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The emergence of *Clostridium difficile* ribotypes 027 and 176 with a predominance of the *Clostridium difficile* ribotype 001 recognized in Slovakia following the European standardized *Clostridium difficile* infection surveillance of 2016

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ABSTRACT

Aim: To obtain standardized epidemiological data for *Clostridium difficile* infection (CDI) in Slovakia.
Methods: Between October and December 2016, 36 hospitals in Slovakia used the European Centre for Disease Prevention and Control (ECDC) *Clostridium difficile* infection (CDI) surveillance protocol.
Results: The overall mean CDI incidence density was 2.8 (95% confidence interval 1.9–3.9) cases per 10 000 patient-days. Of 332 CDI cases, 273 (84.9%) were healthcare-associated, 45 (15.1%) were community-associated, and 14 (4.2%) were cases of recurrent CDI. A complicated course of CDI was reported in 14.8% of cases ($n = 51$). CDI outcome data were available for 95.5% of cases ($n = 317$). Of the 35 patients (11.1%) who died, 34 did so within 30 days after their CDI diagnosis.
Of the 78 isolates obtained from 12 hospitals, 46 belonged to PCR ribotype 001 (59.0%; 11 hospitals) and 23 belonged to ribotype 176 (29.5%; six hospitals). A total of 73 isolates (93.6%) showed reduced susceptibility to moxifloxacin (ribotypes 001 and 176; $p < 0.01$). A reduced susceptibility to metronidazole was observed in 13 isolates that subsequently proved to be metronidazole-susceptible when, after thawing, they were retested using the agar dilution method. No reduced susceptibility to vancomycin was found.
Conclusions: These results show the emergence of *C. difficile* ribotypes 027 and 176 with a predominance of ribotype 001 in Slovakia in 2016. Given that an almost homogeneous reduced susceptibility to moxifloxacin was detected in *C. difficile* isolates, this stresses the importance of reducing fluoroquinolone prescriptions in Slovak healthcare settings.

Introduction

Clostridium difficile, recently reclassified as *Clostridioides difficile* (Oren and Rupnik, 2018), is an important nosocomial pathogen (ECDC, 2013). In the countries of the European Union and European Economic Area (EU/EEA), the burden of hospital-associated *C. difficile* infection (CDI) in acute care hospitals was estimated at 123 997 (95% confidence interval (CI) 61 018–284 857) cases annually between 2011 and 2012. In addition, during the European Centre for Disease Prevention and Control (ECDC) point prevalence survey of healthcare-associated infections (HAI) and antimicrobial use in European acute care hospitals, *C. difficile* was the eighth most frequently found microorganism in HAI and the most common causative agent in gastrointestinal system HAIs (ECDC, 2013).

Slovakia participated in two international studies on the incidence density of CDI in the acute healthcare setting (Bauer

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et al., 2011; Davies et al., 2014). In 2008, two of the three participating hospitals provided CDI incidence density data, and the weighted mean CDI incidence density was 1.4 (range 0.0 to 2.1) CDI cases per 10 000 patient bed-days (Bauer et al., 2011). In a prospective multicentre biannual point prevalence study of CDI in hospitalized patients with diarrhoea (EUCLID), six Slovakian hospitals reported a fluctuating CDI incidence density between two reporting periods. While the incidence density of CDI was 5.3 cases per 10 000 patient bed-days between September 2011 and August 2012, the reported rates between September 2012 and August 2013 fell to 1.2 cases per 10 000 patient bed-days (Davies et al., 2014).

Before the 2016 study, the *C. difficile* isolates derived from stool samples of patients hospitalized in Slovakia were characterized in three studies performed between 2011 and 2013. All three studies were in agreement and showed a low diversity of ribotypes, except for a predominance of PCR ribotype 001 (60.1%, 85.0%, 70%) (Davies et al., 2016; Nyc et al., 2015; Freeman et al., 2018).

After the successful testing of a standardized CDI surveillance protocol in 37 European hospitals in 2013 (van Dorp et al., 2016), the ECDC started coordinating the surveillance of CDI in EU/EEA countries in 2016 (Krutova et al., 2018a). Slovakia participated, in order to obtain comparable CDI density data and information on prevailing PCR ribotypes, as well as data on antimicrobial susceptibility to the first-line CDI treatment drugs.

Methods

Study protocol

Between October and December 2016, a total of 36 hospitals in Slovakia participated in a national CDI surveillance. A 'light surveillance option' of the ECDC-CDI surveillance protocol (ECDC, 2015) was applied to 24 hospitals across Slovakia, with the collection of hospital aggregated numerator and denominator data and information on each CDI case. Twelve hospitals applied an 'enhanced surveillance option' that also collects microbiological data including characterization of *C. difficile* isolates (ECDC, 2015).

The mean CDI incidence density was calculated from the mean CDI incidence densities of participating hospitals. Each patient's outcome was followed until the patient was discharged or died. There was no post-discharge follow-up regarding readmission or death of the patients.

Microbiological investigation

Testing for CDI was performed only when requested by a physician, and only unformed stool samples (taking the shape of the container) were tested in the microbiology departments of the participating hospitals. Thirty-two hospitals used a recommended multi-step testing algorithm; stool samples were tested for the presence of glutamate dehydrogenase (GDH) and toxins A/B by enzyme immunoassay (EIA), and in GDH-positive and toxins A/B-negative stool samples, the presence of toxin genes was determined by nucleic acid amplification test (NAAT) (Crobach et al., 2016). Two hospitals used only *C. difficile* toxins A/B EIA detection and two hospitals used *C. difficile* toxins A/B EIA detection with NAAT in positive samples.

Clostridium difficile *culture and characterization of isolates*

Toxigenic *C. difficile*-positive stool samples sent from the 12 hospitals that followed the 'enhanced surveillance option' were cultured anaerobically on selective agar for *C. difficile* (Brazier, Oxoid). Antibiotic susceptibility of the *C. difficile* isolates was determined by Etest for the following antibiotics: metronidazole, vancomycin, and moxifloxacin. The minimum inhibitory concentration (MIC) breakpoints for metronidazole, vancomycin (2 mg/l), and moxifloxacin (4 mg/l) were based on epidemiological cut-off values (ECOFFs) and were applied according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, version 9.0, 2019). *C. difficile* isolates showing a reduced susceptibility to metronidazole and vancomycin were retested using the agar dilution method. A capillary electrophoresis-based PCR was used for ribotyping (Fawley et al., 2015) and multiplex PCR for the detection of genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*) for toxin production (A, B, and binary) (Persson et al., 2008).

Results

Participating hospitals

A total of 36 Slovak hospitals, covering 17 721 hospital beds and 1 100 418 patient-days, voluntarily participated in a 3-month CDI surveillance (October 2016–December 2016). Eleven (30.6%) are tertiary care institutions, 12 (33.3%) are secondary care facilities, and 13 (36.1%) are primary care institutions. Thirty-two participating hospitals (88.9%) used the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) laboratory CDI diagnosis algorithm (Crobach et al., 2016). The mean testing frequency in all 36 hospitals was 36.5 (95% CI 27.9–45.0) per 10 000 patient-days.

Clostridium difficile *infections*

Patient data were collected for 332 CDI episodes. The median age of the patients was 75 years; nine patients were younger than 18 years and five of them were 2 years old or younger. One hundred and seventy-six patients were female (53.0%).

The overall mean CDI incidence density was 2.8 (95% CI 1.9–3.9) cases per 10 000 patient-days. Of 332 reported CDI cases, 273 (84.9%) were healthcare-associated (HA) with an incidence density of 2.3 (95% CI 1.8–2.7) cases per 10 000 patient-days, and 45 (15.1%) were community-associated (CA) with an incidence density of 0.4 (95% CI 0.2–0.6) cases per 10 000 patient-days. Fourteen (4.2%) of the CDI cases were classified as recurrent cases with an incidence density 0.1 (95% CI 0.02–0.2) cases per 10 000 patient-days. For HA CDIs, the origin of the infection was the same healthcare facility in 264 cases (96.7%), another hospital in five cases (1.8%), and a long-term care facility in four cases (1.5%). Eight hospitals reported zero CDI cases during the surveillance period.

A complicated course of CDI (admission for CDI from the community; admission to an intensive care unit; surgery for toxic megacolon or death) was reported in 15.4% ($n = 51$) of CDIs; in three cases (0.9%), the severity of the course of the CDI was unknown. CDI outcome data were available for 317 cases (95.5%). Thirty-five patients (11.1%) died, and CDI definitely contributed to death in one case (0.3%). Thirty-four patients died within 30 days after a CDI diagnosis. The McCabe score of fatal cases indicated that nine patients (25.7%) had 'rapidly fatal' and 18 patients (51.4%) had 'ultimately fatal' underlying disease. The overall results are summarized in Table 1.

Microbiological data

A total of 78 strains were available for further characterization. Of these, 46 isolates belonged to PCR ribotype 001 (59.0%) and 23 isolates belonged to PCR ribotype 176 (29.5%). Other PCR ribotypes identified were 017 ($n = 3$; 3.8%), 020, 027, 049 and 070 ($n = 1$; 1.3% each). Two ribotyping profiles remained unrecognized. The distribution of ribotypes 001 and 176 identified in Slovakian hospitals is shown in Figure 1. All 78 *C. difficile* isolates were

Table 1
Results from CDI surveillance in Slovakia.

	Number (%)	Mean hospital incidence density (95% CI)
Clostridium difficile infections by type, 36 hospitals, 10–12/2016		
CDI cases	332 (100)	2.8 (1.9–3.9)
Healthcare-associated CDIs	273 (84.9)	2.3 (1.8–2.7)
Community-associated CDIs	45 (15.1)	0.4 (0.2–0.6)
Recurrent CDI	14 (4.2)	0.1 (0.02–0.2)
Complicated course	51 (15.4)	
Death (data for 317 cases, 95.5%)	35 (11.1)	
30-day mortality	34 (10.7)	
Characterization of *C. difficile* isolates ($n = 78$, 100%), 12 hospitals, 10–12/2016		
Ribotype 001	46 (59.0)	
Ribotype 176	23 (29.5)	
Others (017 $n = 3$; 020, 027, 049, 070 $n = 1$ each; unrecognized $n = 2$)	9 (11.5)	
Binary toxin genes positive (027, 176, and one unrecognized)	25 (32.1)	
Moxifloxacin reduced susceptibility (017, 027, 176 (100%) and 001 (97.8%))	73 (93.6)	

CDI, *Clostridium difficile* infection; CI, confidence interval.

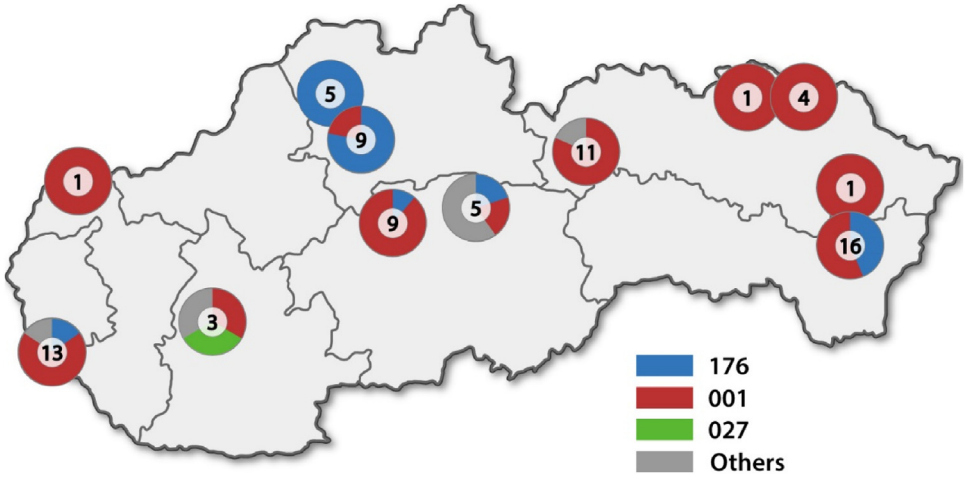

Figure 1. Distribution of Slovak hospitals providing stool samples for *Clostridium difficile* culture. The pie charts show the representation of *C. difficile* ribotypes 001, 027, and 176 identified per hospital. The numbers in the centre represent the number of *C. difficile* isolates cultured for molecular characterization.

positive for the *tcdB* gene and 25 isolates (32.1%; ribotypes 027, 176, and one unrecognized profile) also carried genes for binary toxin (*cdtA*, *cdtB*).

A total of 73 isolates (93.6%) showed reduced susceptibility to moxifloxacin (MIC $\geq$ 32 mg/l; ribotypes 176 $n = 23/23$, 001 $n = 45/46$, 017 $n = 3/3$, 027 $n = 1/1$). A reduced susceptibility to metronidazole (MIC 8–256 mg/l) was observed in 13 isolates (ribotypes 001 $n = 9$, 017 $n = 1$, 020 $n = 1$, 027 $n = 1$, 176 $n = 1$), which subsequently were found to be metronidazole-susceptible when retested using the agar dilution method after thawing of *C. difficile* isolates. No reduced susceptibility to vancomycin was observed. The overall results are summarized in Table 1.

Discussion

In 2016, the mean hospital CDI incidence density in 20 EU/EEA countries (579 surveillance periods from 556 hospitals) was 3.19 cases per 10 000 patient-days (ECDC, 2018). The Slovak CDI incidence density data of 2.8 cases per 10 000 patient-days (36 hospitals) is lower; however, the mean testing frequency in Slovakia of about 8.8 tests per 10 000 patient-days is lower than the European mean (45.3 vs. 36.5 tests per 10 000 patient-days). Interestingly, eight Slovak hospitals reported zero CDI cases during the 3-month surveillance period. The reason for the suboptimal testing frequency in Slovakia may be because not all diarrhoeal stool samples from hospitalized patients were screened for the presence of toxigenic *C. difficile*, since only physician-requested testing of stool samples for CDI were included in the study (Davies et al, 2014; Alcalá et al., 2012).

In 2012, two studies reported the ribotyping data of the Slovak *C. difficile* isolates, and *C. difficile* ribotypes 027 and/or 176 were not detected (Davies et al., 2016; Nyc et al., 2015). In contrast, ribotype 001 was identified as predominant in four CDI studies within the period 2012 to 2017 (Davies et al., 2016; Nyc et al., 2015; Freeman et al., 2018; Krehelova et al., 2019) and moreover clonal relatedness of ribotype 001 isolates was confirmed in hospital and between hospitals by multilocus variable number tandem repeats analysis and whole-genome sequencing (Nyc et al., 2015; Krehelova et al., 2019; Eyre et al., 2018).

Although the occurrence of ribotype 001 is endemic in Slovakia, the emergence of ribotypes 027 and 176 in a Slovak healthcare setting is reported for the first time in this CDI surveillance period (October 2016–December 2016). Identified CDI cases of ribotype 027 ($n = 1$) and ribotype 176 ($n = 23$) were located in six hospitals, but it should be noted that ribotyping data were available for only 12 out of the 36 hospitals. Ribotype 176 is suggested to be related to ribotype 027, because they belong to the same multilocus sequence type (ST1/clade 2) and express a similar proteome profile (Knetsch et al., 2012; Dresler et al., 2017). Importantly for laboratory diagnostics, ribotypes 027 and 176 shared the molecular markers

(binary toxin gene(s) *cdtA*/*cdtB* and the specific deletion at position 117 of *tcdC* gene) that are used in commercial tests for the differentiation of ribotype 027 and other ribotypes (Krutova et al., 2018b).

The CDI epidemiology patterns found in this study, the co-predominance of ribotypes 001 and 176, is similar to that reported in the Czech Republic in 2014 (Krutova et al., 2016), a country that neighbours Slovakia. Interestingly, in Hungary and Poland, countries that also share a border with Slovakia, a high occurrence of ribotype 176 CDIs and a predominance of ribotype 027 were reported in October–December 2014 and 2011–2013 (Tóth et al., 2016; Pituch et al., 2015). Moreover, recent data from South-Eastern Europe captured an outbreak of ribotype 176 CDIs in a Croatian hospital in 2015 (Rupnik et al., 2016). In Slovakia, ongoing CDI surveillance, including ribotyping of *C. difficile* isolates, is needed in order to monitor further the development of CDI epidemiology and the possible emergence of newly recognized epidemic ribotypes such as ribotype 018 in France and Italy (Berger et al., 2019; Gateau et al., 2019).

Antimicrobial susceptibility testing identified a reduced susceptibility to metronidazole in fresh *C. difficile* cultures by Etest, but this was not confirmed after thawing of *C. difficile* isolates and retesting with the agar dilution method. The same phenomenon, when an initial metronidazole-resistant *C. difficile* isolate becomes susceptible after thawing or after serial passages, has been described in *C. difficile* isolates from Spain and Canada (Peláez et al., 2008; Martin et al., 2008). Moreover, variations in MICs using different methods and culture media for metronidazole susceptibility testing have been shown (Baines et al., 2008).

During the surveillance period, only five isolates were susceptible to moxifloxacin. The reduced susceptibility to moxifloxacin identified in ribotypes 001, 017, 027, and 176 was also demonstrated in European *C. difficile* isolates collected during the ClosER study between July 2011 and July 2014 (Freeman et al., 2018) and in national Polish and Czech studies on *C. difficile* ribotype 176 isolates (Lachowicz et al., 2015; Krutova et al., 2015). In the United Kingdom, falls in the incidence of fluoroquinolone-resistant *C. difficile* were observed after the use of fluoroquinolone was restricted (Dingle et al., 2017), even though the numbers of fluoroquinolone-susceptible *C. difficile* remained stable; however, it is probable that other factors also contributed to the stabilizing of CDI epidemiology in the UK, such as the optimizing of laboratory diagnostics, active CDI surveillance, and the availability of ribotyping in several laboratories.

Antimicrobial stewardship is an important key component in the prevention of CDI in acute healthcare settings (Tschudin-Sutter et al., 2018). Except for fluoroquinolones, antimicrobial stewardship should focus on 4C antibiotics (ciprofloxacin/fluoroquinolones, clindamycin, co-amoxiclav, and cephalosporins). A reduction in prescribing could reduce the incidence of multidrug-resistant ribotypes, e.g., 001 and 027 (Lawes et al., 2017).

In conclusion, the results of standardized CDI surveillance in Slovakia showed a similar CDI incidence density but a lower testing frequency compared to standardized European CDI surveillance data. Microbiological investigations revealed the emergence of the *C. difficile* ribotype 176 with the predominance of ribotype 001. Given that an almost homogeneous reduced susceptibility to moxifloxacin was detected in *C. difficile* isolates, this stresses the importance of antibiotic stewardship that focuses primarily on reducing fluoroquinolone prescriptions in Slovak healthcare settings.

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Ethical approval

For this type of study, formal consent was not required.

Conflict of interest

The authors declare that they have no conflicts of interest.

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