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ORIGINAL ARTICLE

A pilot study in Serbia by European *Clostridium difficile* Infection Surveillance Network

MILICA JOVANOVIĆ[1]*, SOFIE M. VAN DORP[2], MITRA DRAKULOVIĆ[3], DUBRAVKA PAPIĆ[4], SLADJANA PAVIĆ[5], SNEŽANA JOVANOVIĆ[1], ALEKSANDAR LEŠIĆ[6,7], MILOŠ KORAĆ[7,8], IVANA MILOŠEVIĆ[7,8] and ED J. KUIJPER[2]

[1] Department of Microbiology, Clinical Center of Serbia, Belgrade, Serbia

[2] National Reference Laboratory for *Clostridium difficile*, Department of Medical Microbiology, Leiden University Medical Center, Leiden, Netherlands

[3] Center for Disease Prevention and Control, National Institute for Public Health "Dr Milan Jovacnović Batut", Belgrade, Serbia

[4] Department of Microbiology, General Hospital Užice, Užice, Serbia

[5] Department of Infectious Diseases, General Hospital Užice, Užice, Serbia

[6] Clinic of Orthopedic Surgery and Traumatology, Clinical Center of Serbia, Belgrade, Serbia

[7] School of Medicine, University of Belgrade, Belgrade, Serbia

[8] Clinic for Infectious and Tropical Diseases, Clinical Center of Serbia, Belgrade, Serbia

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ABSTRACT

Clostridium (Clostridioides) difficile infections (CDIs) are among the most frequent healthcare-associated infections in Serbia. In 2013, Serbia participated in the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) who launched a pilot study to enhance laboratory capacity and standardize surveillance for CDI. Two clinics of Clinical Center of Serbia [Clinic for Infectious and Tropical Diseases (CITD) and Clinic of Orthopedic Surgery and Traumatology (COT)] from Belgrade and one general hospital from another metropolitan area of Serbia, Užice, participated. During a period of 3 months in 2013, all patients with diagnosed CDI were included. The CDI incidence rates in CITD, COT, and General Hospital Užice were 19.0, 12.2, and 3.9 per 10,000 patient-days, respectively. In total, 49 patients were enrolled in the study with average age of 72 years. A complicated course of CDI was found in 14.3% of all patients. Six (12.2%) of 49 patients died, but not attributable to CDI. Of 39 *C. difficile* isolates, available for ribotyping, 78.9% belonged to ribotype 027; other PCR ribotypes were 001, 015, 002, 005, 010, 014, and 276. Antimicrobial susceptibility testing revealed low levels of MIC_{50} and MIC_{90} for metronidazole (0.5 μg/ml both) and vancomycin (0.25 and 0.5 μg/ml), while 28 strains of ribotype 027 were resistant to moxifloxacin with MIC ≥4 μg/ml. National surveillance is important to obtain more insight in the epidemiology of CDI and to compare the results with other European countries. This study by ECDIS-Net gives bases for a national surveillance of CDI in Serbia.

KEYWORDS

Clostridium difficile, healthcare-associated infections, typing

* Corresponding author:
Milica Jovanović
Department of Microbiology, Clinical Center of Serbia, Bulevar oslobodjenja 16, 11000 Belgrade, Serbia
Phone: +381 11 3619 750;
Fax: +381 2684 272
E-mail: mijovan@eunet.rs

INTRODUCTION

Clostridium (Clostridioides) difficile is an important cause of nosocomial acquired diarrhea and pseudomembranous colitis. It mainly affects older people who have been admitted in a healthcare setting and received broad-spectrum antibiotics. Since 2003, *C. difficile* PCR ribotype 027 has been responsible for a great number of hospital outbreaks [1], beginning in US and Canada and spreading to Europe, first reported in 2005 [2]. Subsequently, many

European countries reported patients with CDI caused by PCR ribotype 027 [3, 4]. *C. difficile* infections (CDI) associated with PCR ribotype 027 result in an increased mortality and higher relapse rates than other ribotypes [5]. PCR ribotype 027 is primarily characterized by an increased production of toxins A and B, contains a binary toxin, and is resistant to fluoroquinolones such as moxifloxacin [6].

Incidence of CDI has been increasing steadily over the past decade in US, and *C. difficile* is currently the most commonly identified bacterial cause of healthcare-associated diarrhea [7]. In a recent study, *C. difficile* was responsible for almost half a million infections and was associated with approximately 29,000 deaths in 2011 in USA [8]. In 2010, a study performed in southeastern United States showed that healthcare CDI exceeded the rate of methicillin-resistant *Staphylococcus aureus* (MRSA) infections, with rates 25% higher than for MRSA in 28 community hospitals in several states [9]. In Europe, an European Centre for Disease Prevention and Control-supported survey for CDI performed in 34 European countries in 2008 showed that CDI incidence was generally higher than that documented in 2005, but varied widely across hospitals and countries [10]. The overall incidence in Europe was approximately 4–5.5/10,000 patient-days.

Since 2009, CDI has been recognized as an increasing nosocomial infection in healthcare facilities in Serbia [11–13]. Serbia has 7,164,000 inhabitants and 67 acute care hospitals. From 2013 to 2017, 21, 15, 12, 13, and 6, respectively, clusters of nosocomial CDI were reported to National Public Health Institute "Dr Milan Jovanović Batut," with 163, 75, 59, 137, and 40 cases, respectively. From 2013 to 2017, overall incidence rates for CDI in Serbia were 26.6/100,000, 38.3/100,000, 37.93/100,000, 38.91/100,000, and 32.23/100,000 population, respectively, and mortality rates were 0.87/100,000, 0.86/100,000, 0.86/100,000, 1.00/100,000, and 0.50/100,000 population, respectively.

PCR ribotyping is the preferred method for *C. difficile* typing, but this facility is not available in Serbia. When a pilot study was launched by European *Clostridium difficile* infection surveillance network (ECDIS-Net), Serbia decided to participate to include microbiological data for standardized surveillance of CDI. In this study, the results and our experiences of the pilot study in Serbia are presented.

MATERIALS AND METHODS

Setting

The study was conducted in the General Hospital Užice (GHU), the Clinic for Infectious and Tropical Diseases (CITD), and the Clinic for Orthopedic and Traumatology (COT). CITD and COT belong to the Clinical Center of Serbia (CCS) in Belgrade and have 277 patient beds. CCS is a tertiary healthcare institution equipped with 3,000 beds. GHU is a secondary healthcare institution with 740 beds and located approximately 200 km from Belgrade. In the period from May 20–August 20 of 2013, data were collected during a period of active surveillance of all inpatients with the diagnosis of CDI. Demographic data, patients' data, and epidemiological data were registered with ECDIS-Net protocols in SPSS database.

Definitions

The definitions were obtained from ECDIS-Net Pilot Surveillance Protocol, version 1.2 (http://www.ecdis-pilot.eu/ecdis/home). The incidence rate was calculated as the number of cases per 10,000 patient-days.

Culture and characterization of *C. difficile* isolates

Two laboratories participated in the study. One laboratory located at CITD provided services for CITD, COT, and all other clinics of CCS. CDI was defined as a patient with diarrhea (>3 unformed stool samples for at least two consecutive days) and a positive culture of *C. difficile*. Stool samples were tested within 2 h of collection and in case the cultivation could not be performed rapidly, they are stored at 4 °C until processing. The ethanol shock method was applied to culture *C. difficile* onto the selective CLO (*C. difficile*) agar (bioMérieux, Marcy l'Etoile, France). *C. difficile* was identified by the characteristic morphology, horse odor, Gram staining, and API 20A biochemical test (bioMérieux) or VITEK® 2 ANC ID card (bioMérieux).

The second laboratory was located in GHU and used an immunoassay to detect free toxins of *C. difficile* in stools. An immunochromatographic test (RIDA QUICK *Clostridium difficile* Toxin A/B; R-Biopharm AG, Darmstadt, Germany) was used as a screening test to diagnose CDI. Positive-tested fecal specimens were stored in a freezer at −20 °C and sent for culturing of *C. difficile* to Bacteriology laboratory of CITD.

All *C. difficile* isolates were sent to the Reference Laboratory at Leiden for PCR ribotyping, detection of toxin genes (*tcdA* and *tcdB*) genes, as well as binary toxin genes (*ctdA* and *ctdB*). *In vitro* susceptibility to metronidazole, vancomycin, moxifloxacin, and minimum inhibitory concentration (MIC) was tested by agar dilution method and interpreted through epidemiological cut-off levels from EUCAST [14].

Statistical analyses

The results are reported as medians with interquartile range, proportions, or rates. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used for determining a normal distribution or whether a distribution differed significantly from a normal distribution. For continuous data that had not been fulfilled to normal distributions, means are compared by Kruskal–Wallis test [15]. Categorical data were compared by Pearson's χ^2 test. A p value <0.05 was considered to be statistically significant. Statistical analysis was performed by using SPSS software (version 19.0, IBM SPSS Inc., Chicago, IL, USA).

RESULTS

Incidence and patients' data

From May 20 to August 20, CDI incidence rates in CITD, COT, and GHU were 21.1, 12.2, and 3.9 per 10,000 patient-days, respectively. Patients' demographic and clinical characteristics are summarized in Table I. In total, 49 patients were enrolled in the study with average age of 72 years. CDI presented as healthcare-onset, healthcare-associated in 96% and healthcare-onset, community-associated in 4%. Of 47 patients with healthcare-associated CDI, 40.4% acquired CDI in another hospital. In 51% of all patients with CDI, symptoms were present on admission, more often in patients admitted at CITD. The recurrence rate of CDI was higher in patients in CITD (42%) than in COT (7.6%) or GHU (6.6%). Significantly more patients in CITD were previously hospitalized in other healthcare institution, received antibiotic treatment in the past 3 months, suffered from heart failure, or immunocompromised than the patients in other two hospitals. Other comorbidities (liver cirrhosis, pulmonary diseases, or chronic dialysis) of patients with CDI did not differ in three healthcare facilities. Seven patients (14.3%) required intensive care treatment during the course of CDI; of six *C. difficile* isolates, three were typed as 027 and one each of ribotypes 005, 015, and unknown 276. Six (12.2%) of 49 patients died, but not attributable to CDI. Two patients who died had an infection caused by *C. difficile* PCR ribotype 027.

Microbiological characterization

In a period of 3 months, two laboratories processed 253 fecal specimens. The overall *C. difficile* recovery rate was 19.4%, yielding 49 isolates. Of 49 samples sent to the Research laboratory of Leiden University Medical Centre in Netherlands, 39 were available for PCR ribotyping. Eight different *C. difficile* ribotypes were identified: 77% (30 strains) were assigned to 027; others were 001 and 015 (two strains each), while PCR ribotypes 002, 005, 010, 014, and 276 were represented by single isolates. All isolates belonging to PCR ribotype 027 contained toxin genes and binary toxin genes. Isolates from other PCR ribotypes were always positive for toxin genes, except for PCR ribotype 10 that lacked these genes. Antimicrobial susceptibility testing revealed low levels of MIC_{50} and MIC_{90} values for metronidazole (0.5 μg/ml both) and vancomycin (0.25 and 0.5 μg/ml). Resistance to moxifloxacin was common, with $MIC_{50} = 8$ μg/ml and $MIC_{90} = 16$ μg/ml, respectively. Twenty-eight strains of ribotype 027 were resistant to moxifloxacin with MIC ≥ 4 μg/ml.

Distribution of PCR ribotype 027

C. difficile PCR Ribotype 027 was the most prevalent in all healthcare institutions, but significantly more other ribotypes were found in CITD compared to two other hospitals ($p < 0.05$; Table I).

DISCUSSION

When a European-wide surveillance study for CDI was performed in 2008 [9], low incidence rates of CDI (2 per 10,000 patient-days) were reported from most of the Eastern European countries, such as Bulgaria, Croatia, Czech Republic, Romania, Slovakia, and Hungary. On the contrary, Poland reported one of the highest incidences (12.5 per 10,000 patient-days). Serbia did not participate since CDI was not considered as an important healthcare-associated infection at that time. This was also illustrated in 2010, when a low CDI incidence rate of 3.3 per 10,000 patient-days was reported in patients at Military Medical Academy in Belgrade [16]. However, in the following years, increasing CDI incidence rates in Eastern European countries were found. A recently completed study revealed a CDI incidence rate of 25.6 per 10,000 patient-days in Hungary [17] and 5.2 per 10,000 bed-days in Slovakia [18]. The high incidence rates of CDI in three participating hospitals in this study in Serbia and the high prevalence rate of PCR ribotype 027 are similar according to the recently reported data from Romania. In Romania, a predominance of CDI associated with *C. difficile* PCR ribotype 027 [19, 20] was found in 68% of all investigated *C. difficile* isolates [19]. Other European countries also experienced spread of 027, as was documented by recently reported outbreaks in Poland, Austria, and Portugal [21–23]. After a pilot study performed in 2015, a European surveillance of CDI has been started in 2016 in which most European countries participate [24].

This study showed a high CDI incidence rate of 21.1 per 10,000 patient-days at CITD. CITD is specialized in infectious diseases and encountered admission of many patients who have been treated with antibiotics in other healthcare facilities in the previous 3 months. Of 21 patients diagnosed with CDI at CITD, 16 acquired CDI in other hospital and one patient in a long-term care facility. However, the overall mortality rate (12.9%) was similar in all participating hospitals and was not higher as found in some other studies: 22% in pan-European study [10], 21.9% in Hungary [17], 64.2% in Portugal [23], and a fifth in the study of Morgan [25]. The percentages from this study were higher than in some studies from the neighborhood, like Romania, where case fatality rate was smaller, amounting 9.2% [26] or 8.8% [27]; conversely, in Western Romania, which is in the closest neighborhood to Serbia, case fatality rate was estimated to have mortality rate from CDI of 22.86% [28]. Our two neighboring countries probably share the similar problems with this type of infections [20, 29].

We found a high incidence rate of *C. difficile* PCR ribotype 027 in all participating hospitals. All isolates had the well-known characteristics of ribotype 027 and were moxifloxacin-resistant but sensitive to metronidazole and vancomycin. A complicated course of CDI was found in 14.3% of all patients, without a clear association with PCR ribotype 027. Although only 49 patients were analyzed in this study, we could not find increased morbidity and mortality in patients infected with type 027 compared to other PCR ribotype. Therefore, our results favor the hypothesis that there is no

Table I. General data of participants in the pilot surveillance study conducted in Serbia from May 20 to August 20 of 2013

Characteristic	Hospital type			*p*
	Clinic for Infectious and Tropical Diseases	Clinic of Orthopedic Surgery and Traumatology	General Hospital Užice	
Sex				
Male	9 (42.9%)	4 (30.8%)	7 (46.7%)	n.s.
Female	12 (57.1%)	9 (69.2%)	8 (53.3%)	
Age (years)				
18–60	6 (28.6%)	2 (15.4%)	0 (0.0%)	n.s.
61–85	14 (66.7%)	10 (76.9%)	12 (80%)	
>85	1 (4.7%)	1 (7.7%)	3 (20.0%)	
Length of stay (days)	19 (13.7)	32 (12.5)	17.5 (19.7)	<0.01**
APACHE score				
Yes	3 (14.3%)	2 (15.4%)	0 (0.0%)	n.s.
No	18 (85.7%)	11 (84.6%)	15 (100.0%)	
Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
McCabe score				
Non-fatal underlying disease	15 (71.4%)	12 (92.3%)	14 (93.3%)	n.s.
Ultimately fatal underlying disease	5 (23.8%)	1 (7.7%)	1 (6.7%)	
Rapid fatal underlying disease	1 (4.8%)	0 (0.0%)	0 (0.0%)	
Heart failure				
No	16 (76.2%)	12 (92.3%)	15 (100.0%)	<0.05*
Yes	5 (23.8%)	1 (7.7%)	0 (0.0%)	
Immunocompromised status				
No	14 (66.7%)	12 (92.3%)	15 (100%)	<0.01*
Yes	7 (33.3%)	1 (12.5%)	0 (0.0%)	
Healthcare admission in the past 3 months				
No	3 (14.3%)	11 (84.6%)	8 (53.3%)	<0.05*
Yes	18 (85.7%)	1 (7.7%)	7 (46.7%)	
Unknown	0 (0.0%)	1 (7.7%)	0 (0.0%)	
Antibiotic treatment in the past 3 months				
No	1 (4.8%)	10 (76.9%)	0 (0.0%)	<0.001*
Yes	20 (95.2%)	2 (14.4%)	14 (93.3%)	
Unknown	0 (0.0%)	1 (7.7%)	1 (6.7%)	

Table I. General data of participants in the pilot surveillance study conducted in Serbia from May 20 to August 20 of 2013 *(Continued)*

Characteristic	Hospital type			*p*
	Clinic for Infectious and Tropical Diseases	Clinic of Orthopedic Surgery and Traumatology	General Hospital Užice	
Courses of antibiotic treatment				
None	1 (4.8%)	11 (84.6%)	1 (7.7%)	<0.001*
One course	15 (71.4%)	2 (15.4%)	0 (0.0%)	
Multiple courses	4 (19.0%)	0 (0.0%)	0 (0.0%)	
Combinatory therapy	1 (4.8%)	0 (0.0%)	14 (93.3%)	
Symptoms present on admission				
No	2 (9.5%)	12 (92.3%)	10 (66.7%)	<0.001*
Yes	19 (90.5%)	1 (7.7%)	5 (33.3%)	
CDI origin				
HA (current hospital)	2 (9.5%)	13 (100%)	13 (86.7%)	<0.001*
HA (other hospital)	16 (76.2%)	0 (0.0%)	2 (13.3%)	
HA (long-term care facility)	1 (4.8%)	0 (0.0%)	0 (0.0%)	
HA (other)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Community-acquired	2 (9.5%)	0 (0.0%)	0 (0.0%)	
Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Recurrent CDI				
No	12 (31.6%)	12 (31.6%)	14 (36.8%)	<0.05*
Yes	9 (81.8%)	1 (9.1%)	1 (9.1%)	
Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Death				
No death	19 (90.4%)	12 (92.3%)	12 (80.0%)	n.s.
Related to CDI	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Unrelated to CDI	1 (4.8%)	1 (7.7%)	1 (6.7%)	
Relationship to CDI unknown	1 (4.8%)	0 (0.0%)	2 (13.3%)	
Ribotypes				
027	11 (52.4%)	11 (84.6%)	8 (53.3)	<0.05*
Other	7 (33.3%)	1 (7.7%)	1 (6.7)	
No growth	3 (14.3%)	1 (7.7%)	6 (40.0)	
Total	21 (43%)	13 (26%)	15 (31%)	

Note: Values are expressed as counts (proportions) or as median (IQR). CDI: *Clostridium difficile* infection; n.s.: not significant; HA: healthcare-associated.
*Pearson's χ^2 test. **Kruskal–Wallis test.

association of specific PCR ribotypes with development of severe diseases and clinical outcomes [25, 26].

Two laboratories provided microbiological services, but did not apply the ESCMID-recommended CDI testing with a two-step algorithm. However, samples tested positive at the laboratory in GHU by rapid immunochromatographic toxin detection test were cultured at the central laboratory. Since 80% of them were positive for culture at the laboratory of CITD, we conclude that this immunochromatographic test performed relatively well. Some cases could have been missed, but taking into account that stools have been collected in a freezer for 3 months and sent to other city for culture at the end of the study, it might affect the recovery of strains. Standardized diagnostics of CDI have become a major priority for CDI surveillance in Serbia and tests with a better sensitivity are required.

Participating in this study resulted in the recognition of a high incidence of CDI due to type 027 in three hospitals. Clearly, boundaries between countries do not stop *C. difficile* PCR ribotype 027. We are currently developing a CDI surveillance program with improvement of CD diagnostics and creating laboratory capacities to perform PCR ribotyping. CDI surveillance will include the other hospitals in Serbia and intervention programs will be developed to combat CDI with emphasizes on more stringent infection control measures and antimicrobial stewardship [30].

Attempts to establish an active surveillance system have already been made, but the country is facing difficulties in many aspects. In 2016, the bylaw document "Regulation on the health care program of the population against infectious diseases" was instituted aiming to minimize the rates of incidence and mortality of CDI among other diseases relevant for healthcare protection of the nation. We hope that our healthcare system in general will be better equipped to combat *C. difficile* in the upcoming years.

Conflict of Interest: The authors declare no conflict of interest.

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