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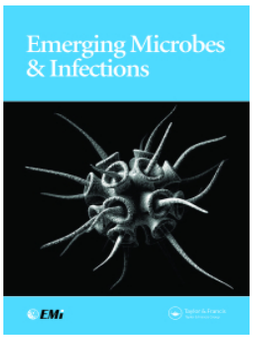

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Emerging *Clostridioides difficile* strains belonging to PCR ribotype 955 in Serbia are distinct from metronidazole-resistant RT955 outbreak isolates from the UK

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ABSTRACT

End 2023, the UK Health Security Agency sent an alert about a new hypervirulent *Clostridioides difficile* PCR ribotype, ribotype 955 (RT955), causing slowly progressing infection clusters in hospitals in the Midlands. Between March 2018 and February 2022, surveillance of *Clostridioides difficile* infections (CDI) was performed in southern Serbia with centres providing medical services for approximately 750,000 inhabitants. Using the ECDC recommended protocol, clinical, epidemiological and microbiological data were collected. *C. difficile* RT955 was identified in 27 (7%) of 383 surveyed patients with CDI. Of 27 patients, 16 (59%) was older than 60 years and 19 (70%) were male. CDI was always associated with previous antibiotic therapy and had a hospital onset in 23 (85%) patients. The clinical presentation was milder than reported in UK. All sequenced strains belonged to multilocus sequence type (ST) 1 and were highly similar, with 0–1 alleles differences in a core genome multilocus sequence typing analysis. The strains differed clearly from the UK RT955 outbreak strain by whole genome sequencing and phenotypic susceptibility to metronidazole, lincosamides and rifampicin. Interestingly, a high-level erythromycin resistance was observed associated with the presence of the *mrmA* gene. Both the UK and Serbian RT955 strains contained *gyrA*_p.T82I associated with resistance to fluoroquinolone antimicrobials and carried the PnimBG promoter mutation, suggestive for haem-dependent metronidazole resistance. We conclude that *C. difficile* RT955 is present in southern Serbia since 2018. The Serbian RT955 strains differed clearly from a representative UK cluster strain.

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KEYWORDS Surveillance; *Clostridioides difficile*; metronidazole resistance; ribotype 955; Serbia

Introduction

Since 2003, *Clostridioides difficile* belonging to PCR ribotype (RT) 027 emerged and spread worldwide. Infections with RT027 were accompanied with higher mortality and morbidity and a more complicated course of the disease with frequent relapses and are for that reason sometimes referred to as hypervirulent [1,2]. RT027 strains belong to multilocus sequence type (ST) 1 and phylogenetic clade 2 and contain a 18 bp deletion in the *tcdC* gene at position 330–347 and a single nucleotide deletion at position 117 [1,3]. This clade also encompasses other PCR ribotypes (such as 016, 036, 176, 181, 198, 244, and 251) that were later found and considered as "hypervirulent" but did not spread yet on a global scale [4,5]. At the end of 2023, the UK Health Security Agency (UKHSA) issued an alert about a new hypervirulent RT955 belonging to ST 1, causing slowly progressing clusters of infections in hospitals in the Midlands involving 50 patients with *C. difficile* infection (CDI) and data related to this PCR ribotype were presented at ESCMID Global conference in 2024 [6].

In response to the CDI epidemiological developments in 2003, the European Centre for Disease Prevention and Control (ECDC) started the European *Clostridium difficile* Infection Surveillance Network (ECDIS-net), with a consortium composed of Universities from Leeds, Leiden, Wales, Berlin and the

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National Institute for Public Health and the Environment in the Netherlands (RIVM) to develop a European surveillance program. This protocol was subsequently tested, optimized, and implemented [7]. In 2018, a special program was added for EU candidate and potential candidate countries, including Serbia (IPA5 contract 4 within framework contract ECDC/2016/016). Of note, in 2013, Serbia already participated in ECDIS-Net who launched a pilot study to enhance laboratory capacity and standardize surveillance for CDI, during which no *C. difficile* RT955 was identified [8]. In a study performed during a 7-year period until 2018 in a different Serbian region, RT955 was also not reported, similar as a study from a university hospital in Belgrade [9,10]. However, RT955 was also detected in 4% of the 93 samples included in a study to CDI in the period from May 2018 to January 2022 [11].

The aim of this descriptive report is to describe the emergence and spread of RT955 in a southern Serbia, with patients characteristics, clinical outcome, response to therapy and molecular findings.

Methods

Location

Between March 2018 and February 2022, CDI was surveyed in southern Serbia by the Institute of Public Health of Niš, Serbia, Centre for Microbiology (National Reference Laboratory for Anaerobic Infections – *C. difficile*). Several health care centres participated such as Community Health Center Niš, various hospitals, military hospitals and one clinical centre in city Niš. These healthcare centres provide medical services for 750,000 individuals in the region. CDI is only tested by request of the physicians. Though a standardized surveillance protocol was lacking, the protocol of ECDC [12] was used as a basis for the diagnostics and case definitions.

Definitions

CDI was defined as the occurrence of diarrhoea (≥3 loose stools per day for at least 2 subsequent days) or the presence of a toxic megacolon and endoscopically diagnosed pseudomembranous colitis (PMC) combined with a positive test for toxin-producing *C. difficile*. Based on the ECDC recommendations, Healthcare Onset-CDI (HO-CDI) was defined as CDI in all patients who had onset of symptoms in a healthcare facility from 72 h after admission until discharge [12]. Community Onset-CDI (CO-CDI) was defined as CDI in all patients who had onset of symptoms outside a healthcare facility at home [12]. A recurrent CDI was defined as a new episode of diarrhoea occurring within 8 weeks after the day of onset of the previous CDI, with a positive microbiological diagnosis. A presumed rCDI was defined as development of clinical symptoms suggestive for CDI, but without a microbiological diagnosis. The severity of CDI was assessed as mild, moderate or severe using clinical characteristics as previously described [13].

Microbiological analysis

Stool samples from patients with presumed CDI were analysed by RIDA GENE *C. difficile* test (real-time multiplex PCR, R-Biopharm AG, Darmstadt, Germany) for the detection of *C. difficile* (16S rDNA) and *C. difficile* toxin A (*tcdA*) and toxin B (*tcdB*) genes. The presence of both free *C. difficile* toxins A and B in stool specimens was determined by the ELISA-RIDASCREEN *C. difficile* Toxin A/B (R-Biopharm AG, Darmstadt, Germany). Positive-tested stool samples for CDI were inoculated on selective cycloserine–cefoxitin–fructose agar (CCFA) (Biomedics, Madrid, Spain) for *C. difficile* culture after an alcohol-shock treatment and incubated at 37°C under anaerobic conditions for 48 h. Suspected *C. difficile* colonies were tested for the presence of glutamate dehydrogenase (GDH) using Rida®QUICK *C. difficile* GDH test (R-Biopharm AG, Darmstadt, Germany). Final identification of *C. difficile* was performed by the API system for anaerobic bacteria (API 20A bioMerieux, France). Isolates of *C. difficile* were sent to Leiden University Medical Center (which hosts Dutch National Expertise Centre for *C. difficile* infections) for PCR ribotyping and molecular characterization [14].

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done both at the Institute of Public Health, Niš and at Leiden University Medical Center (LUMC). In Serbia, susceptibilities to five antibiotics were determined on Brucella Agar plates supplemented with haemin and vitamin K (Biomedics, Madrid, Spain) by E-test gradient strips (LIOFILCHEM ® srl, Italy), for vancomycin, tigecycline, fusidic acid, rifampicin, and moxifloxacin. Epidemiological cut-off values (ECOFF) of tested antibiotics were defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [15]. At the LUMC, isolates were retested for metronidazole susceptibility using a standardized and recommended agar dilution method (CLSI) on EUCAST-recommended Fastidious Anaerobe Agar supplemented with horse blood (FAA-HB) [16]. In addition, three randomly selected but unrelated RT955 strains were used for susceptibility testing to cefuroxime, erythromycin, clindamycin, imipenem, moxifloxacin, metronidazole, rifampicin, and

tetracycline using E-test gradient strips (bioMérieux) on Brucella Blood Agar.

Molecular characterization

C. difficile isolates were typed at the Dutch National Expertise Centre for *C. difficile* infections according to standard procedures by capillary PCR ribotyping [14]. A standardized protocol was used with primers designed by Bidet et al. as described in [14]. PCR products were analysed on either an Applied Biosystems 3500xl or Genetic analyser using 24 cm capillary array with POP-7 separation matrix. The Leeds-Leiden database with various PCR ribotypes was used as reference. The presence of pCD-METRO was analysed as described previously [17,18]. Whole genome sequencing was performed on an Illumina Novaseq 6000 platform and analysed using the *C. difficile* core genome multilocus sequence typing (cgMLST) v2 and AMRFinderPlus routines implemented in SeqSphere+ 9.0.10 (Ridom GmbH, Germany). A minimal spanning tree (MST) was generated in SeqSphere+ with an MST cluster distance threshold of 6 [4]. Mutations in the *nimB* gen and its promoter were analysed by manually identifying the *nimB* gene and aligning the DNA sequence of the gene and its upstream region against reference sequences obtained for the historic RT027 strain CD196 (CD196_1331 in NC_013315.1) and the epidemic strain R20291 (the CDR20291_1308 (in NC_013316.1) in Geneious R10.2.6. (Biomatters Ltd). A SNP tree was generated based on 1833 SNPs in CSIPhylogeny 1.4 [19] using the genome sequence of strain CD196 as a reference; the resulting tree was rooted on strain CD196 (GBR-29-027 in our data). The tree was visualized in iTol [20] in rectangular mode ignoring branch lengths.

Table 1. Demographic data and clinical characteristics of Serbian patients with CDI and RT955.

	Patients with CDI and RT 955 *N* = 27
Age	
Above 80	1 (4%)
Between 60 and 80	15 (56%)
Between 50 and 60	9 (33%)
Younger than 50	2 (7%)
Male gender	19 (70%)
Onset of CDI	
Hospital	23 (85%)
Community	4 (15%)
Clinical presentation of CDI	
Mild	11 (41%)
Moderate	13 (48%)
Severe	3 (11%)
Previous use of antibiotics	
Cephalosporines	20 (74%)
Fluoroquinolones	7 (26%)
Macrolides	6 (22%)
Combination of various antibiotic classes	17 (63%)
Concomitant disease	
COVID-19	16 (59%)
Gastrointestinal	5 (19%)
Solid tumour	3 (11%)
Chronic kidney disease	2 (7%)
Cardiovascular disease	1 (4%)
Outcome after 90 days	
Recovered	25 (93%)
All-cause mortality	2 (7%)
Recurrence	3 (11%)
Treatment of CDI	
Only metronidazole	6 (22%)
Only vancomycin	11 (41%)
Only fidaxomicin	1 (4%)
Metronidazole switch to vancomycin	9 (33%)

Reuse of published WGS of C. difficile

Previously published genome sequences of *C. difficile* strains belonging to ST1 with frequently found PCR ribotypes (027, 176, 181) from various countries were included for comparison with the UK RT955 strain (GBR-30-955 in our analyses; corresponding to GenBank accession ERR12670107). A strain identified in Enterobase as RT955 (GBR-83-955 in our analyses) was also included. Most strains have been used in previous studies to compare PCR ribotyping with whole genome sequence typing and from an outbreak in Greece [4,21] (see Supplemental Table 1).

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of the Institute of Public Health Nis.

Results

In total, 383 patients (290 HO and 93 CO cases; 424 isolates) with CDI were included in the surveillance of which the majority derived from Clinical center Niš (284 patients) and Community Health Center Niš (93 patients). Six patients were diagnosed at the Military Hospital Nis. *C. difficile* RT 955 was identified in 28 of 424 isolates (7%), representing 27 of 383 patients (7%) since one patient has been included twice. Four patients with CDI and RT 955 were also part of a previous report: SRB-02-955, SRB-05-955, SRB-09-955, and SRB-15-955 [11]. Of the 27 patients with CDI due to RT955, 59% were older than 60 years and 70% were male (Table 1). The similarity in PCR ribotyping patterns among RT 955, RT 027, RT 176, and RT 181 – all of which belong to ST1 – is illustrated in the Supplemental Figure 1.

It was not possible to identify outbreaks of CDI due to RT955, since many hospitals were partially converted into COVID-19 centres with many movements of patients. CDI had a community onset in 4 (15%) patients and was always associated with previous antibiotic therapy, mostly including a cephalosporin in 20

patients (74%). Antibiotic combinations were used in 17 (63%) patients, mostly a combination of cephalosporines with fluoroquinolones ($n = 5$) or with a macrolide ($n = 6$). Of 27 patients, 16 (59%) had COVID-19 as concomitant disease whereas 5 patients (19%) had gastrointestinal disease, 3 (11%) a solid malignant tumour, 2 (7%) chronic kidney disease, and 1 (4%) cardiovascular disease as underlying disease. One patient with HO-CDI presented as a recurrent episode of CDI. CDI was categorized as severe in 3 (11%) patients. At 90 days follow up, all patients recovered, 2 patients died but not related to CDI, and 3 (11%) developed a recurrence of which one as a presumed recurrence. Recurrences developed at 2 weeks ($n = 2$) and 4 weeks ($n = 1$) after the diagnosis of the initial episode. Of 15 patients treated with metronidazole, 9 (60%) patients switched to vancomycin.

Twenty-seven strains were available for WGS with cgMLST analysis using SeqSphere+. All sequenced Serbian strains except one, were identified as ST1, CT 5259. The Serbian isolates were found to be highly related, with only 0–1 alleles differences in these analyses, highly suggestive of clonal spread [4,22]. Similar to other ST1 strains, the Serbian RT955 contained genes encoding toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtAB*), and carried a single base pair deletion at position 117 and an 18-bp deletion in the *tcdC* gene. A minimal spanning tree suggested that the Serbian strains are distinct from the UK RT955 cluster (Figure 1), as they fall in a different MST cluster. The UK strains of RT 955 belonged to ST1, CC 6496.

The phylogenetic tree based on whole genome SNP analysis using CSIPhylogeny (Figure 2) confirmed the high genomic relatedness of the Serbian RT955 strains (SRB), despite these mostly not being epidemiologically related. For three isolates, we could identify an epidemiological link (i.e. the clinical data show a link in time and place) and the SNP analysis suggests that transmission may have occurred (Figure 1, strains SRB-25-955, SRB-26-955, and SRB-27-955). All three patients were diagnosed in October 2021 at Clinical Center Niš and were nursed in two adjacent rooms. Interestingly, two samples (SRB-15-955 and SRB-16-955) clearly differ from each other on a genomic level, but are derived from a single patient with a primary (SRB-15-955) and a recurrent CDI (SRB-16-955) after 4 weeks.

An analysis of the antimicrobial resistance gene content (using AMRFinderPlus as implemented in SeqSphere+) shows that all RT955 isolates (both Serbian and UK) contain *gyrA*_p. Thr82Ile allele associated with fluoroquinolone resistance (Figure 2). In addition, manual analysis showed that all RT955 strains carried a T-to-G mutation in the −43 nt position in the promoter region of the nimB gene ($PnimB^G$) implicated in medium-dependent metronidazole resistance [23] (Figure 2). We also note that – in comparison with the historic RT027 strain CD196 – all RT955 (both UK and Serbia) carry a *nimB*_p.Leu155ILe mutation but only the UK outbreak RT955 has an additional *nimB*_p.Tyr130Ser mutation. Other genomic resistance markers identified by AMRFinderPlus in the Serbian RT955 strains were for aph(2″)-Ih (aminoglycoside resistance) and *blaCDD* (beta-lactam resistance).

Using the ECOFF criteria of EUCAST [15], 27 Serbian RT955 strains were resistant to moxifloxacin and susceptible to metronidazole, vancomycin, tigecycline, fusidic acid, and rifampicin by E tests on Brucella Blood Agar tested at the Institute for Public Health Niš. Additional testing by agar dilution on FAA-HB at LUMC showed that all 27 strains were susceptible to metronidazole with MIC = 0.5 mg/l. Three different strains at randomly selected confirmed these findings and also showed low MIC (0.016) for tetracycline, high erythromycin MIC (>256 ug/mL), low clindamycin MIC (0.016–0.064), high imipenem MIC (8–32), and high cefuroxime MIC (>256) (see Supplemental Figure 2). Interestingly, AMRFinderPlus did not identify erythromycin resistance determinants, but manually screening of the Serbian RT955 revealed the presence of the recently describes *mrmA* gene, encoding resistance to macrolides [24,25].

Discussion

The global burden of CDI has steadily increased over the past 30 years, particularly in regions with high to middle sociodemographic index (SDI) levels. A strong positive correlation has been observed between global antibiotic consumption and CDI incidence. Worldwide, CDI is the fastest-growing cause of diarrheal deaths, with mortality rates increasing more than fourfold [26]. The global spread and increased mortality have been associated with fluoroquinolone-resistant and metronidazole-resistant *C. difficile* strains belonging to clade 2, commonly referred to as hypervirulent strains, such as such as the multidrug-resistant *C. difficile* PCR ribotype 176 isolates [23, 25]

The UKHSA sent an alert of a new hypervirulent metronidazole-resistant RT955 causing slowly progressing outbreaks in hospitals in Midlands in the period between September 2021 and December 2023, involving 50 patient with CDI. This raises the question whether RT955 has been circulating prior to these outbreaks and are present in other countries than the UK. Here we address these questions by showing that RT955 was present in Serbia prior to 2021. Subsequently, this RT was also found in Poland in at least three different hospitals [27]. The Polish isolates in that study strongly resembled the UK outbreak strain. By contrast, we find that Serbian isolates

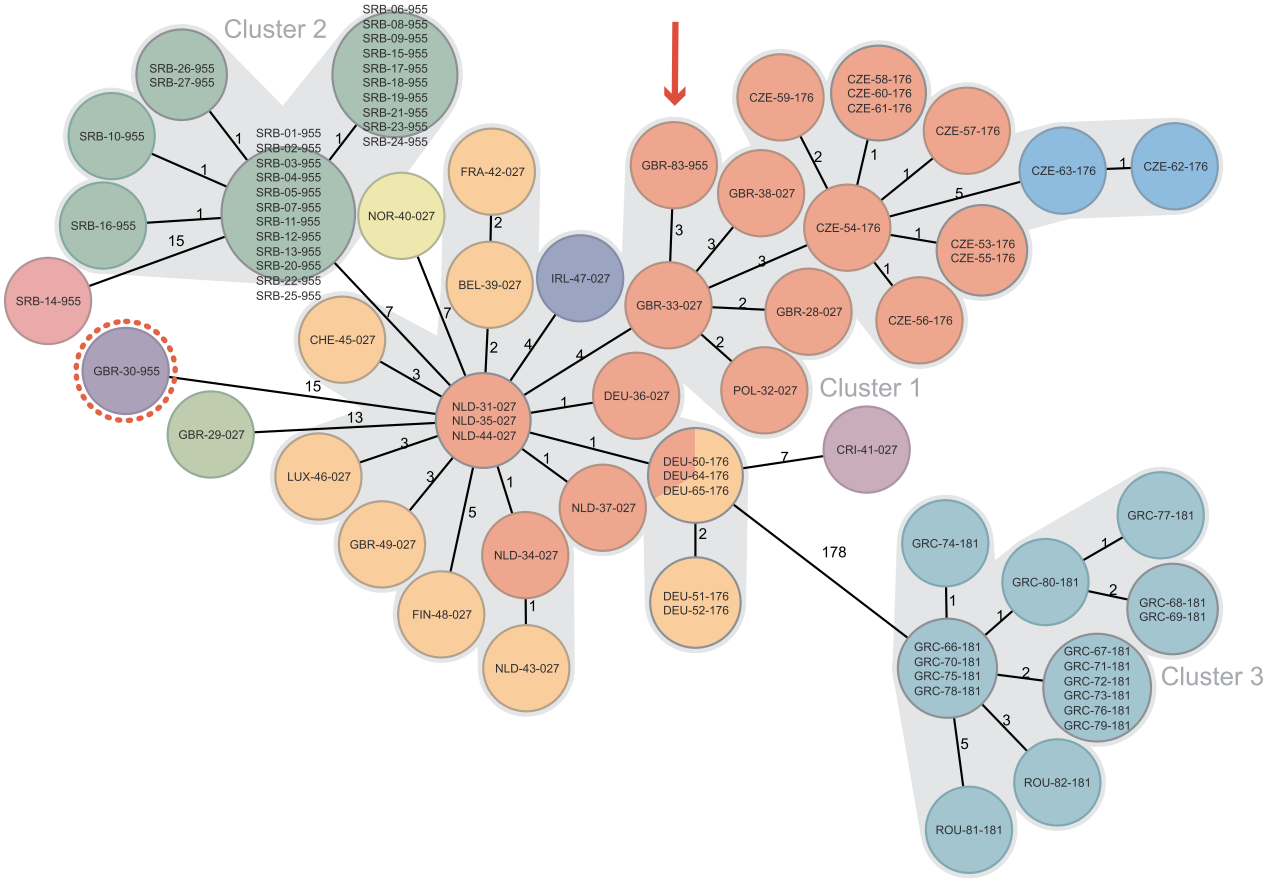

Figure 1. A minimal spanning tree based on cg MLST analysis with al Serbian RT955 strains, the UK RT955, and various RT027, RT176, and RT181 strains from various countries [2]. Strain IDs include country of origin (first three letters per alpha-3 code), see Appendix Table 1. Each colour represents a specific complex type. The numbers on the lines represent the number of allele differences between isolates. Three separate MST clusters are recognized, comprising RT027 and RT176 strains from various countries in Cluster 1, the majority of Serbian RT955 strains in Cluster 2 and RT181 strains from Romania and Greece in Cluster 3. The UK outbreak RT955 strain (GBR-30-955; purple with red dotted ring) shows 15 alleles difference from the RT027 cluster. The UK RT955 identified in Enterobase (GBR-83-955) is indicated with a red arrow

differed in both a cgMLST and SNP analysis, despite belonging to the same ST (ST1). Importantly, the antibiotic susceptibility also differed with susceptibility of the Serbian strains to lincosamides and rifampicin, both phenotypically and genetically. The Serbian strains contained a similar gyrA_T82I mutation (associated with fluoroquinolones resistance) and a mutation in the promoter of the *nimB* gene (P*nimB*^{G}) as did the UK outbreak strain. Phenotypical testing of antibiotic susceptibility, however, did not reveal metronidazole resistance on FAA-HB (MIC = 0.5ug/mL) [16]. Additional testing on different media with presumably higher levels of heme (e.g. chocolate agar with 1% polyvitex, PVX), we observed similar metronidazole MICs, demonstrating that lack of metronidazole resistance is likely not related to limiting haem concentrations (data not shown). We note, however, that the UK RT955 outbreak strain and the Poland strains contains an additional *nimB*_p.Y130S mutation [6,27]. In contrast, the Serbian RT 955 isolates lack the *nimB*_p.Y130S mutation and this could explain the lack of resistance to metronidazole. We hypothesize that it negatively affects the activity of nitroimidazole reductase. In addition, a recent study suggested that an RlmN-type 23S rRNA methyltransferase, designated MrmA, is associated with high-level macrolide resistance in *C. difficile* isolates RT 176 without *ermB* gene [25]. Notably, all Serbian strains showed resistance to erythromycin without the presence of *ermB* gene and contained *mrmA*. However, further studies are necessary to establish a direct link between *mrmA* with increased erythromycin resistance in *C. difficile*. Overall, our genomic and antimicrobial susceptibility data suggest that the Serbian isolates form a separate phylogroup.

Our clinical and epidemiological analyses are limited as a result of the impact of the COVID-19 pandemic. The Serbian RT955 described here were collected in the context of ECDC-supported CDI surveillance in southern Serbia. Though during the COVID-19 period this surveillance continued, the collection of clinical and epidemiological data to study outbreaks and transmission was hampered as many hospitals were converted into COVID centres. As a result it was not possible to determine the exact movement of all patients and to distinguish between local

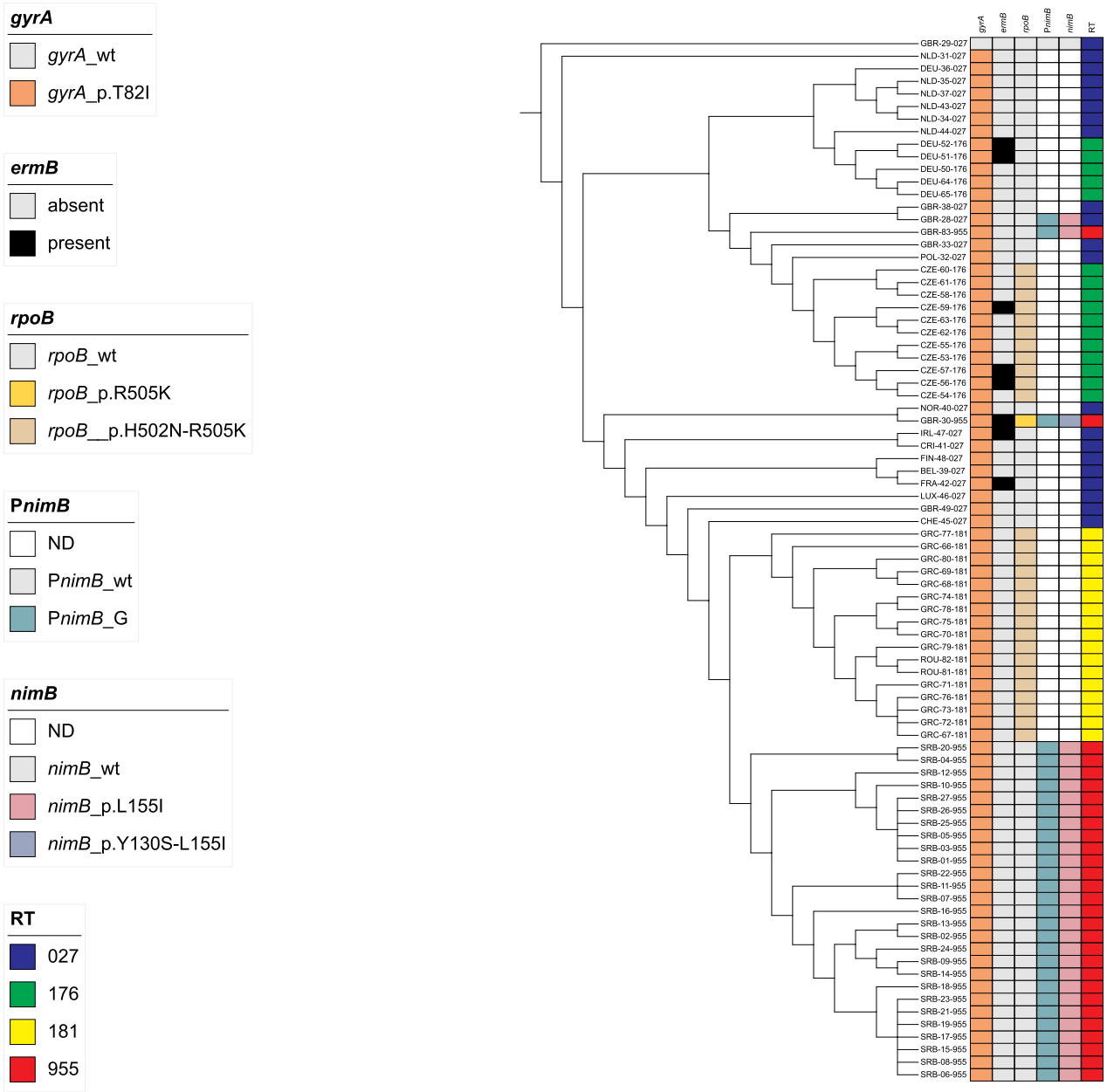

Figure 2. SNP-based phylogenetic tree of selected ST1 isolate. A SNP tree was generated based on 1833 SNPs in CSIPhylogeny 1.4 and visualized in iTOL v 6.9.2 [9]. Strain IDs include country of origin (first three letters per alpha-3 code), see appendix Table 1. Historic and epidemic reference strains for RT027 (CD196 – GBR-29-027 and R20291 – GBR-028-027) are included, and CD196 was set as the root of the tree. Abbreviations: ND; not determined, *gyrA*; DNA gyrase A gene, *ermB*: erythromycin resistance gene, *rpoB*; RNA polymerase B gene, P*nimB*; promotor of nitroreductase gene, *nimB*; nitroreductase gene, RT: PCR ribotype.

sustained transmission and multiple independent importations. In addition, infection prevention measures were reinforced at different stages in different hospitals to prevent spread of COVID-19. Nevertheless, spread and clustering of CDI with RT955 were suggested by our limited epidemiological data as well as our genomic analyses. The absence of an appropriate local control group (patients with CDI caused by other PCR ribotypes) hinders the interpretation of the clinical and epidemiological data..

A few important observations on CDI due to the Serbian RT955 can be made. CDI affected mainly the elderly male patients and only 2 (7%) of 27 patients were found below the age of 50 years. CDI was mainly diagnosed as HO-CDI (85%) and always associated with previous antibiotic use. COVID-19 was reported as concomitant disease in 59% of the patients with CDI, and other well-known predisposing conditions for CDI were found in the remaining group. The high previous use of cephalosporines, fluoroquinolones, and macrolides probably reflects the use of antibiotics for presumed bacterial pulmonary infections. The clinical presentation and outcome of CDI associated with RT955 differed from the UK experiences as reported at ESCMID Global 2024 [6]. Most notably, between September 2021 and December 2023, 50

CDI cases of 48 patients were diagnosed in the UK, of which 11 died within 30 days. In the Serbian study, all-cause mortality was 7% and not attributed to CDI. Though only 11% of the Serbian patients reported a severe presentation of CDI, a treatment change from metronidazole to vancomycin was made in 50% of the patients who started with metronidazole. This may suggest a delayed response to metronidazole, potentially associated with the P*nimB*^{G} mutation, despite the fact that we did not observe metronidazole resistance under the conditions tested. Of note, elevated metronidazole MICs have been associated with clinical failure [28]. However, our study did not differentiate between treatment switches caused by therapeutic failure, adverse effects of anti-CDI therapy, or physician preference.

In summary, CDI with *C. difficile* RT955 is present in southern Serbia since 2018 with a milder clinical presentation than reported in the UK and without large outbreaks. The Serbian strain clearly differed from the UK reference strain at a genomic and phenotypic level.

Part of this work has been presented at 8th International *C. difficile* symposium at Bled, 17–19 September 2024.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Next-generation sequencing data related to this manuscript are available through GenBank (BioProject PRJNA1225735) and detailed in Supplemental Table 1.

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