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Title: Molecular characterisation of Czech *Clostridium difficile* isolates collected in 2013-2015

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Molecular characterisation of Czech *Clostridium difficile* isolates collected in 2013-2015

Abstract

Clostridium difficile is a leading nosocomial pathogen and molecular typing is a crucial part of monitoring its occurrence and spread. Over a three-year period (2013-2015), clinical *Clostridium difficile* isolates from 32 Czech hospitals were collected for molecular characterisation. Of 2,201 *C. difficile* isolates, 177 (8%) were non-toxigenic, 2,024 (92%) were toxigenic (*tcdA* and *tcdB)* and of these, 677 (33.5%) carried genes for binary toxin production (*cdtA*, *cdtB*). Capillary-electrophoresis (CE) ribotyping of the 2,201 isolates yielded 166 different CE-ribotyping profiles, of which 53 were represented by at least two isolates for each profile. Of these, 29 CE-ribotyping patterns were common to the Leeds-Leiden *C. difficile* reference strain library and the WEBRIBO database (83.7% isolates), and 24 patterns were recognized only by the WEBRIBO database (11.2% isolates). Isolates belonging to these 53 CE-ribotyping profiles comprised 94.9% of all isolates. The ten most frequent CE-ribotyping profiles were 176 (n=588, 26.7%), 001 (n=456, 20.7%), 014 (n=176, 8%), 012 (n=127, 5.8%), 017 (n=85, 3.9%), 020 (n=68, 3.1%), 596 (n=55, 2.5%), 002-like (n=45, 2.1%), 010 (n=35, 1.6%) and 078 (n=34, 1.6%). Multi-locus sequence typing (MLST) of seven housekeeping genes performed in one isolate of each of 53 different CE-ribotyping profiles revealed 40 different sequence types (STs). We conclude that molecular characterisation of Czech *C. difficile* isolates revealed a high diversity of CE-ribotyping profiles; the prevailing RTs were 001 (20.7%) and 176 (027-like, 26.7%).

Introduction

Clostridium difficile is a major causative agent of hospital-acquired diarrhoea. Molecular typing of clinically significant *C. difficile* isolates is a crucial tool for surveillance and spread control of *C. difficile* infections (CDI). The typing approaches are focused on conserved parts, repetitive regions or entire genomes (Knetsch *et al.,* 2013). They include PCR-ribotyping (Bidet *et al.*, 1999, Stubbs *et al.*, 1999; Indra *et al.*, 2008; Fawley *et al*., 2015), multi-locus sequence typing (MLST) (Griffiths *et al*., 2010), and toxinotyping (Rupnik, 2010). Multi-locus variable tandem-repeats analysis (MLVA) (van den Berg et al., 2007) and whole-genome sequencing (Eyre *et al.,* 2013).

The Czech Republic participated in the European *Clostridium difficile* infection surveillance Network (ECDIS-net), a European Centre for Disease Prevention and Control (ECDC) supported project that started in 2011 and focused on building laboratory capacity for pan-European *Clostridium difficile* infection (CDI) surveillance. In relation to this project, the department of Medical Microbiology of

Motol University Hospital introduced CE-ribotyping, and 2,201 Czech *C. difficile* isolates were sent from 32 hospitals for molecular typing over a three-year period (2013-2015).

The aim of this study was to use molecular methods to characterise *C. difficile* isolates circulating in the Czech Republic from 2013-2015.

Material and methods

C. difficile strain collection

Microbiology laboratories in 32 Czech healthcare facilities (7 tertiary care hospitals, 24 secondary care hospitals and 1 specialized care hospital), covering 39% of the hospital beds in the Czech Republic, were invited to cooperate voluntarily in this three-year project (2013-2015). Information about the participating hospitals, CDI testing algorithm used, and the number of submitted isolates is shown in in the supplementary material: Characterisation of hospitals in the study. *C. difficile* isolates were cultured from stool samples taken from hospitalised patients of all ages suspected of CDI, including community-acquired and hospital-acquired CDI. The number of isolates sent for molecular characterisation from each hospital was not strictly determined. A total of 2,201 *C. difficile* isolates was received or cultured at the department of Medical Microbiology of Motol University Hospital and characterised by molecular methods.

Molecular characterisation of *C. difficile* strain collection

Ribotyping (ECDIS-net protocol)

Amplification of 16S-23S intergenic spacer regions was performed using the ECDIS-net protocol, using primers described by Stubbs *et al*. (Stubbs *et al.*, 1999). Capillary electrophoresis was performed using an ABI 3130 Genetic Analyser (Applied Biosystems), a 36 cm array length, default fragment analysis, POP7 polymer and LIZ1200 (Applied Biosystems) as a size standard. The ribotypes were determined using the freely available WEBRIBO database (https://webribo.ages.at/) (Indra *et al.*, 2008) after Gene Mapper® v4.0 (Applied Biosystems) software processing. Subsequently, the CE-ribotyping profiles obtained were also compared with the Leeds-Leiden *C. difficile* reference strain set of CE-ribotyping profiles (n=70) generated using Gene Mapper® v4.0 software (Applied Biosystems) from *.fsa files used at the first stage of the CE-ribotyping validation study (Fawley *et al.*, 2015).

Presence of genes for toxin production

The presence of genes (*tcdA, tcdB, cdtA* and *cdtB*) for toxin production (A, B and binary) was investigated in all isolates (n=2,201) by a multiplex PCR (Persson *et al.*, 2008, 2009), including a

Leeds-Leiden reference strain (RT 027) as a positive control. The *tcdA*-negative strains (due to their 3´-end deletion) could not be identified because the location of the primers is upstream from the repetitive region. These strains revealed positive *tcdA* fragment PCR amplification (Persson *et al.*, 2008, 2009).

Molecular characterisation of 53 selected CE-ribotyping profile *C. difficile* isolates

Ribotyping (new consensus protocol)

Selected isolates of 53 CE-ribotyping profiles were reinvestigated according to the recently published consensus CE-ribotyping protocol (Fawley *et al.*, 2015), which applies primers described by Bidet *et al*. (Bidet *et al.*, 1999). We carried out a cluster analysis of these CE-ribotyping profiles using the Unweighted Pair Group Method, with Arithmetic Mean (UPGMA) distance analysis based on the presence of CE-ribotyping peaks of defined molecular weight (Bionumerics v7.1 – Applied Maths; the UPGMA figure is in the supplementary material).

MLST

The MLST was performed by amplification and sequencing of seven housekeeping genes: *adk1, atpA1, dxr3, glyA1, recA2, sodA5* and *tpi2* (Griffiths *et al.*, 2010)**.** The sequences obtained were uploaded to the MLST database (http://pubmlst.org/cdifficile) to determine the appropriate alleles of the genes. The sequence type was determined by the combination of identified alleles. A maximum-likelihood tree was generated from the alignment of concatenated DNA sequences of seven housekeeping loci using the MEGA5 software available at http://www.megasoftware.net/ (Tamura *et al.*, 2011).

Presence of deletions in the tcdC gene

The *tcdC* gene was amplified with primers C1 and C2 (Spigaglia and Mastrantonio, 2002) and sequenced in a reverse direction. The sequences obtained were compared with the NCBI reference sequence *Peptoclostridium difficile* 630, NC_009089.1.

Results

A total of 2,201 *C. difficile* isolates was collected from 32 hospitals from 2013-2015. The geographical distribution of participating hospitals and the number of *C. difficile* isolates available for molecular characterization is shown in Figure 1. The mean age of patients was 65.7 years (range 30 days - 97 years). Of 2,201 isolates, 82 (3.7%), 103 (4.7%), 509 (23.1%) and 1507 (68.5%) were from patients aged $\leq$2, 3-18, 19-64 and $\geq$65 years, respectively.

Ribotyping and the presence of toxin genes

Of 2,201 *C. difficile* isolates, CE-ribotyping revealed 53 profiles in 2,088 isolates (94.9%) when at least two isolates per profile were identified. Of the 53 CE-ribotyping profiles, 29 were recognized both by the Leeds-Leiden reference set and the WEBRIBO database and comprised 1,841 (83.7%) of all isolates (n=2,201). The remaining 24 CE-ribotyping profiles (247, 11.2%, of all isolates) were only identified by the WEBRIBO database and were designated as WEBRIBO types (WRTs). The remaining 113 (5.1%) isolates yielded unique single profiles. An overview of the RTs and WRTs identified is shown in Table 1. The highest diversity was found among 1,507 isolates derived from patients of age ≥65 years, from whom 28 RTs, 24 WRTs and 58 single profiles were identified. In the 19-64 years age group, 509 isolates yielded 27 RTs, 17 WRTs and 38 single profiles; in the 3-18 years group, 103 isolates showed 24 RTs, 9 WRTs and 12 single profiles, and in the two years and younger group 11 RTs, 4 WRTs and 5 single profiles were found for 82 isolates.

Of 2,201 *C. difficile* isolates, 2,024 (92%) were toxigenic (*tcdA* and *tcdB)* and of these, 677 (33.5%) isolates carried genes for binary toxin production (*cdtA*, *cdtB*) and the remaining 177 (8%) isolates were non-toxigenic. The highest ratio of non-toxigenic to toxigenic isolates (64:18) was found for the group of patients two years old and younger. By comparison, the non-toxigenic to toxigenic isolate ratio was 27:76 for patients of age 3-18 years, 33:476 for those of age 19-64 years, and 53: 1,454 for those ≥65 years.

The most frequently identified toxigenic CE-ribotyping profiles were RTs 176 (n=588, 29.1%), 001 (n=456, 22.5%), 014 (n=176, 8.7%), 012 (n=127, 6.3%), 017 (n=85, 4.2%), 020 (n=68, 3.4%), 078 (n=34, 1.7%), 005 (n=30, 1.5%) and WRT 002-like (n=45, 2.2%). The distribution of predominant RTs 001 and 176 differs distinctly within age groups of patients. Whereas in group of patient two years old and younger is the presence of these RTs rare (1.2% of RT 001 only), in group of patients 3-18 years was 11.7% (10.7%, 1%), in group of patient 19-64 years was 35.5% (14.7%, 20.8%) and in group of patients ≥65 years was 56.4% (24.5%,31.9%)

The most frequent non-toxigenic CE-profiles were WRT 596 (n=55, 31.1%) and RT 010 (n=35, 19.8%). WRT 596 was identified in isolates derived from all patient age groups, but the majority (39/55) were detected in isolates from paediatric patients aged ≤2 years. The presence of the 11 most common CE-ribotyping profiles in individual hospitals is shown in Figure 1, and its distribution according to patient age in Figure 2.

Of the 53 CE-ribotyping profiles, one isolate from each profile was reinvestigated by the new consensus CE-ribotyping protocol (Fawley *et al.*, 2015). Of these, four CE-ribotyping profiles showed

a change in their CE-ribotyping profile due to an additional amplification of the 326 bp fragment. WRT 203 changed to WRT 209 and RT 002 to WRT 002-like, whereas WRT AI-60 and WRT AI-75 retained the same designation in the WEBRIBO database. The additional amplification of a 326 bp fragment, observed in RT 002, was not noticed in the Leeds-Leiden reference RT 002 strain, suggesting that only a local Czech RT 002 variant showed this difference.

The UPGMA analysis of CE-ribotyping profiles and the CE-ribotyping profiles together with their band sizes are shown in the supplementary material (UPGMA, Supplementary material – Molecular data on Czech *C. difficile* strain collection).

MLST and the presence of deletions in the *tcdC* gene

The application of the MLST of seven housekeeping genes in isolates from 53 ribotypes revealed 40 different STs clustering to 5 clades (Table 1, Figure 3). The isolates revealing similar ST but different RT or WRT are listed in Table 2. Clade 1 was heterogeneous and consisted of 44 CE-ribotyping profiles, 25 RTs and 19 WRTs, 37 toxigenic (*tcdA*+, *tcdB*+) and 7 non-toxigenic. Clade 2 included only two RTs, both of ST1: RTs 027 and 176. Clade 3 contained two isolates with an identical ST5: RT 023 and WRT 438. Clade 4 consisted of two isolates belonging to RT 017 (ST37) and WRT 498 (ST170). In Clade 5, three isolates of ST11 (RTs 078 and 126, WRT 413) were recognized.

RTs 027 and 176 had the one base pair deletion at nucleotide position 117, and the 18 bp deletion in the *tcdC* gene. RT 023 and WRT 438 had the 54 bp deletion in the *tcdC* gene. RTs 078, 126 and WRT 413 showed the 39 bp deletion in the *tcdC* gene. The isolates harbouring 54 bp and 39 bp deletions (except for WRT 413) revealed a nonsense mutation C184T. All isolates belonged to RT 023, 027, 126, 176 and WRTs 413 and 438 were also binary toxin gene positive.

Discussion

During a three-year period (2013-2015) a total of 32 hospitals voluntarily participated in this project, but only 11 hospitals sent isolates for molecular characterisation in each year of the study. Eight percent of *C. difficile* isolates were non-toxigenic although they were cultured from patients suspected of CDI. These isolates were sent from hospitals with a suboptimal CDI diagnostic algorithm, which means the absence of a confirmatory test for GDH-tested positive only samples (Debast *et al*., 2014), or they were cultured in our laboratory, where all cultured *C. difficile* isolates are ribotyped and tested for the presence of genes for toxin production. In our study, 12 hospitals did not confirm the production of toxins by *C. difficile* isolates cultured from GDH-tested positive only stool samples. Additionally, one laboratory did not test for the presence of toxins in stool samples because they use the nucleic acid amplification technique (NAAT) as the first diagnostic step.

CE-ribotyping of 2,201 Czech isolates revealed 166 different CE-ribotyping profiles. Of these, 113 CE-ribotyping profiles (5.1%) were represented by only a single isolate, and its clinical and/or epidemiological significance is unclear. Fifty-three different CE-ribotyping profiles contained at least two isolates per profile. Of the 53 CE-ribotyping profiles, 29 profiles comprising 83.7% of all isolates were recognized identically by two large, frequently used databases. The spectrum of the most frequently found toxigenic RTs found in our study is similar to the most frequently found toxigenic RTs in the European hospital-based survey (Bauer *et al.*, 2011). The exception is RT 176, with its specifically geographic-epidemiological occurrence in the Czech Republic (Krutova *et al.*, 2014b) and Poland (Pituch *et al.*, 2015). RT 176 belongs to the RT 027 "family" (Valiente *et al*., 2012). Data on CDI patients infected by RT 176 outcomes have recently been published in two single-centre studies, including 30 and 111 patients, respectively. The results showed a higher rate of severe CDI (11/7 and 13/3) and mortality (5/2 and 16/8) in patients infected by RT 176 compared with patients infected by non-176 ribotypes (Drabek *et al*., 2015; Polivkova *et al*., 2016). While RT 027 is distributed worldwide (He *et al*., 2013), its occurrence is rare to date in the Czech Republic (Krutova *et al*., 2014b). We identified only five isolates in four different hospitals over three years; however, hospitals from border areas with Germany and Poland (Figure 1) did not participate in this study, and both countries have high prevalence rates of RT 027 (Arvand *et al*., 2014, Pituch *et al.*, 2015). The second most common CE-ribotyping profile was RT 001 (n=456). In contrast with RT 176, RT 001 is frequently found in many European countries (Bauer *et al.*, 2011; Wiuf *et al*., 2011; Arvand *et al*., 2014; Taori *et al*., 2014; Nyc *et al*., 2015; Freeman *et al*., 2015). In our study, the simultaneous presence of ribotypes 001 and 176 was detected in 28 of the 32 hospitals.

Of 53 CE-ribotyping profiles, 24 were recognized only by the WEBRIBO database and these isolates comprised 11.2% (n=247) of our collection. The occurrence of several WRTs identified in our study (209, 220, 404, 416, 438, 500, 555, AI-12, AI-20, AI-21, AI-75, AI-9-1) has been reported as human clinical isolates (Novak *et al.*, 2015; Indra *et al.*, 2015; Fang *et al.*, 2014; Rafila *et al.*, 2014; Hell *et al.*, 2011; Indra *et al.*, 2008) or as animal isolates WRTs 203, 209, 413, 446, 596, AI-12, AI-60, AI-8/1, AI-9-1 (Janezic *et al.*, 2014; Schneeberg *et a.l*, 2013; Indra *et al.*, 2009; Goldova *et al.*, 2012; Indra *et al.*, 2008). Four of these WRTs (AI-82/1, AI-9-1, AI-60, AI-12) have recently been identified in the UK Ribotyping Reference Laboratory (Leeds, UK) as RTs 103, 013, 097 and 150 respectively (Janezic *et al.*, 2014).

WRTs AI-82/1, AI-9-1 and AI-60 showed the same ST as was published by Dingle *et al*. in RTS 103, 013 and 097 (Dingle *et al.*, 2011). WRTs 015 and 002 were assigned as WRTs 015-like and 002-like due to slight changes in their CE-ribotyping profiles; however, the ST of WRT 002-like (ST8) was identical to that of RT 002 in the studies of Knetsch *et al*. and Dingle et al. (Knetsch *et al.*, 2012; Dingle *et al.*,

2011). The ST of WRT 015-like (ST44) corresponds with the findings of Dingle *et al*., who identified two STs in RT 015 isolates: ST44-*tcdC* wild type and ST10, similarly to Knetsch *et al*. (Knetsch *et al.*, 2012), with the presence of 18 bp deletion in the *tcdC* gene (Dingle *et al.*, 2011).

The distribution isolates depending on the age of the patients revealed the highest ratio of non-toxigenic to toxigenic ribotypes (64:18) and low presence and absence of two predominant toxigenic RTs 001 (1.2%) and 176 (0%) in patients two years old and younger. In other age groups (3-18 years, 19-64 years and >65 year), the non-toxigenic and toxigenic isolates ratio decreases (27:76, 33:476 and 53: 1,454), while the occurrence of RTs 001 and 176 increases (11.7%, 35.5%, to 56.4%), respectively. The predominant occurrence of RTs 001 and 027 in older population was also found in the study authors von Müller et al., where RT 027 was not present and RT 001 was present in 9.6 % in group of patients 0-17 years and these ratios increased to 30.7% for RT 027 and 38.6% for RT001 in oldest group of patients (>85 years). (von Müller *et al*., 2015).

The application of the new CE-ribotyping protocol (Fawley *et al*., 2015) changed the CE-ribotyping profile in 7.5% of profiles (n=4) with a subsequent change of identification by the WEBRIBO database in two profiles. The WEBRIBO database provides a broad spectrum of available CE-ribotyping profiles, but the raw data are obtained by different protocols (primer design, polymer type) and some of the CE-ribotyping profiles are designated only by a WEBRIBO number or by a combination of letter and number. This stresses the importance of the use of a standardized protocol and also the standardisation of an appropriate dataset of reference *C. difficile* strains uploaded to the WEBRIBO database.

The MLST of seven housekeeping genes of 53 ribotypes revealed 40 different STs clustering to 5 clades. Although the MLST was performed only in one isolate of each identified CE-ribotyping profile, we found the correlation with STs identified in ribotypes represented in the Leeds-Leiden *C. difficile* reference strain collection published by Knetsch *et al.* (Table 1, marked with*) (Knetsch *et al.*, 2012). The most heterogeneous was MLST clade 1, which included 44 CE-ribotyping profiles of 53 CE-ribotyping profiles. MLST clade 1 heterogeneity was also observed in the study by Stabler *et al*., who found that this clade contained 106 STs of the 141 studied STs (Stabler *et al*., 2012). Knetsch *et al*. typed 35 STs out of 56 as belonging to clade 1 (Knetsch *et al*., 2012), whereas Griffiths *et al*. concluded that 31 STs out of 40 belonged to clade 1 (Griffiths *et al*., 2010). Similarly, Dingle *et al*. found 60 STs out of 69 belonging to clade 1 (Dingle *et al*., 2011).

Several isolates belonging to a different RT or WTR revealed the same ST (clade) and the specific deletion in *tcdC* gene that suggests their phylogenetic relationship. RTs 027 and 176 revealed ST1 (clade 2), as was published by Knetsch *et al*. (Knetsch *et al.*, 2012), as well as the presence of one

base pair deletion at nucleotide position 117, which is a target site for commercial molecular systems (Krutova *et al*., 2014a; Mentula *et al*., 2015), and 18 bp deletions in the *tcdC* gene. RT023 and WRT 438 revealed ST5 (clade 3) and had 54 bp deletions in the *tcdC* gene. RTs 078, 126 and WRT 413 showed ST11 and 39 bp deletions in the *tcdC* gene. Isolates harbouring 54 bp and 39 bp deletions (except WRT 413) as previously described above revealed a nonsense mutation C184T (Spigaglia and Mastrantonio, 2002; Curry *et al*., 2007). All these isolates (RT 023, 027, 126, 176 and WRTs 413 and 438) revealed the presence of binary toxin genes, another important *C. difficile* virulence factor (Gerding *et al*., 2014).

The Czech Republic is a country with increasing CDI incidence (1.1 cases per 10,000 patient bed-days in 2008 to 4.4 cases in 2011 – 2012 and 6.2 cases per 10,000 patient bed-days in 2012 – 2013) (Bauer *et al*., 2008; Davies *et al*., 2014) and relatively high rates of antibiotic resistant *C. difficile* strains (Freeman *et al*., 2015). Implementation of CDI surveillance based on the recently released CDI surveillance protocol Control (ECDC, 2015) in the Czech Republic would fill the gap in Czech CDI epidemiology with national CDI incidence data, including clinical case information and *C. difficile* isolate antibiotic susceptibility results.

Conclusion

The molecular characterisation of 2,201 Czech clinical *C. difficile* isolates revealed 53 different CE-ribotyping profiles and 40 multi-locus sequence types. Of 2,201 *C. difficile* isolates, 2,024 were toxigenic (*tcdA* and *tcdB*), and of these, 677 isolates carried genes for binary toxin production (*cdtA*, *cdtB*). The results of molecular characterisation showed a high diversity of *C. difficile* strains circulating in the Czech Republic with prevailing representation of RTs 001 and 176 (027-like).

CE-ribotyping applied on a Czech *C. difficile* isolate collection demonstrates its high discrimination capability and the results highlight the need to use a standardised protocol as well as a standardised CE-ribotyping profile library to gain inter-laboratory comparable data on clinically and/or epidemiologically significant *C. difficile* isolates.

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Ethical statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

Conflicts of interest

EJK, FA, ON, JM, MK declare no conflict of interests. MHW has received: consulting fees from Actelion, Astellas, bioMerieux, MedImmune, Merck, Pfizer, Qiagen, Sanofi-Pasteur, Seres, Summit, Synthetic Biologics and Valneva; lecture fees from Alere, Astellas, Merck & Pfizer; and grant support from Actelion, Astellas, bioMerieux, Da Volterra, Merck, Sanofi-Pasteur, Seres and Summit. There is no relationship between above mentioned companies and the study presented in this manuscript.

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Table 1: Distribution of toxigenic and non-toxigenic ribotypes (bold) and WEBRIBO types of Czech C. difficile isolates as identified by Leeds-Leiden database and WEBRIBO database in a Czech C. difficile collection.

CE-ribotyping profile	Presence of toxin genes*	ST (clade)	Number of Isolates (%)	Number of hospitals	Number of isolates in age groups (%) ≤2	>2-≤18	>18-≤64	≥65
176	A, B, Bin	1*(2)	588 (26.7)	30	0	1 (0.1)	106 (4.8)	481 (21.9)
001	A, B	3*(1)	456 (20.7)	30	1 (0.1)	11 (0.5)	75 (3.4)	369 (16.8)
014	A, B	2*(1)	176 (8.0)	26	5 (0.2)	10 (0.5)	51 (2.3)	110 (5.0)
012	A, B	54*(1)	127 (5.8)	14	1 (0.1)	2 (0.1)	44 (2.0)	80 (3.6)
017	A, B	37*(4)	85 (3.9)	18	0	1 (0.1)	15 (0.7)	69 (3.1)
020	A, B	110 (1)	68 (3.1)	21	5 (0.2)	6 (0.3)	21 (1.0)	36 (1.6)
596	non-toxigenic	48 (1)	55 (2.5)	8	39 (1.8)	4 (0.2)	3 (0.1)	9 (0.4)
002-like	A, B	8 (1)	45 (2.1)	16	1 (0.1)	1 (0.1)	14 (0.6)	29 (1.3)
010	non-toxigenic	15*(1)	35 (1.6)	13	3 (0.1)	7 (0.3)	10 (0.5)	15 (0.7)
078	A, B, Bin	11*(5)	34 (1.6)	15	0	2 (0.1)	18 (0.8)	14 (0.7)
005	A, B	6* (1)	30 (1.4)	14	0	5 (0.2)	14 (0.7)	11 (0.5)
029	A, B	16* (1)	27 (1.2)	13	0	4 (0.2)	7 (0.3)	16 (0.7)
070	A, B	55* (1)	26 (1.2)	14	1 (0.1)	3 (0.1)	5 (0.2)	17 (0.8)
023	A, B, Bin	5* (3)	26 (1.2)	14	0	4 (0.2)	7 (0.3)	15 (0.7)
015-like	A, B	44 (1)	25 (1.1)	8	0	2 (0.1)	7 (0.3)	16 (0.7)
081	A, B	9* (1)	23 (1.0)	11	0	1 (0.1)	3 (0.1)	19 (0.9)
449	A, B	2 (1)	21 (1.0)	14	1 (0.1)	1 (0.1)	8 (0.4)	11 (0.5)
039	non-toxigenic	26 (1)	21 (1.0)	6	8 (0.4)	5 (0.2)	2 (0.1)	6 (0.3)
011	A, B	325 (1)	20 (0.9)	11	0	1 (0.1)	8 (0.4)	11 (0.5)
003	A, B	12* (1)	15 (0.7)	6	0	1 (0.1)	4 (0.2)	10 (0.5)
018	A, B	17* (1)	15 (0.7)	8	0	5 (0.2)	3 (0.1)	7 (0.3)
AI-61	non-toxigenic	27 (1)	14 (0.6)	5	8 (0.4)	2 (0.1)	2 (0.1)	2 (0.1)
087	A, B	46* (1)	10 (0.5)	8	0	1 (0.1)	4 (0.2)	5 (0.2)
046	A, B	35 (1)	10 (0.5)	4	1 (0.1)	1 (0.1)	6 (0.3)	2 (0.1)
126	A, B, Bin	11* (5)	9 (4.5)	6	0	1 (0.1)	3 (0.1)	5 (0.2)
498	A, B	170 (4)	9 (0.4)	4	0	0	2 (0.1)	7 (0.3)
AI-75	A, B	8 (1)	8 (0.4)	6	0	0	4 (0.2)	4 (0.2)
009	non-toxigenic	3* (1)	8 (0.4)	5	1 (0.1)	1 (0.1)	2 (0.1)	4 (0.2)
031	non-toxigenic	29* (1)	7 (0.3)	4	1 (0.1)	1 (0.1)	2 (0.1)	3 (0.1)
AI-21	A, B	44 (1)	7 (0.3)	6	0	1 (0.1)	4 (0.2)	2 (0.1)
220	A, B	35 (1)	7 (0.3)	3	0	1 (0.1)	2 (0.1)	4 (0.2)
446	A, B	58 (1)	6 (0.3)	4	0	1 (0.1)	1 (0.2)	4 (0.2)
AI-9-1 (013)	A, B	45 (1)	6 (0.3)	6	0	0	1 (0.1)	5 (0.2)
AI-12 (150)	A, B	92 (1)	6 (0.3)	4	0	0	1 (0.1)	5 (0.2)
054	A, B	43* (1)	5 (0.2)	4	0	0	2 (0.1)	3 (0.1)
027	A, B, Bin	1* (2)	5 (0.2)	4	0	0	1 (0.1)	4 (0.2)
AI-82/1 (103)	A, B	53 (1)	5 (0.2)	4	0	0	1 (0.1)	4 (0.2)
203/209	A, B	8 (1)	5 (0.2)	3	0	1 (0.1)	1 (0.1)	3 (0.1)
076	A, B	2* (1)	4 (0.2)	3	0	0	0	4 (0.2)
051	non-toxigenic	101 (1)	4 (0.2)	2	0	2 (0.1)	1 (0.1)	1 (0.1)
500	A, B	42 (1)	4 (0.2)	3	0	0	1 (0.1)	3 (0.1)
026	A, B	7* (1)	3 (0.1)	3	1 (0.1)	1 (0.1)	1 (0.1)	0
236	A, B	33 (1)	3 (0.1)	3	0	0	2 (0.1)	1 (0.1)
404	A, B	13 (1)	3 (0.1)	3	0	0	0	3 (0.1)
434	A, B	91 (1)	3 (0.1)	1	0	0	0	3 (0.1)
555	A, B	286 (1)	3 (0.1)	3	0	0	0	3 (0.1)
AI-60 (097)	A, B	21 (1)	3 (0.1)	3	0	0	1 (0.1)	2 (0.1)
413	A, B, Bin	11 (5)	3 (0.1)	2	0	0	0	3 (0.1)
043	A, B	103* (1)	2 (0.1)	1	0	0	0	2 (0.1)
053	A, B	63* (1)	2 (0.1)	1	0	0	1 (0.1)	1 (0.1)
212	A, B	2 (1)	2 (0.1)	2	0	0	0	2 (0.1)
416	A, B	6 (1)	2 (0.1)	2	0	0	0	2 (0.1)
438	A, B, Bin	5 (3)	2 (0.1)	1	0	0	0	2 (0.1)

*Table 1 footnotes: MLST and tcdC sequencing were performed in representative isolates of each CE-ribotyping profile (n=53). Knetsch et al. identified STs-RTs marked with *. (ST: sequence type; tcdA/B: genes for toxin A/B production; cdtA/B: genes for binary toxin production; tcdC: toxin gene expression negative regulator). *Primers used to amplify tcdA are located upstream of the repetitive region in the 3´-end. The TcdA-negative strains due to 3´-end deletion revealed positive PCR amplification [Persoon et al., 2008, 2009].*

Table 2: Ribotypes and WEBRIBO types (italics) revealing identical sequence type

ST	Ribotype	Clade
1	027, 176	2
2	014, 076, 212, *449*	1
3	001, 009	1
5	023, *438*	3
6	005, *416*	1
8	*002-like, 203/209, AI-75*	1
11	078, 126, *413*	5
35	046, *220*	1
44	*015-like, AI-21*	1

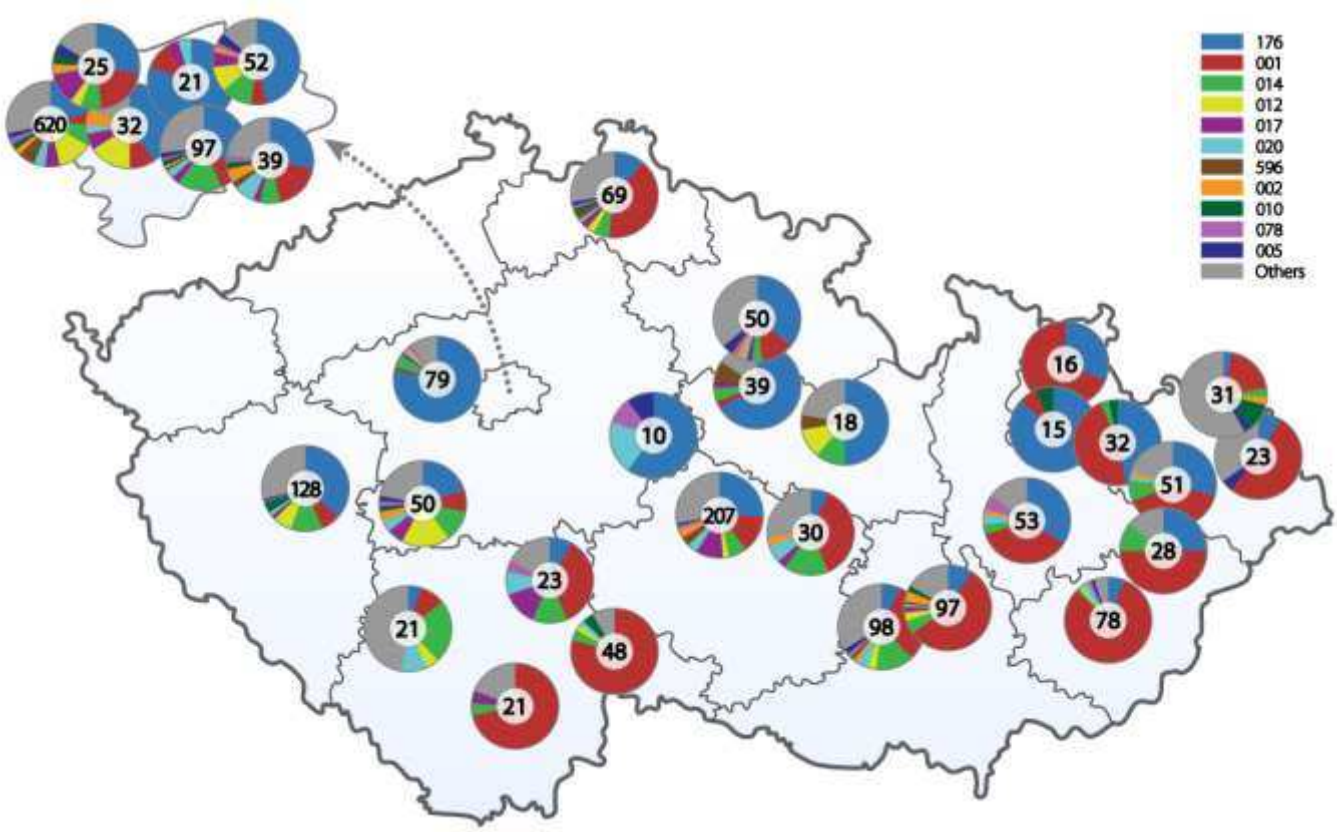

Figure 1: Distribution of participating hospitals in the study. Pie charts show the most common CE-ribotyping profiles identified per hospital. The numbers in the centre represent number of C. difficile isolates sent for molecular characterisation.

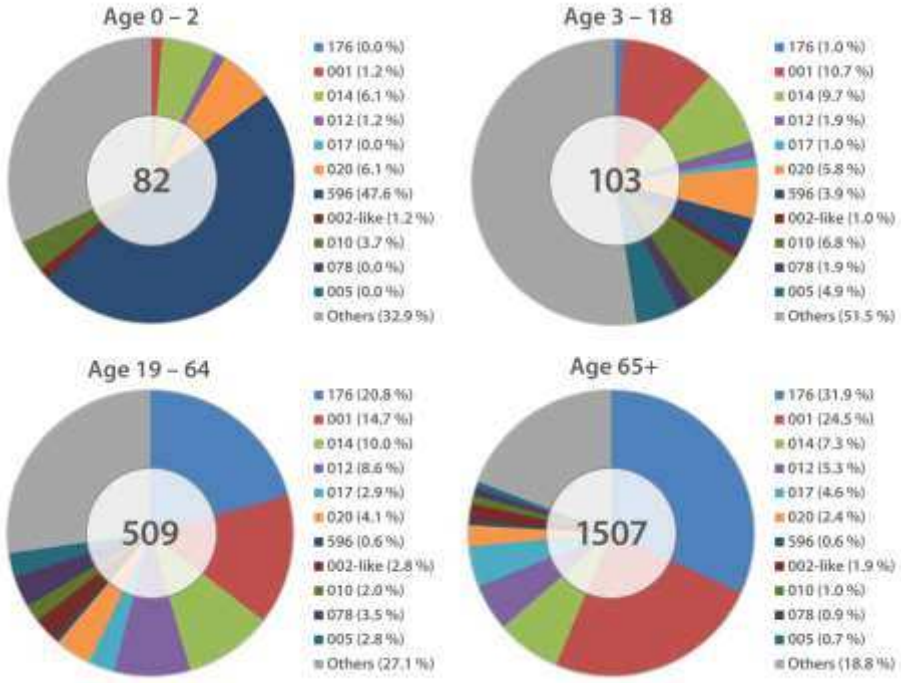

Figure 2: Distribution of commonest CE-ribotyping profiles depending on age of patients.

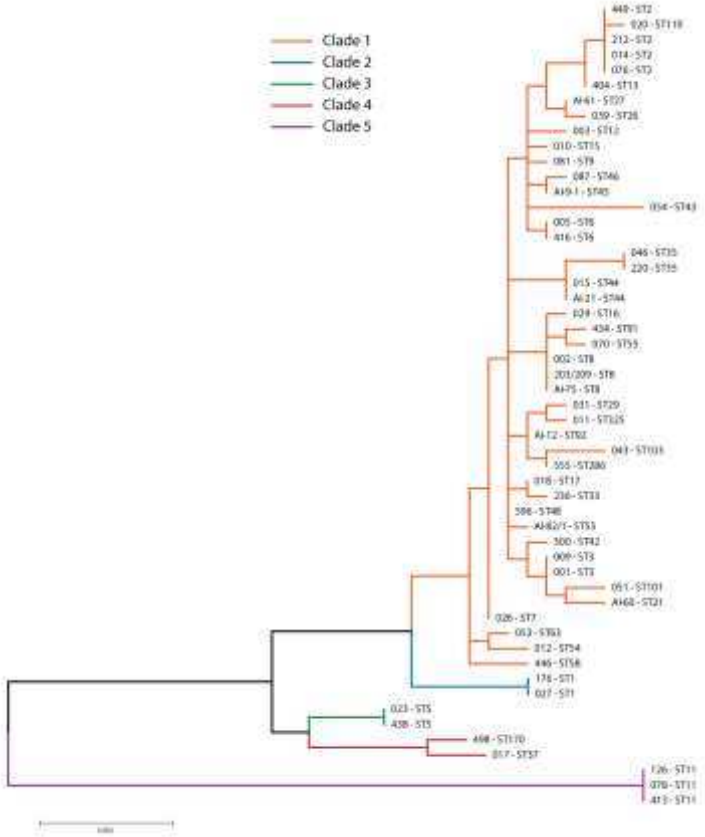

Figure 3: Maximum likelihood tree generated using alignment of concatenated DNA sequences of seven housekeeping loci (ribotype or WEBRIBO type-sequence type).