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Molecular typing and antimicrobial susceptibility testing to six antimicrobials of *Clostridium difficile* isolates from three Czech hospitals in Eastern Bohemia in 2011–2012

V. Beran[1] · E. J. Kuijper[2] · C. Harmanus[2] · I. M. Sanders[2] · S. M. van Dorp[2] · C. W. Knetsch[2] · J. Janeckova[3] · A. Seidelova[4] · L. Barekova[5,6] · J. Tvrdik[7] · D. Chmelar[1] · I. Ciznar[1]

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Abstract In 2011–2012, a survey was performed in three regional hospitals in the Czech Republic to determine the incidence of *Clostridium difficile* infections (CDIs) and to characterize bacterial isolates. *C. difficile* isolates were characterized by PCR ribotyping, toxin genes detection, multiple-locus variable-number tandem-repeat analysis (MLVA), and antimicrobial susceptibility testing to fidaxomicin, vancomycin, metronidazole, clindamycin, LFF571, and moxifloxacin using agar dilution method. The incidence of CDI in three studied hospitals was 145, 146, and 24 cases per 100,000 inhabitants in 2011 and 177, 258, and 67 cases per 100,000 inhabitants in 2012. A total of 64 isolates of *C. difficile* was available for molecular typing and antimicrobial susceptibility testing. 60.9% of the isolates were classified as ribotype 176. All 41 isolates of ribotypes 176 and 078 were positive for the presence of binary toxin genes. Ribotype 176 also carried 18-bp deletion in the regulatory gene *tcdC*. Tested isolates of *C. difficile* were fully susceptible to vancomycin and metronidazole, whereas 65.1% of the isolates were resistant to moxifloxacin. MLVA results indicated that isolates from three different hospitals were genetically related, suggesting transmission between healthcare facilities.

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✉ V. Beran
vladimir.beran@osu.cz

[1] Czech Anaerobic Bacteria Reference Laboratory, Department of Biomedical Sciences, Faculty of Medicine, University of Ostrava, Ostrava, Czech Republic

[2] Department of Medical Microbiology, Leiden University Medical Centre, Leiden, Netherlands

[3] Department of Infection Diagnosis, Litomysl Hospital, The Hospital of Pardubice Region, Pardubice, Czech Republic

[4] Department of Clinical Immunology and Microbiology, Regional Hospital of Nachod, Nachod, Czech Republic

[5] Department of Clinical Microbiology, Pardubice Hospital, The Hospital of Pardubice Region, Pardubice, Czech Republic

[6] Department of Epidemiology, Faculty of Military Health Sciences, Hradec Kralove, University of Defense, Brno, Czech Republic

[7] Department of Computer Science, Faculty of Science, University of Ostrava, Ostrava, Czech Republic

Introduction

Clostridium difficile is the most common nosocomial pathogen of diarrhea in humans. *C. difficile* infection (CDI) is often triggered by antimicrobial therapy. The prevalence and severity of the disease increased worldwide in the past 10–15 years (Rupnik et al. 2009). Stubbs et al. (1999) described more than 100 different PCR ribotypes. The so-called hypervirulent ribotype 027 of *C. difficile* is associated with a more severe disease course, a higher production of toxins, presence of a binary toxin, and a higher resistance to fluoroquinolones than other ribotypes (Razavi et al. 2007). In Central European countries, such as Poland and the Czech Republic, *C. difficile* ribotype 176 is frequently found (Krutova et al. 2014; Obuch-Woszczatynski et al. 2014). *C. difficile* ribotype 176 is highly related to ribotype 027 and differs only in one band by PCR ribotyping (Nyc et al. 2011; Valiente et al. 2012). In contrast to the worldwide spread of ribotype 027, ribotype 176 was reported in Poland, the Czech Republic, and now also in Croatia (Rupnik et al. 2016).

Since 2011, we noticed an increase in the CDI incidence in three hospitals in Eastern Bohemia (Czech Republic). A retrospective analysis to the incidence of CDI was performed with

characterization of the cultured *C. difficile* isolates. *C. difficile* isolates were characterized by PCR ribotyping, toxin genes detection, multiple-locus variable-number tandem-repeat analysis (MLVA), and antimicrobial susceptibility testing to fidaxomicin, vancomycin, metronidazole, clindamycin, LFF571, and moxifloxacin using the CLSI (Clinical and Laboratory Standards Institute) agar dilution method.

Material and methods

The study design

In 2011, the Czech Anaerobic Bacteria Reference Laboratory in Ostrava requested diagnostic laboratories in the Czech Republic to send *C. difficile* isolates for typing and characterization. In the period 2011–2012, three hospitals in Eastern Bohemia participated to this survey; a regional hospital in Nachod (609 beds), a regional hospital in Litomysl (632 beds), and a regional hospital in Pardubice (932 beds). All three hospitals are located within a radius of about 100 km. Inclusion criteria for testing patients for CDI were diarrhea and previous antimicrobial treatment. Diagnosis of patients with CDI was performed at the microbiological departments with toxin A/B and glutamate dehydrogenase (GDH) detection using rapid tests (C. DIFF QUIK CHEK COMPLETE®, Techlab) as a routine part of CDI diagnostic algorithm. Toxin A/B and/or GDH positive stool samples were cultured anaerobically at 37 °C for 48 h. The stool samples were tested only in patients on the physician request. The microbiological laboratories were asked to send strain and patient information to the Department of Biomedical Sciences, Faculty of Medicine at the University of Ostrava. Patients diagnosed with CDI were used to determine CDI incidence rate per 100,000 inhabitants in Eastern Bohemia region.

Culture of isolates and DNA isolation

C. difficile isolates were transferred from the hospital laboratories to our laboratory at the University of Ostrava and then from the Czech Republic to Leiden University Medical Center in Leiden, Netherlands, using Amies transport swabs (COPAN, Italy). Swabs were cultured on selective *C. difficile* agar (M836, Himedia, India) at 37 °C in anaerobic workstation for 48 h. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen).

PCR ribotyping

PCR ribotyping using both the agarose gel electrophoresis and capillary electrophoresis detection system (automated sequencer and fragment analysis system ABI-PRISM™ 3100, POP-4™ Polymer, Applied Biosystems) was performed as described by Bidet et al. (1999) and Fawley et al. (2015). Fragment analysis was performed using GeneMapper® Software Version 5.0 (Applied Biosystems) and identification of ribotypes was carried out using the PCR ribotyping library (the database of the National Reference Laboratory for *Clostridium difficile* of the Netherlands in Leiden) with BioNumerics® Software Version 7.1 (Applied Maths).

Detection of toxin genes

Multiplex PCR was employed for the detection of *gluD* (*C. difficile* identification) and toxin genes *tcdA*, *tcdB*, *cdtA*, and *cdtB* as described by Paltansing et al. (2007) and Persson et al. (2008).

Detection of deletions in the *tcdC* gene and the presence of *ermB*

A monoplex PCR was used for the detection of *tcdC* gene deletions according to Spigaglia and Mastrantonio (2002) modified by the National Reference Laboratory in Leiden as follows: primers CD-tcdC-1-S (5′-CATATCCTTCTTCTCCTCTTC-3′) and CD-tcdC-2-AS (5′-AATTGTCTGATGCTGAACC-3′) were used. External control strains of *C. difficile* were used with no deletion (159 bp, strain 630), Δ18 bp (141 bp, strain UK 027), and Δ39 bp (120 bp, strain Δ39) deletion.

A monoplex PCR was used for the detection of *ermB* genes according to Farrow et al. (2000).

MLVA typing

Multiple-locus variable-number tandem-repeat analysis (MLVA) was performed according to van den Berg et al. (2007). The genetic relationship among the genotypes was determined by clustering them according to MLVA-type using the number of differing loci and the summed absolute distance as coefficients for calculating the minimum-spanning tree, as described by Marsh et al. (2006) using the BioNumerics software program (version 7.1, Applied Maths, Belgium). Briefly, the summed absolute distance between two MLVA-typed isolates is the summed tandem-repeat difference (STRD) at all seven variable number of tandem-repeat (CDR) loci. Isolates with a STRD ≤10 were defined as genetically related, irrespective of the number of differing loci. Clonal complexes were defined by an STRD ≤2, provided that isolates were single locus variants (SLV's) or double locus variants (DLV's) of each other (Marsh et al. 2006).

Antimicrobial susceptibility testing

Susceptibility of *C. difficile* strains to six antimicrobial agents was tested: fidaxomicin, vancomycin, metronidazole, clindamycin, moxifloxacin, and LFF571 (Aplichem or Fluka, Germany). The strains were tested using the agar dilution

method according to the CLSI guidelines (2007). The antimicrobials were diluted into Brucella blood agar (pancreatic digest of casein, peptic digest of animal tissue, yeast extract, glucose, sodium chloride, sodium bisulfite, and agar) supplemented with 5% sheep blood, hemin, $CaCl_2$, and vitamin K1. Bacterial isolates were cultured on blood agar plates at 37 °C and after 48 h, suspended to a concentration of 0.5 McFarland in phosphate-buffered saline (PBS). The strains were inoculated onto solid media using multipoint inoculator to a final concentration of 10^4 CFU per spot. Plates were incubated in an anaerobic chamber (Don Whitley Scientific; 10% H_2, 5% CO_2, 85% N_2) at 37 °C and read after 24 and 48 h for growth. The MIC_{50} and MIC_{90} were determined. The following antimicrobial concentration series were used: 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.06 μg/mL (except of clindamycin: 64, 32, 16, 8, 4, 2, 1, 0.5 μg/mL). The breakpoints for *C. difficile* and Gram-positive anaerobes according to EUCAST (2016) are (mg/L) vancomycin (2), metronidazole (2), moxifloxacin (4), clindamycin (4), fidaxomicin (−), and LFF571 (−).

Statistical analysis

Frequency distribution of selected results was statistically compared in groups of strains in order to find significant differences. Statistical analysis was processed with NCSS software (Hintze 2012). Dependence of binary toxin gene and Δ*tcdC* gene occurrence and moxifloxacin resistance on ribotype 176 occurrence was assessed by Fisher's exact two-side test in four-fold tables. Variable ribotyping, Δ*tcdC*, and moxifloxacin were dichotomized according to the rules: ribotyping = 176 vs. others, Δ*tcdC* = 18 bp vs. others, and moxifloxacin resistance by cutpoint 4.

Results and discussion

Patient characteristics

The incidence of CDI in three studied hospitals was 145, 146, and 24 cases per 100,000 inhabitants in 2011 and 177, 258, and 67 cases per 100,000 inhabitants in 2012. In 2012, the average incidence of CDI in these three hospitals was 136 cases per 100,000 persons per year. This is higher than that of reported recently in Germany, where an incidence rate was found of 5–20 cases per 100,000 persons per year (Luebbert et al. 2014). In the USA, the incidence of CDI was 35 cases per 100,000 persons in 2009 (Burke and Lamont 2014). Recent results of an extensive surveillance program in the USA estimated the CDI prevalence at 0.5% in acute care hospitals. In our study, we probably found out a local increase in the number of cases compared with that of normal occurrence, but data from other hospitals in the Czech Republic are not comparable because of different assessment methodologies.

In total, 817 patients with CDI were diagnosed of which 64 (7.8%) were included in this study. Only limited clinical and demographic data were available. CDI was most commonly diagnosed in the departments of internal medicine (61.5%) and long-term care departments (26.2%). CDI was mainly diagnosed in elderly (mean age 76 years) and at a higher rate in women (72.3%). According to the primary diagnosis (before the onset of diarrhea), 44.6% of patients had infectious etiology, of which 62.1% had gastroenteritis and diarrhea of various infectious etiologies.

C. difficile ribotypes and toxin detection

Of 817 patients diagnosed with CDI in this study, 64 (7.8%) *C. difficile* isolates were available for further analysis. These isolates derived from hospitals Nachod (n = 28), Litomysl (n = 32), and Pardubice (n = 4). Of 64 isolates, 39 belonged to ribotype 176, six to ribotype 014, and 4 to ribotype 002. Ribotype 078 was found in two cases (Fig. 1). The frequency of ribotype 014 (9.4%) and 002 (6.3%) was similar as reported for instance in the USA (Tickler et al. 2014) or England (Wilcox et al. 2012). *C. difficile* ribotype 176 is according to some researchers recently considered as a "hypervirulent ribotype" since it resembles genetically ribotype 027 very much and clinical findings suggest increased virulence (Drabek et al. 2015; Polivkova et al. 2016).

The occurrence of *C. difficile* ribotype 176 in the Czech Republic was first described by Nyc et al. (2011). A recent report indicated that ribotype 176 had increased in the Czech Republic, since 40% of 624 typed isolates in a study performed in 2013 belonged to ribotype 176 (Krutova et al. 2014). This ribotype was found also in Poland (Obuch-Woszczatynski et al. 2014) and in the USA (Valiente et al. 2012). Another ribotype, which is considered as a "hypervirulent" (Goorhuis et al. 2008) and was found in our

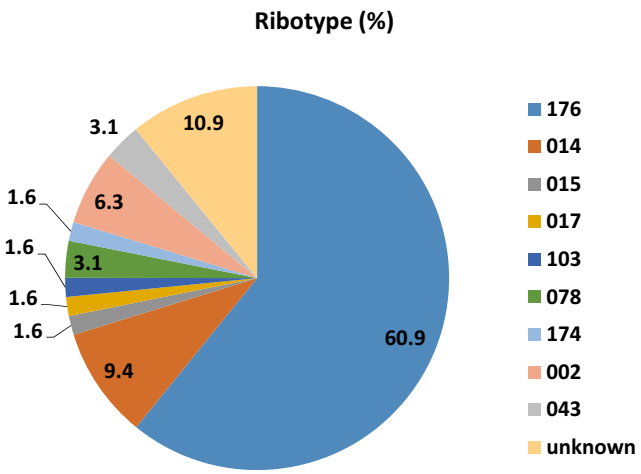

Fig. 1 Distribution of *C. difficile* ribotypes in three Czech hospitals (n = 64)

study, is ribotype 078 (3.1%). Researchers in the USA in a comprehensive study reported 2.0% prevalence of this ribotype (Tickler et al. 2014) and in Europe, even 8% (Bauer et al. 2011). Goorhuis et al. (2008) report even 13% incidence in the Netherlands and note that the occurrence of ribotype 078 is more closely associated with community-acquired disease and younger patients.

Of all 64 isolates, 62 contained the gene for toxin A (*tcdA*) and 63 the gene for toxin B (*tcdB*). A single isolate of ribotype 017 contained only *tcdB* and not *tcdA* and also had no genes for binary toxin *cdtA* and *cdtB* or a deletion in the gene *tcdC*. All 39 isolates of ribotype 176 and two isolates of ribotype 078 were positive for the presence of binary toxin genes (*cdtA* and *cdtB*). All other samples were negative for the presence of the binary toxin genes except one undetermined ribotype. This difference was statistically significant ($p < 0.001$). Isolates belonging to ribotype 176 also carried a possible 18-bp deletion in the regulatory gene *tcdC* in most cases (with the exception of two isolates), whereas ribotype 078 had a possible 39-bp deletion and other ribotypes had no deletion in this gene except for ribotype 015 (18-bp deletion). This difference was also statistically significant ($p < 0.001$).

Both *C. difficile* ribotypes 176 and 078 have genes for binary toxin, which would correspond with their increased virulence (Cowardin et al. 2016). Stewart et al. (2013) suggested that binary toxin could be responsible for recurrent colitis since statistically significant association was found between the presence of this virulence factor and recurrent disease. Similarly, both ribotypes have deletions in the gene *tcdC*. *tcdC* gene is responsible for negative regulation of expression of genes for toxins and a deletion therein causes an increased production of toxins (Matamouros et al. 2007). According to Bakker et al. (2012), the role of *tcdC* in regulation of toxin expression is unclear.

MLVA analysis

Of 39 isolates belonging to ribotype 176, 30 were selected for MLVA analysis; 13 from Litomysl, 16 from Nachod and 1 from Pardubice. MLVA results are depicted in Fig. 2 and

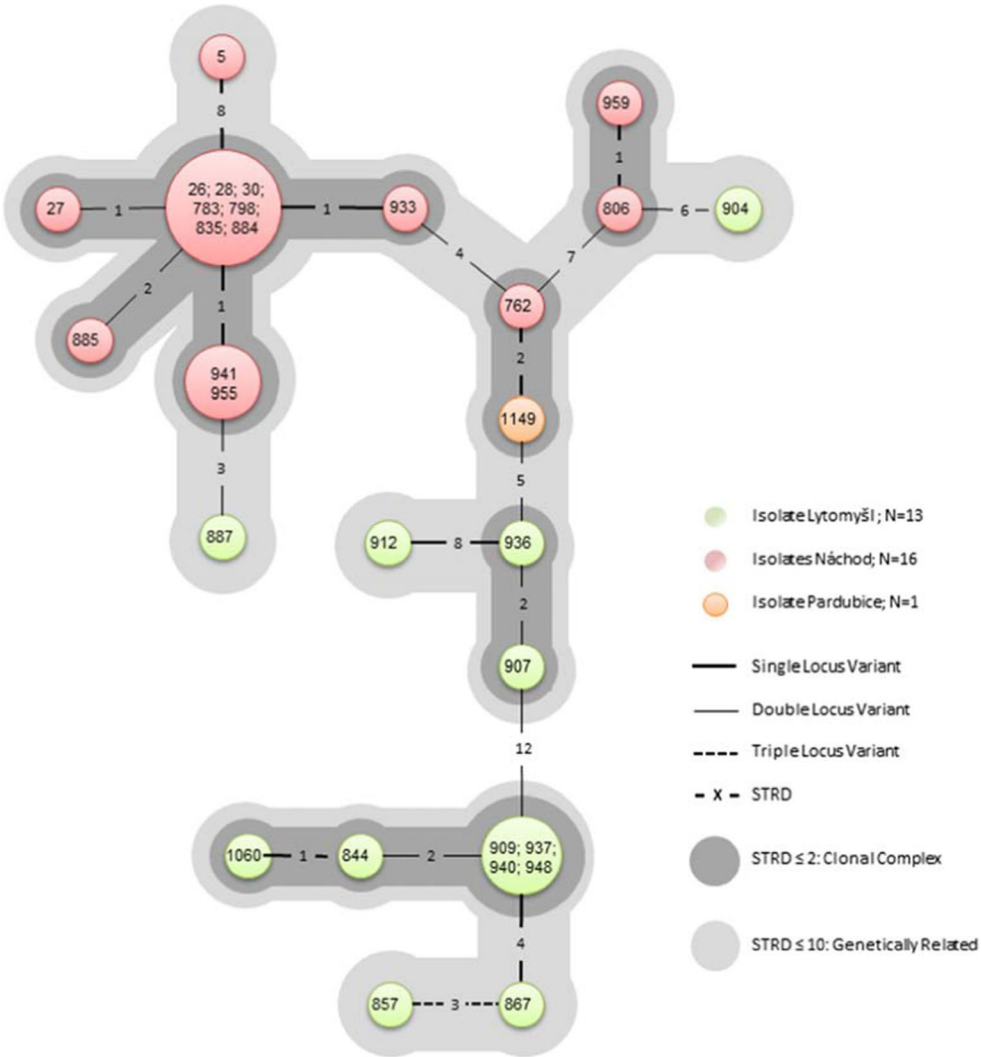

Fig. 2 MLVA typing of 30 isolates (ribotype 176) from three hospitals. A total of seven loci were used. A clonal complex is defined as isolates differing in two or less summed tandem-repeats (*dark gray area*). Strains are genetically related if the isolates differ in 10 or less summed tandem-repeats (*light gray area*)

Table 1 Susceptibility of *C. difficile* to six antimicrobials (μg/mL)

	MIC_{50}[a]	MIC_{90}[b]	MIC range	Breakpoint[c]
Fidaxomicin	0.06	0.125	0.06–0.25	–
Vancomycin	0.125	0.25	0.125–0.25	2
Metronidazole	0.25	0.5	0.06–0.5	2
LFF571	0.06	0.125	0.06–0.125	–
Clindamycin	2	4	1–64	−(4)[d]
Moxifloxacin	8	8	0.06–8	4

[a] Minimum inhibitory concentration required to inhibit the growth of 50% of organisms

[b] Minimum inhibitory concentration required to inhibit the growth of 90% of organisms

[c] Breakpoint values according to EUCAST (2016)

[d] Breakpoint value in parentheses shown in the table for Gram-positive anaerobes

revealed that isolates originating from all three hospitals were genetically related (STRD ≤10), whereas a second complex of genetically related strains only had isolates from one hospital (Litomysl). Five clonal complexes (STRD ≤2) were found of which one contained isolates from two hospitals. Ribotype 176 was the most commonly found ribotype (60.9%) and MLVA results indicated that isolates from three different hospitals were genetically related, suggesting transmission between healthcare facilities.

Antimicrobials susceptibility testing

All 64 tested isolates of *C. difficile* were susceptible to vancomycin and metronidazole. In contrast, 65.1% of the isolates were resistant to moxifloxacin. Ribotype 176 had a high level of resistance to moxifloxacin (100%) compared with other ribotypes (12%; $p < 0.001$). Only two isolates of ribotype other than 176 were resistant to clindamycin. MIC_{50} and MIC_{90} values are depicted in Table 1.

Clinical breakpoints presented in the database EUCAST (2016) are listed for Gram-positive anaerobes and *C. difficile* separately. For newly introduced antimicrobials, such as fidaxomicin and LFF571 (new semisynthetic thiopeptide), breakpoints have not yet been set. However, the MIC values determined for these antimicrobials are very low. MIC ranges for fidaxomicin and LFF571 are 0.06–0.5 mg/L, for both antimicrobials (Debast et al. 2013). Corbett et al. (2015) provide reference MIC for fidaxomicin 0.25 mg/L. For clindamycin, there is no breakpoint regarding *C. difficile*, but it is shown in Table 1 for Gram-positive anaerobes.

Susceptibility testing of *C. difficile* revealed no or low resistance to vancomycin and metronidazole (Freeman et al. 2015). In addition, LFF571 and fidaxomicin that were tested in some studies display MICs even lower than those for vancomycin and metronidazole (Debast et al. 2013). Interestingly, a significant difference in resistance to moxifloxacin was found between ribotype 176 and other ribotypes ($p < 0.001$). High resistance of ribotype 176 to moxifloxacin was also observed by Lachowicz et al. (2015) and by Krutova et al. (2015). This finding is in agreement with ribotype 027 which is also frequently high-level resistant to new fluoroquinolones (Razavi et al. 2007). Only 3.2% of all isolates in our study were resistant to clindamycin. One previous study in the Czech Republic (Beran et al. 2014) states resistance of *C. difficile* isolates to clindamycin (4.8%). Published reports from the Central European countries showed resistance to clindamycin generally higher even up to 57% (Fenner et al. 2008; Indra et al. 2008; Terhes et al. 2009). The *ermB* gene, the marker for resistance to macrolides, lincosamides, and streptogramin B, was detected in two isolates only (MIC to clindamycin was in these isolates 64 and 4 μg/mL). Another isolate, which had MIC to clindamycin 64 μg/mL, did not contain this gene. Interestingly, the resistance to clindamycin was observed in non-RT027 isolates and not in presumptive RT027 isolates in the study of Beran et al. (2014). Conversely, in this work, a higher resistance to moxifloxacin was confirmed in ribotype 176.

Ribotype 176 was the most frequently found ribotype in three hospitals participating in a CDI survey. The close relatedness among ribotype 176 isolates determined by MLVA suggests the transmission among health-care facilities. Our isolates corresponding to the ribotype 176 showed resistance to moxifloxacin.

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Authors' contributions VB: the author of the article and scenario of experiments, and article writing.

EJK: organization of experimental work in Leiden laboratories, suggestions, and checking the manuscript.

Compliance with ethical standards

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

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