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Clostridioides difficile Ribotype 027 (RT027) Outbreak Investigation Due to the Emergence of Rifampicin Resistance Using Multilocus Variable-Number Tandem Repeat Analysis (MLVA)

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Objective: The aim of this study was *Clostridioides difficile* outbreak investigation due to the emergence of rifampicin resistant ribotype 027 (RT 027) fecal isolates from patients of Polish tertiary care hospital between X. 2017 and II. 2018 using multilocus variable tandem repeat analysis (MLVA).

Materials and Methods: Twenty-nine *C. difficile* fecal isolates from patients of tertiary care hospital in Southern Poland were ribotyped and analyzed by MLVA. Multiplex PCR (mPCR) for genes encoding GDH (*gluD*), toxins A (*tcdA*) B (*tcdB*), 16S rDNA and binary toxin genes (*ctdA* and *ctdB*) was performed. The antibiotic susceptibility profile was determined by E-test.

Results: The A, B and binary toxins encoding genes were detected in all 29 *C. difficile* strains which were sensitive to metronidazole, vancomycin and were resistant to erythromycin, clindamycin, and moxifloxacin; resistance to imipenem demonstrated 97%, to rifampicin – 45% isolates. *C. difficile* strains could be grouped by MLVA into 5 distinct clusters, and the largest cluster II contains 16 strains. The comparison of rifampicin GM MIC of cluster II (n=16 strains) with all others (n=13) showed that strains from clusters I, III, IV and V possessed significantly ($p < 0.005$) higher GM MIC and were more resistant to rifampicin.

Conclusion: MLVA analysis proved transmission and recognized outbreak due to multidrug-resistant RT 027 *C. difficile* among patients of tertiary care hospital in Southern Poland. The reason for this is probably the widespread occurrence of spores in the hospital environment, which includes, among others, neglect of hygienic procedures and epidemic supervision. High resistance to imipenem (97%) and to rifampicin (45%) among *C. difficile* RT 027 Silesian isolates is threatening and requires further studies to elucidate this phenomenon.

Keywords: *Clostridioides difficile*, epidemiology, MLVA, RT 027, rifampicin resistance

Introduction

Clostridioides difficile is responsible for 15% of all health care-associated infections (HAI) in the USA and is the main etiologic factor of epidemic outbreaks in Poland.^{1,2} An ECDC (European Centre of Disease Control) report from 2018 stated that 74.6% *C. difficile* infection (CDI) cases were HAI.³

C. difficile is a Gram-positive, anaerobic, sporulating bacterium. Infections caused by *C. difficile* range from the milder forms of antibiotic-associated diarrhea to the more severe pseudomembranous colitis, toxic megacolon and sepsis. The

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symptoms of CDI correlated with production of toxins: A - enterotoxin, B - cytotoxin and binary toxin (~20% of strains). Infections with the *C. difficile* hypervirulent strain RT 027 have a more dramatic course and are associated with high mortality.⁴ The main reason for the development of CDI is the use of antibiotics, especially broad-spectrum (fluoroquinolones, clindamycin, III generation cephalosporins, penicillin), older age (> 65) and long-term hospitalization.⁵ *C. difficile* spores are resistant to many factors (alcohol - present in disinfectants, UV radiation, gamma radiation, etc.).⁶ *C. difficile* spores, after being released by infected patients, are the main cause of the CDI spreading among hospitalized patients.⁷ To recognize hospital transmission of *C. difficile* and outbreak the multi-locus variable number tandem repeat analysis (MLVA) technique is proposed. The MLVA technique allows the performance of simultaneous analysis of several loci during one PCR reaction. This is very important for an epidemiological point of view for analyzing outbreaks in hospitals as well as comparing and characterizing the strains causing infections in a given area.^{8,9} According to Eyre et al¹⁰ overall findings using MLVA and whole genome sequencing (WGS) were very similar despite the fact that Authors analyzed different parts of the bacterial genome. The MLVA technique and WGS have similar performances, in our setting MLVA technique is preferred.

The aim of this study was *C. difficile* outbreak investigation due to the emergence of rifampicin resistant ribotype 027 (RT 027) fecal isolates from patients of Polish tertiary care hospital between X. 2017 and II. 2018 using MLVA, since in our previous study the dominance of multidrug-resistant *C. difficile* RT 027 strains in Silesia, Southern Poland was described.⁵

Materials and Methods

Twenty nine *C. difficile* strains belonging to the PCR RT 027, cultured from the stool of patients with post-antibiotic diarrhea, hospitalized in the tertiary care hospital of Silesia between October 2017 and February 2018 were analyzed. All cases meet the conditions for CDI according to the ECDC definition.¹¹ This study was conducted in accordance with Good Clinical and Laboratory Practice Guidelines and the Declaration of Helsinki. The protocols were approved by the Ethical Committee of Medical University of Silesia in Katowice, Poland (KNW/0022/KB/127/I/12, KNW/0022/KB/252/18). Permission to use patient-specific materials and medical data for analysis

was obtained from patients (all patients provided written informed consent).

Stool samples were studied according to multistep algorithm, in direction of glutamate dehydrogenase (GDH) antigen and *C. difficile* A/B toxins by TechLab C diff Quick Check Complete (TechLab, Blacksburg, USA). All positive samples were cultured using CDIF-chromID and CLO selective media (bioMérieux, Marcy L'Etoile, France) and identified by using the VITEK2 Compact System (bioMérieux, Marcy L'Etoile, France).⁵

Multiplex PCR (mPCR) was performed for detection of genes encoding GDH (*gluD*), toxin A (*tcdA*) and toxin B (*tcdB*) and bacterial 16S rDNA in *C. difficile* isolates.¹² Binary toxin genes (*ctdA* /*ctdB*) were detected according to Stubbs et al¹³ using HotStarTaq Plus PCR Master Mix Kit (QIAGEN, Germany). After isolation of DNA (QIAamp DNA Mini Kit) from a 24 h *C. difficile* BHI (bioMérieux, Marcy L'Etoile, France) culture, PCR was performed in a GeneAmp PCR System 9700 thermocycler (Thermo Fisher Scientific, USA) with a volume of 25 μ L, containing approximately 25 ng of template DNA. Results of electrophoresis in a 2% agarose gel were visualized in apparatus G: BOX F3 (Syngene, UK). To determine the presence of gene (*ermB*) encoding *C. difficile* resistance mechanisms MLS_B, a PCR was performed.⁵

MLVA analysis and ribotyping were performed at the Department of Microbiology Leiden University Medical Center in the Netherlands, according to van den Berg et al and Fawley et al, respectively, using the primers presented in Table 1.^{14,15} There are 7 regions within the genome of *C. difficile*, recognized by MLVA, designated as A6_{Cd}, B7_{Cd}, C6_{Cd}, E7_{Cd}, F3_{Cd}, G8_{Cd} and H9_{Cd}.^{14,16} The Minimum Spanning Tree (MST) was based on summed tandem repeat differences (STRD) between studied strains. If the STRD number was ≤ 2 (clonal complexes), consideration that "direct transmission is very likely" was made. Also genetically related complexes with a STDR number ≤ 10 were considered as "transmission is likely."

The antibiotic susceptibility of the isolated strains was determined by E-test (bioMérieux, Marcy L'Etoile, France) for 7 antibiotics: metronidazole, vancomycin, moxifloxacin, erythromycin, clindamycin, imipenem and rifampicin. Plates were incubated at 37°C for 48 h in an anaerobic chamber (Whitley A35 Workstation, UK) according to the manufacturer's instructions.¹⁷ The antibiotic susceptibility results were interpreted in accordance with the recommendations of EUCAST (European Committee on Antimicrobial Susceptibility Testing, Version 11.0, valid from 2021-01-

Table 1 Primers, Detecting the Appropriate *C. difficile* Regions in MLVA¹⁴

Region	Forward Primer (5'→3')	Reverse Primer (3'→5')
A6 _{Cd}	FAM-TTAATTGAGGGAGAATGTAAAA	AAATACTTTTCCCACCTTTCATAA
B7 _{Cd}	FAM-CTTAATACTAAACTAACTCTAACCCAGTAA	TTATATTTTATGGGCATGTAAAA
C6 _{Cd}	HEX- GTTTAGAATCTACAGCATTATTGTA	ATTGGAATTGAATGTAACAAAA
E7 _{Cd}	FAM-TGGAGCTATGGAAATTGATAA	CAAATACATCTTGCAATAATTCTT
F3 _{Cd}	HEX-TTTTTGAAACTGAACCAACATA	ACAAAAGACTGTGCAAATATACTAA
G8 _{Cd}	NED-TGTATGAAGCAAGCTTTTTATT	AATCCAGCAATCTAATAATCCA
H9 _{Cd}	VIC-GTTTTGAGGAAACAAACCTATC	GATGAGGAAATAGAAGAGTTCAA

01) for Gram positive anaerobes and *C. difficile*; for erythromycin breakpoints CLSI criteria were used.¹⁸

The statistical analysis was carried out with the Mann-Whitney *U*-test (StatSoft Polska Sp. z o.o. 2021 www.statsoft.pl). Differences were considered as statistically significant for *p*-values < 0.05.

Results

In studied Polish tertiary care hospital the evidence of the endemic rates of CDI was 6.4 cases per 10.000 patient/days. 29 *C. difficile* strains were cultured from the feces of patients with antibiotic-associated diarrhea, hospitalized in a 504-bed clinical hospital in 4 departments: Internal Medicine, Urology, General Surgery and Nephrology in the period October 2017–February 2018. Table 2 demonstrates a list of the *C. difficile* strains cultured along with the place, date of stool sampling and patients age.

The toxins A (*tcdA*), B (*tcdB*), and binary toxin encoding genes (*ctdA* /*ctdB*) were detected in all studied 29 strains, belonging to RT 027.

The minimum spanning tree (MST, Figure 1) shows the strains grouped according to their genetic sequence similarity – all strains in one circle are 100% clonally identical (the same STRD in the 7 studied *loci*).

The one such cluster includes strains no: 296, 297, 299, 305, 309 and 313 derived from patients hospitalized the Internal Medicine Ward. Strains no. 296, 297, 299 were cultured from patients' fecal samples collected at a 3-day time interval in November 2017 (on November 16th, 19th and 21st, respectively), indicating that an outbreak caused by the same *C. difficile* clone occurred.

The next two samples no. 305, 309 were collected in December 2017 (December 3rd and 17th), but sample no. 313 was collected on February 2nd 2018, suggesting that from November to February *C. difficile* spores persisted in the hospital environment.

The next clonally identical *C. difficile* strains no. 284 and 286 were cultured from patients hospitalized in the Internal Medicine Ward (October 25th and 27th, 2017), as well as no. 303 and 310 cultured from the Nephrology Ward patients (feces collected on December 4th and 24th, 2017). In Figure 1 the 5 clonal groups can be distinguished (see also Table 2).

Cluster I contains isolates no. 312 and 314 from patients of Nephrology Ward (feces collected at January 1st, 2018 and December 2nd, 2017). Both isolates demonstrated rifampicin resistance (100%).

The largest cluster II contains 16 strains no. 283, 284, 285, 286, 289, 296, 297, 299, 300, 304, 305, 306, 309, 313–14 strains from the Internal Medicine; and one strain each from the Urology (291) and Nephrology (302) Wards, rifampicin resistance (6.3%).

Cluster III contains 7 *C. difficile* strains, 3 (294, 288, 295) from patients of the Urology Ward, 3 (303, 310, 290) from patients of the Nephrology, and 1 (293) from patient of Internal Medicine, rifampicin resistance (85.7%).

Clusters IV and V contain 2 *C. difficile* strains (307, 308) from Internal Medicine patients and 2 strains (no. 292 and 287) from General Surgery patients, respectively. All 4 isolates were rifampicin resistant (100%).

Strains no. 286 and 293 were isolated from feces of the same patient hospitalized in the Internal Medicine (stools collected on October 27th, 2017 and November 13th, 2017). The MLVA shows that the strains belong to two different clonal clusters, the II and III, suggesting that this is not a reinfection but a new infection.

Stools no. 305 and 306 were obtained from another patient hospitalized in the Internal Medicine. Both strains belong to the same clonal group (II), with strain 305 being 100% genetically identical to the other 5 strains, suggesting that this patient initially acquired a nosocomial infection and then got reinfected.

Table 2 Sources, Isolation Time and Age of Patients Infected with *C. difficile* RT 027 Strains Divided by MLVA into 5 Clusters

Clusters	<i>C. difficile</i> Strain Numer	Patient's Age	Date of Stool Collection	Departments
I	312	79	12.02.2018	Nephrology
	314	57	11.01.2018	Nephrology
II	296	79	16.11.2017	Internal
	297	65	19.11.2017	Internal
	299	83	21.11.2017	Internal
	305#	65	05.12.2017	Internal
	309	56	17.12.2017	Internal
	313	81	03.02.2018	Internal
	284	91	25.10.2017	Internal
	286*	87	27.10.2017	Internal
	285	87	25.10.2017	Internal
	306#	65	11.12.2017	Internal
	302	87	02.12.2017	Nephrology
	291	80	05.11.2017	Urology
	283	88	24.10.2017	Internal
	304	88	05.12.2017	Internal
	300	84	22.11.2017	Internal
289	33	30.10.2017	Internal	
III	288	19	30.10.2017	Urology
	293*	87	13.11.2017	Internal
	294	81	14.11.2017	Urology
	295	71	15.11.2017	Urology
	290	76	31.10.2017	Nephrology
	303	50	04.12.2017	Nephrology
	310	82	24.12.2017	Nephrology
IV	307	80	11.12.2017	Internal
	308	90	16.12.2017	Internal
V	287	72	27.10.2017	General Surgery
	292	72	13.11.2017	General Surgery

Notes: Strains no *286 and *293 came from the same patient, similarly no #305 and #306.

Among the examined strains, all were sensitive to metronidazole (range 0.016–256 µg/mL) and vancomycin (0.016–256 µg/mL). All strains demonstrated high level resistance to erythromycin (0.016–256 µg/mL), clindamycin (0.016–256 µg/mL) (also confirmed by *ermB* gene) and moxifloxacin (0.002–32 µg/mL). Resistance to imipenem (0.002–32 µg/mL) was demonstrated by nearly 97% of the strains, and to rifampicin (0.002–32 µg/mL) by almost 45% (13/29) (Table 3). The comparison of rifampicin GM MIC (GM MIC – the geometric mean for MIC = 0.0037) between the 16 strains from the cluster II with all other strains from clusters I, III, IV, V (GM MIC = 15.2) showed that *C. difficile* strains from clusters I, III, IV, V were significantly ($p < 0.005$) more resistant to rifampicin.

Discussion

According to CDI surveillance in 37 European acute care hospitals in 2013, medium incidence rate of HA-CDI in Poland was 7.6 (7.0–8.2), and distribution of RT 027 dominates among 16 identified ribotypes.¹⁹ The increase in CDI cases caused by the hyperepidemic RT 027 strain was observed in Canada and the USA, and also in Europe.²⁰ Now *C. difficile* RT 027 is dominant in Eastern Europe, including Poland. This is evidenced by the analysis carried out in 2012–13.²¹ In Eastern Europe, the dominance of RT 027 was found at the level of 35%, while in Western Europe it was below 20%, in Southern Europe it was below 10% and in Northern Europe the presence of RT 027 was not found at all. Compared to a similar analysis from 2008 for 34 participating countries, the presence of 64 ribotypes was found: RT

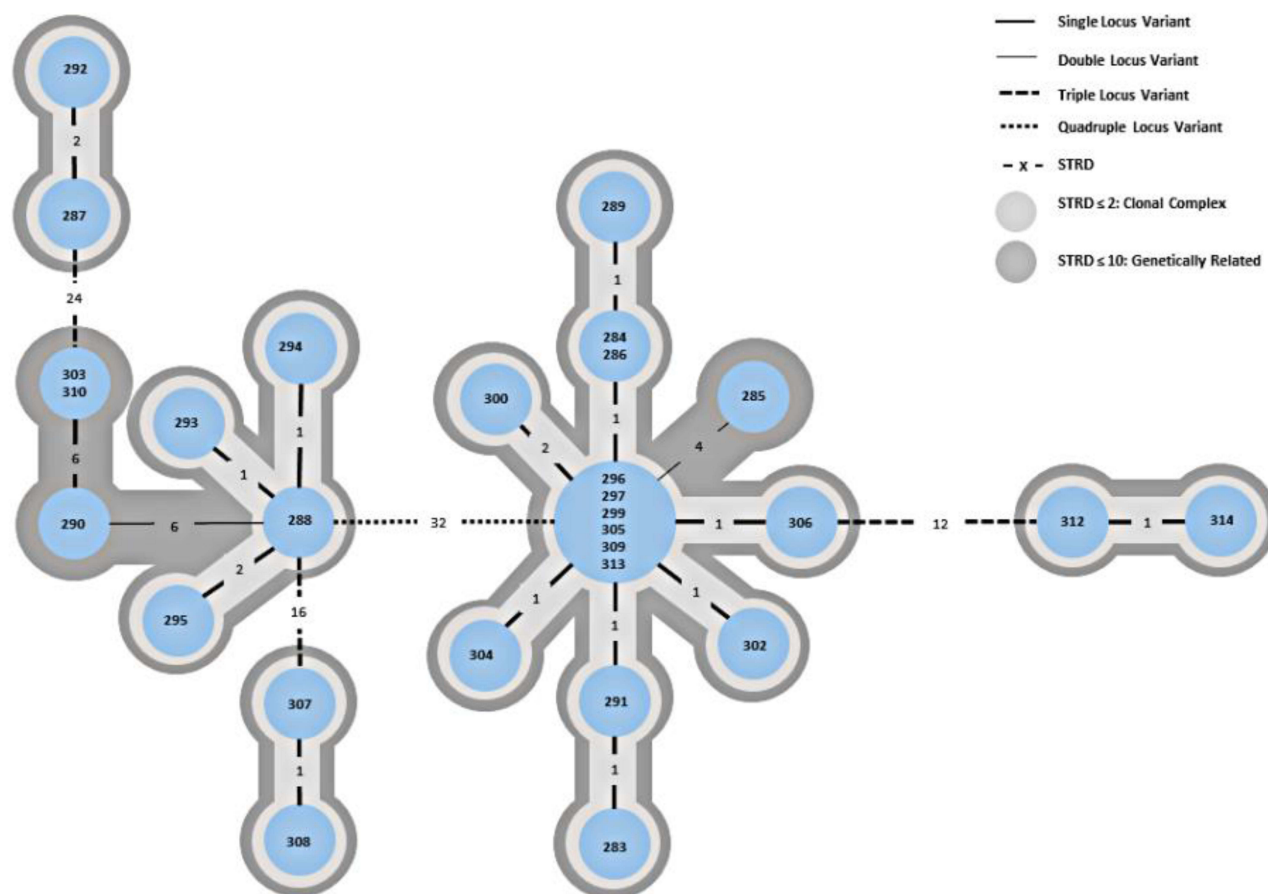


Figure 1 Minimum spanning tree (MST) of isolates from a hospital outbreak of *C. difficile* in Silesia, Southern Poland by MLVA. Each circle represents either an unique isolate or more isolates that are 100% homologous. The numbers between the circles indicate the summed tandem repeat difference (STRD) on a certain number of alleles (specified by the line style). Within the spanning tree, clonal complexes with a STRD ≤ 2 are marked in light grey (direct transmission very likely) and genetically related complexes with a STRD ≤ 10 are marked in dark grey (transmission likely).

014/020 (16%) was dominant, and RT 027 accounted for only 5% of the strains tested.²² However, more recent analyses by Popescu et al in Romania and Korać et al in Serbia also indicated the dominance of RT 027.^{23,24}

CDI analysis in Silesia (Southern Poland) from 2016–17 demonstrated the dominance of the *C. difficile* RT 027 strain in about 80%.⁵ *C. difficile* RT 027 is responsible for

more severe infections, with a higher mortality rate, with the ability to increase sporulation, overproduce toxins, including binary toxin.^{25,26} All strains analyzed in our study had the A⁺/B⁺/CDT⁺ phenotype. Such a widespread presence of *C. difficile* RT 027 in Polish hospitals is a serious challenge for epidemiological surveillance, and one of the tools that make it possible is the

Table 3 MIC₅₀, MIC₉₀, Geometric Mean (GM) MIC Ranges for Antimicrobials Used for *C. difficile* Isolates

Antibiotic	MIC ₅₀ [μg/mL]	MIC ₉₀ [μg/mL]	GM	Range [μg/mL]	%Strains Resistant [EUCAST]	EUCAST [μg/mL]*
Metronidazole	1.5	1.5	1.06	0.016–256	0	>2
Vancomycin	0.38	0.5	0.31	0.016–256	0	>2
Moxifloxacin	32	32	32	0.002–32	100	4
Erythromycin	256	256	256	0.016–256	100	IE
Clindamycin**	256	256	256	0.016–256	100	>4
Imipenem**	32	32	25.4	0.002–32	96.6	>4
Rifampicin	0.002	32	0.18	0.002–32	44.8	0.004

Notes: *Resistance according EUCAST; IE- lack of limit value. **MICs for Gram-positive anaerobes were used, because lack of them according EUCAST.

MLVA technique. This technique can be used to analyze genetic similarities not only among hospital strains but also strains obtained from different sources (environment, food, CDI patients feces, medical staff shoes, animal feces, others).²⁷ Analyzing the outbreak in 2 hospitals in Southern Germany, caused by an unusual *C. difficile* RT 018 strain, MLVA was used among other methods.²⁸ Authors concluded that outbreak strain was probably transferred from Northern Italy, because clonal similarity with the strains from the Milan region was shown in the MLVA and not with the strains from Germany. Additionally, this strain possessed similar to the Italian strains, resistance to rifampicin. High incidence of RT 027 *C. difficile* with transmission and outbreaks and also clusters with rifampicin resistant strains in European countries were also described.^{29,30} Studies from Germany (2014–2019)³¹ and from USA (2011–2017)³² also noted that *C. difficile* RT 027 overtook other ribotypes and these authors found resistance to rifampicin among 19.2% and 12.1% of strains, respectively. However both publications noted decreased resistance to rifampicin during years depending on changed antibiotic stewardship and other procedures. About 45% resistance to rifampicin was found in our studied strains, with the GM MIC for rifampicin significantly lower within cluster II ($p < 0.005$). According to the data collected as part of the tasks of the Polish National Focal Point for Antibiotic Consumption, operating under the European Surveillance of Antibiotic Consumption Network (ESAC-Net), at ECDC, the total consumption of rifampicin and rifaximin, presented by daily doses defined per 1000 inhabitants (DDD) as at 2018 and 2019 amounted to 0.2776 and 0.3596, respectively. Since in Poland patients with tuberculosis are diagnosed and treated in specialized infectious hospitals, no use of rifampicin has been reported in the studied hospital.

Rifampicin resistance of RT 046 *C. difficile* strains was described in 2013 in Silesian hospital, which was specialized in treatment of patients with tuberculosis and other lung diseases by using rifampicin.³³ In Germany strains of *C. difficile* belonging to RT 027 resistant to rifampicin were isolated among patients with osteoarticular infections who were frequently treated with rifampicin.³⁴

Nyc et al³⁵ conducted a CDI analysis in 10 academic hospitals in Slovakia by MLVA, testing RT 001 *C. difficile* fecal isolates of patients from 14 Departments. RT 001, occurred in the years 2007/9 in high percentages in various regions of Europe (Germany, Scotland, Croatia). Based on the MLVA, the possibility of one *C. difficile* clone

spreading within the same Department, but also the clonal similarity between strains found in 3 different hospitals were shown. Krehelova et al³⁶ showed that RT 001 is the dominant ribotype in Slovakia, spreads between hospitals, opposed to the neighboring Silesian region, Southern Poland. In this publication, a high percentage (90%) of the *ermB* gene presence, defining resistance MLS_B type, was shown similarly to the results described in our study. Couturier et al³⁷ report studies conducted in 2005/14 in France, where 103 RT 027 *C. difficile* strains were tested by MLVA. A clear regionalization of infections was demonstrated on the basis of clonal similarities. The most shocking conclusion from this study is that after many years in a given hospital, infection with the same *C. difficile* clone may occur, proving that spores can survive not only for many months but also for years.

Our study included MLVA analysis of RT 027 *C. difficile* strains, isolated from different departments in one tertiary care hospital. Five clusters were distinguished, where the most understandable seems to be the occurrence of genetic relationships in the I, IV and V clusters, because all of them are located within the same ward (I – Nephrology, IV – Internal Medicine, V – General Surgery). Samples from the Internal Medicine and Nephrology Wards were taken at a monthly interval, while from General Surgery Ward were taken at shorter time interval.

The II and III clusters seem to be disturbing (Figure 1), because genetically closely related strains come from different Wards (Internal Medicine, Urology, Nephrology). In this hospital, the Urology Ward is located on the same floor as the Internal Medicine, while the Nephrology Ward is located far away. Infections are probably transmitted between Wards, possibly through spores transmitted by cleaning/medical staff, and patients transferred between wards. This conclusion is also in line with the conclusions of the above mentioned publications.^{28,35,36}

C. difficile RT 027 strains are resistant to fluoroquinolones, and possess the MLS_B resistance mechanism due to the presence of the *ermB* gene – multi-drug-resistance.^{37,38} Among our studied strains, all 29 were resistant to erythromycin (MIC > 256 µg/mL) and clindamycin (MIC > 256 µg/mL) and possessed the *ermB* gene and also were resistant to moxifloxacin. Nearly 97% resistance to imipenem among *C. difficile* RT 027 isolates we described, earlier *C. difficile* resistance to imipenem was noted in Portugal.³⁹ Portuguese authors stated 12.6% imipenem resistance among RT 017 *C. difficile* strains cultured from 15 hospitals in

Portugal between 2012–2015. As a main cause of this resistance authors noted carriage an extra penicillin-binding protein gene, *pbp5* and additional substitutions near the transpeptidase active sites of *pbp1* and *pbp3* in imipenem-resistant strains. The presence of *pbp*'s among our isolates was not studied; however we are planning to do this in the future.

There are some limitations in our investigation. We studied only 29 *C. difficile* isolates from patients hospitalized in one tertiary care Silesian hospital during 5 months and it is impossible to generalize these data. However, obtained data will enable to combine of a specific MLVA cluster/clusters with rifampicin resistance in the future.

Conclusions

MLVA analysis proved transmission and recognized outbreak due to multidrug-resistant RT 027 *C. difficile* among patients of tertiary care hospital in Southern Poland. The reason for this is probably the widespread occurrence of spores in the hospital environment, which includes, among others, neglect of hygienic procedures and epidemic supervision. High resistance to imipenem (97%) and to rifampicin (45%) among *C. difficile* RT 027 Silesian isolates is threatening and required further studies to elucidate this phenomenon.

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Disclosure

The authors report no conflicts of interest for this work.

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