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ORIGINAL ARTICLE

First genotypic characterization of toxigenic *Clostridioides difficile* in Lithuanian hospitals reveals the prevalence of the hypervirulent ribotype 027/ST1

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Abstract

Clostridioides difficile has become the leading nosocomial Gram-positive pathogen in the developed countries. In Lithuania, the national surveillance program for *C. difficile* started in 2017. Enzyme immunoassay, the real-time PCR system, and culture are used for laboratory confirmation of *C. difficile* infection in Lithuanian clinical laboratories. No reference laboratory for *C. difficile* is present in Lithuania. Fifty-eight isolates of *C. difficile* were collected in 2016 and 2017 in two hospitals using real-time PCR and culture methods. Agarose gel–based PCR ribotyping, multilocus variable number tandem repeats analysis (MLVA), and multilocus sequence typing (MLST) were used for the genotypic characterization of 28 isolates. PCR ribotyping and MLST showed that 78.6% of the tested toxigenic isolates belong to the ribotype RT027/ST1. Using MLVA, 95.5% of RT027 isolates were genetically related. MLVA revealed three clonal complexes in RT027. Six non-RT027 isolates showed four different electrophoretic profiles in PCR ribotyping and were assigned to the MLST sequence types ST2, ST13, ST54, and ST63. The highest discriminatory power showed the genotyping by MLVA. In total, 20 MLVA profiles were identified. This genotyping technique allowed to identify four groups of RT027/ST1 isolates that were indistinguishable by PCR ribotyping and MLST. Our study is the first genotypic characterization of *C. difficile* isolates in Lithuania. We observed a high prevalence of presumptive RT027 that suggests unfavorable epidemiological situation in Lithuania. Our results stress for implementation of genotyping of *C. difficile* isolates in Lithuanian surveillance.

Keywords *Clostridioides difficile* · Lithuania · MLST · MLVA · Ribotype 027 · Surveillance

Introduction

Clostridioides difficile [1] are strictly anaerobic Gram-positive endospore-forming members of the phylum *Firmicutes*. They are found in the environment as well as in the intestinal tract of humans and animals [2]. Pathogenicity of these bacteria is strongly associated with the production of thermolabile exotoxins [3]. Symptoms of *C. difficile* infection (CDI) range from diarrhea (from mild to severe) to pseudomembranous colitis, toxic megacolon, or even death [2–4]. These bacteria became the leading nosocomial Gram-positive pathogens in the developed countries [2]. It was determined that ~48% of healthcare institutions associated gastrointestinal infections in acute care hospitals in Europe with *C. difficile* [2, 5].

Characterization of *C. difficile* isolates by molecular methods is an important part of CDI surveillance [6]. Currently, PCR ribotyping—both agarose gel based and capillary—is the most common method used for genotyping of *C. difficile* isolates in Europe [5, 7]. Schemes for multilocus variable number tandem repeats analysis (MLVA) [8], multilocus sequence typing (MLST) [9], whole genome sequencing [10], and toxinotyping [11] were also developed and are commonly used.

National surveillance program for *C. difficile* in Lithuania started in 2017. Currently, no reference laboratory for *C. difficile* is present in Lithuania. Enzyme immunoassay, the real-time PCR system, and culture are used for laboratory confirmation of *C. difficile* infection in Lithuanian clinical laboratories. Neither ribotyping nor other genotyping methods were used in Lithuania for the epidemiology of this important

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pathogen until now [5, 6]. Here, we report the first genotypic characterization of *C. difficile* isolates collected in 2016 and 2017 in two Lithuanian hospitals as well as evaluate the diagnostic strategies for the further surveillance of CDI in Lithuania.

Materials and methods

C. difficile isolate collection and identification

Unformed stool specimens from patients suspected of having CDI were analyzed during 2016 and 2017 at two Lithuanian hospitals A and B. Both hospitals are municipal acute care hospitals of medium size. The presence of genes coding for toxin B (*tcdB*) and binary toxin (*cdt*) and deletion in the *tcdC* gene were evaluated using the Xpert *C. difficile* assay (Cepheid, USA). Samples positive by Xpert assay were cultured anaerobically using a chromogenic medium chromID® *C. difficile* (bioMérieux, France) at 35 °C for 24 h. Presumptive *C. difficile* colonies were identified using MALDI-TOF MS (VITEK®MS, bioMérieux, France).

Characterization of *C. difficile* isolates

The toxigenic isolates were additionally tested for the presence of the *tcdA* gene coding for toxin A. For these experiments, genomic DNA was extracted from the vegetative cells of *C. difficile* using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). PCR-based screening for *tcdA* gene was performed in 50 μL of reaction mixture using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific). Primers and PCR conditions were as described by Griffiths et al. [9]. Products of amplifications were analyzed by electrophoresis through 1% agarose gel.

Ribotyping, MLVA, and MLST

Genomic DNA for ribotyping, MLVA, and MLST was extracted from the vegetative cells as described above. Primers and PCR conditions for standard PCR ribotyping were chosen according to the previously published method [12, 13]. Primers and PCR conditions for MLVA were selected according to van den Berg et al. [8]. PCR reaction mixtures for ribotyping, MLVA, and MLST were identical to that described above for *tcdA*-PCR. Products of PCR ribotyping and MLVA were analyzed by electrophoresis through 3% high-resolution MetaPhor™ agarose (Lonza, Switzerland) gel. In MLVA, the number of tandem repeats in each of the seven loci was calculated manually based on the size of the PCR products. For calculations, the genome of *C. difficile* strain 630 was used according to van den Berg et al. [8]. The size of the PCR products was the sum of the primers, tandem repeats, and the sequences between the primers and tandem repeats. A few different PCR products were sequenced at the DNA Sequencing Centre (Vilnius University, Life Sciences Center, Lithuania) in order to confirm the correctness of the calculations. MLVA profile for each toxigenic *C. difficile* isolate was defined by the number of repeats in all seven loci. MLVA profiles were compared using the web server DendroUPGMA [14]. Similarity index was calculated using the Pearson correlation coefficient. The dendrogram was constructed by the unweighted-pair group method with arithmetic mean (UPGMA) grouping method. A minimum spanning tree based on MLVA profiles was constructed in BioNumerics 7.6 by a temporary BioNumerics evaluation license from Applied Maths (Belgium) using the categorical coefficient. The permission to publish these results was received from Applied Maths. A clonal cluster was defined by ≤ 2 repeat differences, and genetically related isolates were defined by ≤ 10 repeat differences [8].

Toxigenic isolates with at least one difference in MLVA profile were selected for MLST. MLST was performed according to Griffiths et al. [9]. Seven housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi*) were amplified; PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and sequenced at the DNA Sequencing Centre (Vilnius University, Life Sciences Center, Lithuania). Alleles of the genes, sequence types (ST), and MLST clades were determined using an official website (https://pubmlst.org/cdifficile/).

Results

In total, 360 stool specimens from patients suspected of having CDI were obtained from two Lithuanian hospitals (119 samples from hospital A and 241 samples from hospital B) in 2016 and 2017. The CDI incidence density (cases/10,000 patient days) was 2.7 in 2016 and 2.73 in 2017 for hospital A and 0.43 in 2016 and 2.78 in 2017 for hospital B. Out of 360 samples, 16% ($n = 58$) were positive by Xpert *C. difficile* assay. All Xpert-positive samples were positive for the presence of *tcdB* gene (toxin B), 81% ($n = 47$) of all positive samples also for *cdtA* (binary toxin), and 78% ($n = 45$) of the samples were positive for the presence of a single nucleotide deletion at nucleotide 117 in the *tcdC* gene. Samples positive for all three targets ($n = 45$) were assigned to the presumptive PCR ribotype 027. Subsequent *C. difficile* culture was positive for 58 Xpert-positive samples.

For screening for *tcdA* gene, 32 toxigenic *C. difficile* isolates have been chosen. Analysis of *tcdA*-PCR products showed that 28 isolates (87.5% of the toxigenic isolates) were positive for the gene of toxin A while four isolates were negative. The latter four isolates were excluded from further analysis.

PCR ribotyping was used to confirm results of the Xpert *C. difficile* assay. Isolates with deletion in the *tcdC* gene were assigned to the presumptive ribotype RT027 in the latter assay. Six toxigenic isolates were not assigned to this ribotype (Table 1). PCR ribotyping revealed identical electrophoretic profiles for previously identified presumptive RT027 isolates, while non-RT027 isolates fell into four electrophoretic clusters designated from U1 to U4 (Fig. 1).

All ($n = 28$) *tcdA*-positive isolates were subjected to MLVA analysis. In total, 20 MLVA profiles were identified (Table 1). Four MLVA profiles were represented by either 2 or 4 completely identical isolates (Fig. 2). Usually, such isolates came from the same hospital, the only exception being isolates 1958, 2719, 5113 (all three from hospital B), and 9629 (hospital A). Three clonal complexes (the summed tandem repeat difference ≤ 2) were determined: 5049-9785, 8044-8116-9133, and 1716-1958-2719-5113-9629-5951-6013 (Fig. 2). Ribotype RT027 isolates showed conservative markers E7*Cd*, F3*Cd*, and H9*Cd*, except isolates 2654 and 3927 that had 9 and 8 repeats for marker E7*Cd*. On the other hand, an identical number of repeats for markers E7*Cd*, F3*Cd*, and H9*Cd* (10, 5, and 2 respectively) were also detected for non-RT027 isolate 3256.

All but one RT027 isolates clustered together in UPGMA dendrogram (Fig. 2). The only exception was isolate 8202 that had an unusual number of repeats for the marker C6*Cd*—16 vs. 30–34 copies in the other RT027 isolates. It should be noted that all RT027 isolates (except isolate 8202) were genetically related (the summed tandem repeat difference ≤ 10) in the minimum spanning tree.

The toxigenic isolates with the different MLVA profiles were subjected to MLST. Out of 16 isolates, 5 were assigned to clade

Table 1 Molecular characteristics of Lithuanian *C. difficile* isolates

Isolate	Hospital	Toxin profile	Ribotype		MLVA profile							ST (clade)
			Xpert assay	PCR ribotyping	A6*Cd*	B7*Cd*	C6*Cd*	E7*Cd*	F3*Cd*	G8*Cd*	H9*Cd*	
880	A	*tcdA*, *tcdB*	Unknown	U1	28	13	32	8	5	7	4	ST54 (1)
1106	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	34	10	5	14	2	
1716	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	28	18	34	10	5	16	2	ST1 (2)
1781	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	34	10	5	14	2	
1958	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	34	10	5	16	2	
2291	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	34	10	5	14	2	ST1 (2)
2654	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	34	9	5	13	2	ST1 (2)
2719	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	34	10	5	16	2	ST1 (2)
3256	B	*tcdA*, *tcdB*, *cdt*	Unknown	U2	11	28	34	10	5	16	2	ST13 (1)
3927	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	16	32	8	5	10	2	ST1 (2)
4270	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	28	18	31	10	5	14	2	ST1 (2)
4278	A	*tcdA*, *tcdB*	Unknown	U1	24	18	16	6	5	9	2	ST63 (1)
4321	A	*tcdA*, *tcdB*	Unknown	U3	14	11	30	6	5	10	2	ST2 (1)
5049	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	28	16	30	10	5	14	2	ST1 (2)
5113	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	34	10	5	16	2	
5951	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	32	10	5	16	2	ST1 (2)
6013	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	32	10	5	16	2	
6181	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	34	10	5	14	2	
8044	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	30	10	5	14	2	ST1 (2)
8116	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	30	10	5	14	2	
8202	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	16	10	5	14	2	ST1 (2)
9133	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	24	18	30	10	5	14	2	
9629	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	26	18	34	10	5	16	2	
9670	B	*tcdA*, *tcdB*	Unknown	U3	33	21	34	6	5	7	2	ST2 (1)
9785	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	28	16	32	10	5	14	2	ST1 (2)
10045	B	*tcdA*, *tcdB*, *cdt*	RT027	RT027	31	17	34	10	5	13	2	
10244	B	*tcdA*, *tcdB*	Unknown	U4	23	13	23	6	5	5	2	ND
11407	A	*tcdA*, *tcdB*, *cdt*	RT027	RT027	23	17	32	10	5	14	2	

ND not determined

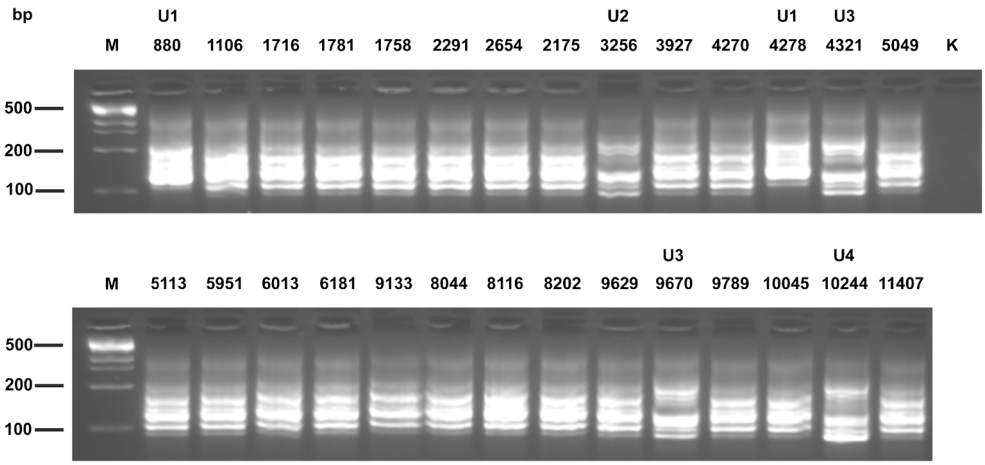


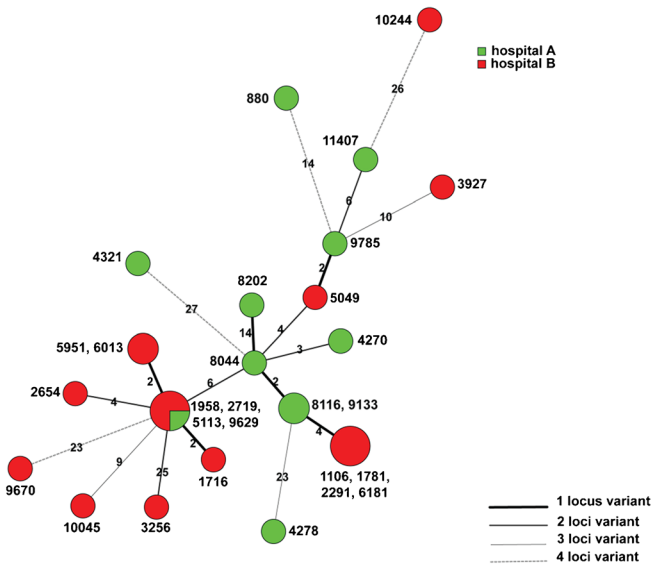
Fig. 1 Standard PCR ribotyping of toxigenic *C. difficile* isolates. M, *Thermo Scientific* GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific); K, PCR control without genomic DNA. Numbers above lanes indicate the numbers of the isolates. U1–U4, unidentified (non-RT027) ribotypes. The isolates without U above their numbers represent RT027 ribotype

1, while others were found to belong to clade 2. All RT027 isolates were assigned to the MLST sequence type ST1 (Table 1).

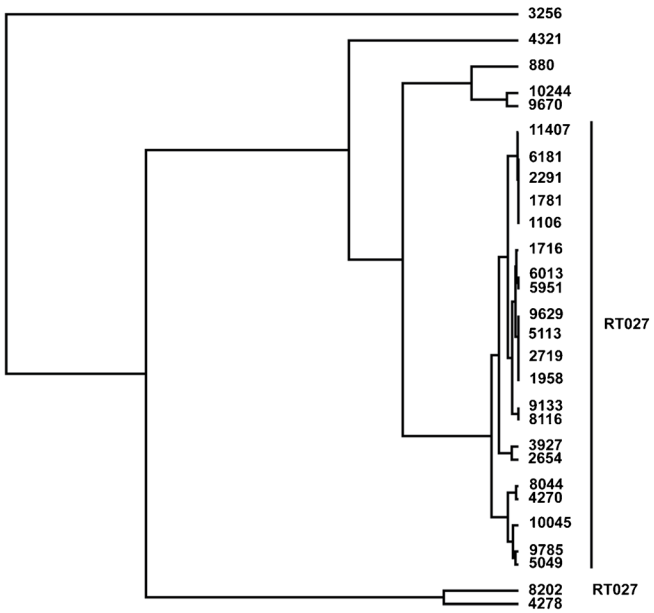
Toxigenic non-RT027 isolates 880 and 4278 were assigned to the sequence types ST54 and ST63 respectively. It should be noted that these two isolates had identical electrophoretic profiles in PCR ribotyping (Fig. 1), but completely different MLVA profiles. The other two non-RT027 isolates (4321 and 9670) with identical electrophoretic profiles in PCR ribotyping (Fig. 1) but with genetically distinct MLVA profiles were assigned to a single sequence type—ST2 (Table 1). Non-RT027 isolate 3256 with the toxin profile *tcdA tcdB cdt* and with the unique electrophoretic ribotyping profile was assigned to ST13 (Fig. 1; Table 1).

Discussion

During the last decades, *C. difficile* has become the prevalent Gram-positive pathogen in healthcare institutions of the developed world. In European countries, the strategy of the national surveillance of this pathogen, diagnostic procedures, and attention given to CDI differ [6, 15]. The European multicentre, prospective, biannual, point-prevalence study of CDI in hospitalized patients with diarrhea (EUCLID) highlighted an increased diversity of *C. difficile* ribotypes across Europe, with considerable intercountry variation in ribotype distribution [16]. The findings of this study emphasized the importance of continuous national and European



a



b

Fig. 2 Analysis of MLVA profiles of toxigenic *C. difficile* isolates. **a** Minimum spanning tree analysis of MLVA profiles. The circles represent unique MLVA profiles. The numbers between the circles represent the summed tandem repeat difference between the MLVA types. **b** UPGMA analysis based on MLVA profiles. RT027. isolates that were assigned to RT027 based on both Xpert and standard PCR ribotyping results

surveillance programs to monitor the changing epidemiology of *C. difficile*.

In 2013, experts from the European CDI Surveillance Network and from the European Centre for Disease Prevention and Control developed a protocol with three options of CDI surveillance for acute care hospitals: a "minimal" option (aggregated hospital data), a "light" option (including patient data for CDI cases), and an "enhanced" option (including microbiological data) [5, 17]. In Lithuania, the national surveillance program, where basic case-based epidemiological data (e.g., age, sex, date of hospital admission and of CDI onset, CDI origin, recurrent CDI, CDI diagnostic algorithm) were included in all CDI cases, started in 2017. According to the latest surveillance program report, CDI diagnostic algorithms significantly vary between different Lithuanian clinical microbiological laboratories [18]. Questionnaires on the local diagnostic capacity for CDI were completed by 27 Lithuanian hospitals, and it was revealed that only less than half (13/27) of the laboratories use 2-step algorithms recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [19]. Out of 27 laboratories, 8 responded using nucleic acid amplification test (NAAT) as a screening test and EIA toxins detection as a confirmatory test and 5 laboratories use GDH EIA and toxins detection tests as well as NAAT or toxigenic culture for confirmation [18]. More than half of the laboratories (14/27) use other diagnostic algorithms that according to the ESCMID recommendations are classified as incomplete [5]. Diversity of the diagnostic algorithms showed that there is no consensus established on how to test CDI among Lithuanian local laboratories. The current work is a pilot study of *C. difficile* to evaluate the situation in Lithuanian hospitals, to test the presence/absence of the hypervirulent ribotypes as well as to compare available methods in order to decide on the further diagnostic procedures.

C. difficile ribotype determination is one of the main microbiological characteristics in CDI surveillance. Both conventional agarose gel–based and capillary gel electrophoresis–based PCR ribotyping can be used according to the surveillance protocol of ESCMID [17]. Currently, Lithuania is among those few European countries that do not have any ribotyping [6]. In 2018, capillary gel electrophoresis–based PCR ribotyping was used for only 1.9% of all tested samples. The Xpert *C. difficile* assay but not PCR ribotyping is usually used for the identification of RT027 [18]. Therefore, the implementation of PCR ribotyping in Lithuanian surveillance is urgently needed. In our study, agarose gel–based PCR ribotyping was used. This method is more suitable for Lithuanian clinical laboratories that are usually not equipped with capillary electrophoresis instruments. Our PCR ribotyping results confirmed those of the Xpert assay—both methods assigned the same isolates to the ribotype RT027. In addition, our results showed that RT027 isolates were the most prevalent (78.6%) among all toxigenic isolates in two Lithuanian hospitals. This ribotype was previously reported to be the most prevalent in Poland, Hungary, and Romania, although it is not the most prevalent in European hospitals in general [15, 16]. Agarose gel–based ribotyping did not allow to determine PCR ribotypes of the non-RT027 isolates because it was impossible to predict the particular ribotypes before the experiments and consequently use the respective controls. On the other hand, agarose gel–based ribotyping allowed to distinguish between the different electrophoretic profiles/ribotypes that were further subjected to MLVA and MLST.

Molecular typing methods are recommended for characterization and comparison of the circulating *C. difficile* isolates [4]. These methods are also valuable for the identification of the outbreak isolates [19]. We used MLVA and MLST in our study. MLVA is considered to be one of the best methods for *C. difficile* genotyping because of the high discriminatory power [4]. MLVA revealed 20 different MLVA profiles among 28 toxigenic isolates. For RT027, four groups of the completely identical isolates were identified by MLVA. As MLVA is considered to be a strain-specific genotyping method [20], these four groups, actually, represent four genetically related strains. Three of these strains/groups of isolates were isolated from the stool specimens of the different patients in the same hospital—either A or B. Therefore, these strains/groups of isolates were supposed to be nosocomial strains involved in the intrahospital transmission. The fourth strain/group of isolates was identified in the specimens of the patients from both hospitals. Although the intrahospital spread of the latter strain was supposed to occur in hospital B, this strain could be also involved in the interhospital transmission. On the other hand, we cannot completely rule out the possibility that this strain could be a community-acquired strain.

Toxigenic sequence types ST2, ST13, ST54, and ST63 from MLST clade 1 were also detected in the examined Lithuanian hospitals. ST2 was previously found to be dominant in both asymptomatic and symptomatic infants in Oxfordshire, UK [21], and the most prevalent sequence type in CDI patients from hospitals in Australia and China [22, 23], among the clinical isolates in the UK [24] as well as in piglets in Australia [22]. Nontoxigenic ST13 isolates were reported to be among the most common sequence types in infant's stool in France [25]; toxigenic isolates of this sequence type were also identified in CDI patients and piglets [22]. It should be noted that both ST2 and ST13 are MLST sequence types of the ribotype RT014, one of the most common ribotypes causing CDI in developed countries, that was previously supposed to be community-acquired, food chain–associated ribotype [21, 22, 25]. As only a few genetically distinct strains of ST2 and ST13 were identified in two Lithuanian hospitals, we concluded that these strains are community-acquired but not nosocomial. The same conclusion was drawn for the strains of the sequence types ST54 and ST63. Although both non-epidemic

and outbreak-causing strains of ST54 were identified previously [21, 26–28], and ST63 isolates were previously detected among the clinical isolates [24], ST54 isolates were among the most common sequence types in community-acquired CDI in the study of Liao et al. [29]. The percentage of the seemingly community-acquired *C. difficile* strains in our study (21.4%) correlates with the previously reported percentage of the community-acquired CDI isolates (20–27% of all CDI cases) in Europe and North America [29].

Although we have achieved our objective to characterize toxigenic *C. difficile* isolates from two Lithuanian hospitals and to compare the available genotyping methods for further diagnostic procedures in our country, some limitations of our work should be pointed out. Firstly, we studied a small collection of the toxigenic non-RT027 isolates, and the discrepancies between the MLVA and the other two genotyping methods could be caused namely by the small number of these isolates. Secondly, the toxigenic *C. difficile* isolates from the patients of the only two hospitals were examined and at least some tendencies (for example, the percentage of the different toxigenic ribotypes, MLST sequence types) could differ in the other hospitals of Lithuania. Therefore, more hospitals from the different Lithuanian cities should be included in the characterization of the toxigenic *C. difficile* isolates in the future.

Our study also revealed limitations of the current Lithuanian *C. difficile* surveillance. Molecular typing methods are not used in the current Lithuanian surveillance in contrast with most European countries [6]. The most frequently used Xpert *C. difficile* assay does not allow to identify other non-RT027 ribotypes/sequence types. Besides, the results of the Xpert assay should be interpreted with caution as misclassifications of RT027 using this assay were previously reported [30, 31]. Diversity of the ribotypes including those with the epidemic potential increases in Europe [16], but it will be impossible to monitor them in Lithuania without the appropriate molecular methods. To begin, we suggest to start from the agarose gel–based ribotyping which should be changed to the capillary gel electrophoresis–based ribotyping in the future because of the higher discriminatory power of the latter method [6]. We successfully applied MLVA and MLST for the characterization of *C. difficile* isolates, and the obtained results showed that it would be reasonable to use these methods in Lithuanian *C. difficile* surveillance. But it is recommended to perform genotyping experiments at a national reference laboratory [6], and this is another serious problem in Lithuanian *C. difficile* surveillance—there is no reference laboratory in Lithuania. It is evident that the national laboratory should be set up for better *C. difficile* surveillance.

In conclusion, genotypic characterization of the toxigenic *C. difficile* isolates from Lithuanian hospitals revealed the prevalence of the ribotype RT027/ST1, and the current work is the first record on this ribotype in Lithuania. Four different non-RT027 electrophoretic profiles were identified through the agarose gel–based PCR ribotyping that were assigned to MLST sequence types ST2, ST13, ST54, and ST63 from clade 1. Our results clearly showed that molecular typing methods should be urgently included in Lithuanian *C. difficile* surveillance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval For this type of study formal consent is not required.

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