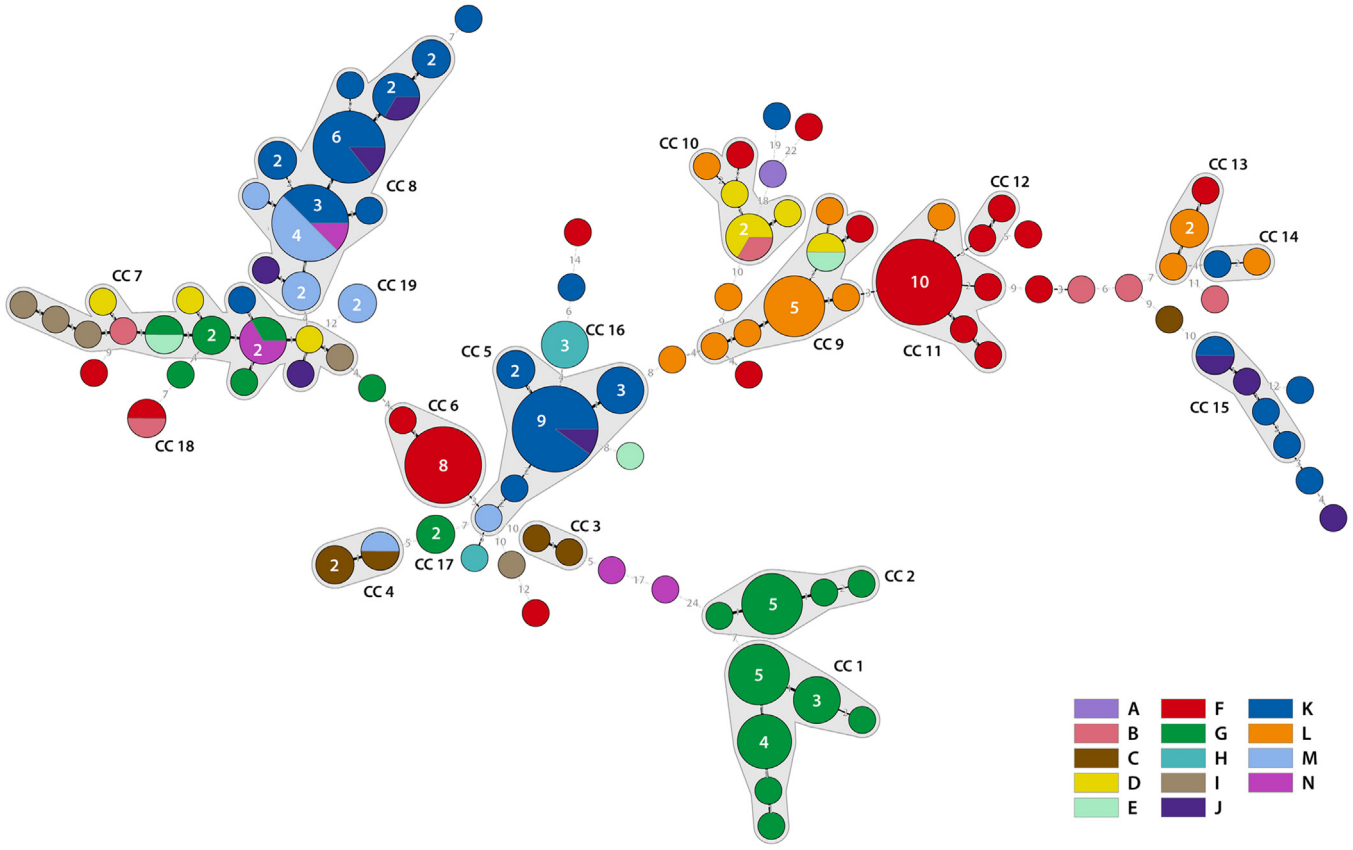

Figure 2. Minimum spanning tree generated from multi-locus variable tandem repeat analysis data of *Clostridioides difficile* polymerase chain reaction (PCR) ribotype 176 isolates from 14 hospitals participating in this study (n=183/185). Each hospital is represented by a different colour. The numbers in the circles represent the number of seven variable-number tandem repeat loci that were 100% identical [sum of tandem repeat differences (STRD)=0] among the *C. difficile* PCR ribotype 176 isolates. The numbers on the lines represent STRD between isolates. cc, clonal complex.

n=15), and a large CC (CC11) with 13/14 isolates from one hospital (Figs 2 and 3; also see online supplementary table). Most hospitals with isolates clustered in the same CC were close geographically (see online supplementary table).

To confirm the observed genetic relatedness, one isolate of RT001 and one isolate of RT176 from each hospital, where applicable, was characterized by WGS.

3.4. Whole-genome sequencing

WGS data were available for 27 isolates (RT001 *n*=13, RT176 *n*=14). RT001 isolates (*n*=13) belonged to ST3 (Clade 1) and RT176 isolates (*n*=14) were ST1 (Clade 2).

All RT001/ST3 isolates were designated as 'type 6335' by cgMLSTFinder, and 0–10 allelic differences between isolates were found using wgMLST (Fig. 4). Three isolates showed only one allelic difference and two isolates had two allelic differences.

In RT176/ST1 isolates, the cgMLSTFinder identified that almost all (*n*=13/14) isolates were designated as 'type 301', and the remaining isolate (4879) was 'type 602'. wgMLST showed 0–64 allelic differences between isolates (Fig. 5). Seven isolates showed one to three allelic differences and three isolates had zero to two allelic differences.

The *erm*(B) gene was detected in 88.9% (24/27) isolates, and their resistance to clindamycin and erythromycin was confirmed phenotypically (≥256 mg/L). The *aac*(6′)-*aph*(2′′) gene was detected in 71.4% (10/14) of RT176 isolates and 46.2% (6/13) of RT001 isolates, with all 16 isolates having MICs ≥256 mg/L for both amikacin and gentamicin. One RT176 isolate carried the *tet*(M) gene and had an MIC for tetracycline of 16 mg/L. In addition, two *cfr* genes – *cfr*(E) in nine of 13 RT176 isolates and *cfr*(C) in one RT001 isolate – were detected but isolates did not have the expected linezolid-resistant phenotype [16–24 mg/L for *cfr*(C) and ≥256 mg/L for *cfr*(E) gene], as published previously [24]. In the present study, one strain (4687) had an MIC of 6 mg/L and the remaining isolates had an MIC <1.5 mg/L.

To investigate the integrity of the *cfr* genes and their surroundings, the short reads were assembled to previously published genomes MH229772 for the *cfr*(C) gene and DF11 for the *cfr*(E) gene [24,25]. In the *cfr*(C) sequence, there was a stop codon at position 23 compared with the MH229772 strain. The *cfr*(E) gene in all nine strains was intact, but the region surrounding the gene differed compared with the DF11 strain with the linezolid-resistant phenotype (Fig. 6a). In all strains, a *lsa*-like gene was located upstream of the *cfr*(E) gene. In *Enterococcus faecalis*, the *lsa* gene confers intrinsic resistance to lincosamides and streptogramin through a ribosome protection mechanism [26,27]. Whereas the *lsa*-like and *cfr*(E) genes are separated by only 17 nt and are most likely co-transcribed in DF11, the two genes were separated by a 790-nt-long region containing a 118-nt-long duplication of the start of the *cfr*(E) gene in all nine isolates from this study (Fig. 6a). In addition, the *lsa*-like gene encoded a protein with only 62.8% amino acid identity to the Lsa protein from the DF11 strain (Fig. 6b).

4. Discussion

In European acute care hospitals (ACHs), CDI is a common cause of morbidity and mortality [28]. In 2016, 556 ACHs in European Union/ European Economic Area countries, including 36 in Slovakia, participated in the ECDC-coordinated surveillance of CDI.

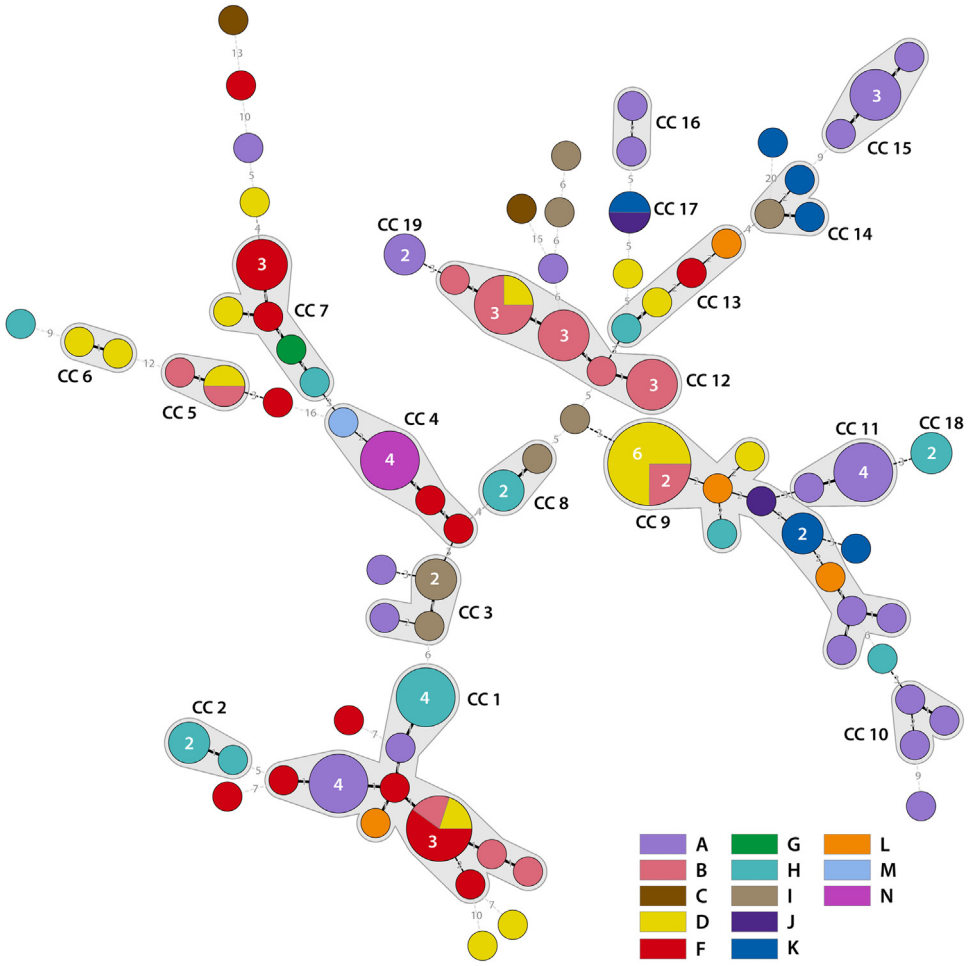

Figure 3. Minimum spanning tree generated from multi-locus variable tandem repeat analysis data of *Clostridioides difficile* polymerase chain reaction (PCR) ribotype 001 isolates from 14 hospitals participating in this study (n=128). Each hospital is represented by a different colour. The numbers in the circles represent the number of seven variable-number tandem repeat loci that were 100% identical [sum of tandem repeat differences (STRD)=0] among the *C. difficile* PCR ribotype 001 isolates. The numbers on the lines represent STRD between isolates. cc, clonal complex.

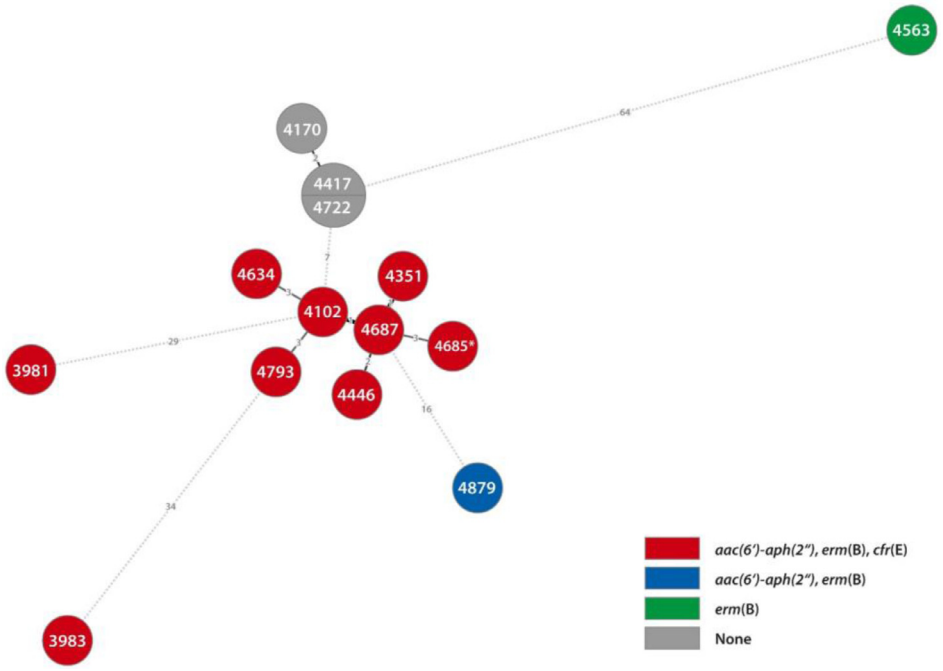

Figure 4. A minimum spanning tree of 14 *Clostridioides difficile ribotype* 176 isolates constructed based on whole-genome multi-locus sequence typing data using Bionumerics v8.1. * *C. difficile* isolate 4685 also carried the *tet* (M) gene.

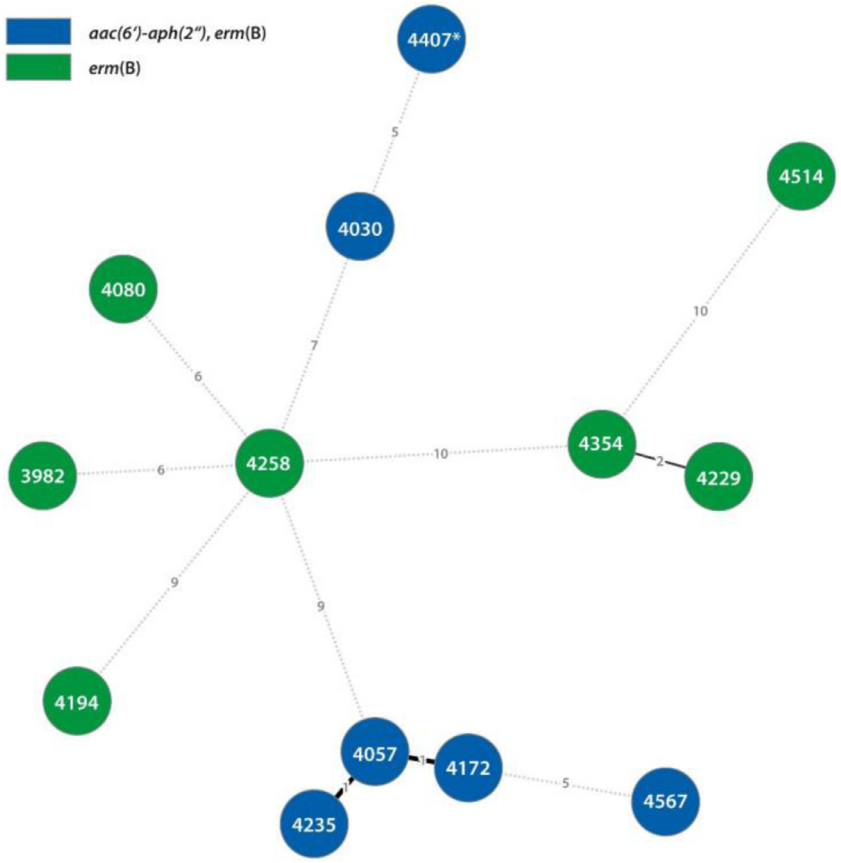

Figure 5. A minimum spanning tree of 13 *Clostridioides difficile ribotype* 001 isolates constructed based on whole-genome multi-locus sequence typing data using Bionumerics v8.1. * *C. difficile* isolate 4407 also carried the *cfr* (C) gene.

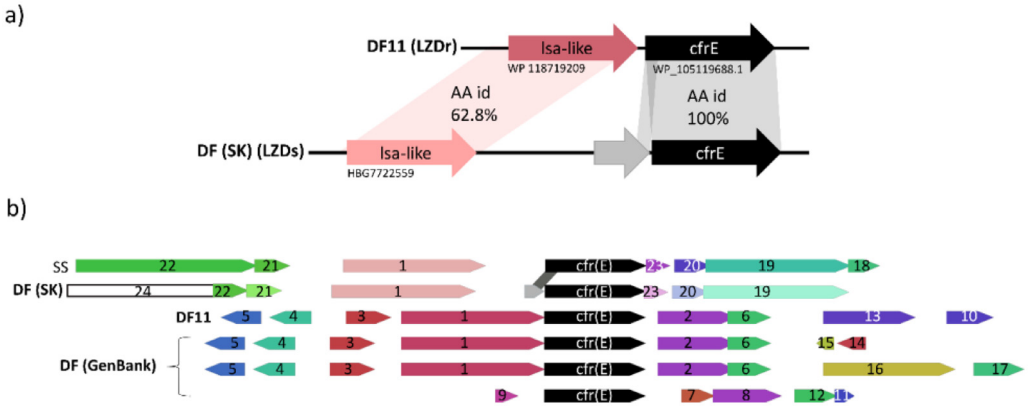

Figure 6. Genetic soundings of the *cfr*(E) gene in *Clostridioides difficile*. (a) Comparison of the *cfr*(E) gene upstream region of linezolid-resistant (DF11 [22]) and linezolid-susceptible (LZDs) isolates from nine of 14 hospitals participating in this study [DF(SK); *n*=9]. The small open reading frame upstream of the *cfr*(E) gene in the LZDs strains contains 114 nk duplication of the *cfr*(E) gene. (b) WebFlaGs comparison of four flanking genes of the *cfr*(E) gene in linezolid-susceptible [DF (SK), *n*=9], linezolid-resistant (LZDr) reference strain DF11, *C. difficile* genomes from GenBank [DF (GenBank); *n*=34], and the genome of *Streptococcus suis* LSM29 (SS). Genes labelled with the same number encode homologous proteins. See online supplementary table for protein annotations.

The European mean hospital incidence of CDI was 4.0 (95% CI 3.45–4.51) cases/10,000 patient-days, with 20.7% of cases dying in the hospital from various causes [29]. That year, in the participating ACHs in Slovakia, the mean hospital incidence of CDI was 2.8 (95% CI 1.9–3.9) cases/10,000 patient-days, with 10.8% cases dying within 30 days of hospitalization from any cause [8]. In 2018–2019, the incidence of CDI in Slovakia increased 1.5-fold compared with 2016 [8]. Furthermore, the crude mortality rate was notably high, with one-quarter of cases (25.7%) dying from any cause within 30 days of diagnosis during hospitalization, compared with one in 10 in 2016 [8]. It is important to note that stool testing for CDI was performed at the request of the physician, so some CDI cases in the participating hospitals may have remained undiagnosed [30].

Over the period from 2016 to 2018, RT176 emerged as the predominant strain in Slovak ACHs, overtaking RT001 which comprised 59% of strains in 2016 [8]. Indeed, in 2018–2019 in Slovakia, a very high proportion (five of six strains) were either RT001 or RT176. There was no strong evidence that either RT001 or RT176 caused a worse course of infection or infection outcome than the other strains (see online supplementary material). Similar findings were observed in a multi-centre study in the Czech Republic in 2017, with an exception for all-cause mortality and RT001 CDIs, but

those associations were not confirmed statistically when mortality within 30 days was analysed [31].

The observed near-exclusive representation of two RTs in the analysed set of isolates suggests epidemic spread. Therefore, MLVA was performed to determine the genetic relatedness of the isolates within each RT, and to identify clones among isolates within and between participating hospitals. Indeed, the close genetic relatedness was confirmed in the randomly selected, geographically diverse, sequenced isolates at wgMLST level. Seven of 14 sequenced RT176 isolates and five of 13 RT001 isolates met a proposed adjusted threshold of zero to three allelic differences for outbreak recognition [32]. The genetic relatedness of isolates from geographically distant hospitals may be explained by reportedly frequent patient transfers between hospitals.

Based on the study results, physicians, including hospital epidemiologists, monitored compliance with infection prevention and control measures. Although compliance with patient isolation and wearing of personal protective equipment was deemed sufficient, bed disinfection was identified as requiring enhancement. Therefore, hospitals introduced swabbing of beds after cleaning and terminal disinfection of rooms using plasma ozone. Swabs were cultured on selective media for *C. difficile* and aerobically on blood agar, with any bacterial growth considered a cleaning failure. Also, antimicrobial stewardship was enhanced through the recommendation to the network of the hospitals in the study for restriction of the use of cephalosporins and fluoroquinolones [21].

At the time of the study, metronidazole was the standard treatment for laboratory-confirmed CDI. Delayed clinical effects at 5 days were reported. Following the publication of updated guidance for CDI treatment [33], the use of fidaxomicin or vancomycin commenced in the hospital network. Metronidazole treatment is associated with longer shedding of *C. difficile* in stool compared with vancomycin and fidaxomicin, which may have contributed to environmental contamination and spore transmission between patients during the study [34]. Poor patient outcomes could be associated with relatively low stool concentration of metronidazole and its reduced antimicrobial bioactivity due to interaction with faecal microbiota. In addition, reduced susceptibility to metronidazole was observed in 16 isolates of epidemic RT001 and RT176. Repeated testing by E-test did not confirm this phenotype; however, Baines et al. also observed higher MICs using agar incorporation compared with E-test [35]. A recently described plasmid-mediated mechanism of metronidazole resistance was not present in these isolates [22,23], but other molecular mechanisms of metronidazole resistance were suggested [36].

Interestingly, the epidemiology of CDI in the Czech Republic, which is a neighbouring country of Slovakia, reported a similarly high prevalence of RT001 (24%) and RT176 (29%) with clonal clustering based on MLVA results in 774 isolates investigated in 2014 [37]. To investigate the intercountry relatedness of European *C. difficile* RT001 and 176 isolates, the WGS data from the European, multi-centre, prospective, biannual, point-prevalence study of CDI in hospitalized patients with diarrhoea (EUCLID study) [38] was used. cgMLST 602 was not detected in RT176 isolates (n=21, four countries), but cgMLST 301 was identified in two isolates from Poland. Both isolates carried the *erm*(*B*) gene. Presence of the *cfr*(E) gene was not observed in all 21 European RT176 isolates. Further, in 119 isolates of RT001 from 14 European countries, the same cgMLST (6335) was only identified in eight of 11 Slovak RT001 isolates and in one of two Czech RT001 isolates, but the Czech isolate did not carry the *erm*(B) gene and/or the *aac*(6′)-*aph*(2′′) gene as was observed in Slovak *C. difficile* RT001 isolates in this study. Unfortunately, the percentage of called alleles in cgMLST analyses of these three genomes was <95%, and wgMLST analyses failed to be performed in Bionumerics. As such, further studies are needed to confirm the suggested genetic relatedness of Slovak epidemic clones with Polish *C. difficile* RT176 and Czech RT001 isolates.

The role of horizontal gene transfer in the evolution of *C. difficile* was documented in RT027 [39]. The majority of sequenced isolates (24/27) acquired the *erm*(B) gene conferring resistance to erythromycin and clindamycin, and 16/24 also carried *aac*(6′)-*aph*(2′′) gene coding resistance to aminoglycosides which is more common than observed in European *C. difficile* isolates of the same RTs in the EUCLID study [38]. Unlike the macrolide–lincosamide–streptogramin B (MLS_B) group of antibiotics, anaerobes are intrinsically resistant to aminoglycosides, with high MICs reported (MIC_{50} 120 mg/L, 95% CI 62–250; MIC90 200 mg/L 95% CI 78–490) [40]. The acquired *aac*(6′)-*aph*(2′′) gene encoding aminoglycoside-modifying enzyme in isolates of RT001 and RT176 had higher MIC (≥256 mg/L) values compared with isolates of the same RTs without this gene (MIC90 64 mg/L, data not shown), but the epidemiological advantage for *C. difficile* remains unknown; however, its potential as a resistance reservoir in the human gut has to be taken into account. This also opens up new significance in the *cfr* genes which encode a methyltransferase that modifies position A-2503 in bacterial 23S rRNA and confers resistance to linezolid, a last resort drug in the treatment of severe infections caused by Gram-positive pathogens. Interestingly, the presence of the *cfr*(E) gene and the *cfr*(C) gene was not associated with the linezolid-resistant phenotype, likely due to genetic variation within and upstream of the *cfr*(C) and *cfr*(E) genes, respectively. In the case of the *cfr*(E) gene, the gene was intact, but the upstream region was distinct from linezolid-resistant strain DF11 (Fig. 6a) and also from all other *C. difficile* genomes with the identical Cfr(E) protein found in the GenBank database using the WebFlags tool for clustering neighbour-encoded proteins [41] (Fig. 6b). Surprisingly, the organization of the region around the *cfr*(E) gene in the isolates from this study was most similar to *Streptococcus suis* LSM29 human isolates from China. In addition to differences in the amino acid sequence of the proteins encoded in the flanking regions, the main difference was the duplication of 114 nt corresponding to the beginning of the *cfr*(E) gene, which was present in all nine linezolid-sensitive isolates from this study, but absent in the *S. suis* LSM29 strain. A subject of further investigation is whether this duplication prevents the *cfr*(E) gene from being expressed. It is also not clear whether an *lsa*-like gene can confer resistance to lincosamides and streptogramin A similar to *Enterococcus faecalis* [26,27], because all nine isolates in the present study co-harboured the *erm*(B) gene which mediates resistance to the MLS_B group of antibiotics.

5. Conclusions

The newly-predominant RT176 and endemic RT001 are driving the epidemiology of CDI in Slovakia. MLVA showed clonal relatedness of predominant RTs within and between hospitals. In wgMLST, seven of 14 sequenced RT176 isolates and five of 13 RT001 isolates showed zero to three allelic differences. In addition to fluoroquinolones, use of the MLS_B group of antibiotics can represent another driving force for the spread of these epidemic lineages in Slovakia. In *C. difficile*, the linezolid-resistant phenotype should be confirmed in strains with detected *cfr* gene(s). A follow-up surveillance study should be performed to evaluate the benefits of changes in infection prevention and control, antimicrobial stewardship and treatment of CDI.

Author contributions

AP: study design, coordination of the study, collection of stool samples and epidemiological data, editing subsequent versions of the manuscript.

MB: *C. difficile* culture, DNA extraction, ribotyping, toxin gene detection, sequencing of *gyrA* gene, confirmation of resistant phenotypes by MIC tests, bioinformatics, editing subsequent versions of the manuscript.

VC: statistical analysis.

PK: data analysis, co-drafting the first version of the manuscript and editing subsequent versions.

JS: collecting epidemiological data and editing subsequent versions of the manuscript.

GBN: bioinformatic analysis of the *cfr*-like genes, co-drafting the first version of the manuscript and editing subsequent versions.

PD: editing subsequent versions of the manuscript, funding acquisition.

AS: study design, coordination of the collection of stool samples, editing subsequent versions of the manuscript.

MK: supervision of the study, bioinformatic analysis, co-drafting the first version of the manuscript and editing subsequent versions.

Competing interests: None declared.

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Ethical approval: Not required.

Data availability: All raw sequence data in this study were submitted to the NCBI Sequence Read Archive under accession number BioProject ID PRJNA918961.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106824.

References

[1] Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. Clostridium difficile infection. Nat Rev Dis Primers 2016;2:16020.
[2] Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP. Importance of toxin A, toxin B, and CDT in virulence of an epidemic Clostridium difficile strain. J Infect Dis 2014;209:83–6.
[3] Krutova M, Kinross P, Barbut F, Hajdu A, Wilcox MH, Kuijper EJ. Survey Contributors. How to: Surveillance of Clostridium difficile infections. Clin Microbiol Infect 2018;24:469–75.
[4] Nyc O, Krutova M, Liskova A, Matejkova J, Drabek J, Kuijper EJ. The emergence of Clostridium difficile PCR-ribotype 001 in Slovakia. Eur J Clin Microbiol Infect Dis 2015;34:1701–8.
[5] Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH, Study Group EUCLID. Diversity of Clostridium difficile PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. Euro Surveill 2016;21 pii=30294.
[6] Freeman J, Vernon J, Pilling S, Morris K, Nicolson S, Shearman S, et al. Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study Group. Five-year Pan-European, longitudinal surveillance of Clostridium difficile ribotype prevalence and antimicrobial resistance: the extended ClosER study. Eur J Clin Microbiol Infect Dis 2020;39:169–77.
[7] Novakova E, Kotlebova N, Gryndlerova A, Novak M, Vladarova M, Wilcox M, et al. An outbreak of Clostridium (Clostridioides) difficile infections within an acute and long-term care wards due to moxifloxacin-resistant PCR Ribotype 176 genotyped as PCR ribotype 027 by a commercial assay. J Clin Med 2020;9:3738.
[8] Novakova E, Stefkovicova M, Kopilec MG, Novak M, Kotlebova N, Kuijper E, et al. The emergence of Clostridium difficile ribotypes 027 and 176 with a predominance of the Clostridium difficile ribotype 001 recognized in Slovakia following the European standardized Clostridium difficile infection surveillance of 2016. Int J Infect Dis 2020;90:111–15.
[9] European Centre for Disease Prevention and Control. European surveillance of Clostridium difficile infectionsSurveillance protocol. Version 2.3. Stockholm: ECDC; 2017.
[10] Core Team R. R: a language and environment for statistical computing, Vienna: R Foundation for Statistical Computing; 2018. Available at: www.R-project.org.
[11] Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et al. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for Clostridium difficile. PLoS One 2015;10:e0118150.
[12] Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect 2008;14:1057–64 Erratum in: Clin Microbiol Infect 2009;15:296.
[13] European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoints. Vaxjo: EUCAST. Available at: http://www.eucast.org/clinical_breakpoints/.
[14] Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. 30th ed. Wayne, PA: CLSI; 2020. M100.
[15] Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC. gyrA and gyrB mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in Clostridium difficile. Antimicrob Agents Chemother 2002;46:3418–21.
[16] Bortolaia V, Kaas RF, Ruppe E, Roberts MC, Schwarz S, Cattoir V, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 2020;75:3491–500.
[17] van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of Clostridium difficile isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol 2007;45:1024–8.
[18] Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, et al. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative Clostridium difficile in a general hospital in Buenos Aires, Argentina. Clin Microbiol Infect 2009;15:1080–1086.
[19] Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 2012;50:1355–61.
[20] Frentrup M, Zhou Z, Steglich M, Meier-Kolthoff JP, Göker M, Riedel T, et al. A publicly accessible database for Clostridioides difficile genome sequences supports tracing of transmission chains and epidemics. Microb Genom 2020;6 mgen000410.
[21] Lawes T, Lopez-Lozano JM, Nebot CA, Macartney G, Subbarao-Sharma R, Wares KD, et al. Effect of a national 4C antibiotic stewardship intervention on the clinical and molecular epidemiology of Clostridium difficile infections in a region of Scotland: a non-linear time-series analysis. Lancet Infect Dis 2017;17:194–206.
[22] Boekhoud IM, Hornung BVH, Sevilla E, Harmanus C, Bos-Sanders IMJG, Terveer EM, et al. Plasmid-mediated metronidazole resistance in Clostridioides difficile. Nat Commun 2020;11:598.
[23] Cizek A, Masarikova M, Mares J, Brajerova M, Krutova M. Detection of plasmid-mediated resistance to metronidazole in Clostridioides difficile from river water. Microbiol Spectr 2022;10:e0080622.
[24] Stojković V, Ulate MF, Hidalgo-Villeda F, Aguilar E, Monge-Cascante C, Pizarro-Guajardo M, et al. cfr(B), cfr(C), and a new cfr-like gene, cfr(E), in Clostridium difficile strains recovered across Latin America. Antimicrob Agents Chemother 2019;64 e01074-19.
[25] Chatedaki C, Voulgaridi I, Kachrimanidou M, Hrabak J, Papagiannitsis CC, Petinaki E. Antimicrobial susceptibility and mechanisms of resistance of Greek Clostridium difficile clinical isolates. J Glob Antimicrob Resist 2019;16:53–8.
[26] Singh KV, Weinstock GM, Murray BE. An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob Agents Chemother 2002;46:1845–50.
[27] Sharkey LK, Edwards TA, O'Neill AJ. ABC-F proteins mediate antibiotic resistance through ribosomal protection. mBio 2016;7:e01975.
[28] Cassini A, Plachouras D, Eckmanns T, Abu Sin M, Blank HP, Ducomble T, et al. Burden of six healthcare-associated infections on European population health: estimating incidence-based disability-adjusted life years through a population prevalence-based modelling study. PLoS Med 2016;13:e1002150.
[29] . European Centre for Disease Prevention and Control. Clostridium difficile infectionsAnnual epidemiological report for 2016. Stockholm: ECDC; 2018.
[30] Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis 2014;14:1208–19.
[31] Krutova M, Capek V, Nycova E, Vojackova S, Balejova M, Geigerova L, et al. The association of a reduced susceptibility to moxifloxacin in causative Clostridium (Clostridioides) difficile strain with the clinical outcome of patients. Antimicrob Resist Infect Control 2020;9:98.
[32] Baktash A, Corver J, Harmanus C, Smits WK, Fawley W, Wilcox MH, et al. Comparison of whole-genome sequence-based methods and PCR ribotyping for subtyping of Clostridioides difficile. J Clin Microbiol 2022;60:e0173721.
[33] van Prehn J, Reigadas E, Vogelzang EH, Bouza E, Hristea A, Guery B, et al. Guideline Committee of the European Study Group on Clostridioides difficile. European Society of Clinical Microbiology and Infectious Diseases: 2021 up-

date on the treatment guidance document for Clostridioides difficile infection in adults. Clin Microbiol Infect 2021;27(Suppl. 2):S1–21.
[34] Krutova M, Wilcox M, Kuijper E. Clostridioides difficile infection: are the three currently used antibiotic treatment options equal from pharmacological and microbiological points of view? Int J Infect Dis 2022;124:118–23.
[35] Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, et al. Emergence of reduced susceptibility to metronidazole in Clostridium difficile. J Antimicrob Chemother 2008;62:1046–52.
[36] O'Grady K, Knight DR, Riley TV. Antimicrobial resistance in Clostridioides difficile. Eur J Clin Microbiol Infect Dis 2021;40:2459–78.
[37] Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc OCzech Clostridium difficile study group. Clostridium difficile PCR ribotypes 001 and 176 – the common denominator of C. difficile infection epidemiology in the Czech Republic, 2014. Euro Surveill 2016;21.
[38] Eyre DW, Davies KA, Davis G, Fawley WN, Dingle KE, De Maio N, et al., EUCLID Study Group Two distinct patterns of Clostridium difficile diversity across Europe indicating contrasting routes of spread. Clin Infect Dis 2018;67:1035–44.
[39] He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated Clostridium difficile. Nat Genet 2013;45:109–13.
[40] Khanafer N, Daneman N, Greene T, Simor A, Vanhems P, Samore M, et al. Susceptibilities of clinical Clostridium difficile isolates to antimicrobials: a systematic review and meta-analysis of studies since 1970. Clin Microbiol Infect 2018;24:110–17.
[41] Saha CK, Sanches Pires R, Brolin H, Delannoy M, Atkinson GC. FlaGs and webFlaGs: discovering novel biology through the analysis of gene neighbourhood conservation. Bioinformatics 2021;37:1312–14.