Univerzita Karlova

2. lékařská fakulta

Studijní program: Mikrobiologie

Mgr. Marcela Krůtová

Clostridium difficile: Molekulární typizace klinicky významných izolátů

Clostridium difficile: Molecular typing of clinically significant isolates

Dizertační práce

Školitel: MUDr. Otakar Nyč, Ph.D.

Praha, 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem řádně uvedl/a a citoval/a všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 28. 03. 2017

Marcela Krůtová

Identifikační záznam:

KRŮTOVÁ, Marcela. *Clostridium difficile*: Molekulární typizace klinicky významných izolátů. *[Clostridium difficile: Molecular typing of clinical significant isolates]*. Praha, 2017. 70 s., 92 příl. Dizertační práce (Ph.D.). Univerzita Karlova, 2. lékařská fakulta, Ústav lékařské mikrobiologie. Školitel: MUDr. Otakar Nyč, Ph.D.

Klíčová slova: *Clostridium difficile*, molekulární typizace, ribotypizace, MLVA, MLST

Poděkování

V první řadě bych upřímně poděkovala svému školiteli Dr. Nyčovi za bezmeznou podporu a rady během řešení celého projektu a poskytnutí prostoru pro realizaci zahraničních projektů.

Mé hluboké díky patří profesoru Kuijperovi za obětavé sdílení zkušeností a cenných rad v problematice *Clostridium difficile* v Evropě.

Dále bych ráda poděkovala kolektivu Ústavu lékařské mikrobiologie, jmenovitě pak Dr. Matějkové za láskyplnou péči o izoláty *Clostridium difficile*.

Mé díky patří také kolektivu profesora Seemana za poskytnutí skvělého technického i přátelského zázemí pro molekulární typizaci izolátů.

Na závěr bych poděkovala svému manželovi za mnohostrannou a obětavou podporu během mého studia a doufám, že tato životní zkušenost byla pozitivním příkladem našemu synovi Matějovi.

Dizertační práce vznikala za finanční podpory Interní grantové agentury Ministerstva zdravotnictví (IGA NT14209-3) a institucionální podpory MZ ČR – RVO, FN v Motole 00064203.

Seznam zkratek

APD	Autoprotease domain
CDI	*Clostridium difficile* infection
CDT	*Clostridium difficile* actin-specific ADP-ribosyltransferase
CLSI	Clinical and Laboratory Standards Institute
CROPS	Combined repetitive oligopeptides
DNA	Deoxyribonucleic acid
GTD	Glucosyltransferase domain
ECCMID	European congress of clinical mikrobiology and infectious diseases
ECDC	European centre for disease infection and control
ECDIS-net	European *Clostridium difficile* infection surveillance network
EIA	Enzyme immunoassay
EHK	Externí hodnocení kvality
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDX	Fidaxomicin
GDH	Glutamátdehydrogenáza
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MLST	Multilocus sequence typing
MLVA	Multilocus variable-number tandem repeats analysis
MTZ	Metronidazol
NAAT	Nucleic acis amplification tests
NCBI	National Center for Biotechnology Information
PaLoc	Pathogenicity locus
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
rRNA	Ribosomal ribonucleic acid
STRD	Sum of tandem-repeats differences
VAN	Vancomycin
VNTR	Variable number tandem repeat

Obsah

1. Úvod

Infekce vyvolané *Clostridium difficile* (CDI) nabývají v současné době na významu jak v souvislosti s nozokomiálním, tak i komunitním výskytem. Klinická manifestace onemocnění je různorodá, od mírných průjmů až po život ohrožující stavy.

Pro zdravotnická zařízení znamená výskyt *C. difficile* zvýšení nákladů na zdravotní péči o pacienta z důvodu nutnosti zavedení bariérových protiepidemických opatření a prodloužení délky hospitalizace. Ze strany pacienta jde o snížení kvality života a značnou psychickou zátěž spojenou s jeho izolací a v neposlední řade komplikaci léčby základního onemocnění.

Globální problém narůstající bakteriální rezistence se týká i *C. difficile*. Dle posledních doporučených dostupů jsou pro léčbu těchto infekcí doporučeny pouze tři preparáty, z nichž jeden je indikován pouze v případě mírného průběhu onemocnění a druhý, ačkoliv s vynikajícím farmakologickým účinkem, je vzhledem k jeho ceně téměř nepoužíván. Vývoj a klinické testování nových preparátů a terapeutických postupů v boji s těmito infekcemi jsou proto v popředí zájmu farmaceutických firem.

Narůstající incidence CDI vedla k sepsání doporučených postupů pro léčbu, diagnostiku a surveillance těchto onemocnění. Zároveň bylo provedeno několik celoevropských studií zaměřených na incidenci a testovací frekvenci CDI, a také na charakterizaci konkrétních kmenů stanovením jejich genotypu, tak i fenotypu testováním citlivosti k antimikrobním látkám.

Diverzita jednotlivých kmenů *C. difficile* je vysoká s dokumentovaným výskytem v prostředí, u zvířat i v potravním řetězci. Studium podstaty molekulárních mechanismů umožňujících rozšíření konkrétních kmenů *C. difficile* v určitém prostředí je nutné pro porozumění epidemiologický konsekvencí.

Molekulární typizace je základním nástrojem pro sledování výskytu jednotlivých kmenů *C. difficile*. Mnoho publikovaných typizačních přístupů znesnadňuje porovnání údajů z jednotlivých studií. V předkládané práci byly použity metody v Evropě zavedené a v případě ribotypizace i doporučené pro surveillance CDI. Výsledky práce předkládají hodnotnou informaci o kmenech cirkulujících v České republice v období 2013-2015.

1.1 Clostridium difficile

Clostridium difficile bylo popsáno v roce 1935 autory Hall a O'Toole jako součást střevní mikroflóry zdravých novorozenců a vzhledem k obtížné kultivovatelnosti bylo nazváno *Bacillus difficilis* (Hall a O'toole, 1935). V roce 1977, bylo *C. difficile* rozpoznáno jako původce pseudomembranózní kolitidy (Bartlett a Gorbach, 1977). Na základě detailní fylogenetické analýzy bylo *C. difficile* v roce 2013 reklasifikováno jako *Peptoclostridium difficile* (Yutin a Galperin, 2013), název, který byl přijat Americkým centrem pro biotechnologické informace (NCBI), ale odbornou veřejností zůstal opomenut. V roce 2016, pak autoři Lawson a kolektiv navrhli, na základě 94,7 % shody v analyzovaných sekvencích s *Clostridium mangenotii,* přejmenování na *Clostridioides difficile* (Lawson et al., 2016), což by umožnilo zachování zažitých zkratek odborných tak komerčních.

Kmen Firmicutes, třída Clostridia, řád Clostridiales, čeleď Peptosreptococcaceae, rod Clostridioides, druh *Clostridioides difficile* je charakterizován jako striktně anaerobní, motilní, gram pozitivní, sporulující bakterie. Spóry jsou oválné, subterminální. Kmeny *C. difficile* fermentují fruktózu, arabinózu, galaktózu, glykogen, inositol, inulin, laktózu, rafinózu a sukrózu. Pro růst jsou využívány prolin, kyselina aspartamová, serin, leucin, alanine, treonin, valin, fenylalanin, metionin a izoleucin. Pyruvát je přeměňován na acetát a butyrát, treonin na propionát, laktát není využíván (Lawson et al., 2016).

1.2 Patofyziologie onemocnění

K přenosu *C. difficile* dochází fekálně-orální cestou. Spóry jsou schopné adherovat na stěnu střeva (Paredes-Sabja et al., 2012) a v přítomnosti žlučových solí dochází k jejich vyklíčení (Paredes-Sabja et al., 2014). Pokud je fyziologické složení mikroflóry narušeno dochází k pomnožení vegetativních forem *C. difficile* a kolonizaci stěny střeva. Protektivní mechanismus střevní flóry zahrnuje stimulaci imunitní odpovědi hostitele, kompetici mikroorganismů o limitované nutrienty, produkci bakteriocinů, které inhibují růst *C. difficile* a modifikaci žlučových solí, které ovlivňují germinaci spór (Britton and Young, 2012). V případě pomnožení toxigenních *C. difficile* může dojít k produkci toxinů, které jsou primárně zodpovědné za klinickou manifestaci *Clostridium difficile* infection (CDI), (Hammond a Johnson, 1995; Braun et al., 1996). Různé kmeny *C. difficile* mohou produkovat různé spektrum toxinů zahrnující toxin A, toxin B a binární toxin.

1.2.1 Toxiny A (TcdA) a B (TcdB)

Geny pro tvorbu velkých klostridiálních toxinů (*tcdA*, *tcdB*) jsou lokalizovány na lokusu patogenicity (PaLoc) spolu s dalšími třemi geny kódujícími produkci proteinů (*tcdR*, *tcdE*, *tcdC*), které se pravděpodobně podílejí na regulaci produkce toxinů (Monot et al., 2015). Oba dva toxiny mají čtyři funkční domény (GTD – glucosyltransferase domain, APD – autoprotease domain, delivery domain, CROPS – combined repetitive oligopeptides (Shen, 2012; Chumbler et al., 2016). Uvolnění aktivního toxinu zahrnuje čtyři kroky zprostředkované jejich jednotlivými doménami.

Toxiny vstupují do buňky pomocí receptorem zprostředkované endocytózy. Z endosomu se toxiny uvolní jeho acidifikací a vytvořením póru v membráně (Pruitt a Lacy, 2012). Poté, inositol hexakisphosphate ($InsP_6$) váže a aktivuje APD uvolněním GTD, která inaktivuje Rho proteiny a ostatní GTPázy (Egerer et al., 2007; Just et al., 1995a; Just et al., 1995b; Shen et al., 2011;). To má za následek interakci se signálními molekulami a s tím narušení životně důležitých signálních drah. Infikované buňky se zakulacují, dochází k apoptóze (Brito et al., 2002) což vede ke ztrátě střevního epitelu a narušení pevných spojů mezi buňkami umožňující migraci neutrofilů. Působení toxinů také stimuluje uvolnění prozánětlivých cytokinů (Shen, 2012). Výskyt epidemicky významných kmenů, které jsou TcdA negativní (v důsledku částečné nebo úplné delece genu *tcdA*), potvrzuje, že izolovaná exprese *TcdB* může vyvolat onemocnění (Cairns et al., 2015; Janezic et al., 2015; Elliot et al., 2011; Goorhuis et al., 2009).

1.2.2 Binární toxin

C. difficile aktin specifická ribosyltransferáza (CDT, binární toxin) je třetím toxinem produkovaným některými kmeny *C. difficile* (například ribotypy 023, 027, 078 a 176). Tento toxin je kódovaný dvěma geny (*cdtA* a *cdtB*), které jsou lokalizovány na lokusu pro binární toxin (CdtLoc) společně s regulačním genem *cdtR* (Perelle et al., 1997; Gonçalves et al., 2004; Carter et al., 2007). Binární toxin se skládá ze dvou komponent (CDTa, CDTb). CDTb komponenta je zodpovědná za vazbu a translokaci enzymatické složky CDTa do buňky, která svojí aktivitou způsobí destrukci aktinového cytoskeletu a s tím související buněčnou smrt (Sundriyal et al., 2009; Sundriyal et al., 2010).

V nedávné době byl u lidí i zvířat publikován výskyt kmenů *C. difficile*, které produkují pouze binární toxin a způsobují klinickou manifestaci CDI (Elliot et al., 2009; Eckert et al, 2014, Androga et al., 2015; Grandesso et al., 2016). Tyto kmeny, pokud

zůstanou laboratorně nerozpoznány (negativní test pro stanovení toxinů A/B v rámci rutinního vyšetření a/nebo negativní molekulární test, pokud je cílovým místem detekce pouze gen pro tvorbu toxinu A a/nebo toxinu B), vedou k nesprávné interpretaci a falešně negativní výsledek může mít závažné klinické i epidemiologické důsledky.

1.2.3 Sporulace

Vzhledem k striktnímu anaerobnímu charakteru *C. difficile*, jeho vegetativní formy nemohou přežít v prostředí obsahujícím kyslík. Vlivem signálů z prostředí (nedostatek živin, přítomnost antibiotik) dochází ke sporulaci, tvorbě metabolicky neaktivních forem *C. difficile*. Sporulace je významným virulenčním faktorem umožňujícím přežití *C. difficile* mimo hostitele po dobu několika měsíců hrající zásadní roli v horizontálním přenosu mezi pacienty. Spóra *C. difficile* je pokryta pláštěm tvořeným peptidoglykanem a dalšími několika vrstvami proteinů, které ji chrání před vysycháním a účinkům kyslíku a chemických látek (Rineh et al., 2014; Paredes-Sabja et al., 2014).

Kultivace *C. difficile* na selektivních půdách inhibuje sporulaci izolátů. Pro dlouhodobé uchovávání izolátů *C. difficile* je nutné provést kultivaci čistého izolátu na neselektivní půdě.

1.3 Klinický průběh CDI

Klinický průběh CDI může být manifestován průjmy různé tíže, kolitidou, pseudomembranózní kolitidou, ileem až po život ohrožující toxickým megakolon. Jako průjem je obecně definována přítomnost neformované stolice s frekvencí tři a více během 24 hodin, nebo méně po sobě jdoucích hodin, nebo častěji, než je běžné pro daného jedince. Klinické známky ileu, vážně narušené funkce střeva, jsou provázené zvracením a absencí stolice, popřípadě s radiologickými známkami dilatace střeva. Toxické megakolon je charakterizováno enormní dilatací kliček tlustého střeva s rizikem jeho ruptury doprovázené známkami těžké systémové zánětlivé odpovědi (Debast et al., 2014).

Rizikovými faktory pro rozvoj onemocnění jsou předchozí hospitalizace, přítomnost základního onemocnění, věk nad 65 let, předchozí nebo souběžná antibiotická terapie (Slimings a Riley, 2014) a také podávání inhibitorů protonové pumpy (McDonald et al., 2015).

Klinické údaje a predispoziční faktory rozvoje CDI u českých pacientů byly sledovány na čtyřech různých pracovištích (Infekční klinika Fakultní nemocnice v Brně v období 2007-2010, (Vojtilová et al., 2014); Fakultní nemocnici v Plzni v období 2006-2008, (Balihar et al., 2014); Interní oddělení, Fakultní nemocnice v Motole v období 2013-2014, (Drábek et al., 2015); Infekční oddělení nemocnice Na Bulovce v roce 2013, (Polívková et al., 2016).

1.3.1 Rekurentní forma onemocnění

Jako rekurentní forma onemocnění je považován nový nástup příznaků CDI během osmi týdnů od počátku příznaků a po ukončení léčby první episody onemocnění (přibližně **2.** - 8. týden). V klinické praxi není možnost rozlišit relaps a reinfekci, termín rekurence tak zahrnuje oboje (Kuijper et al., 2006; Debast et al., 2014). Po prodělání první episody CDI je riziko další episody 15-25 %. Pro pacienta, který prodělal další episodu CDI, se riziko další episody onemocnění zvyšuje na 40-65 % (Kelly, 2012).

Rizikovými faktory pro vznik rekurence jsou věk nad 65let, podávání další antibiotické terapie v průběhu léčby nebo po léčbě CDI, základní diagnóza těžkého onemocnění (komorbidity) a předchozí episoda CDI v anamnéze pacienta (D'Agostino et al., 2014; Debast et al., 2014). Kuriózním rizikovým faktorem z pohledu stravovacích návyků pro rozvoj rekurence CDI bylo označeno pití čaje (Oman et al., 2016).

1.3.2 Extra intestinální formy CDI

Extra intestinální formy CDI jsou vzácné, zahrnující bakteriémie, intra abdominální infekce a extra abdominální abscesy (Bedimo a Weinstein, 2003). Proběhlá CDI může být také spouštěcím faktorem reaktivní artritidy (Cappella et al., 2016). V prostředí se zvýšenou možností kontaminace spórami *C. difficile* také narůstá riziko infekce rány (Mattila et al., 2013).

Naše vlastní zkušenost s extra intestinální formou CDI se týkala pacienta hospitalizovaného na oddělení Spinální jednotky Fakultní nemocnice v Motole. Tento pacient byl přijat s mnohočetnými dekubity, které byly chirurgicky řešeny plastikou. Z jedné dlouhodobě se nehojící pooperační rány bylo vykultivováno *C. difficile* určené jako ribotyp 078. Po nasazení orální formy metronidazolu, došlo ke zklidnění a zhojení rány. K určení možného zdroje infekce byla vyšetřena i stolice pacienta s nálezem odlišného ribotypu (014).

Tato klinická zkušenost upozorňuje na uplatnění *C. difficile* v etiologii infekce kůže a přilehlých struktur a na nebezpečí environmentální kontaminace rány (Nyč et al., 2015a).

1.4 Asymptomatické nosičství *C. difficile*

Vysoké procento nosičství *C. difficile* je publikováno u dětí v prvním roce věku (25-35 %), a u starších dětí 1-8 let 15 % (Enoch et al., 2011; Leibowitz et al., 2015) z důvodů nestabilní střevní mikroflóry umožňující *C. difficile* osídlení střevního traktu (Bergstrom et al., 2014). Kolonizace pacientů v nemocničním prostředí se pohybuje kolem 8 % (Zacharioudakis et al., 2015) s narůstající incidencí v následné péči (Riggs et al., 2007; Ziakas et al., 2015). Asymptomatičtí nosiči mohou být rizikem pro vnímavé pacienty z hlediska přenosu *C. difficile* jehož spóry se uvolňují do prostředí (Curry et al., 2013; Eyre et al., 2013).

1.5 Prevence a kontrola šíření *C. difficile*

V případě výskytu CDI na oddělení je nutné zavést protiepidemická opatření, jejichž základními součástmi jsou izolace pacienta (optimálně na pokoji s individuální toaletou), používání jednorázových bariérových pomůcek, hygiena rukou prováděná mýdlem pod tekoucí vodu, úklid prováděný několikrát denně s použitím sporocidních přípravků. Doprovodným, ale ne méně důležitým opatřením je omezení expozice rizikových skupin pacientů k antibiotikům (Vonberg et al., 2008).

1.6 Evropské a české doporučené postupy ve vztahu k CDI

V roce 2008 byl vydán doporučený postup pro kontrolu šíření *C. difficile* (Vonberg et al., 2008). V roce 2009, následoval evropský doporučený postup pro léčbu a diagnostiku CDI (Bauer et al., 2009), který byl podpořen evropskou společností pro klinickou mikrobiologii a infekční lékařství (ESCMID). V roce 2014, byl tento dokument aktualizován (Debast et al., 2014). V návaznosti na výše uvedené dokumenty, byly publikovány v letech 2012 a 2014 také české národní doporučené postupy pro diagnostiku a léčbu kolitidy vyvolané *C. difficile* (Beneš et al., 2012; Beneš et al., 2014). Za účelem standardizace surveillance CDI v Evropě, byl evropským centrem pro prevenci a kontrolu onemocnění (ECDC) v roce 2015 uvolněn surveillance protokol pro CDI (ECDC, 2015). Nejnovějším

závazným dokumentem je aktualizované ESCMID doporučení pro diagnostiku CDI (Crobach et al., 2016).

1.7 Laboratorní diagnostika

Laboratorní diagnostika CDI je založena na vyšetření průjmovité stolice pacienta (vzorek kopíruje tvar kontejneru). Formovanou stolici se nedoporučuje vyšetřovat, výjimku tvoří pacienti s paralytickým ileem. Pro ileosní pacienty je přípustné provést vyšetření i z rektálního výtěru. Diskutabilní je spodní věková hranice pacientů pro vyšetření CDI, kdy v rámci evropských doporučení pro diagnostiku a léčbu CDI byla stanovena na tři roky. Doporučení má v tomto ohledu omezenou váhu, protože CDI nelze zcela vyloučit i u jedinců mladších tří let (Crobach et al., 2016).

Podle posledního evropského doporučení pro diagnostiku CDI, je u pacientů s podezřením na CDI základním vyšetřením průkaz glutamátdehydrogenázy (GDH), toxinů A/B a/nebo přítomnosti toxigenního *C. difficile* ve vzorku neformované stolice (Crobach et al., 2016).

1.7.1 Stanovení glutamátdehydrogenázy a toxinů A/B

Glutamátdehydrogenáza je enzym metabolismu kódovaný genem *gluD*, produkovaným jak toxigenními, tak netoxigenními kmeny *C. difficile* (Carman et al., 2012). Metody stanovení jsou imunoenzymatické (EIA).

Přítomnost toxinů A/B může být detekována taktéž pomocí EIA nebo neutralizačním testem na buněčných kulturách. Komerčně dostupné testy se liší svým uspořádáním, principem detekce tak svojí sensitivitou, která by měla být vzata v potaz při interpretaci výsledků klinickému pracovišti a korelována s klinickým stavem pacienta a dalšími laboratorními nálezy.

Z naší zkušenosti můžeme uvést výsledky porovnávací studie dvou komerčních metod na stanovení GDH (glutamát dehydrogenázy) a toxinů A/B (Liaison, Diasorin, USA a Quik Chek Complete Techlab, USA). Jako referenční metoda byla použita toxigenní kultivace a komerční PCR test (*C. difficile* Elite MGB kit, Nanogen). O 7 % vyšší sensitivita pro detekci GDH tak i toxinů A/B byla zjištěna u souprav Liaison (Diasorin). Toto zjištění si vysvětlujeme větším vazebným povrchem na paramagnetických částicích v porovnání s membránovým uspořádáním (Krůtová et al., 2014a).

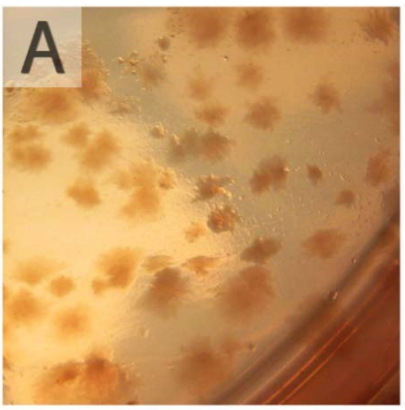
1.7.2 Kultivace *C. difficile* a testování citlivosti k lékům volby

Přítomnost *C. difficile* může být zjišťováno anaerobní kultivací na selektivním agaru (pro potlačení růstu průvodní střevní mikroflóry) nebo průkazem DNA toxigenních *C. difficile* pomocí molekulárních metod (NAAT-Nucleic Acid Amplification Techniques).

Před kultivací vzorku stolice na průkaz *C. difficile* provádíme alkoholový šok, který indukuje germinaci spór. Vzorek stolice je smíchán v poměru 1:1 se 70 % metyl alkoholem a následně inkubován 30-60 minut při pokojové teplotě.

Selektivní půdy pro kultivaci *C. difficile* obsahují látky podporující germinaci spór (kyselina cholová) a do jisté míry potlačující růst průvodní mikroflóry (cykloserin a cefoxitin), (Riley et al., 1987; Bowman RA a Riley, 1988; Brazier, 1995).

Na selektivní půdě se kolonie *C. difficile* projevují charakteristickým růstem (plochý vzhled s nerovnými okraji, bez hemolýzy, kolonie různých velikostí, (viz obrázek číslo 1 – A), s charakteristickým zápachem připomínajícím koňský trus (tvorba p-kresolu na půdách s kyselinou p-hydroxyfenyloctovou). Na půdách obsahující koňskou krev můžeme pozorovat žluto-zelenou fluorescenci pod UV lampou (Brazier, 1995).



Obrázek 1: Kultivační nález C. difficile ze vzorku stolice na selektivním agaru pro C. difficile (Oxoid) po 48 hod. kultivaci v anaerobním boxu (Bentley), foto M. Krůtová.

Suspektní kolonie *C. difficile* mohou být určeny pomocí biochemických testů, aglutinace nebo hmotnostní spektrometrie (MALDI-TOF MS – matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), (Coltella et al., 2013).

Izoláty *C. difficile*, kultivované z GDH pozitivních a toxin A/B negativních vzorků, by měly být dále testovány na přítomnost genů pro tvorbu toxinů nebo na produkci toxinů

samotných pomocí EIA nebo neutralizačního testu na buněčných kulturách (toxigenní kultivace) k odlišení toxigenních a netoxigenních kmenů (Crobach et al., 2016).

Dále mohou být izoláty *C. difficile* testovány na citlivost k antimikrobním látkám. Jako referenční postup pro stanovení antimikrobní citlivosti u izolátů *C. difficile* je doporučená agarová diluční metoda (CLSI, 2007). Vzhledem k tomu, že tato metoda je poměrně pracná a časově náročná s nutností připravovat testovací média denně čerstvá nebyla do rutinního mikrobiologického provozu adaptována. Jako alternativa je používán epsilometrový test (Etest), který uvolňuje do kultivačního média antimikrobiální látku v koncentračním gradientu. Klinické mezní koncentrace (breakpoints) pro metronidazol a vankomycin jsou ≤2 mg/l. Pro fidaxomicin nejsou mezní koncentrace prozatím stanoveny, protože dostupná data ukazují velkou variabilitu minimálních inhibičních koncentrací mezi studiemi (EUCAST, 2016).

1.7.3 Molekulární detekce toxigenních *C. difficile*

Molekulární testy se liší podle cílového místa v genomu, které detekují a také podle principu metody. Některé testy jsou cílené pouze na gen pro produkci toxinu B (BDmax, BD; GenomEra, Abacus Diagnostica; Simplexa, 3M) jiné upřednostňují detekci genu pro tvorbu toxinu A (Illumigene, Meridian Bioscience; AmpliVue, Quidel Molecular) a další volí kombinaci hned několika cílových míst v genomu toxigenních *C. difficile* (gen pro produkci toxinu B, gen pro produkci binárního toxinu a delece v pozici 117 genu *tcdC*; Xpert, Cepheid). Většina systémů využívá klasickou polymerázovou reakci v real-time uspořádání (amplifikace v reálném čase). Přístroj AmpliVue, Quidel Molecular ji kombinuje s denaturací DNA závislé na helikázové aktivitě. Systém Illumigene (Meridian Bioscience) funguje na principu izotermální amplifikace nukleových kyselin (Loop-Mediated Isothermal Amplification technology – LAMP), (Paitan et al., 2017).

1.7.4 Laboratorní diagnostické schéma

Jako vyhledávací (screeningový) test je doporučeno použít citlivou EIA metodu pro průkaz glutamátdehydrogenázy GDH nebo test založený na amplifikaci nukleových kyselin (NAAT). V případě pozitivity prvního testu by mělo následovat stanovení přítomnosti volných toxinů A/B ve stolici pomocí EIA.

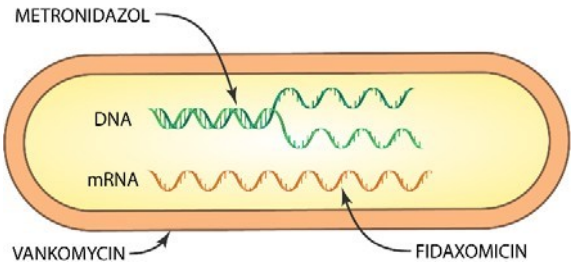
V případě pozitivity obou testů je tento nález hodnocen jako CDI pravděpodobně přítomna. V případě, kdy výsledek prvního testu je pozitivní a druhý test (EIA na průkaz

toxinů) je negativní, je doporučeno vyšetření doplnit třetím konfirmačním testem, kterým může být NAAT, pokud nebyl tento test použit jako vyhledávací, nebo toxigenní kultivace, kdy z narostlé kultury *C. difficile* prokazujeme buď produkci toxinů, nebo přítomnost genů pro tvorbu toxinů (Crobach et al., 2016).

Současný stav diagnostiky v ČR ukázal průzkum vycházející z výsledků webového dotazníku, na který odpovědělo 61 mikrobiologických laboratoří v období duben až červenec 2014). Zjistili jsme, že 8,2 % (n=5) laboratoří nepoužívalo doporučenou citlivou screeningovou metodu a 21,3 % (n=13) laboratoří nemá k dispozici konfirmační metodu v případě výsledku testu GDH pozitivní, toxin A/B negativní (Krůtová a Nyč, 2015).

1.8 Léčba

Volba léčebné strategie u CDI závisí na tíži onemocnění a počtu episod CDI, které pacient prodělal. V současné době jsou pro léčbu CDI doporučeny tři antimikrobiální přípravky s různým cílovým místem účinku: metronidazol, vankomycin a fidaxomicin (obrázek 2). Z neantibiotických postupů je nutné zmínit fekální bakterioterapii, kterou je třeba vnímat spíše jako prevenci dalších rekurencí, nikoli jako metodu volby pro terapii akutní infekce.



Obrázek 2: Schématické znázornění cílových míst v bakteriální buňce (vegetativní forma C. difficile) používaných antimikrobiálních přípravků v léčbě CDI.

1.8.1 Metronidazol

Metronidazol je podáván jako inaktivní prekurzor léčiva. K jeho aktivaci je zapotřebí redukce nitroskupiny po přestupu v nezměněné formě přes buněčnou membránu za vzniku toxického metabolitu. Nestabilní radikály metronidazolu působí cytotoxicky na DNA, RNA a proteiny (Goldman, 1982). In *vitro* rezistence k MTZ byla zjištěna jak u izolátů *C. difficile* humánního, tak i zvířecího původu (Goudarzi et al., 2013; Norman et al., 2014; Adler et al., 2015; Orden et al., 2016; Freeman et al., 2015; Spigaglia, 2016). Mechanismus rezistence

k MTZ nebyl prozatím objasněn. Studie provedené na izolátech ribotypů 027 a 010 (netoxigenní) naznačují, že se jedná o multifaktoriální proces, který zahrnuje různé změny v metabolických drahách jako je aktivita nitroreduktázy, příjem železa a opravné mechanismy DNA (Moura et al., 2014; Lynch et al., 2013).

1.8.2 Vankomycin

Vankomycin patří mezi glykopeptidová antibiotika, která inhibují syntézu buněčné stěny a zároveň narušují permeabilitu buněčné membrány bakterií a syntézu RNA. Rezistence k vankomycinu je popisována, ale s nízkou frekvencí (Adler et al., 2015; Freeman et al., 2015). Mechanismus rezistence u *C. difficile* není prozatím objasněn.

1.8.3 Fidaxomicin

Fidaxomicin (FDX) je makrocyklické antibiotikum, které inhibuje syntézu RNA vazbou na DNA dependentní RNA polymerázu (Johnson a Wilcox, 2012). Rezistence k FDX je vzácná, prozatím byl publikován výskyt jednoho izolátu s minimální inhibiční koncentrací 16 mg/l (Goldstein et al., 2011). Je nutné, ale podotknout že testování citlivosti k fidaxomicinu se vzhledem k absenci Etestů na trhu a doporučených breakpointů rutinně neprovádí.

1.8.4 Fekální transplantace

CDI je charakterizována snížením mikrobiální diverzity střevní mikroflóry (Chang et al., 2008) a transplantace stolice (FMT) jedním z efektivních způsobů jak rychle „resetovat kriticky narušený mikrobiální ekosystém“ (Fuentes et al., 2014), a to obzvláště u pacientů s vícečetnými rekurencemi (van Nood et al., 2013; Cammarota et al., 2015), kde je podle doporučených postupů FMT indikována jako lék (postup) volby (Debast et al., 2014; Beneš et al., 2014). Při FMT je stolice od zdravého (většinou příbuzenského) dárce naředěna, mixována, filtrována do formy roztoku nebo kapslí. Forma roztoku se aplikuje rektální nebo nasoduodenální sondou. Stolice dárce a krev (sérum) dárce je testováno na přítomnost definovaných patogenů (Cammarota et al., 2017; Woodworth et al., 2017).

České pozitivní zkušenosti s podáváním fekální bakterioterapie na Klinice infekčních chorob Fakultní nemocnice Brno u pacientů s rekurentní formou infekce byly popsány v prospektivní studii z let 2010-2014 (Polák et al., 2015).

1.8.5 Léčebné postupy

V následujícím textu jsou uvedena terapeutická schémata vycházející z posledních doporučených postupů (Debast et al., 2014; Beneš et al., 2014) která podle počtu dostupných klinických studií dosáhla úrovně doporučení A nebo B (A – silně doporučeno, B – středně doporučeno).

Při první epizodě CDI s nezávažným průběhem onemocnění je lékem volby metronidazol, podávaný v dávce 3x 500 mg, p. o. (A-I) nebo i. v., kde není možné perorální podávání (A – II). Alternativně je možné podat vankomycin v dávce 4x 125 mg (B-I) nebo fidaxomicin v dávce 2x 200 mg (B-I).

Při těžké formě CDI je lékem volby vankomycin v dávce 4x 125 mg (A-I) nebo fidaxomicin v dávce 2x 200 mg (B-I). Pokud perorální podání není možné, podává se metronidazol i. v. v dávce 3x 500 mg (A-II) v kombinaci s vankomycinem podávaným 4x 500 mg enterálně (B-III).

Při první rekurenci nebo u pacientů s rizikem rekurence CDI je doporučeno podání fidaxomicinu v dávce 2x 200 mg (B-I) nebo vankomycinu v dávce 4x 125 mg (B-I).

Doporučená doba léčby u všech tří preparátů je 10 dní.

Při mnohočetných rekurencích CDI je uveden buď medikamentózní způsob léčby, tj. fidaxomicin v dávce 2x 200 mg po dobu 10 dní (B-I) nebo vankomycin v sestupném dávkování nebo provedení fekální bakterioterapie kombinované s předchozí antibiotickou léčbou (A-I), (Debast et al., 2014; Beneš et al, 2014).

1.8.6 Nové a budoucí možnosti léčby CDI

Nové terapeutické možnosti CDI zaměřují několik cílů: antimikrobiální terapii CDI (surotomycin, cadazolid, ridinilazol), CDI profylaxi (ribaxamase), aktivní a pasivní (protektivní) imunizaci proti *C. difficile* a bioterapii (RBX2660, SER-109 a kolonizaci netoxigenními kmeny *C. difficile),* (Martin a Wilcox, 2016).

1.9 Epidemiologie CDI

C. difficile je významným nozokomiálním a v řadě zemí i komunitním patogenem současnosti. Infekce vyvolané *C. difficile* jsou charakterizovány vysokou mortalitou a jsou spojeny s narůstající ekonomickou zátěží pro zdravotnická zařízení (Freeman et al., 2010).

Nárůst CDI v souvislosti s výskytem ribotypu 027 byl zaznamenán v Kanadě a USA od roku 2003 (Warny et al., 2005). V roce 2005 byl výskyt ribotypu 027 hlášen v Nizozemí a Anglii (Kuijper et al., 2006; Kuijper et al., 2007).

Česká republika se zúčastnila dvou evropských studií zaměřených na incidenci CDI. První evropská studie zahrnující 106 laboratoří z 34 zemí byla organizována v roce 2008. Průměrná incidence CDI byla vypočtena na 4,1 případů na 10 000 ošetřovacích dní s prevalentním výskytem ribotypů 014/020 (16%), 001 (9%) a 078 (8%), (Bauer et al., 2011). Druhá evropská studie byla navržena jako dvoudenní sběr neformovaných stolic, které byly vyšetřeny v národní centrální laboratoři (Ústav lékařské mikrobiologie FN v Motole). Sběr vzorků probíhal jeden den v zimě (prosinec 2012 nebo leden 2013) a jeden den v létě (červenec nebo srpen 2013) a zúčastnilo se jí 482 nemocnic z 20 zemí. Průměrná incidence CDI vzrostla na 7 případů na 10 000 ošetřovacích dní (Davies et al., 2014) s prevalentním výskytem ribotypů 027 (19 %), 001 (11 %) a 014/020 (10 %), (Davies et al., 2016).

Česká republika vykazovala v první evropské studii, které se zúčastnily tři české nemocnice, velmi nízkou incidenci CDI (1,1 případů na 10 000 ošetřovacích dní) s prevalencí ribotypu 017 (n=3 z šesti zaslaných izolátů), (Bauer et al., 2011). V druhé studii, která zahrnovala data z 10 českých nemocnic, byl zaznamenán markantní nárůst incidence CDI na 4,4 případů 10 000 ošetřovacích dní v období 2011-2012 a 6,2 případů na 10 000 ošetřovacích dní v období 2012–2013 s prevalencí ribotypu 176 (12 z 34 zaslaných izolátů), (Davies et al., 2014).

1.9.1 Molekulární typizace izolátů *C. difficile*

Metody molekulární typizace jsou důležitým nástrojem umožňujícím sledování a kontrolu výskytu *C. difficile*. Typizační přístupy jsou zaměřené na analýzu konzervovaných nebo variabilních oblastí genomu nebo celého genomu. Metody založené na porovnání elektroforetického profilu jsou pulzní gelová elektroforéza (PFGE) a restrikční nukleázová analýza (REA), kdy před gelovou separací dochází k enzymatickému naštěpení genomické DNA a ribotypizace využívající polymerázovu řetězovou reakci (PCR) k amplifikaci specifického úseku v genomu *C. difficile*, konkrétně prostoru mezi geny 16S a 23S rDNA, který je u jednotlivých kmenů *C. difficile* variabilní jak v délce, tak i v počtu kopií. Serparace naamplifikovaných fragmentů je možná pomocí agarózové gelové elektroforézy nebo v kapilární elektroforéze (Knetsch et al., 2013). Různorodost typizačních technik ztěžuje orientaci v označení jednotlivých kmenů. Například nejvíce zkoumaný

ribotyp 027 bývá v literatuře označován jako BI/NAP1/027, přičemž BI označuje REA typ, NAP1 je pulzní typ a 027 je označení ribotyp.

Dalšími přístupy jsou metody sekvenační jako je multilocus variable-number tandem repeats analysis (MLVA) metoda založená na rozdílech počtu opakování v repetitivních oblastech genomu, multilocus sequence typing (MLST) porovnávající varianty ve vysoce konzervovaných udržovacích genech a celogenomové sekvenování (WGS), (Knetsch et al., 2013).

Vysokokapacitní sekvenování nové generace je typizačním přístupem, kterým můžeme porovnávat u jednotlivých kmenů jednonukleotidové varianty (SNVs) v nerepetitivní části genomu (Eyre et al., 2013).

1.9.2 Rezistence izolátů *C. difficile* k antimikrobním látkám

Kumulace mechanismů rezistence je jedním z významných faktorů napomáhající rozvoji CDI a šíření epidemických ribotypů (Rupnik et al., 2009). Poslední evropská studie zaměřená na výskyt antibiotické rezistence u prevalentních izolátů *C. difficile* zahrnovala 22 zemí (39 laboratoří) a celkem bylo shromážděno 953 izolátů, které byly charakterizovány ribotypizací a následně testovány na citlivost k metronidazolu, vankomycinu, fidaxomicinu, rifampicinu, moxifloxacinu, klindamycinu, imipenemu, chloramfenikolu a tigecyklinu. Nejčastějšími ribotypy ve studii byly 027, 014, 001 a 078. Během studie nebyla zjištěna rezistence k fidaxomicinu a k tigecyklinu. Rezistence k metronidazolu a k vankomycinu byla zjištěna ojediněle (0,11 % a 0,87 % izolátů). Nejvyšší procento izolátů bylo rezistentní ke klindamycinu (49,62 %) a k moxifloxacinu (39,99 %). K rifampicinu bylo rezistentních 13,4 % izolátů, k imipenemu 7,4 % izolátů a k chloramfenikolu 3,7 % izolátů (Freeman et al., 2015).

České izoláty byly ve výše uvedené studii reprezentovány majoritním zastoupením ribotypů 001 (31,8 %) a 176 (38 %). V součtu zjištěných rezistencí (2 body) a snížených citlivostí (1 bod) jsme u 9 testovaných antimikrobních látek dosáhli skóre 4-5, výsledek shodný se Slovenskem, Maďarskem, Bulharskem a Itálií. Vyššího, méně příznivého skóre (5-6) dosáhlo Polsko a Lotyšsko (Freeman et al., 2015).

Ve studii Krůtová et al., (2015) byla testována citlivost k deseti antimikrobiálním látkám u vybraných dvaceti izolátů *C. difficile* ribotypu 176 z 10 různých nemocnic v ČR. U izolátů byla předem vyloučena klonální příbuznost pomocí MLVA. U všech izolátů byla

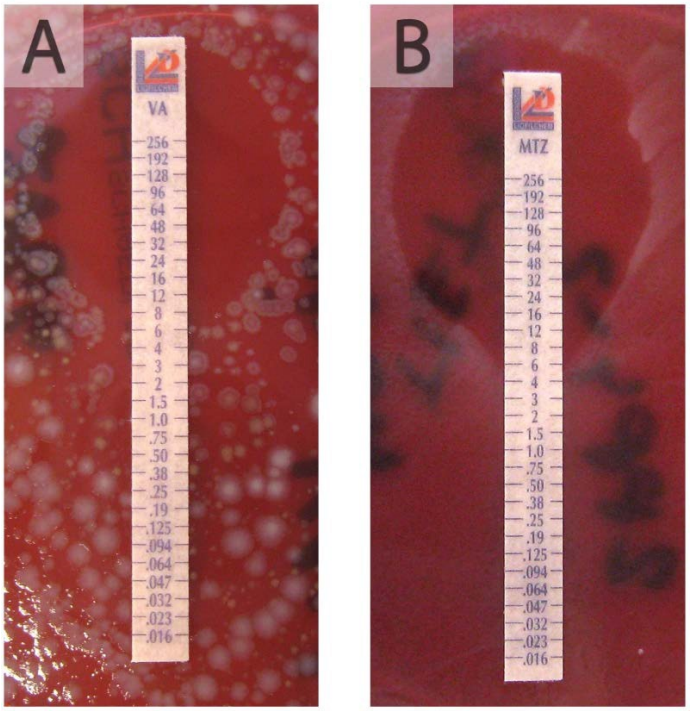
zjištěna rezistence k erytromycinu (MIC≥256 mg/l), ciprofloxacinu a moxifloxacinu (MIC≥32mg/l). K rifampicinu bylo rezistentních 13 izolátů (MIC≥256mg/l) a klindamycin rezistentní byly dva izoláty (MIC=16 mg/l). K ostatním testovaným antimikrobním látkám (metronidazol, vankomycin, imipenem, tetracyklin, tigecyklin) bylo všech 20 izolátů *C. difficile* ribotyp 176 citlivých.

Molekulární mechanismus rezistence k fluorochinolonům a rifampicinu byl potvrzen přítomností aminokyselinové záměny Thr82Ile v GyrA a His502Asn společně s Arg505Lys v RpoB (Krůtová et al., 2015). Vysoké procentuální zastoupení výše uvedených aminokyselinových záměn bylo zjištěno i ve studii Polívková et al. (2016), kde 57,7 % izolátů příslušelo k ribotypu 176 (Polívková et al., 2016).

Další soubor českých izolátů *C. difficile* byl testován ve studii Beran et al. (2014). Jednalo se o 62 izolátů *C. difficile* z let 2011-2012 kultivovaných ve východočeských nemocnicích. Celkem 33 izolátů bylo pozitivních na přítomnost genů pro tvorbu toxinů B a binárního toxinu a současně delece v pozici 117 genu *tcdC*. Ribotypizace nebyla provedena. Izoláty byly testovány na citlivost ke klindamycinu, metronidazolu, vankomycinu a amoxicilinu s kyselinou klavulanovou. U 10,34 % izolátů byla zjištěna rezistence ke klindamycinu (Beran et al., 2014).

V rámci rutinního testování antimikrobiální citlivosti k lékům volby jsme na našem pracovišti zaznamenali u tří externích izolátů rezistenci k metronidazolu. Dvakrát se jednalo o izoláty ribotypu 176 zaslané ze stejné nemocnice, kdy rezistence (MIC = 4 mg/l) byla prokázána nemocniční servisní mikrobiologickou laboratoří a opakovanou kultivací potvrzena i na našem pracovišti. U třetího izolátu ribotypu 027 byla rezistence (MIC = 6mg/l) prokázána na našem pracovišti, zatímco v odesílající laboratoři byl izolát k metronidazolu opakovaně citlivý. U vankomycinu jsme zachytili jeden sporadický případ (MIC = 6mg/l). Jednalo se externí izolát ribotypu 001. Tento izolát byl v odesílající laboratoři k vankomycinu citlivý s použitím diskové difuzní metody, která není metodou doporučenou.

Výsledky testování citlivosti k metronidazolu a vankomycinu u dvou izolátů jsou uvedeny na obrázku č. 3.



Obrázek 3: Testování citlivosti C. difficile k A-vankomycinu (MIC=6 mg/l), B – metronidazolu (MIC=6 mg/l). Schaedler anaerobe agar (Oxoid), Etest s koncentrací antimikrobní látky v rozpětí 0,16-256 mg/l (Liofilchem), 48 hod kultivace v anaerobním boxu (Bentley), foto M. Krůtová.

1.9.3 Nehumánní výskyt *C. difficile*

U zvířat, je výskyt *C. difficile* hlášen jak u domácích, divoce žijících tak i u hospodářských zvířat (Moono et al., 2016), kde s nárůstem mortality v chovech a snížením přírůstku nabývá na ekonomické významnosti. Diverzita zvířecích ribotypů je vysoká (Janezic et al, 2014) s predominancí ribotypu 078, a to obzvláště u prasat (Andrés-Lasheras et al., 2017; Wu et al., 2016; Spigaglia et al., 2015; Knetsch et al., 2014; Usui et al., 2014).

Přímo v potravním řetězci bylo *C. difficile* izolováno ze vzorků syrové zeleniny (Eckert et al., 2013; Rodriguez-Palacios et al., 2014; Yamoudy et al., 2015), jatečních králíků (Drigo et al., 2015), hamburgerech (Esfandiari et al., 2014) a mase určeného k prodeji (de Boer et al., 2011; Rodriguez et al., 2014).

Dvě nově publikované studie zaměřené na výskyt *C. difficile* v prostředí, zachytily *C. difficile* v 14,4 % vzorků odebraných z louží a 36,7 % půdních vzorků, kde osmnáct z třiceti čtyř identifikovaných ribotypů patřilo mezi ribotypy s výskytem jak u lidí, tak i/nebo

zvířat (Janezic et al., 2016) a také ve vzorcích trávy v 59 % s predominancí ribotypu 014/020 (39%), (Moono et al., 2017).

Pilotní data o českých nehumánních izolátech *C. difficile* pocházejí ze studie Goldová et al., (2012), kdy bylo *C. difficile* kultivováno z rektálních výtěrů selat. Prevalentním (jediným ribotypem) identifikovaným v této studii byl WEBRIBO typ AI-12 (Goldová et al., 2012), který byl identifikován jako ribotyp 150 ve studii Janezic et al. (2014).

2. Cíle práce

Hlavní náplní předkládané práce byla molekulární analýza klinicky významných izolátů *C. difficile* kultivovaných ze stolic pacientů s podezřením na CDI z různých nemocničních zařízení s cílem prohloubení znalostí o možných zdrojích a zákonitostech šíření epidemických kmenů *C. difficile* v České republice.

Zvyšující se incidence a závažnost CDI na území ČR podle mezinárodních zkušeností a dílčích lokálních dat souvisí právě s výskytem epidemických kmenů. Zvolený přístup s využitím v Evropě používaných molekulárně typizačních metod umožňuje zmapovat aktuální epidemiologickou situaci a zasadit získaná data do evropského kontextu surveillance CDI.

3. Použitá metodika

Jako základní typizační metoda byla zvolena Polymerase Chain Reaction (PCR) ribotypizace, typizační metoda doporučená evropským centrem pro kontrolu infekčních onemocnění (ECDC), doplněná o detekci fragmentů genů pro produkci toxinů (A, B, binární) pro získání toxigenního profilu kmene. Jako subtypizační metoda byla použita multilocus variable-number tandem repeats analysis (MLVA) k určení vzájemné genetické příbuznosti u izolátů prevalujícího ribotypu. Fylogenetická analýza zástupců jednotlivých ribotypizačních profilů byla provedena pomocí multilocus sequence typing (MLST).

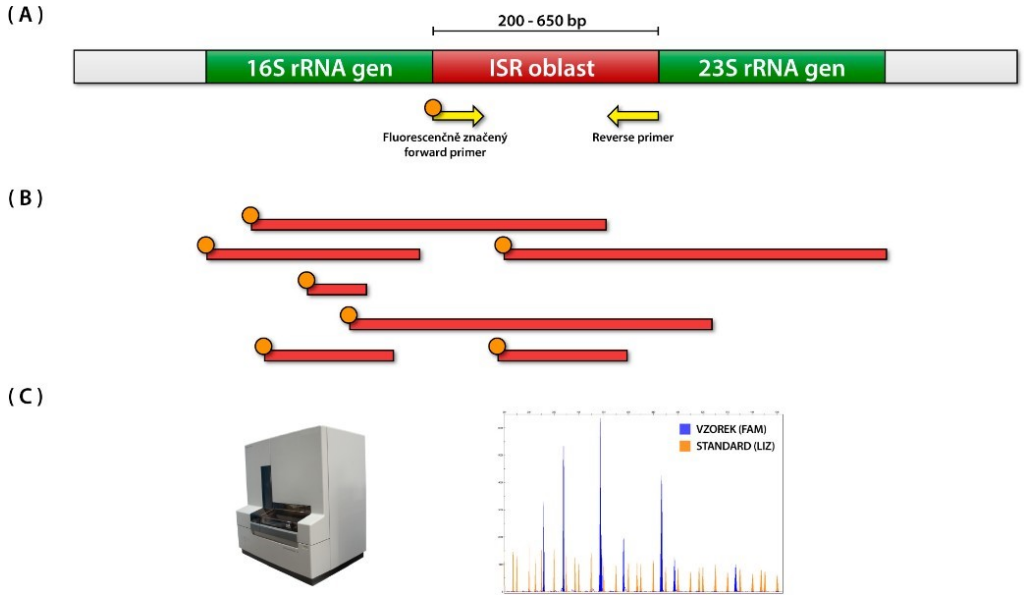
3.1 DNA extrakce

DNA byla izolována pomocí chelexových částic (Chelex® 100 Resin, Bio-Rad) ze 48 hodin starých izolátů *C. difficile* narostlých na selektivním médiu (Brazier's *Clostridium difficile* Selective agar PB5191A, Oxoid).

3.2 PCR ribotypizace

PCR ribotypizace je metoda založená na rozdílech v délce a počtu kopií prostoru mezi geny pro 16S a 23S kódujících rRNA. Metoda byla vyvinuta Národní referenční laboratoří pro Anaeroby v Cardiffu (Stubbs et al., 1999) a pro použití automatické fragmentační analýzy byla modifikována fluorescenčním značením jednoho z primerů (Indra et al., 2008). PCR ribotypizace byla provedena podle Standardního operačního postupu dostupného po přihlášení na stránkách: http://www.ecdisnet.eu/. Fragmentační analýza proběhla na přístroji 3130 Genetic Analyzer (Applied Biosystems) s použitím polymeru POP7 a velikostního standardu LIZ1200 (Applied Biosystems). Hrubá data (*fsa soubory) byla zpracována softwarem GeneMapper v4.0 (Applied Biosystems) s použitím defaultní mikrosatelitové analýzy s oříznutím profilů od 200 do 650bp. Elektroforetické profily byly porovnány s profily dostupnými ve WEBRIBO databázi (https://webribo.ages.at/), (Indra et al., 2008). Zástupci jednotlivých profilů byly opětovně analyzováni podle nového konsensuálního protokolu (Fawley et al., 2015), který používá odlišný pár primerů (Bidet et al., 1999). Tyto elektroforetické profily byly následně porovnány s elektroforetickými profily Leeds-Leiden databáze, které byly použity v rámci validační studie (n=70), (Fawley et al., 2015).

Schématické znázornění PCR ribotypizace je zobrazeno na obrázku 4.



Obrázek 4: Schématické znázornění PCR ribotypizace. A. V prvním kroku dochází k amplifikaci ISR oblastí pomocí jednoho fluorescenčně značeného páru primerů. B, C) amplifikované fragmenty DNA jsou separovány kapilární elektroforézou a velikost jednotlivých fragmentů je určena v porovnání s velikostním standardem značeným odlišnou fluorescenční barvou.

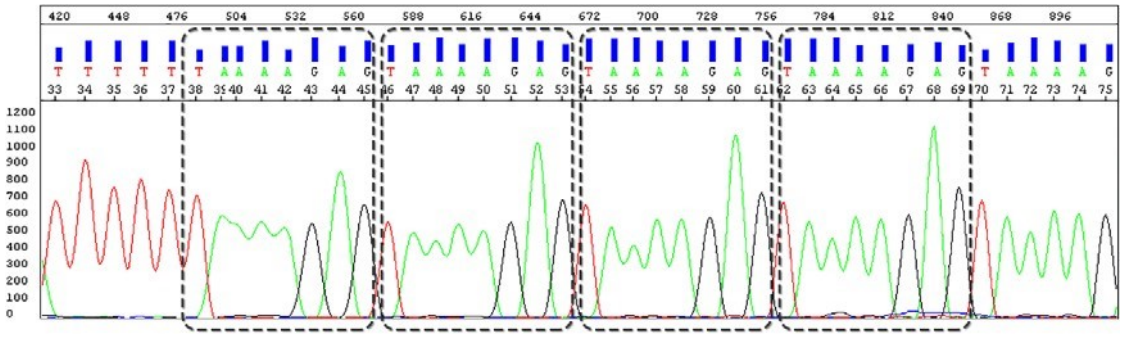
V letech 2013 a 2014 jsme se zúčastnili celoevropského externího hodnocení kvality (EHK) v ribotypizaci v rámci projektu European *Clostridium difficile* infection surveillance network (ECDIS-net). Každá EHK sada obsahovala 10 slepých lyofilizovaných izolátů *C. difficile*. V obou kolech jsme dosáhli 100 % shody v ribotypizaci těchto vzorků. Tohoto výsledku dosáhlo v prvním kole 9 z 20 a v druhém kole 12 z 19 zúčastněných laboratoří.

3.3 MLVA

MLVA metoda je založena na amplifikaci a sekvenaci úseků v genomu *C. difficile*, který obsahuje repetitivní sekvence (VNTR-variable number tandem-repeats). Nejběžněji používané MLVA schéma zahrnuje 7 VNTR lokusů (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd), (van den Berg et al., 2007) se změnou reverzního primeru pro lokus G8Cd (Goorhuis et al., 2009). V několika projektech jsme použili další VNTR lokus a to CDR60 (Marsh et al., 2006).

Amplifikované úseky byly sekvenovány konvenčním Sangerovo sekvenováním a počet repetic byl počítán manuálně po zanalyzování hrubých dat pomocí Sequence analysis softwaru (Applied Biosystems). Součet rozdílů v počtu repetic v analyzovaných úsecích (STRD – Sum of tandem-repeats differences) udává míru příbuznosti izolátů *C. difficile*. Pokud je STRD≤2, jedná se o klonální komplex, STRD>2 a ≤10 je hodnoceno jako geneticky příbuzný izolát (van den Berg et al., 2007).

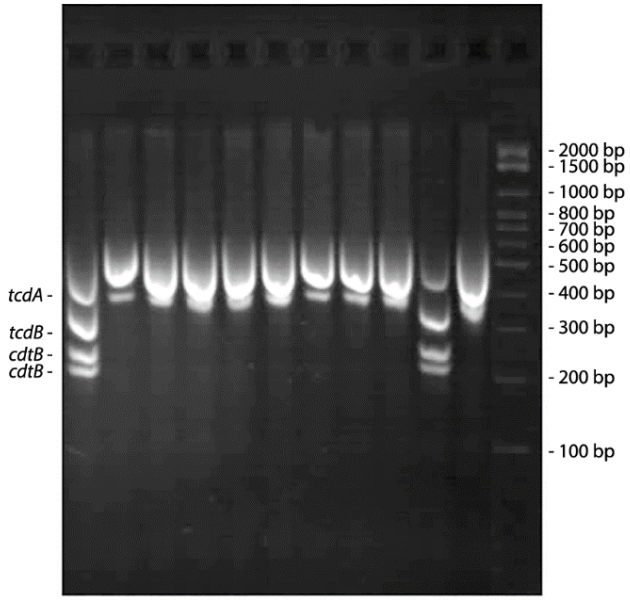
Ke grafickému vyjádření vzájemné příbuznosti izolátů *C. difficile* slouží dendrogram (Minimum spanning tree) sestrojený pomocí softwaru Bionumerics v5.0 (Applied Maths). Příklad tandemové repetice je uveden na obrázku č. 5.



Obrázek 5: Příklad tandemové repetice (Lokus G8Cd, repetitivnÍ motiv TAAAAGAG).

3.4 Přítomnost genů pro tvorbu toxinů a delece v *tcdC*

K detekci přítomnosti genů pro tvorbu toxinů byla použita multiplexová reakce zahrnující primery pro detekci *tcdA* (toxin A), *tcdB* (toxin B), *cdtA* a *cdtB* (binární toxin), (Persson et al., 2008, 2009). Primery navržené pro amplifikaci *tcdA* jsou umístěné nad repetitivní oblastí, která je u některých kmenů deletována. Kmeny s částečnou delecí v *tcdA* (ribotyp 017) tedy vykazují také pozitivní PCR amplifikaci (Persson et al., 2008, 2009). Vizualizace PCR produktů byla provedena na 2 % agarózovém gelu barveném pomocí GelRed (Biotium), obrázek 6.



Obrázek 6: Elektroforetická separace PCR produktů (multiplex PCR reakce – detekce genů pro tvorbu toxinů A, B, binární). 2 % agaróza, 80 V, 50 min., barveno GelRed (Biotinum), velikostní standard LowRanger 100bp (Norgen Biotek).

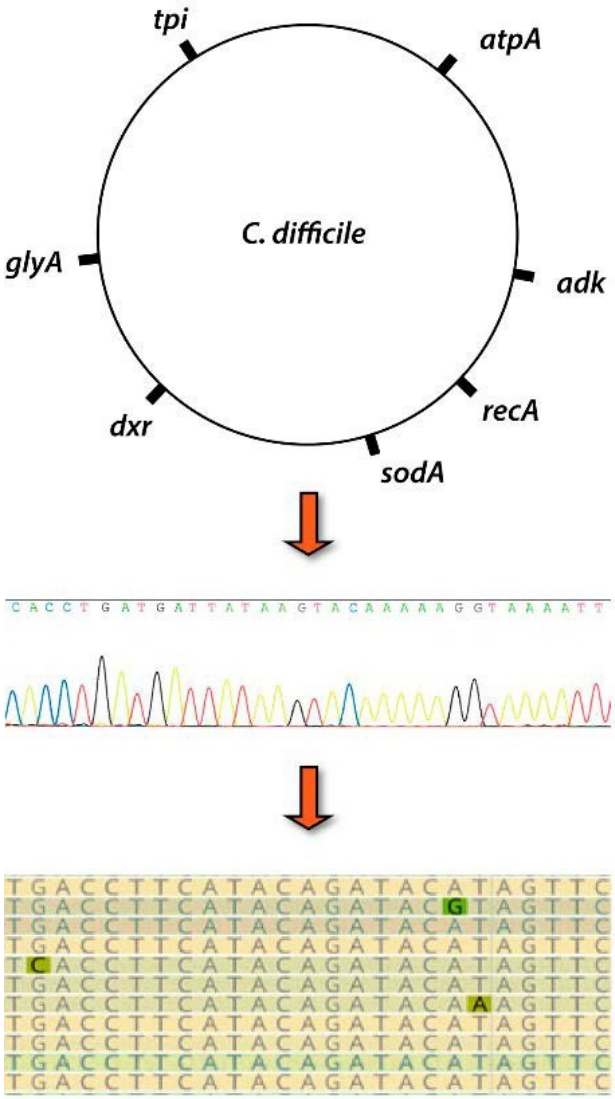
Delece v *tcdC* (toxin gene expression negative regulator) byly detekovány Sangerovo sekvenováním fragmentu genu amplifikovaném primery C1 a C2 (Spigaglia et al., 2002). Výsledné sekvence byly porovnány s referenční sekvencí *Clostridioides difficile* 630, NC_009089.1 dostupné v NCBI databázi (https://www.ncbi.nlm.nih.gov).

3.5 MLST

Pro fylogenetickou analýzu MLST bylo Sangerovo sekvenováním osekvenováno sedm udržovacích (house-keeping) genů (*adk* – adenylate kinase, *atpA* – ATP synthase subunit A, *dxr* – 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *glyA* – serine hydroxymethyl transferase, *recA* – recombinase A, *sodA* – superoxid dismutase, *tpi* – triosepho-

sphate isomerase), (Griffiths et al., 2010). Jednotlivé alely genů byly určeny porovnáním našich sekvencí se sekvencemi dostupnými v MLST databázi (http://pubmlst.org/cdifficile/), (Jolley et al., 2010). Kombinací alel byl zjištěn sekvenační typ izolátu *C. difficile*. Dendrogram (Maximum likelihood tree) byl generován pomocí softwaru Mega5 (http://www.megasoftware.net/), kdy byly vzájemně porovnány spojené sekvence všech sedmi genů (Tamura et al., 2011).

Schématické znázornění MLST je zobrazeno na obrázku 7.



Obrázek 7: Schématické znázornění MLST. Sedm udržovacích genů rozmístěných v genomu C. difficile je osekvenováno a spojené sekvence jsou vzájemně porovnány.

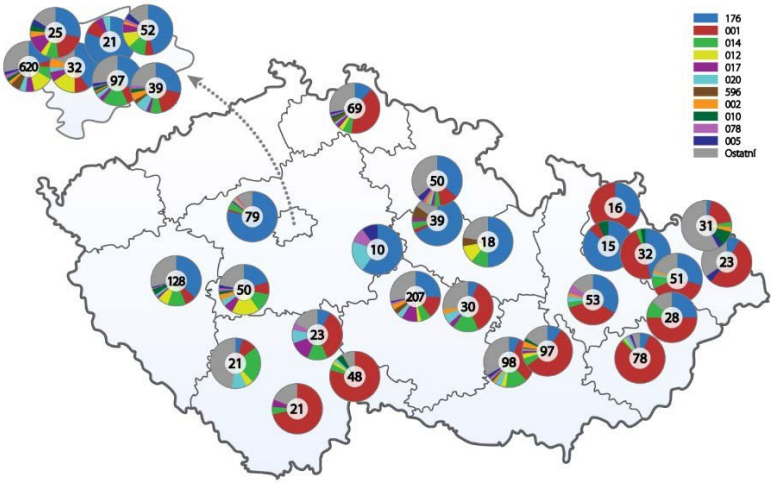
PCR ribotypizace a detekce genů pro produkci toxinů byly provedeny u všech izolátů *C. difficile*. Detekce delece v *tcdC* genu, MLVA a MLST byly provedeny u vybraných izolátů *C. difficile*.

4. Souhrnné výsledky k anotovaným cílům projektu

4.1 Molekulární typizace českých izolátů

4.1.1 PCR ribotypizace

Během řešení projektu jsme pomocí PCR ribotypizace vyšetřili celkem 2201 českých izolátů z 32 nemocnic a shromáždili tak jedinečnou sbírku klinických izolátů *C. difficile* (geografické rozložení je zobrazeno na obrázku 8).



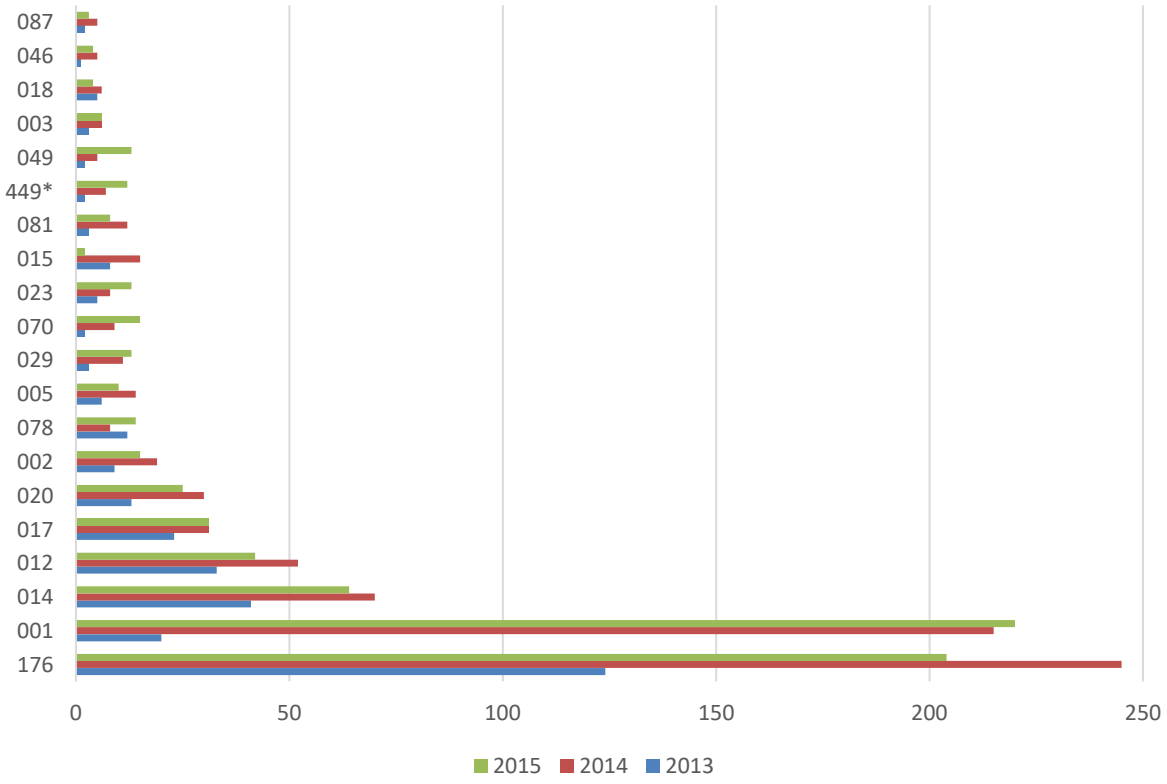
Obrázek 8: Geografické rozložení 32 nemocnic, které v letech 2013-2015 zaslaly alespoň 10 izolátů C. difficile. Číslice uvnitř koláčového grafu udává počet izolátů C. difficile zaslaných k typizaci. Barevná škála zobrazuje proporci nejčastěji zachycených ribotypizačních profilů ve studii. (Krůtová et al., 2016b)

PCR ribotypizací bylo identifikováno 53 různých ribotypizačních profilů, kdy byly zachyceny alespoň dva izoláty reprezentující jeden profil. Z těchto 53 ribotypizačních profilů, 29 profilů (1841 izolátů, 83,7 %) bylo shodných s profily referenčních kmenů (Leeds – Leiden sbírky) a 24 profilů (n=247, 11,2 %) bylo určeno pouze pomocí volně dostupné WEBRIBO databáze. PCR ribotypy a WEBRIBO typy zachycené v České republice v letech 2013 jsou uvedeny v tabulce 1. U zbývajících 113 izolátů (5,1 %) byl získán unikátní ribotypizační profil.

Ribotypy	Geny pro produkci toxinů	Počet izolátů (%)	Počet nemocnic	WEBRIBO typ	Geny pro produkci toxinů	Počet izolátů (%)	Počet nemocnic
176	A, B, Bin	588 (26,7)	30	**596**	netoxigenní	55 (2,5)	8
001	A, B	456 (20,7)	30	**002like**	A, B	45 (2,1)	16
014	A, B	176 (8,0)	26	**015like**	A, B	25 (1,1)	8
012	A, B	127 (5,8)	14	**449**	A, B	21 (1,0)	14
017	A, B	85 (3,9)	18	**AI-61**	netoxigenní	14 (0,6)	5
020	A, B	68 (3,1)	21	**498**	A, B	9 (0,4)	4
010	netoxigenní	35 (1,6)	13	**AI-75**	A, B	8 (0,4)	6
078	A, B, Bin	34 (1,6)	15	**AI-21**	A, B	7 (0,3)	6
005	A, B	30 (1,4)	14	**220**	A, B	7 (0,3)	3
029	A, B	27 (1,2)	13	**446**	A, B	6 (0,3)	4
070	A, B	26 (1,2)	14	**AI-9-1**	A, B	6 (0,3)	6
023	A, B, Bin	26 (1,2)	14	**AI-12**	A, B	6 (0,3)	4
081	A, B	23 (1,0)	11	**AI-82/1**	A, B	5 (0,2)	4
039	netoxigenní	21 (1,0)	6	**203/209**	A, B	5 (0,2)	3
011	A, B	20 (0,9)	11	**500**	A, B	4 (0,2)	3
003	A, B	15 (0,7)	6	**236**	A, B	3 (0,1)	3
018	A, B	15 (0,7)	8	**404**	A, B	3 (0,1)	3
087	A, B	10 (0,5)	8	**434**	A, B	3 (0,1)	1
046	A, B	10 (0,5)	4	**555**	A, B	3 (0,1)	3
126	A, B, Bin	9 (0,5)	6	**AI-60**	A, B	3 (0,1)	3
009	netoxigenní	8 (0,4)	5	**413**	A, B, Bin	3 (0,1)	2
031	netoxigenní	7 (0,3)	4	**212**	A, B	2 (0,1)	2
054	A, B	5 (0,2)	4	**416**	A, B	2 (0,1)	2
027	A, B, Bin	5 (0,2)	4	**438**	A, B, Bin	2 (0,1)	1
076	A, B	4 (0,2)	3				
051	netoxigenní	4 (0,2)	2				
026	A, B	3 (0,1)	3				
043	A, B	2(0,1)	1				
053	A, B	2 (0,1)	1				

Tabulka 1: Přehled C. difficile ribotypů a WEBRIBO typů zachycených v letech 2013-2015(Nyč et al., 2016a).

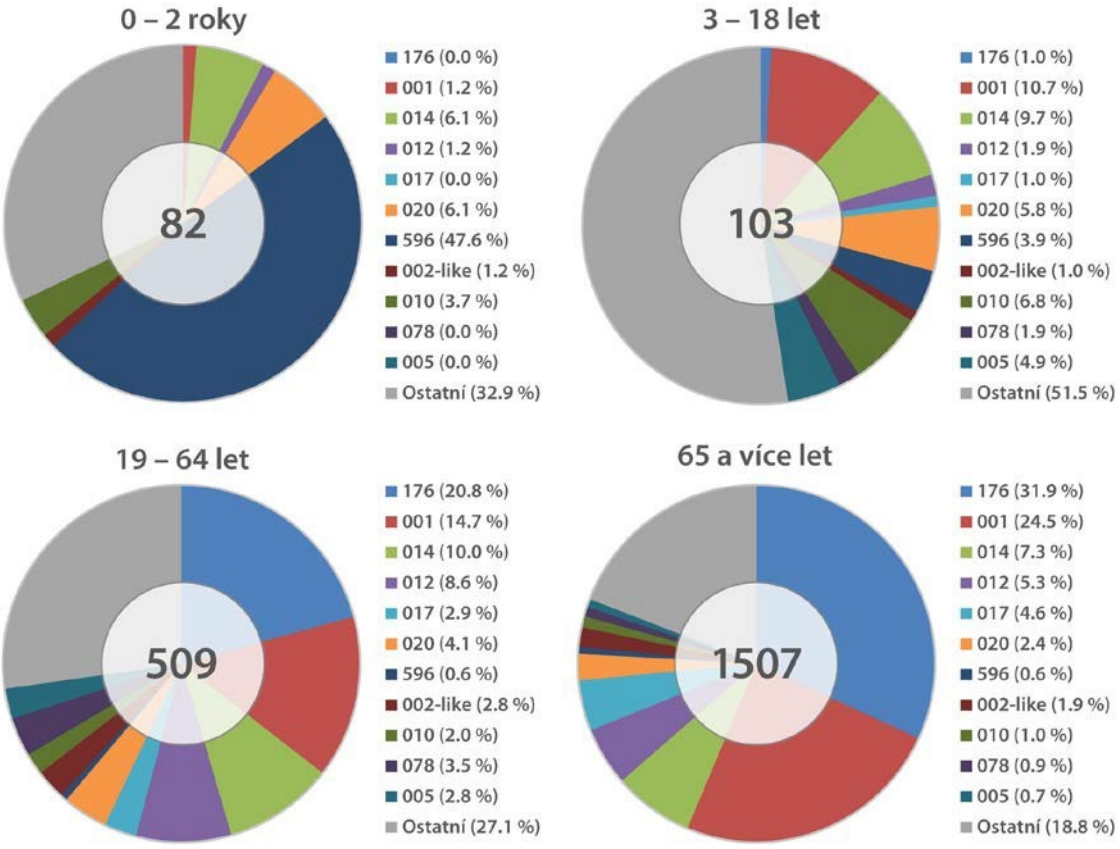
Nejčastěji zachycenými ribotypy v ČR byly 001 (n=456) a 176 (n=588) následovány ribotypy 014 (n=176), 012 (n=127), 017 (n=85) a 020 (n=68). Ostatní elektroforetické profily (ribotypy nebo WEBRIBO typy) nepřesáhly 3 % hranici výskytu. Geografické rozložení 32 nemocnic a počet izolátů, které zaslaly k typizaci společně s výskytem nejčastějších ribotypizačních profilů jsou znázorněny na obrázku 8 (Krůtová et al., 2016b). Grafický přehled nejčastěji se vyskytujících toxigenních ribotypů v jednotlivých letech řešení projektu je zobrazen v grafu 1.



*Graf 1: Distribuce nejčastěji zachycených toxigenních ribotypizačních profilů v jednotlivých letech řešení projektu. (*449 je WEBRIBO typ, ostatní jsou ribotypy). (Nyč et al., 2016a)*

Rozdíly v distribuci nejčastěji zachycených ribotypizačních profilů v závislosti na věku pacientů jsou zobrazeny na grafech obrázku 9 (Krůtová et al., 2016b). Zatímco ve skupině pacientů do dvou let byla přítomnost prevalentních ribotypů 001 a 176 vzácná (1,2 % RT 001), ve skupině pacientů 3-18 let dochází k nárůstu podílu ribotypu 001 na 11,7 % (10,7 %, 1 %). Ve skupině pacientů 19–64 let prevalence ribotypů 001 a 176 stoupla na 35,5 % (14,7 %, 20,8 %) a ve skupině pacientů ve věku 65 let dosáhla již 56,4 % (24,5 %, 31,9 %).

Nejčastější netoxigenním ribotypizačním profilem byly WEBRIBO typ 596 (n = 55, 31,1 %) a ribotyp 010 (n = 35, 19,8 %). WEBRIBO typ 596 byl identifikován u izolátů kultivovaných ze vzorků stolic všech věkových skupin pacientů, ale většina z nich (39/55) pocházela od pacientů do dvou let věku.

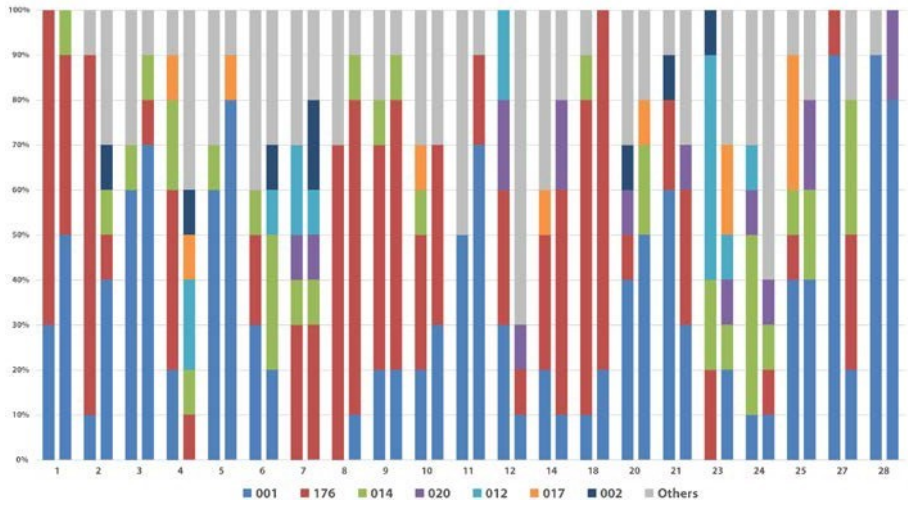


Obrázek 9: Distribuce nejčastěji zachycených ribotypizačních profilů ve studii v závislosti na věku pacientů. Číslice uvnitř koláčového grafu udává počet izolátů C. difficile pro danou věkovou skupinu. Barevná škála reprezentuje nejčastěji zachycené ribotypizační profily. Ribotyp 010 a WEBRIBO typ 596 patří mezi netoxigenní kmeny C. difficile. (Krůtová et al., 2016b)

V návaznosti na připravovaný ECDC surveillanční protokol pro CDI (ECDC, 2015), který v tzv. rozšířené verzi surveillance zahrnuje i ribotypizaci izolátů *C. difficile*, jsme na začátku roku 2015 oslovili spolupracující mikrobiologická pracoviště o zaslání dvaceti po sobě jdoucích izolátů *C. difficile* získaných od pacientů s CDI a celkem 21 nemocnic splnilo toto kritérium. Porovnáním prvních deseti a druhých deseti po sobě jdoucích izolátů byly v některých nemocnicích zjištěny výrazné odchylky v distribuci epidemických

ribotypů, což poukazuje na nutnost dlouhodobější typizace izolátů pro účely surveillance CDI.

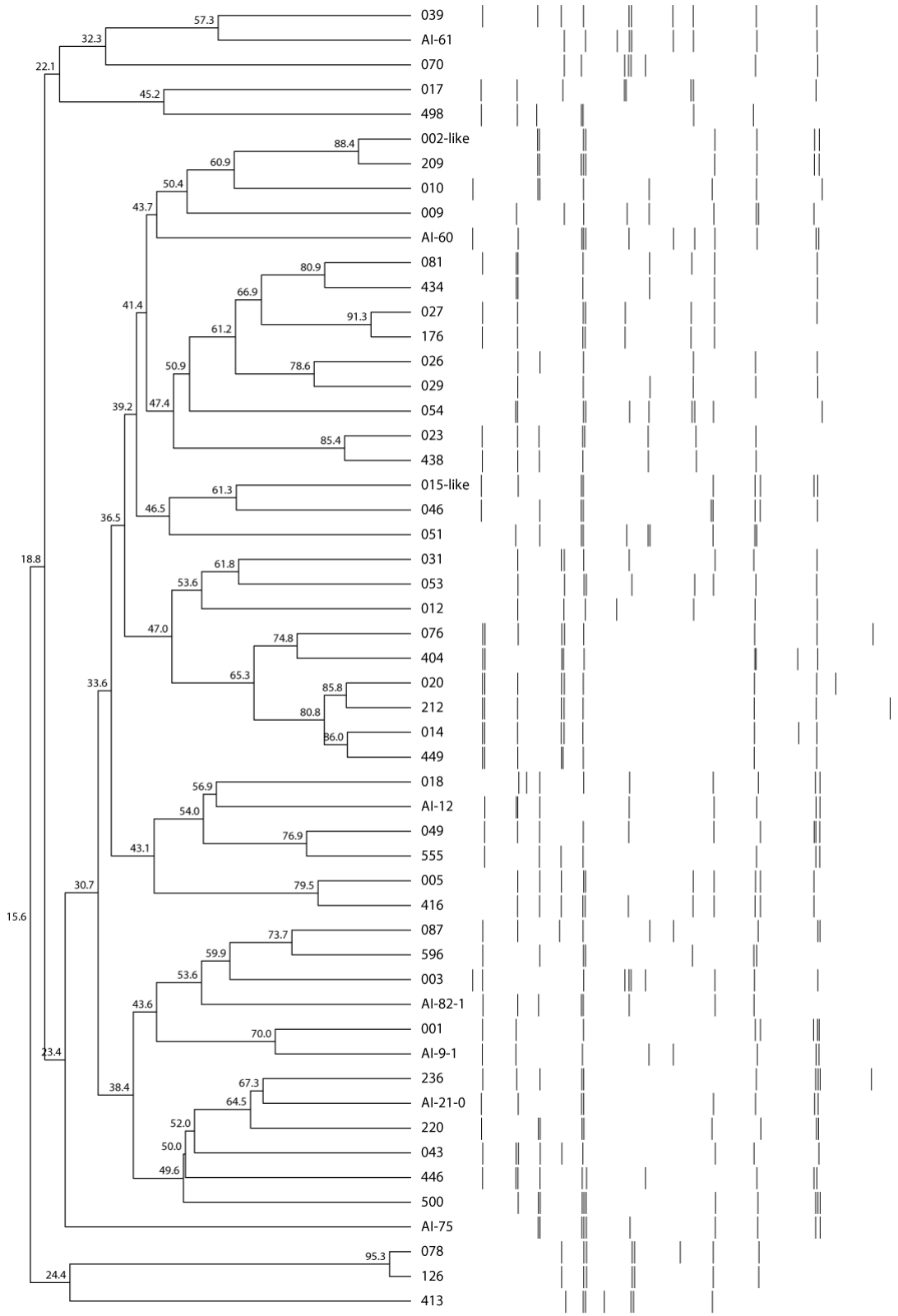
Distribuci nejčastěji identifikovaných ribotypů při analýze prvních deseti (1-10) a druhých deseti (11-20) izolátů *C. difficile* a v rámci jednotlivých nemocnic v roce 2015 je znázorněno na grafu 2.



Graf 2: Distribuce nejčastěji zachycených ribotypů v 21 nemocnicích při analýze deseti a dvaceti po sobě jdoucích izolátů C. difficile. Každá nemocnice je reprezentována jedním číslem a dvěma sloupci. První sloupec zahrnuje analýzu prvních deseti (1-10) po sobě jdoucích izolátů C. difficile a druhý sloupec zahrnuje analýzu dalších deseti (11-20) po sobě jdoucích izolátů C. difficile. Barevná škála reprezentuje nejčastěji zachycené toxigenní ribotypizační profily (Krůtová et al., v recenzním řízení).

Celkem 53 izolátů odlišných ribotypizačních profilů bylo opětovně analyzováno v souladu s novým konsensuálním protokolem pro PCR ribotypizaci (Fawley et al., 2015), ve kterém je použit odlišný pár primerů (Bidet et al., 1999). U čtyřech izolátů *C. difficile* byla pozorována změna v získaném ribotypizačním profilu, konkrétně byl navíc v elektroforeogramu přítomný band (fragment) o velikosti 326 bp. Jednalo se o izoláty určené původně jako ribotyp 002, WEBRIBO typy 203, AI-60, AI-75. Tato změna se projevila ve WEBRIBO databázi změnou označení z ribotypu 002 na WEBRIBO typ 002 - podobný a z WEBRIBO typu 203 na WEBRIBO typ 209 zatímco označení pro AI-60, AI-75 zůstalo nezměněno.

Ribotypizační elektroforetické profily 53 zástupců českých *C. difficile* izolátů společně s údaji o frekvenci výskytu, přítomnosti či absenci genů pro tvorbu toxinů a delece v *tcdC* jsou součástí publikace Krůtová et al., 2016b, doplňková data. Dendrogram zahrnující 53 elektroforetických profilů získaných při použití konsensuálního ribotypizačního protokolu (Fawley et al., 2015) je zobrazen na obrázku 10.



Obrázek 10: Dendrogram zahrnující 53 různých ribotypizačních profilů zkonstruovaný metodou UPGMA – Unweighted Pair Group Method with Arithmetic mean (Bionumerics v5.0). (Krůtová et al., 2016b)

4.1.2 Přítomnost genů pro tvorbu toxinů

Multiplexová PCR reakce na přítomnost genů pro tvorbu toxinů (A, B, binární) byla provedena vždy paralelně s PCR ribotypizací. V průběhu testování jsme nezachytili změnu genetické výbavy ve smyslu přítomnosti či absence genů pro tvorbu toxinů v rámci určeného ribotypu.

Z celkem 2201 izolátů *C. difficile* bylo 2024 (92 %) izolátů toxigenních (neslo geny pro produkci toxinů A, B) a z těchto izolátů, bylo 677 pozitivních i pro přítomnost genů pro tvorbu binárního toxinu. Zbývajících 177 (8 %) izolátů bylo netoxigenních (nebyl u nich detekován ani jeden z genů pro tvorbu toxinů).

Toxigenní potenciál izolátů *C. difficile* vzhledem k ribotypizačnímu profilu byl u izolátů patřících do výše uvedených 53 různých ribotypizačních profilů následující: u 46 profilů jsme prokázali přítomnost genů pro produkci toxinů A, B (*tcdA, tcdB*) a u sedmi těchto profilů byla potvrzena navíc přítomnost genů pro tvorbu binárního toxinu (*cdtA, cdtB*). Sedm profilů patřilo k netoxigenním izolátům *C. difficile* (tabulka 1).

V závislosti na věku pacientů byl nejvyšší poměr netoxigenních a toxigenních izolátů *C. difficile* (64:18) nalezen u skupiny pacientů mladších dvou let. Ve vyšších věkových skupinách se poměr netoxigenních a toxigenních izolátů snižoval na 27:76 u pacientů ve věku 3-18 let, 33: 476 pro věkové skupiny 19-64 let, a 53: 1454 pro pacienty starší 65 let.

4.1.3 Delece v genu *tcdC*

V průběhu roku 2013 jsme vyšetřili 29 izolátů *C. difficile*, kultivovaných ze vzorků stolic, které byly analyzovány pomocí komerčního testu *C. difficile* Epi assay na přístroji Xpert (Cepheid) s výsledkem pravděpodobné detekce ribotypu 027 na základě současné pozitivity genu pro tvorbu binárního toxinu (*cdtB*) a delece v genu *tcdC* v pozici 117.

Všech 29 izolátů *C. difficile* bylo určeno PCR ribotypizací jako ribotyp 176. Sekvenování fragmentu *tcdC* potvrdilo přítomnost výše uvedené delece v *tcdC*. Jako kontrolní vzorek jsme osekvenovali ještě dalších 120 izolátů *C. difficile* ribotypu 176 se stejným výsledkem. Vzhledem ke shodě ve všech třech cílových místech (*tcdB*, *cdtB*, Δ117 v *tcdC*) nelze pomocí výše uvedeného testu odlišit *C. difficile* ribotyp 027 a ribotyp 176 (Krůtová et al., 2014b).

Sekvenování fragmentu v genu *tcdC* bylo také provedeno u 27 izolátů *C. difficile* ze studie Drábek a kol. (2015), kde 14 izolátů (ribotyp 176) potvrdilo přítomnost obou výše

zmíněných delecí (18bp a 1bp), jeden izolát (ribotyp 078) měl 39 bp dlouhou deleci a zbylých 12 izolátů (ribotypy 001, 002, 005, 012, 014, 017, 020, 015, 049 a WEBRIBO typ 434) nevykazovalo deleci v sekvenovaném fragmentu genu *tcdC* v porovnání s referenční sekvencí.

Dále byla delece v genu *tcdC* vyšetřena v rámci charakterizace české sbírky izolátů *C. difficile* u vybraných 53 izolátů patřící k různým ribotypizačním profilům. Delece byla zjištěna u izolátů ribotypů 027 a 176 (1bp a 18bp); ribotypů 078, 126 a WEBRIBO typu 413 (39bp); ribotypu 023 a WEBRIBO typu 438 (54bp), (Krůtová et al., 2016b).

4.1.4 Multilocus variable-number tandem repeats analysis

MLVA, příbuzenská analýza izolátů v rámci shodného ribotypu, byla použita v několika projektech. V publikaci autorů Krůtová *et al*., (2014c) jsme použili publikované schéma sedmi VNTR úseků k analýze českého izolátu *C. difficile* ribotyp 027 od pacientky dlouhodobě žijící v Německu a sedmi německých izolátů *C. difficile* ribotypu 027 ze stejné nemocnice, kde byla pacientka v minulosti hospitalizována a z další nemocnice ve stejném regionu. Mezi českým a jedním z německých izolátů se nám podařilo potvrdit blízkou genetickou příbuznost (STRD=9), což ve spojení s ojedinělostí výskytu *C. difficile* ribotypu 027 v ČR v době studie potvrzuje hypotézu, že se jednalo o importovanou infekci (Krůtová et al., 2014c).

Shodných sedm VNTR úseků bylo použito i u MLVA dvaceti izolátů *C. difficile* ribotypu 176 z deseti různých nemocnic a dvou časových období (2012 a 2014-2015), tentokrát ale s opačným záměrem, a to k vybrání klonálně nepříbuzných izolátů *C. difficile*. Případná klonální příbuznost izolátů by ovlivňovala výsledky testované antimikrobiální citlivosti (Krůtová et al., 2015).

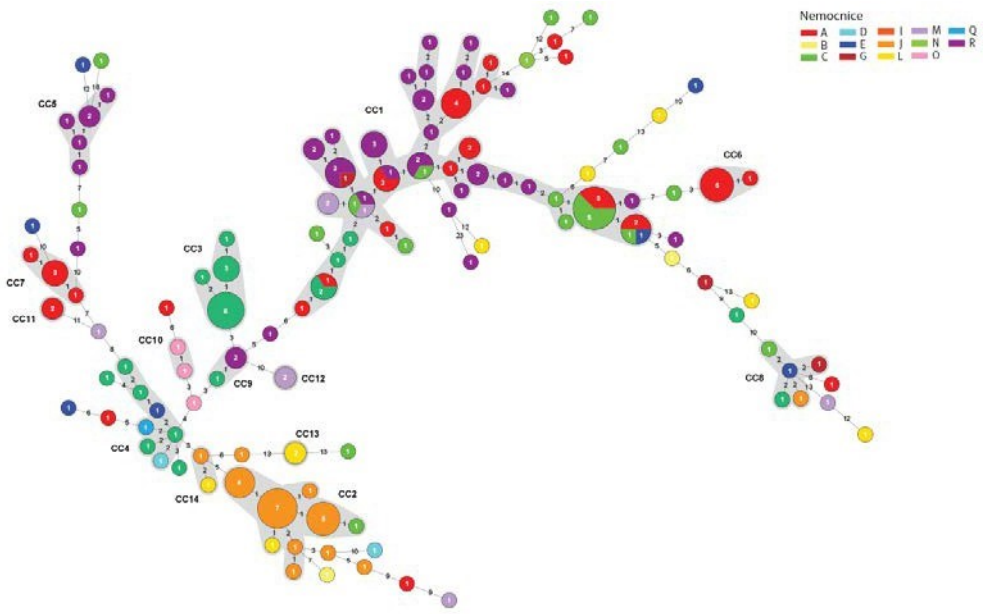
V publikacích Drábek et al. (2015) Polívková et al. (2016) jsme použili MLVA k subtypizaci izolátů *C. difficile* ribotypu 176 z období únor 2013 – únor 2014 (Drábek et al., 2015) a z roku 2013 (Polívková et al., 2016). V prvním projektu bylo použito schéma pěti VNTR lokusů: A6Cd, B7Cd, C6Cd, G8Cd, CDR60 (van den Berg, 2007; Goorhuis et al., 2009; March et al., 2006), v druhém projektu bylo použito osm VNTR lokusů, konkrétně kompletní schéma van den Berg et al. (2007) doplněné o lokus CDR 60 (March et al., 2006). V obou případech se jednalo o izoláty *C. difficile* z jednoho oddělení (Interní oddělení FN v Motole a Infekční oddělení nemocnice Na Bulovce). Na obou pracovištích bylo zjištěno klonální šíření a zároveň přítomnost několika MLVA profilů *C. difficile* ribotypu 176

upozorňující jak na zvýšený potenciál tohoto ribotypu k šíření v nemocničním prostředí, tak na cirkulaci několika MLVA profilů *C. difficile* ribotypu 176 v ČR (Drábek et al., 2015; Polívková et al., 2016).

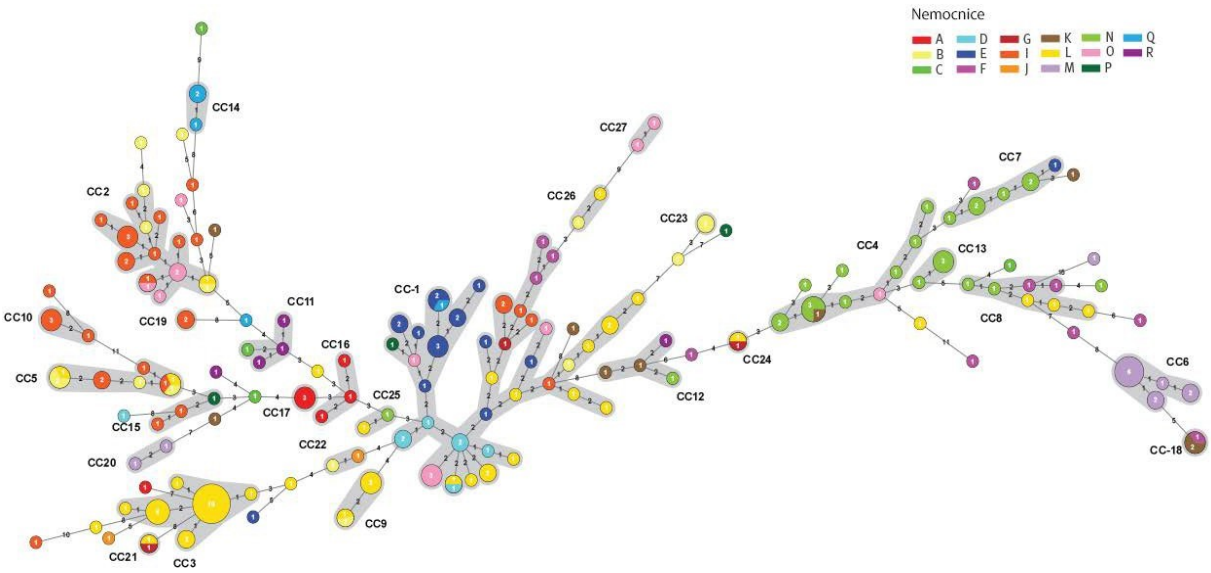
Vzhledem k narůstající incidenci CDI v ČR a výrazné prevalenci ribotypů 001 a 176 v roce 2014, jsme u izolátů *C. difficile* těchto ribotypů provedli MLVA celkem 5 VNTR úseků genomu *C. difficile* (A6Cd, B7Cd, C6Cd, G8Cd a CDR60). Celkem bylo vyšetřeno 409 izolátů (225 izolátů *C. difficile* ribotypu 176 ze 17 nemocnic a 184 izolátů *C. difficile* ribotypu 001 ze 14 nemocnic). Celkový počet nemocnic ve studii byl 18 (Krůtová et al., 2016a).

Minimum spanning tree (MST) vygenerovaný pro oba dva ribotypy je zobrazen na obrázcích číslo 11, 12.

Izoláty *C. difficile* ribotypu 176 klastrovaly do 27 klonálních komplexů tvořených 84,5 % izolátů a izoláty *C. difficile* ribotypu 001 klastrovaly do 14 klonálních komplexů tvořených 76,6 % izolátů. Blízká genetická příbuznost (STRD >2 a ≤10) byla prokázána u 33 izolátů *C. difficile* ribotypu 176 a 32 izolátů *C. difficile* ribotypu 001, zatímco STRD>10 bylo zjištěno jen u 3 izolátů *C. difficile* ribotypu 176 a 15 izolátů ribotypu 001.



Obrázek 11: Dendrogram (Minimum spanning tree) izolátů C. difficile ribotypu 001 kultivovaných v roce 2014 ve 14 nemocnicích z 18 zařazených ve studii. Každá nemocnice je reprezentována odlišnou barvou. Čísla uvnitř kruhu udávají počet izolátů C. difficile ribotyp 001 se shodným počtem tandemových repetic v pěti sekvenovaných úsecích. Čísla na spojnicích udávají počet nalezených rozdílů v součtu tandemových repetic (STRD). (Krůtová et al., 2016a).



Obrázek 12: Dendrogram (Minimum spanning tree) izolátů C. difficile ribotypu 176 kultivovaných v roce 2014 v 17 nemocnicích z 18 zařazených ve studii. Každá nemocnice je reprezentována odlišnou barvou. Čísla uvnitř kruhu udávají počet izolátů C. difficile ribotyp 001 se shodným počtem tandemových repetic v pěti sekvenovaných úsecích. Čísla na spojnicích udávají počet nalezených rozdílů v součtu tandemových repetic (STRD), (Krůtová et al., 2016a).

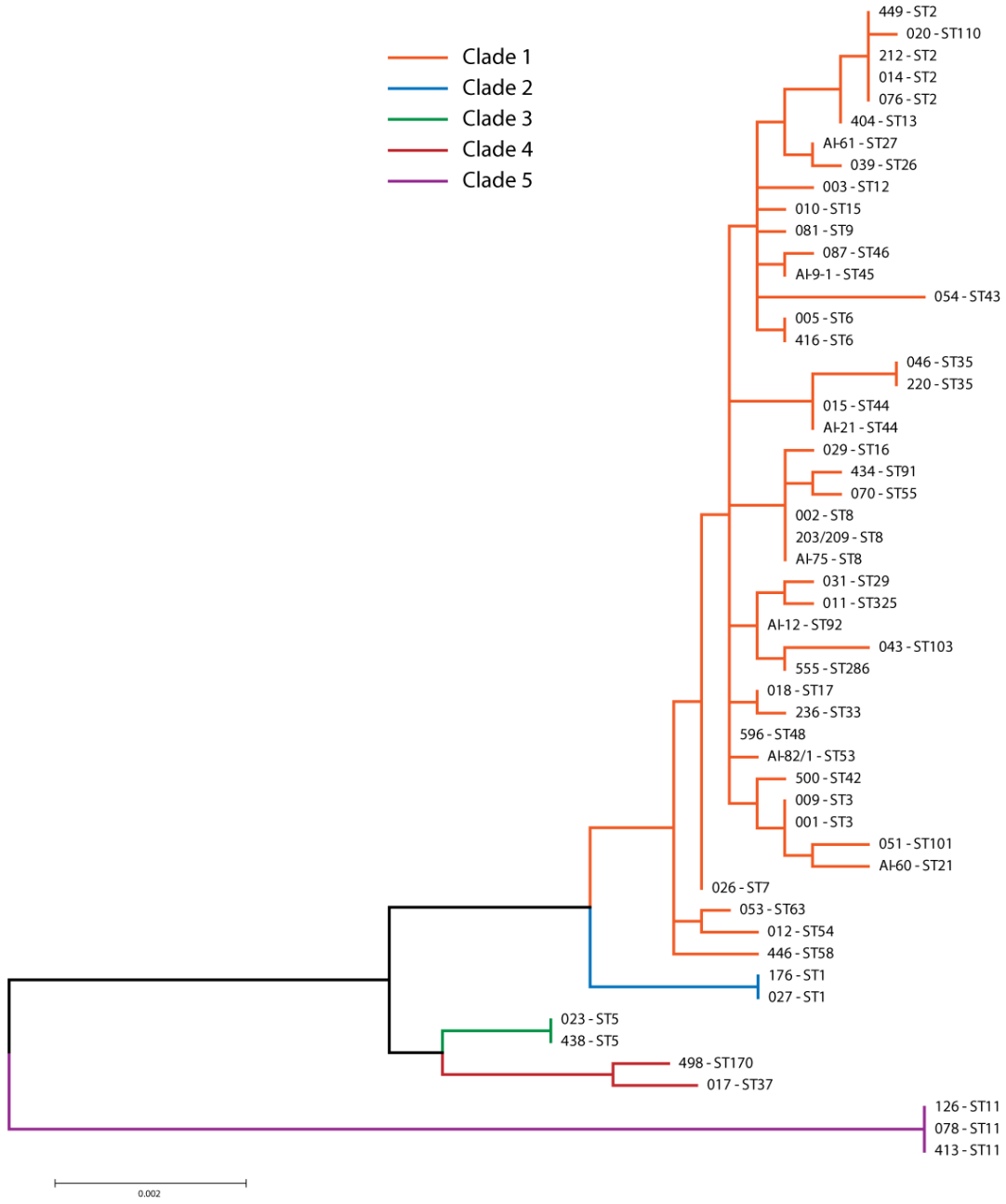
Dále byla MLVA (van den Berg et al., 2007 schéma) použita při mikrobiologicko-epidemiologické studii pětinásobně zvýšeného výskytu CDI na chirurgickém oddělení Fakultní nemocnice u svaté Anny v Brně v roce 2014. Studie zahrnovala jedenáct případů CDI vyvolaných shodným ribotypem 001. MLVA těchto jedenácti izolátů odhalila výskyt dvou nepříbuzných MLVA profilů (pět a šest izolátů). Klonální šíření MLVA profilu A zahrnovalo i případ CDI s měsíčním odstupem od posledního výskytu CDI na oddělení, kdy bylo oddělení na několik dní zavřeno a byla provedena kompletní sanitace (Nyč et al., 2016b).

4.1.5 Multilocus sequence typing

MLST bylo provedeno u 53 zástupců jednotlivých ribotypizačních profilů (ribotypů a WEBRIBO typů). Porovnáním sekvencí sedmi udržovacích genů bylo identifikováno 40 různých sekvenčních typů (ST). Sekvenační typy shodné pro více ribotypizačních profilů jsou uvedeny v tabulce č. 2.

Zjištěných 40 sekvenančních typů se v rámci dendrogramu rozřadilo do 5 různých kladů (clades). Nejvíce heterogenní byl klad 1, který zahrnoval izoláty 44 různých ribotypizačních profilů, 37 z nich bylo toxigenních 7 netoxigenních. Klad 2 zahrnoval

izoláty *C. difficile* ribotypů 027 a 176, klad 3 pak izoláty *C. difficile* ribotyp 023 a WEBRIBO typ 438 a klad 5 izoláty *C. difficile* ribotypů 078, 126 a WEBRIBO typ 413, izoláty ribotypizačních profilů, u kterých byly detekovány geny pro tvorbu toxinů A, B a binární. V kladu 4 byly *C. difficile* ribotyp 017 a WEBRIBO typ 498 nesoucí geny pro tvorbu toxinů A i B (Krůtová et al., 2016b), obrázek 13.



Obrázek 13: Nezakořeněný Dendrogram (Maximum likelihood tree) zahrnující 53 izolátů různých ribotypizačních profilů zkonstruovaný porovnáním spojených sekvencí 7 udržovacích genů. Délka jednotlivých větví odpovídá počtu variant v sekvenovaných genech. (Krůtová et al., 2016b)

Sekvenační typ	Ribotyp / WEBRIBO typ	Klad (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 - podobný, 203/209, AI-75*	1
11	078, 126, *413*	5
35	046, *220*	1
44	*015 - podobný, AI-21*	1

Tabulka 2: Souhrn sekvenačních typů shodných pro více ribotypizačních profilů. WEBRIBO typy jsou uvedeny kurzívou.

4.2 Molekulární typizace slovenských izolátů *C. difficile*

V rámci pilotní měsíční surveillanční studie CDI, která proběhla v září 2012 v deseti univerzitních nemocnicích na Slovensku, jsme otypovali 20 izolátů *C. difficile* z nichž 17 bylo určeno jako *C. difficile* ribotyp 001 (85 % izolátů). U těchto izolátů byla provedena MLVA, která odhalila dva klonální komplexy a blízkou genetickou příbuznost mezi *C. difficile* izoláty z šesti různých nemocnic. Zbývající tři izoláty byly určeny jako *C. difficile* ribotypy 017, 078 and WEBRIBO typ 449.

Molekulární analýza genů a mutací asociovaných s rezistencí k antibiotikům ukázala, že 85 % izolátů *C. difficile* neslo Thr82Ile v GyrA, aminokyselinovou záměnu asociovanou s rezistencí k fluorochinolonům a 90 % *C. difficile* izolátů bylo pozitivních pro přítomnost *erm*(B), genu asociovaného s rezistencí k MLS_B skupině antibiotik. Tyto výsledky upozorňují na závažnou epidemiologickou situaci *C. difficile* infekcí na Slovensku, a na významný potenciál *C. difficile* ribotypu 001 ke klonálnímu šíření a akumulaci mechanismů rezistence k několika antibiotikům. (Nyč et al., 2015b).

4.3 Molekulární typizace finských izolátů *C. difficile*

Ve spolupráci s National Institute for Health and Welfare, Finland, Helsinki jsme provedli detailní analýzu 28 finských izolátů *C. difficile*, které byly vybrány z finské národní sbírky pro svoji genetickou podobnost s celosvětově rozšířeným ribotypem 027. Izoláty *C. difficile* byly vybrány na základě přítomnosti genů pro produkci binárního toxinu a 18bp delece v regulačním genu *tcdC*). Izoláty byly typovány pomocí PCR ribotypizace (kapilární elektroforéza), MLVA, MLST a sekvenování fragmentu genu *tcdC*.

Mezi vyšetřovanými 28 izoláty *C. difficile* jsme identifikovali 12 různých ribotypizačních profilů a 11 sekvenačních typů. Analýza variabilních oblastí genomu (MLVA) potvrdila regionální klonální šíření u izolátů *C. difficile* třech ribotypů a také u izolátů *C. difficile* ribotypu 027, které byly zařazeny jako kontrolní kmeny. Navíc 22 izolátů *C. difficile* osmi různých ribotypů neslo deleci v pozici 117 genu *tcdC*, která je cílovým místem některých komerčních systémů pro odlišení *C. difficile* ribotypu 027 od ostatních ribotypů (Přijato k publikaci v Journal of Microbiology, Immunology and Infection dne 14. 3. 2017).

4.4 Molekulární typizace iránských izolátů *C. difficile*

Ve spolupráci s Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti a University of Medical Sciences, Tehran, Iran jsme otypovali 23 vzorků DNA extrahované izolátů *C. difficile* kultivovaných ze stolic hospitalizovaných pacientů s CDI a také ze stěrů z nemocničního prostředí včetně zdravotnických pomůcek a přístrojů.

C. difficile ribotyp 126 byl nejčastějším ribotypem zachyceným ve studii (21,7 %). Další identifikované ribotypy byly: 001, 003, 014, 017, 029, 039, 081, 103 a 150. *C. difficile* ribotypy 001, 126 a 150 byly identifikovány u izolátů *C. difficile* kultivovaných jak ze vzorků stolic pacientů, tak i ze stěrů z prostředí (pacientské lůžko) a zdravotnických pomůcek (kolonoskop, endoskop), (v recenzním řízení).

Navzdory odlišné geografické poloze této studie, nalezené *C. difficile* ribotypy patří k humánním *C. difficile* ribotypům vyskytujících se v Evropě (Bauer et al., 2011). A navíc, stejné spektrum *C. difficile* ribotypů a WEBRIBO typů (kromě WRT AI-29) bylo nalezeno také v mezinárodní sbírce zvířecích izolátů *C. difficile* (Janezic et al., 2014).

4.5 Česká typizační data v evropském kontextu

Průběžné výsledky byly prezentovány jak v ČR, tak i na Evropském kongresu klinické mikrobiologie a infekčních onemocnění (ECCMID) v Barceloně formou publikovaného příspěvku R476 „Emergence of *Clostridium difficile* 027 - like PCR ribotype 176 in the Czech Republic (2012-2013)", v Kodani formou posteru EV0870 „Emergence of *Clostridium difficile* PCR ribotypes 001 and 176 in the Czech Republic" a v Amsterdamu formou tištěných posterů P0315 „Practical experiences with capillary electrophoresis ribotyping applied on Czech *Clostridium difficile* isolates collected over 3 years

(2013-2015)“, P1088 Update on the molecular epidemiology of *Clostridium difficile* infections in the Czech Republic“ a P1089 „Different distribution of *Clostridium difficile* PCR ribotypes in acute care and long term care wards of Czech hospitals”.

Všechna výše zmíněná abstrakta a postery jsou dostupné bez registrace na https://www.escmid.org/escmid_publications/escmid_elibrary/.

Molekulárně typizační data izolátů *C. difficile* získaná v průběhu projektu jsou součástí deseti zahraničních impaktovaných publikací a další dvě publikace jsou v recenzním řízení (příloha 1-12).

5. Souhrnná diskuze k jednotlivým publikacím

PCR ribotypizace je v Evropě dobře zavedenou a doporučenou metodou typizace klinických izolátů *C. difficile* pro účely surveillnace (ECDC, 2015). Pro sjednocení variability v používaných postupech a designech primerů byl nedávno publikován konsensuální protokol pro PCR ribotypizaci (Fawley et al., 2015). Tento protokol byl pro porovnání použit i u 53 zástupců různých ribotypů (Krůtová et al., 2016b). Pozorovaná změna v ribotypizačním profilu (extra band 326 bp u čtyřech izolátů) bude pravděpodobně souviset s jejich vzájemnou genetickou příbuzností (tři měly stejný sekvenační typ) a také pravděpodobně se vztahem k lokálnímu výskytu (kontrolní izolát *C. difficile* ribotypu 002 z Leeds – Leiden sbírky tuto změnu nevykazoval), (Krůtová et al., 2016b). Konsensuální protokol s primery navrženými Bidet et al. (1999) byl od 2016 adoptován na ÚLM v Motole pro ribotypizaci izolátů *C. difficile*.

C. difficile ribotypy 176 a 001 patřily mezi nejčastěji zachycené ribotypy (26,7 % a 20,7 %) v našem souboru izolátů. *C. difficile* ribotyp 176 byl na území ČR prvně identifikován v polovině roku 2009 (Nyč et al., 2011), krátce po ukončení první evropské studie zaměřené na incidenci *C. difficile* v roce 2008 (Bauer et al., 2011). V druhé celoevropské prevalenční studii CDI (Davies et al., 2014; Davies et al. 2016), byl *C. difficile* ribotyp 176 identifikován mezi izoláty *C. difficile* z České republiky (12/34 izolátů), Maďarska (1/66 izolátů), Německa (5/626 izolátů), Polska (5/87 izolátů) a Rumunska (1/65 izolátů), (osobní komunikace prof. Wilcox, Leeds, UK). V Maďarsku byl výskyt *C. difficile* ribotypu 176 zachycen také v roce 2014 v Budapešťském regionu (Tóth et al., 2016). Z Polska je epidemicky významný výskyt *C. difficile* ribotypu 176 současně s výskytem *C. difficile* ribotypu 027 hlášen již od roku 2008 (Obuch-Woszczatyński et al.,

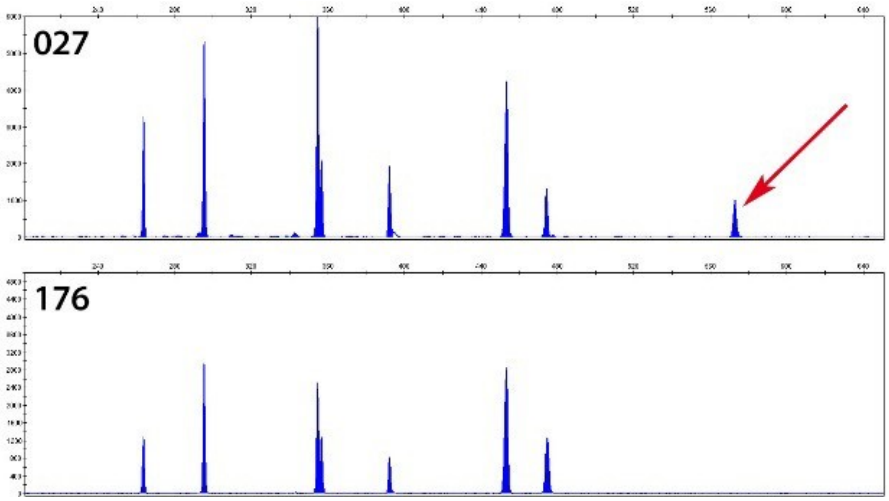
2014; Pituch et al., 2015). V roce 2015 byl výskyt *C. difficile* ribotypu 176 zachycen v jedné chorvatské nemocnici (n=18/19 izolátů), (Rupnik et al., 2016). Pět izolátů *C. difficile* ribotypu 176 (čtyři z roku 2008 a jeden z roku 2009) bylo identifikováno při příbuzenské analýze 28 finských izolátů *C. difficile* k ribotypu 027 (přijato k publikaci v JMII, 14. 3. 2017).

Vzhledem ke sdílení několika molekulárních charakteristik je usuzována genetická příbuznost mezi *C. difficile* ribotypy 176 a 027 (Valiente et al., 2012; Knetsch et al., 2012; Krůtová et al., 2014b; Krůtová et al., 2016b). Izoláty *C. difficile* ribotypu 176 byly dokonce použity k fylogeneticko-geografickému mapování rozšíření *C. difficile* ribotypu 027 (He et al., 2013), který je celosvětově rozšířen a jeho výskyt je asociován s nemocničním šířením a závažným průběhem onemocnění (Kuijper et al., 2006; He et al., 2013; Rao et al., 2015).

Ribotypy 027 a 176 nesou ve svém genomu geny pro tvorbu binárního toxinu (*cdtA* a *cdtB*), deleci v regulačním genu *tcdC* (18pb v pozici 330-347 a 1bp v pozici 117), oba patří ke stejnému sekvenačnímu typu (ST1, Clade 2) a ve svém elektroforetickém profilu se liší absencí jednoho bandu (fragmentu), (obrázek 14), (Valiente et al, 2012; Knetsch et al., 2012; Krůtová et al., 2014b; Krůtová et al., 2016b). Zmíněná delece v pozici 117 genu *tcdC* je používaná jak v komerčních (Carroll et al., 2013; Krůtová et al., 2014b; Mentula et al., 2015; McMillen et al., 2016), tak v in-house molekulárních metodách (Wolff et al., 2009, de Boer et al., 2010) jako marker pro odlišení *C. difficile* ribotypu 027 od ostatních ribotypů.

Vzhledem k raritnímu výskytu *C. difficile* ribotypu 027, kdy v roce 2013 byl zachycen první případ importované infekce (Krůtová et al., 2014c) s dalšími čtyřmi v následujících dvou letech a oproti tomu výraznému výskytu *C. difficile* ribotypu 176, kdy v letech 2013-2015 tvořil 26,7 % izolátů (n=588) zaslaných k ribotypizaci (Krůtová et al., 2016b), je nutné dodat k výsledku těchto molekulárních testů patřičný komentář.

Navíc, přítomnost shodné delece v *tcdC* genu současně s přítomností genů pro tvorbu binárního toxinu nebyly prokázány pouze u *C. difficile* ribotypu 176 (Krůtová et al., 2014b), ale i u dalších izolátů různých ribotypů (Kok et al., 2011; Mentula et al., 2015), což potvrzuje nutnost provedení PCR ribotypizace ke spolehlivému určení, zda se jedná o *C. difficile* ribotyp 027.



Obrázek 14: Rozdíl v elektroforetických profilech C. difficile ribotypu 027 a 176 (Krůtová et al., 2014b)

C. difficile ribotyp 001 obsadil v obou evropských studiích druhou příčku ve výskytu toxigenních ribotypů. Desetiprocentní výskyt za *C. difficile* ribotypy 014/020 (16 %) v roce 2008 (Bauer et al., 2011) a jedenáctiprocentní výskyt za *C. difficile* ribotypem 027 (19 %) v letech 2012-2013. Výskyt tohoto ribotypu je v porovnání jednotlivých evropských zemí častější v západní a východní Evropě, kde zároveň prevaluje *C. difficile* ribotyp 027 (Davies et al., 2016).

U prevalentních ribotypů *C. difficile* (001 a 176) v ČR byly zjištěny rozdíly v jejich distribuci závislosti na věku pacientů. Zatímco výskyt *C. difficile* ribotypu 001 byl u českých pacientů pozorován již ve skupině dětí do dvou let (1,2 %) a ve skupině 3-18 let dosáhl 10,7 %, výskyt *C. difficile* ribotypu 176 byl v těchto věkových skupinách vzácný (0 % a 1 %). Naše pozorování ve vztahu k výskytu *C. difficile* ribotypu 001 u dětí jsou ve shodě s německou studií zahrnující 1067 izolátů *C. difficile*, kde ribotyp 001 u dětí tvořil 9,6 % izolátů (von Müller et al., 2015) a s nizozemskou studií, kde u dětí tvořil 10,5 % izolátů z analyzovaných 3 671 izolátů *C. difficile* (van Dorp et al., 2017). Ačkoliv nemůžeme považovat *C. difficile* ribotyp 176 za ribotyp 027, jeho raritní výskyt u českých pediatrických izolátů je opět ve shodě s výše uvedenými studiemi (von Müller et al., 2015; van Dorp et al., 2017).

C. difficile ribotyp 001, na rozdíl od ribotypu 176, nese ve své genetické výbavě pouze geny pro produkci toxinů A, B. Jedním společným epidemiologickým znakem pro oba tyto ribotypy (001 a 176) může být popsaná rezistence hned k několika

antimikrobním přípravkům a s tím spojená kumulace mechanismů rezistence. U obou ribotypů byla popsána rezistence k moxifloxacinu (Spigaglia et al., 2011; Freeman et al., 2015, Krůtová et al., 2015; Lachowicz et al., 2015) a s ní spojená přítomnost záměny aminokyseliny Thr82Ile v GyrA (Dridi et al., 2002; Spigaglia et al., 2011; Krůtová et al., 2015).

Závažnější průběh CDI vyvolaných *C. difficile* ribotypy 001 a 176 byl indikován v německé, polské a slovenské studii (Arvand et al., 2009; Obuch-Woszczatyński et al., 2014, Nyč et al., 2015b). U českých pacientů s infekcí vyvolanou *C. difficile* ribotypem 176 byly klinické údaje o závažnosti průběhu CDI analyzovány ve dvou studiích (Drábek et al., 2015; Polívková et al., 2016). Výsledky ukázaly vyšší poměr jak závažnějšího průběhu onemocnění 11/7 a 13/3 tak i mortality 5/2 a 16/8 u pacientů s infekcí vyvolanou *C. difficile* ribotypem 176 v porovnání s pacienty s infekcí vyvolanou jiným ribotypem (Drábek et al., 2015; Polívková et al., 2016).

Různorodá klinická manifestace CDI zahrnující mírný i závažný průběh a rekurenci onemocnění, byla popsána u 11 pacientů infikovaných dvěma různými kmeny *C. difficile* ribotyp 001 (Nyč et al., 2016b).

S výskytem *C. difficile* ribotypů 001 a 176 na našem území pravděpodobně souvisí také zvýšená incidence CDI v ČR (6,2 případů na 10 000 ošetřovacích dní v roce 2013 a 6,1 případů na 10 000 ošetřovacích dní v roce 2014, kdy byla zpracována data z 10 a 18 nemocnic), (Davies et al., 2014; Krůtová et al., 2016a).

MLVA je používanou subtypizační metodou u izolátů *C. difficile* shodného ribotypu jak humánního (van den Berg, 2007; Goorhuis et al. 2009; Eckert et al., 2011; van Dorp et al., 2017) tak i nehumánního původu (Baker et al., 2010; Schneeberg et al., 2012; Schneeberg et al., 2013a,b; Usui et al., 2014; Rodriguez et al., 2015; Wu et al., 2016).

Rozlišovací schopnost MLVA pro detekci přenosu *C. difficile*, při použití schématu zahrnující 7 VNTR lokusů (van den Berg et al., 2007), je srovnatelná s WGS i s rozdílem v odlišné části genomu, která je analyzována (Eyre et al., 2013). Rozlišovací schopnost MLVA se zvyšuje s počtem použitých úseků až na srovnatelnou úroveň s ribotypizací (Manzoor et al., 2011). Tím se ale navyšuje i cena typizace a obtížnost interpretace (Knetsch et al., 2013).

MLVA byla jako subtypizační metoda poprvé použita u českých a polských izolátů *C. difficile* ribotypu 176 v publikaci Nyč *et al*. (Nyč at al., 2011), které byly vzájemně porovnány s evropskými izoláty *C. difficile* ribotyp 027. Blízká genetická příbuznost byla potvrzena mezi třemi českými a dvěma polskými izoláty *C. difficile* ribotypu 176 (Nyč at al., 2011).

U českých izolátů *C. difficile* ribotypu 176 z let 2013-2014 výsledky MLVA prokázaly klonální šíření v rámci nemocničního zařízení (Drábek et al., 2015; Polívková et al., 2016; Krůtová et al., 2016a), tak i mezi jednotlivými nemocnicemi (Krůtová et al., 2016a), což ukazuje na nutnost zavést striktní protiepidemická opatření při výskytu tohoto ribotypu.

Ačkoliv *C. difficile* ribotyp 001 patří k rozšířeným ribotypům v Evropě, subtypizace pomocí MLVA byla použita v omezeném počtu studií. U českých izolátů *C. difficile* ribotypu 001 z roku 2014 bylo potvrzeno klonální šíření v sedmi z osmnácti nemocnic v ČR (Krůtová et al., 2016a). Dále byla tato metoda použita k epidemiologickému šíření zvýšeného výskytu CDI na oddělení (Nyč et al., 2016b). U slovenských izolátů *C. difficile* z roku 2013, byla odhalena blízká genetická příbuznost mezi izoláty *C. difficile* ribotypu 001 v šesti nemocnicích (Nyč et al., 2015b). U britských izolátů *C. difficile* ribotypu 001 z let 2010-2011 byla provedena MLVA zahrnující 12 různých VNTR úseků (Hardy et al., 2012). Z důvodu potvrzení klonality izolátů *C. difficile* spojené s antimikrobiální rezistencí byla MLVA použita u britských a španělských izolátů *C. difficile* ribotypu 001 (Baines et al., 2008; Marín et al., 2015).

MLST určila 40 sekvenačních typů u 53 různých ribotypizačních profilů (Krůtová et al., 2016b). Ačkoliv byl vždy sekvenován jen jeden izolát *C. difficile* od každého ribotypizačního profilu, u 24 dvojic (ST a RT) byla nalezena shoda v porovnání s výsledky MLST a PCR ribotypizace izolátů *C. difficile* z Leeds – Leiden sbírky (Knetsch et al., 2012). Některé izoláty *C. difficile* s různým elektroforetickým profilem patřily do stejného kladu a zároveň u nich byla detekována shodná delece v *tcdC* genu a přítomnost shodných genů pro tvorbu toxinů, což naznačuje jejich genetickou příbuznost.

6. Souhrn

Clostridium difficile je významným nozokomiálním patogenem současnosti v souvislosti s rozšířením epidemických kmenů. Molekulární typizace klinických izolátů je nedílnou součástí kontroly výskytu a šíření *C. difficile* v nemocničním prostředí a v komunitě.

Soubor 2201 klinických izolátů *C. difficile* z 32 nemocničních zařízení z období 2013-2015 byl charakterizován pomocí PCR ribotypizace doplněné o průkaz genů pro tvorbu toxinů. Identifikovali jsme 166 různých ribotypizačních profilů a u 53 profilů byly zachyceny alespoň dva izoláty reprezentující jeden profil. Nejčastěji zachycenými ribotypy byly 176 (n=588, 26,7 %) a 001 (n=456, 20,7 %), následovány ribotypy 014 (n=176, 8 %), 012 (n=127 5,8 %), 017 (n=85, 3,9 %) a 020 (n=68, 3,1 %). Celkem 2024 (92 %) izolátů bylo toxigenních (neslo geny pro produkci toxinů A, B) a z těchto navíc 677 neslo geny pro tvorbu binárního toxinu. Zbývajících 177 (8 %) izolátů bylo netoxigenních.

Subtypizace izolátů *C. difficile* pomocí MLVA (multilocus variable number tandem repeats analysis) porovnávající počet repetitivních úseků byla provedená u izolátů ribotypu 176 (n=225, 17 nemocnic) a u izolátů ribotypu 001 (n=184, 14 nemocnic) kultivovaných v roce 2014. Klonální příbuznost izolátů v rámci ribotypu byla zjištěna u 76,6 % izolátů ribotypu 001, které tvořily 14 klonálních komplexů a u 84,5 % izolátů ribotypu 176, které tvořily 27 klonálních komplexů. Dále byla MLVA použita v lokálních retrospektivních epidemiologických šetřeních na třech odděleních (78 izolátů ribotypu 176 z roku 2013 a 11 izolátů ribotypu 001 z roku 2014) s podobnými zjištěními. Klonální příbuznost izolátů *C. difficile* v rámci ribotypu 001 nebo 176, pozorována v jednotlivých nemocničních zařízeních tak i mezi nimi, potvrzuje vyšší potenciál těchto ribotypů k šíření v nemocničním prostředí.

Fylogenetický vztah zástupců různých ribotypizačních profilů byl zkoumán pomocí MLST (multilocus sequence typing). Vybraných 53 izolátů *C. difficile* patřilo k 40 sekvenačním typům. Ribotypy se shodným sekvenačním typem vykazují i podobný ribotypizační profil a mají i shodnou výbavu genů pro produkci toxinů, což naznačuje jejich fylogenetickou příbuznost.

Výrazné zastoupení pouze dvou ribotypů (001 a 176) zjištěné v reprezentativním počtu zúčastněných zdravotnických zařízení lze považovat za epidemiologicky zásadní skutečnost. Získaná data mohou sloužit v dalších letech jako podklad pro další sledování a posouzení dynamiky šíření a zastoupení jednotlivých ribotypů *C. difficile* v ČR.

7. Summary

Currently, *Clostridium difficile* is a leading nosocomial pathogen due to the spread of epidemic strains. Molecular typing of clinical isolates is an important part of *C. difficile* occurrence and spread control in hospitals as well as in the community.

A total of 2201 clinical *C. difficile* isolates from 32 hospitals cultured between 2013-2015 were characterized by PCR ribotyping and toxin gene multiplex PCR. A total of 166 different ribotyping profiles were identified, of which 53 ribotyping profiles were represented by at least two isolates for each profile. The most frequently found ribotypes were 176 (n=588, 26.7%) and 001 (n=456, 20.7%) followed by 014 (n=176, 8%), 012 (n=127, 5.8%), 017 (n=85, 3.9%) and 020 (n=68, 3.1%). Out of 2201 isolates, 2024 (92%) isolates were toxigenic and carried genes for toxin A and B, and of these, 677 (33.5%) also carried genes for binary toxin. The remaining 177 (8%) isolates were non-toxigenic.

Subtyping of *C. difficile* isolates using a multilocus variable-number tandem repeats analysis (MLVA), that compared the sum of tandem repeats differences, was performed in *C. difficile* isolates of ribotype 176 (n=225, 17 hospitals) and in *C. difficile* isolates of ribotype 001 (n=184, 14 hospitals) cultured in 2014. The clonal relatedness in *C. difficile* isolates belonging to the same ribotype was found in 76.6% of ribotype 001 isolates forming 14 clonal complexes and in 84.5% of ribotype 176 isolates forming 27 clonal complexes. MLVA was also used in three retrospective local department epidemiologic investigations (78 isolates of ribotype 176 from 2013 and 11 isolates of ribotype 001 from 2014) with similar results. That clonal relatedness of *C. difficile* isolates of ribotypes 001 or 176 was observed within and between hospitals confirms a higher potential of these ribotypes to spread in a hospital environment.

The phylogenetic relationship of selected isolates belonging to different profiles was investigated using multilocus sequence typing (MLST). The selected 53 *C. difficile* isolates revealed 40 different sequence types. Ribotypes with the same sequence type also had a similar ribotyping profile and carried the same toxin genes, which suggest a phylogenetic relatedness.

The significant representation of the two ribotypes revealed in a representative number of hospitals, could be regarded as an epidemiologically important situation. The data obtained can be used for further the monitoring and assessment of the dynamics of distribution and representation of individual *C. difficile* ribotypes in the country.

7. Závěr

Molekulární typizace izolátů *C. difficile* je nepostradatelným nástrojem pro studium epidemiologie infekcí vyvolaných tímto patogenem. V předkládané práci byly použity tři různé metody cílené na různá místa v genomu *C. difficile*.

Výsledky projektu potvrzují, že PCR ribotypizace s detekcí na kapilární elektroforéze je metodou s velkou diskriminační schopností, vhodnou pro typizaci velkého množství izolátů *C. difficile*. Shromážděním ribotypizačních dat z více než 2 tisíc izolátů *C. difficile* za tříleté období jsme získali cenné informace o kmenech *C. difficile* cirkulujících na území ČR.

Velký počet analyzovaných izolátů dává získaným datům vysokou validitu. Výrazné zastoupení dvou ribotypů je i v rámci Evropy neobvyklé a zřejmě svědčí o rezervách v kontrole těchto infekcí. Příslušné zjištění by mělo být chápáno jako zásadní pro zkvalitnění prevence šíření původců infekcí spojených s nemocniční péčí. Diversita ribotypů *C. difficile* v konkrétním zařízení může sloužit jako pomocný indikátor kvality nemocniční péče. Získaná robustní data mohou sloužit v dalších letech jako podklad pro další sledování a posouzení dynamiky šíření a zastoupení jednotlivých ribotypů a z těchto informací i odvodit účinnost případných protiepidemických opatření.

MLVA je vhodnou subtypizační metodou při podezření na nozokomiální šíření *C. difficile*, která umožňuje poměrně přesné analyzování konkrétní situace včetně retrospektivního hodnocení. Může tak být účinným nástrojem efektivní analýzy proběhlých „outbreaků“ a tím i prevencí těchto situací do budoucna.

MLST byla použita ke studiu bližší příbuznosti izolátů *C. difficile* v rámci různých ribotypů. Výsledky poukazují na nižší rozlišovací schopnost metody v porovnáním s ribotypizací. Fylogenetická příbuznost několika ribotypů příslušející ke shodnému sekvenačnímu typu musí být potvrzena celogenomou analýzou většího počtu zástupců těchto ribotypů.

Tříletá spolupráce s rozsáhlou sítí mikrobiologických laboratoří v ČR nám pomohla vymezit situace, v jakých je vhodné přistoupit k zasílání izolátů *C. difficile* k typizaci do referenční laboratoře nebo provádění typizace samotné. Na základě získaných zkušeností by charakterizace kmene, a to nejen na molekulární úrovni, ale také fenotypově stanovením

antimikrobní citlivosti k lékům volby, měla být prováděna při náhlém zvýšení incidence CDI na oddělení, při těžkém průběhu onemocnění nebo selhání specifické terapie onemocnění.

Prezentované metody představují komplexní strategii vyšetření, které mohou dnešní specializované laboratoře nabídnout a tím podstatně přispět k diagnostice a řešení jak akutních, tak i rekurentních forem CDI. Porozumění epidemiologii, mezinárodní kontext a podklady pro efektivní prevenci jsou další aspekty molekulárních metod aplikovaných v diagnostice a typizaci *C. difficile* a použitých v této práci.

Přístrojové vybavení a určité nároky na kvalifikaci a zkušenost s danou problematikou mohou být limitujícími faktory pro rutinní použití uvedených typizačních metod. Z těchto důvodů bude příslušná komplexní diagnostika do budoucna centralizována v rámci referenční laboratoře nebo spolupracující sítě několika specializovaných pracovišť.

8. Použitá literatura

1. Adler A, Miller-Roll T, Bradenstein R, Block C, Mendelson B, Parizade M, Paitan Y, Schwartz D, Peled N, Carmeli Y, Schwaber MJ. A national survey of the molecular epidemiology of *Clostridium difficile* in Israel: the dissemination of the ribotype 027 strain with reduced susceptibility to vancomycin and metronidazole. Diagn Microbiol Infect Dis. 2015;83(1):21-4.

2. Andrés-Lasheras S, Bolea R, Mainar-Jaime RC, Kuijper E, Sevilla E, Martín-Burriel I, Chirino-Trejo M. Presence of *Clostridium difficile* in pig faecal samples and wild animal species associated with pig farms. J Appl Microbiol. 2017;122(2):462-472.

3. Androga GO, Hart J, Foster NF, Charles A, Forbes D, Riley TV. Infection with Toxin A-Negative, Toxin B-Negative, Binary Toxin-Positive *Clostridium difficile* in a Young Patient with Ulcerative Colitis. J Clin Microbiol. 2015;53(11):3702-4.

4. Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G. Clostridium difficile ribotypes 001, 017, and 027 are associated with lethal *C. difficile* infection in Hesse, Germany. Euro Surveill. 2009;14(45). pii: 19403.

5. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, Kuijper EJ, Wilcox MH. Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J Antimicrob Chemother. 2008;62(5):1046-52.

6. Balihar K, Kozak F, Kozeluhova J, Hejda V, Fremundova L, Krcma M, Geigerova L, Bergerova T, Matejovic M. *Clostridium difficile* infection in hospitalized patients at a Czech tertiary center: analysis of epidemiology, clinical features, and risk factors of fulminant course. Eur J Gastroenterol Hepatol. 2014;26(8):880-7.

7. Bartlett JG, Gorbach SL. Pseudomembranous enterocolitis (antibiotic-related colitis). Adv Intern Med 1977;22:455-76.

8. Bauer MP, Kuijper EJ, van Dissel JT; European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for *Clostridium difficile* infection (CDI). Clin Microbiol Infect. 2009;15(12):1067-79.

9. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ; ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. 2011;377(9759):63-73.

10. Bakker D, Corver J, Harmanus C, Goorhuis A, Keessen EC, Fawley WN, Wilcox MH, Kuijper EJ. Relatedness of human and animal *Clostridium difficile* PCR ribotype 078 isolates determined on the basis of multilocus variable-number tandem-repeat analysis and tetracycline resistance. J Clin Microbiol. 2010;48(10):3744-9.

11. Bedimo R, Weinstein J. Recurrent extraintestinal *Clostridium difficile* infection. Am J Med. 2003;114(9):770-1.

12. Beneš J, Husa P, Nyč O. Doporučený postup diagnostiky a léčby kolitidy vyvolané *Clostridium difficile*. Klin Mikrobiol Infekc Lek. 2012;18(5):160-7.

13. Beneš J, Husa P, Nyč O, Polívková S. Doporučený postup diagnostiky a léčby kolitidy vyvolané *Clostridium difficile*. Klin Mikrobiol Infekc Lek. 2014;20(2):56-66.

14. Beran V, Chmelar D, Vobejdova J, Konigova A, Nemec J, Tvrdik J. Sensitivity to antibiotics of *Clostridium difficile* toxigenic nosocomial strains. Folia Microbiol (Praha). 2014;59(3):209-15.

15. Bergström A, Skov TH, Bahl MI, Roager HM, Christensen LB, Ejlerskov KT, Mølgaard C, Michaelsen KF, Licht TR. Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. Appl Environ Microbiol. 2014;80(9):2889-900.

16. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett. 1999;175(2):261-6.

17. Bowman RA, Riley TV. Laboratory diagnosis of *Clostridium difficile*-associated diarrhoea. Eur J Clin Microbiol Infect Dis. 1988;7(4):476-84.

18. Braun V, Hundsberger T, Leukel P, Sauerborn M, von Eichel-Streiber C. Definition of he single integration site of the pathogenicity locus in *Clostridium difficile*. Gene. 1996;181(1-2):29-38.

19. Brazier JS. The laboratory diagnosis of *C. difficile* associated disease. Reviews in Medical Microbiology. 1995;6:236-245.

20. Britton RA, Young VB. Interaction between the intestinal microbiota and host in *Clostridium difficile* colonization resistance. Trends Microbiol. 2012;20(7):313-9.

21. Brito GA, Fujji J, Carneiro-Filho BA, Lima AA, Obrig T, Guerrant RL. Mechanism of *Clostridium difficile* toxin A-induced apoptosis in T84 cells. J Infect Dis. 2002;186(10):1438-47.

22. Cairns MD, Preston MD, Lawley TD, Clark TG, Stabler RA, Wren BW. Genomic Epidemiology of a Protracted Hospital Outbreak Caused by a Toxin A-Negative *Clostridium difficile* Sublineage PCR Ribotype 017 Strain in London, England. J Clin Microbiol. 2015;53(10):3141-7.

23. Cammarota G, Masucci L, Ianiro G, Bibbò S, Dinoi G, Costamagna G, Sanguinetti M, Gasbarrini A. Randomised clinical trial: faecal microbiota transplantation by colonoscopy vs. vancomycin for the treatment of recurrent *Clostridium difficile* infection. Aliment Pharmacol Ther. 2015;41(9):835-43.

24. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, Sokol H, Arkkila P, Pintus C, Hart A, Segal J, Aloi M, Masucci L, Molinaro A, Scaldaferri F, Gasbarrini G, Lopez-Sanroman A, Link A, de Groot P, de Vos WM, Högenauer C, Malfertheiner P, Mattila E, Milosavljević T, Nieuwdorp M, Sanguinetti M, Simren M, Gasbarrini A; European FMT Working Group. European consensus conference on faecal microbiota transplantation in clinical practice. Gut. 2017 Jan 13. pii: gutjnl-2016-313017.

25. Cappella M, Pugliese F, Zucchini A, Marchetti F. *Clostridium difficile* Enterocolitis and Reactive Arthritis: A Case Report and Review of the Literature. Case Rep Pediatr. 2016;2016:1591753.

26. Carman RJ, Wickham KN, Chen L, Lawrence AM, Boone JH, Wilkins TD, Kerkering TM, Lyerly DM. Glutamate dehydrogenase is highly conserved among *Clostridium difficile* ribotypes. J Clin Microbiol. 2012;50(4):1425-6.

27. Carroll KC, Buchan BW, Tan S, Stamper PD, Riebe KM, Pancholi P, Kelly C, Rao A, Fader R, Cavagnolo R, Watson W, Goering RV, Trevino EA, Weissfeld AS, Ledeboer NA. Multicenter evaluation of the Verigene *Clostridium difficile* nucleic acid assay. J Clin Microbiol. 2013;51(12):4120-5.

28. Carter GP, Lyras D, Allen DL, Mackin KE, Howarth PM, O'Connor JR, Rood JI. Binary toxin production in *Clostridium difficile* is regulated by CdtR, a LytTR family response regulator. J Bacteriol. 2007;189(20):7290-301.

29. Clinical and Laboratory Standards Institute. Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved standard M11-A7. 7th ed. Wayne, PA: CLSI; 2007.

30. Coltella L, Mancinelli L, Onori M, Lucignano B, Menichella D, Sorge R, Raponi M, Mancini R, Russo C. Advancement in the routine identification of anaerobic bacteria by MALDI-TOF mass spectrometry. Eur J Clin Microbiol Infect Dis. 2013;32(9):1183-92.

31. Crobach MJ, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, Wilcox MH, Kuijper EJ. European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for *Clostridium difficile* infection. Clin Microbiol Infect. 2016;22 Suppl 4:S63-81.

32. Curry SR, Muto CA, Schlackman JL, Pasculle AW, Shutt KA, Marsh JW, Harrison LH. Use of multilocus variable number of tandem repeats analysis genotyping to determine the role of asymptomatic carriers in *Clostridium difficile* transmission. Clin Infect Dis. 2013;57(8):1094-102.

33. D'Agostino RB Sr, Collins SH, Pencina KM, Kean Y, Gorbach S. Risk estimation for recurrent *Clostridium difficile* infection based on clinical factors. Clin Infect Dis. 2014;58(10):1386-93.

34. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, Delmée M, Fitzpatrick F, Ivanova K, Kuijper E, Macovei IS, Mentula S, Mastrantonio P, von Müller L, Oleastro M, Petinaki E, Pituch H, Norén T, Nováková E, Nyč O, Rupnik M, Schmid D, Wilcox MH. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis. 2014;14(12):1208-19.

35. Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH; EUCLID study group. Diversity of *Clostridium difficile* PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. Euro Surveill. 2016;21(29).

36. Debast SB, Bauer MP, Kuijper EJ; European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. Clin Microbiol Infect. 2014;20 Suppl 2:1-26.

37. de Boer RF, Wijma JJ, Schuurman T, Moedt J, Dijk-Alberts BG, Ott A, Kooistra-Smid AM, van Duynhoven YT. Evaluation of a rapid molecular screening approach for the detection of toxigenic *Clostridium difficile* in general and subsequent identification of the *tcdC* Δ117 mutation in human stools. J Microbiol Methods. 2010;83(1):59-65.

38. de Boer E, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ. Prevalence of *Clostridium difficile* in retailed meat in the Netherlands. Int J Food Microbiol. 2011;144(3):561-4.

39. Drabek J, Nyc O, Krutova M, Stovicek J, Matejkova J, Keil R. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study. Ann Clin Microbiol Antimicrob. 2015;14:55.

40. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC. gyrA and gyrB mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother. 2002;46(11):3418-21.

41. Drigo I, Mazzolini E, Bacchin C, Tonon E, Puiatti C, Bano L, Spigaglia P, Barbanti F, Agnoletti F. Molecular characterization and antimicrobial susceptibility of *Clostridium difficile* isolated from rabbits raised for meat production. Vet Microbiol. 2015;181(3-4):303-7.

42. Eckert C, Vromman F, Halkovich A, Barbut F. Multilocus variable-number tandem repeat analysis: a helpful tool for subtyping French *Clostridium difficile* PCR ribotype 027 isolates. J Med Microbiol. 2011;60(Pt 8):1088-94.

43. Eckert C, Burghoffer B, Barbut F. Contamination of ready-to-eat raw vegetables with *Clostridium difficile* in France. J Med Microbiol. 2013;62(Pt 9):1435-8.

44. Eckert C, Emirian A, Le Monnier A, Cathala L, De Montclos H, Goret J, Berger P, Petit A, De Chevigny A, Jean-Pierre H, Nebbad B, Camiade S, Meckenstock R, Lalande V, Marchandin H, Barbut F. Prevalence and pathogenicity of binary toxin-positive *Clostridium difficile* strains that do not produce toxins A and B. New Microbes New Infect. 2014;3:12-7.

45. Egerer M, Giesemann T, Jank T, Satchell KJ, Aktories K. Auto-catalytic cleavage of *Clostridium difficile* toxins A and B depends on cysteine protease activity. J Biol Chem. 2007;282(35):25314-21.

46. Elliott B, Reed R, Chang BJ, Riley TV. Bacteremia with a large clostridial toxin-negative, binary toxin-positive strain of *Clostridium difficile*. Anaerobe. 2009;15(6):249-51.

47. Elliott B, Squire MM, Thean S, Chang BJ, Brazier JS, Rupnik M, Riley TV. New types of toxin A-negative, toxin B-positive strains among clinical isolates of *Clostridium difficile* in Australia. J Med Microbiol. 2011;60(Pt 8):1108-11.

48. Enoch DA, Butler MJ, Pai S, Aliyu SH, Karas JA. *Clostridium difficile* in children: colonisation and disease. J Infect. 2011;63(2):105-13.

49. Esfandiari Z, Weese S, Ezzatpanah H, Jalali M, Chamani M. Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran. BMC Microbiol. 2014;14:283.

50. European Centre for Disease Prevention and Control. European Surveillance of *Clostridium difficile* infections. Surveillance protocol version 2.2. Stockholm: ECDC; 2015.

51. European Committee on Antimicrobial Susceptibility Testing, EUCAST, Clinical Breakpoints for bacteria, 2016.

52. Eyre DW, Fawley WN, Best EL, Griffiths D, Stoesser NE, Crook DW, Peto TE, Walker AS, Wilcox MH. Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. J Clin Microbiol. 2013;51(12):4141-9.

53. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, Paulick A, Anderson L, Kuijper EJ, Wilcox MH. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One. 2015;10(2):e0118150.

54. Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH. The changing epidemiology of *Clostridium difficile* infections.Clin Microbiol Rev. 2010;23(3):529-49.

55. Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, Wilcox MH; Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent *Clostridium difficile* Ribotypes' Study Group. Pan-European longitudinal surveillance

of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin Microbiol Infect. 2015;21(3):248.e9-248.e16.

56. Fuentes S, van Nood E, Tims S, Heikamp-de Jong I, ter Braak CJ, Keller JJ, Zoetendal EG, de Vos WM. Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent *Clostridium difficile* infection. ISME J. 2014;8(8):1621-33.

57. Goldman P. The development of 5-nitroimidazoles for the treatment and prophylaxis of anaerobic bacterial infections. J Antimicrob Chemother. 1982;10 Suppl A:23-33.

58. Goldová J, Malinová A, Indra A, Vítek L, Branny P, Jirásková A. *Clostridium difficile* in piglets in the Czech Republic. Folia Microbiol (Praha). 2012;57(2):159-61.

59. Goldstein EJ, Citron DM, Sears P, Babakhani F, Sambol SP, Gerding DN. Comparative susceptibilities to fidaxomicin (OPT-80) of isolates collected at baseline, recurrence, and failure from patients in two phase III trials of fidaxomicin against *Clostridium difficile* infection. Antimicrob Agents Chemother. 2011;55(11):5194-9.

60. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, Lumelsky G, Kuijper EJ. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. Clin Microbiol Infect. 2009;15(12):1080-6.

61. Goudarzi M, Goudarzi H, Alebouyeh M, Azimi Rad M, Shayegan Mehr FS, Zali MR, Aslani MM. Antimicrobial susceptibility of *Clostridium difficile* clinical isolates in Iran. Iran Red Crescent Med J. 2013;15(8):704-11.

62. Grandesso S, Arena F, Eseme FE, Panese S, Henrici De Angelis L, Spigaglia P, Barbanti F, Rossolini GM. *Clostridium difficile* ribotype 033 colitis in a patient following broad-spectrum antibiotic treatment for KPC producing *Klebsiella pneumoniae* infection, Italy. New Microbiol. 2016;39(3):235-236.

63. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, Golubchik T, Harding RM, Jeffery KJ, Jolley KA, Kirton R, Peto TE, Rees G, Stoesser N, Vaughan A, Walker AS, Young BC, Wilcox M, Dingle KE. Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol. 2010;48(3):770-8.

64. Gonçalves C, Decré D, Barbut F, Burghoffer B, Petit JC. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from *Clostridium difficile*. J Clin Microbiol. 2004;42(5):1933-9.

65. Hall IC, O'toole E. Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am J Dis Child. 1935;49(2):390-402.

66. Hammond GA, Johnson JL. The toxigenic element of *Clostridium difficile* strain VPI 10463. Microb Pathog. 1995;19(4):203-13.

67. Hardy K, Manzoor S, Marriott C, Parsons H, Waddington C, Gossain S, Szczepura A, Stallard N, Hawkey PM. Utilizing rapid multiple-locus variable-number tandem-repeat analysis typing to aid control of hospital-acquired *Clostridium difficile* Infection: a multicenter study. J Clin Microbiol. 2012;50(10):3244-8.

68. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet. 2013;45(1):109-13.

69. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB. Decreased diversity of the fecal Microbiome in recurrent *Clostridium difficile*-associated diarrhea. J Infect Dis. 2008;197(3):435-8.

70. Chumbler NM, Rutherford SA, Zhang Z, Farrow MA, Lisher JP, Farquhar E, Giedroc DP, Spiller BW, Melnyk RA, Lacy DB. Crystal structure of *Clostridium difficile* toxin A. Nat Microbiol. 2016;1:15002.

71. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. 2008;57(Pt 11):1377-82.

72. Janezic S, Zidaric V, Pardon B, Indra A, Kokotovic B, Blanco JL, Seyboldt C, Diaz CR, Poxton IR, Perreten V, Drigo I, Jiraskova A, Ocepek M, Weese JS, Songer JG, Wilcox MH, Rupnik M. International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. BMC Microbiol. 2014;14:173.

73. Janezic S, Marín M, Martín A, Rupnik M. A new type of toxin A-negative, toxin B-positive *Clostridium difficile* strain lacking a complete *tcdA* gene. J Clin Microbiol. 2015;53(2):692-5.

74. Janezic S, Potocnik M, Zidaric V, Rupnik M. Highly Divergent *Clostridium difficile* Strains Isolated from the Environment. PLoS One. 2016;11(11):e0167101.

75. Johnson AP, Wilcox MH. Fidaxomicin: a new option for the treatment of *Clostridium difficile* infection. J Antimicrob Chemother. 2012;67(12):2788-92.

76. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11:595.

77. Just I (a), Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature. 1995;375(6531):500-3.

78. Just I (b), Wilm M, Selzer J, Rex G, von Eichel-Streiber C, Mann M, Aktories K. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J Biol Chem. 1995;270(23):13932-6.

79. Kelly CP. Can we identify patients at high risk of recurrent *Clostridium difficile* infection? Clin Microbiol Infect. 2012;18 Suppl 6:21-7.

80. Knetsch CW, Terveer EM, Lauber C, Gorbalenya AE, Harmanus C, Kuijper EJ, Corver J, van Leeuwen HC. Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages. Infect Genet Evol. 2012;12(7):1577-85.

81. Knetsch CW, Lawley TD, Hensgens MP, Corver J, Wilcox MW, Kuijper EJ. Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections. Euro Surveill. 2013;18(4):20381.

82. Knetsch CW, Connor TR, Mutreja A, van Dorp SM, Sanders IM, Browne HP, Harris D, Lipman L, Keessen EC, Corver J, Kuijper EJ, Lawley TD. Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. Euro Surveill. 2014;19(45):20954.

83. Kok J, Wang Q, Thomas LC, Gilbert GL. Presumptive identification of *Clostridium difficile* strain 027/NAP1/BI on Cepheid Xpert: interpret with caution. J Clin Microbiol. 2011;49(10):3719-21.

84. Krůtová M (a), Matějková J, Zajac M, Hubáček P, Nyč O. Diagnostika *Clostridium difficile* infekcí – porovnávací studie dvou imunoenzymatických metod s konfirmací pomocí PCR a kultivace s následnou ribotypizací kmene. Epidemiol Mikrobiol Imunol. 2014;63(2):99-102.

85. Krutova M (b), Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha). 2014;59(6):523-6.

86. Krutova M (c), Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, Arvand M. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 2014;30:153-5.

87. Krůtová M, Nyč O. Diagnostika infekcí vyvolaných *Clostridium difficile* v České republice – dostupnost, možnosti, interpretace laboratorních nálezů. Epidemiol Mikrobiol Imunol. 2015;64(2):92-7.

88. Krutova M, Matejkova J, Tkadlec J, Nyc O. Antibiotic profiling of *Clostridium difficile* ribotype 176--A multidrug resistant relative to *C. difficile* ribotype 027. Anaerobe. 2015;36:88-90.

89. Krutova M (a), Matejkova J, Kuijper EJ, Drevinek P, Nyc O; Czech *Clostridium difficile* study group. *Clostridium difficile* PCR ribotypes 001 and 176 - the common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014. Euro Surveill. 2016;21(29).

90. Krutova M (b), Nyc O, Matejkova J, Allerberger F, Wilcox MH, Kuijper EJ. Molecular characterisation of Czech *Clostridium difficile* isolates collected in 2013-2015. Int J Med Microbiol. 2016;306(7):479-485.

91. Kuijper EJ, Coignard B, Tüll P; ESCMID Study Group for *Clostridium difficile*.; EU Member States.; European Centre for Disease Prevention and Control. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect. 2006;12 Suppl 6:2-18.

92. Kuijper EJ, Coignard B, Brazier JS, Suetens C, Drudy D, Wiuff C, Pituch H, Reichert P, Schneider F, Widmer AF, Olsen KE, Allerberger F, Notermans DW, Barbut F, Delmée M, Wilcox M, Pearson A, Patel BC, Brown DJ, Frei R, Akerlund T, Poxton IR, Tüll P. Update of *Clostridium difficile*-associated disease due to PCR ribotype 027 in Europe. Euro Surveill. 2007;12(6):E1-2.

93. Lachowicz D, Pituch H, Obuch-Woszczatyński P. Antimicrobial susceptibility patterns of *Clostridium difficile* strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012. Anaerobe. 2015;31:37-41.

94. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prévot 1938. Anaerobe. 2016;40:95-9.

95. Leibowitz J, Soma VL, Rosen L, Ginocchio CC, Rubin LG. Similar proportions of stool specimens from hospitalized children with and without diarrhea test positive for *Clostridium difficile*. Pediatr Infect Dis J. 2015;34(3):261-6.

96. Lynch T, Chong P, Zhang J, Hizon R, Du T, Graham MR, Beniac DR, Booth TF, Kibsey P, Miller M, Gravel D, Mulvey MR; Canadian Nosocomial Infection Surveillance Program (CNISP). Characterization of a stable, metronidazole-resistant *Clostridium difficile* clinical isolate. PLoS One. 2013;8(1):e53757.

97. Manzoor SE, Tanner HE, Marriott CL, Brazier JS, Hardy KJ, Platt S, Hawkey PM. Extended multilocus variable-number tandem-repeat analysis of *Clostridium difficile* correlates exactly with ribotyping and enables identification of hospital transmission. J Clin Microbiol. 2011;49(10):3523-30.

98. Marín M, Martín A, Alcalá L, Cercenado E, Iglesias C, Reigadas E, Bouza E. *Clostridium difficile* isolates with high linezolid MICs harbor the multiresistance gene cfr. Antimicrob Agents Chemother. 2015;59(1):586-9.

99. Marsh JW, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN, Muto CA, Harrison LH. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in Hospitals. J Clin Microbiol. 2006;44(7):2558-66.

100. Martin J, Wilcox M. New and emerging therapies for *Clostridium difficile* infection. Curr Opin Infect Dis. 2016;29(6):546-554.

101. Mattila E, Arkkila P, Mattila PS, Tarkka E, Tissari P, Anttila VJ. Extraintestinal *Clostridium difficile* infections. Clin Infect Dis. 2013;57(6):e148-53.

102. McDonald EG, Milligan J, Frenette C, Lee TC. Continuous Proton Pump Inhibitor Therapy and the Associated Risk of Recurrent *Clostridium difficile* Infection. JAMA Intern Med. 2015;175(5):784-91.

103. McMillen T, Kamboj M, Babady NE. Comparison of Multilocus Sequence Typing and the Xpert *C. difficile*/Epi Assay for Identification of *Clostridium difficile* 027/NAP1/BI. J Clin Microbiol. 2016;54(3):775-8.

104. Mentula S, Laakso S, Lyytikäinen O, Kirveskari J. Differentiating virulent 027 and non-027 *Clostridium difficile* strains by molecular methods. Expert Rev Mol Diagn. 2015;15(9):1225-9.

105. Monot M, Eckert C, Lemire A, Hamiot A, Dubois T, Tessier C, Dumoulard B, Hamel B, Petit A, Lalande V, Ma L, Bouchier C, Barbut F, Dupuy B. *Clostridium difficile*: New Insights into the Evolution of the Pathogenicity Locus. Sci Rep. 2015;5:15023.

106. Moono P, Foster NF, Hampson DJ, Knight DR, Bloomfield LE, Riley TV. *Clostridium difficile* Infection in Production Animals and Avian Species: A Review. Foodborne Pathog Dis. 2016;13(12):647-655.

107. Moono P, Lim SC, Riley TV. High prevalence of toxigenic *Clostridium difficile* in public space lawns in Western Australia. Sci Rep. 2017;7:41196.

108. Moura I, Monot M, Tani C, Spigaglia P, Barbanti F, Norais N, Dupuy B, Bouza E, Mastrantonio P. Multidisciplinary analysis of a nontoxigenic *Clostridium difficile* strain with stable resistance to metronidazole. Antimicrob Agents Chemother. 2014;58(8):4957-60.

109. Norman KN, Scott HM, Harvey RB, Norby B, Hume ME. Comparison of antimicrobial susceptibility among *Clostridium difficile* isolated from an integrated human and swine population in Texas. Foodborne Pathog Dis. 2014;11(4):257-64.

110. Nyč O, Pituch H, Matějková J, Obuch-Woszczatynski P, Kuijper EJ. *Clostridium difficile* PCR ribotype 176 in the Czech Republic and Poland. Lancet. 2011;377(9775):1407.

111. Nyc O (a), Krutova M, Kriz J, Matejkova J, Bebrova E, Hysperska V, Kuijper EJ. *Clostridium difficile* ribotype 078 cultured from post-surgical non-healing wound in a patient carrying ribotype 014 in the intestinal tract. Folia Microbiol (Praha). 2015;60(6):541-4.

112. Nyc O (b), Krutova M, Liskova A, Matejkova J, Drabek J, Kuijper EJ. The emergence of *Clostridium difficile* PCR-ribotype 001 in Slovakia. Eur J Clin Microbiol Infect Dis. 2015;34(8):1701-8.

113. Nyč O (a), Krůtová M, Matějková J, Dřevínek P. *Clostridium difficile*: genotypizace a fenotypizace klinicky významných izolátů - mapování epidemiologické situace v ČR. Závěrečná zpráva o řešení grantu Interní grantové agentury MZ ČR. Číslo grantové zprávy: NT14209. 2016.

114. Nyc O (b), Tejkalova R, Kriz Z, Ruzicka F, Kubicek L, Matejkova J, Kuijper E, Krutova M. Two Clusters of Fluoroquinolone and Clindamycin-Resistant *Clostridium difficile* PCR

Ribotype 001 Strain Recognized by Capillary Electrophoresis Ribotyping and Multilocus Variable Tandem Repeat Analysis. Microb Drug Resist. 2016 Nov 18.

115. Obuch-Woszczatyński P, Lachowicz D, Schneider A, Mól A, Pawłowska J, Ożdżeńska, Milke E, Pruszczyk P, Wultańska D, Młynarczyk G, Harmanus C, Kuijper EJ, van Belkum A, Pituch H. Occurrence of *Clostridium difficile* PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010. Anaerobe. 2014;28:13-7.

116. Oman Evans Ii M, Starley B, Galagan JC, Yabes JM, Evans S, Salama JJ. Tea and Recurrent *Clostridium difficile* Infection. Gastroenterol Res Pract. 2016;2016:4514687.

117. Orden C, Blanco JL, Álvarez-Pérez S, Garcia-Sancho M, Rodriguez-Franco F, Sainz A, Villaescusa A, Harmanus C, Kuijper E, Garcia ME. Isolation of *Clostridium difficile* from dogs with digestive disorders, including stable metronidazole-resistant strains. Anaerobe. 2016;43:78-81.

118. Paredes-Sabja D, Sarker MR. Adherence of *Clostridium difficile* spores to Caco-2 cells in culture. J Med Microbiol. 2012;61(Pt 9):1208-18.

119. Paredes-Sabja D, Shen A, Sorg JA. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol. 2014;22(7):406-16.

120. Paitan Y, Miller Roll T, Adler A. Comparative performance study of six commercial molecular assays for rapid detection of toxigenic *Clostridium difficile*. Clin Microbiol Infect. 2017 Feb 18. pii: S1198-743X(17)30104-0.

121. Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect Immun. 1997;65(4):1402-7.

122. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA*/*cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect. 2008;14(11):1057-64. Erratum in: Clin Microbiol Infect. 2009;15(3):296.

123. Pituch H, Obuch-Woszczatyński P, Lachowicz D, Wultańska D, Karpiński P, Młynarczyk G, van Dorp SM, Kuijper EJ; Polish *Clostridium difficile* Study Group. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro Surveill. 2015;20(38).

124. Polák P, Freibergerová M, Husa P, Juránková J, Svačinka R, Mikešová L, Kocourková H, Mihalčin M, Skalická R, Stebel R, Porubčanová M. Fekální bakterioterapie v léčbě rekurentní kolitidy způsobené *Clostridium difficile* na klinice infekčních chorob fakultní nemocnice Brno v letech 2010–2014 – prospektivní studie. Epidemiol Mikrobiol Imunol. 2015;64(4):232-5.

125. Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. *Clostridium difficile* ribotype 176 - A predictor for high mortality and risk of nosocomial spread? Anaerobe. 2016;40:35-40.

126. Pruitt RN, Lacy DB. Toward a structural understanding of *Clostridium difficile* toxins A and B. Front Cell Infect Microbiol. 2012;2:28.

127. Rao K, Micic D, Natarajan M, Winters S, Kiel MJ, Walk ST, Santhosh K, Mogle JA, Galecki AT, LeBar W, Higgins PD, Young VB, Aronoff DM. *Clostridium difficile* ribotype 027: relationship to age, detectability of toxins A or B in stool with rapid testing, severe infection, and mortality. Clin Infect Dis. 2015;61(2):233-41.

128. Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents. Clin Infect Dis. 2007;45(8):992-8.

129. Riley TV, Brazier JS, Hassan H, Williams K, Phillips KD. Comparison of alcohol shock enrichment and selective enrichment for the isolation of *Clostridium difficile*. Epidemiol Infect. 1987;99(2):355-9.

130. Rineh A, Kelso MJ, Vatansever F, Tegos GP, Hamblin MR. *Clostridium difficile* infection: molecular pathogenesis and novel therapeutics. *Expert review of anti-infective therapy*. 2014;12(1):131-150.

131. Rodriguez C, Taminiau B, Avesani V, Van Broeck J, Delmée M, Daube G. Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans in Belgium. Food Microbiol. 2014;42:166-71.

132. Rodriguez C, Avesani V, Taminiau B, Van Broeck J, Brévers B, Delmée M, Daube G. Investigation of *Clostridium difficile* interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing. Vet J. 2015;206(3):349-55.

133. Rodriguez-Palacios A, Ilic S, LeJeune JT. *Clostridium difficile* with Moxifloxacin/Clindamycin Resistance in Vegetables in Ohio, USA, and Prevalence Meta-Analysis. J Pathog. 2014;2014:158601.

134. Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol. 2009;7(7):526-36.

135. Rupnik M, Tambic Andrasevic A, Trajkovska Dokic E, Matas I, Jovanovic M, Pasic S, Kocuvan A, Janezic S. Distribution of *Clostridium difficile* PCR ribotypes and high proportion of 027 and 176 in some hospitals in four South Eastern European countries. Anaerobe. 2016;42:142-144.

136. Shen A, Lupardus PJ, Gersch MM, Puri AW, Albrow VE, Garcia KC, Bogyo M. Defining an allosteric circuit in the cysteine protease domain of *Clostridium difficile* toxins. Nat Struct Mol Biol. 2011;18(3):364-71.

137. Shen A. *Clostridium difficile* toxins: mediators of inflammation. J Innate Immun. 2012;4(2):149-58.

138. Schneeberg A, Rupnik M, Neubauer H, Seyboldt C. Prevalence and distribution of *Clostridium difficile* PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany. Anaerobe. 2012;18(5):484-8.

139. Schneeberg A (a), Neubauer H, Schmoock G, Grossmann E, Seyboldt C. *Presence of Clostridium difficile* PCR ribotype clusters related to 033, 078 and 045 in diarrhoeic calves in Germany. J Med Microbiol. 2013;62(Pt 8):1190-8.

140. Schneeberg A (b), Neubauer H, Schmoock G, Baier S, Harlizius J, Nienhoff H, Brase K, Zimmermann S, Seyboldt C. *Clostridium difficile* genotypes in piglet populations in Germany. J Clin Microbiol. 2013;51(11):3796-803.

141. Slimings C, Riley TV. Antibiotics and hospital-acquired *Clostridium difficile* infection: update of systematic review and meta-analysis. J Antimicrob Chemother. 2014;69(4):881-91.

142. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40(9):3470-5.

143. Spigaglia P, Barbanti F, Mastrantonio P; European Study Group on *Clostridium difficile* (ESGCD). Multidrug resistance in European *Clostridium difficile* clinical isolates. J Antimicrob Chemother. 2011;66(10):2227-34.

144. Spigaglia P, Drigo I, Barbanti F, Mastrantonio P, Bano L, Bacchin C, Puiatti C, Tonon E, Berto G, Agnoletti F. Antibiotic resistance patterns and PCR-ribotyping of *Clostridium difficile* strains isolated from swine and dogs in Italy. Anaerobe. 2015;31:42-6.

145. Spigaglia P. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection. Ther Adv Infect Dis. 2016;3(1):23-42.

146. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 1999;37(2):461-3.

147. Sundriyal A, Roberts AK, Shone CC, Acharya KR. Structural basis for substrate recognition in the enzymatic component of ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*. J Biol Chem. 2009;284(42):28713-9.

148. Sundriyal A, Roberts AK, Ling R, McGlashan J, Shone CC, Acharya KR. Expression, purification and cell cytotoxicity of actin-modifying binary toxin from *Clostridium difficile*. Protein Expr Purif. 2010;74(1):42-8.

149. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 1999;37(2):461-3.

150. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-9.

151. Tóth J, Urbán E, Osztie H, Benczik M, Indra A, Nagy E, Allerberger F. Distribution of PCR ribotypes among recent *Clostridium difficile* isolates collected in two districts of Hungary using capillary gel electrophoresis and review of changes in the circulating ribotypes over time. J Med Microbiol. 2016;65(10):1158-1163.

152. Usui M, Nanbu Y, Oka K, Takahashi M, Inamatsu T, Asai T, Kamiya S, Tamura Y. Genetic relatedness between Japanese and European isolates of *Clostridium difficile* originating from piglets and their risk associated with human health. Front Microbiol. 2014;5:513.

153. Yamoudy M, Mirlohi M, Isfahani BN, Jalali M, Esfandiari Z, Hosseini NS. Isolation of toxigenic *Clostridium difficile* from ready-to-eat salads by multiplex polymerase chain reaction in Isfahan, Iran. Adv Biomed Res. 2015;4:87.

154. Yutin N, Galperin MY. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ Microbiol. 2013;15(10):2631-41.

155. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol. 2012;61(Pt 1):49-56.

156. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol. 2007;45(3):1024-8.

157. van Dorp SM, Smajlović E, Knetsch CW, Notermans DW, de Greeff SC, Kuijper EJ. Clinical and Microbiological Characteristics of *Clostridium difficile* Infection Among Hospitalized Children in the Netherlands. Clin Infect Dis. 2017;64(2):192-198.

158. van Nood E, Dijkgraaf MG, Keller JJ. Duodenal infusion of feces for recurrent *Clostridium difficile*. N Engl J Med. 2013;368(22):2145.

159. Vojtilová L, Freibergerová M, Juránková J, Bortlíček Z, Husa P. Epidemiological factors influencing the development of relapsing and severe *Clostridium difficile* infection. Epidemiol Mikrobiol Imunol. 2014;63(1):27-35.

160. Vonberg RP, Kuijper EJ, Wilcox MH, Barbut F, Tüll P, Gastmeier P; European *C. difficile*-Infection Control Group; European Centre for Disease Prevention and Control (ECDC), van den Broek PJ, Colville A, Coignard B, Daha T, Debast S, Duerden BI, van den Hof S, van der Kooi T, Maarleveld HJ, Nagy E, Notermans DW, O'Driscoll J, Patel B, Stone S, Wiuff C. Infection control measures to limit the spread of *Clostridium difficile*. Clin Microbiol Infect. 2008;14 Suppl 5:2-20.

161. von Müller L, Mock M, Halfmann A, Stahlmann J, Simon A, Herrmann M. Epidemiology of *Clostridium difficile* in Germany based on a single center long-term surveillance and German-wide genotyping of recent isolates provided to the advisory laboratory for diagnostic reasons. Int J Med Microbiol. 2015;305(7):807-13.

162. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. Toxin production by an emerging strain of *Clostridium difficile* associated

with outbreaks of severe disease in North America and Europe. Lancet. 2005 Sep 24-30;366(9491):1079-84.

163. Wolff D, Brüning T, Gerritzen A. Rapid detection of the *Clostridium difficile* ribotype 027 *tcdC* gene frame shift mutation at position 117 by real-time PCR and melt curve analysis. Eur J Clin Microbiol Infect Dis. 2009;28(8):959-62.

164. Woodworth MH, Neish EM, Miller NS, Dhere T, Burd EM, Carpentieri C, Sitchenko KL, Kraft CS. Laboratory Testing of Donors and Stool Samples for Fecal Microbiota Transplantation for Recurrent *Clostridium difficile* Infection. J Clin Microbiol. 2017 Apr;55(4):1002-1010.

165. Wu YC, Lee JJ, Tsai BY, Liu YF, Chen CM, Tien N, Tsai PJ, Chen TH. Potentially hypervirulent *Clostridium difficile* PCR ribotype 078 lineage isolates in pigs and possible implications for humans in Taiwan. Int J Med Microbiol. 2016;306(2):115-22.

166. Zacharioudakis IM, Zervou FN, Pliakos EE, Ziakas PD, Mylonakis E. Colonization with toxinogenic *C. difficile* upon hospital admission, and risk of infection: a systematic review and meta-analysis. Am J Gastroenterol. 2015;110(3):381-90; quiz 391.

167. Ziakas PD, Zacharioudakis IM, Zervou FN, Grigoras C, Pliakos EE, Mylonakis E. Asymptomatic carriers of toxigenic *C. difficile* in long-term care facilities: a meta-analysis of prevalence and risk factors. PLoS One. 2015;10(2):e0117195.

9. Publikace které jsou podkladem dizertační práce

Příloha 1

Krutova M, Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha). 2014;59(6):523-6.

Příloha 2

Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, Arvand M. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 2014;30:153-5.

Příloha 3

Krutova M, Matejkova J, Tkadlec J, Nyc O. Antibiotic profiling of *Clostridium difficile* ribotype 176--A multidrug resistant relative to *C. difficile* ribotype 027. Anaerobe. 2015;36:88-90.

Příloha 4

Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc O; Czech *Clostridium difficile* study group. *Clostridium difficile* PCR ribotypes 001 and 176 - the common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014. Euro Surveill. 2016;21(29).

Příloha 5

Krutova M, Nyc O, Matejkova J, Allerberger F, Wilcox MH, Kuijper EJ. Molecular characterisation of Czech *Clostridium difficile* isolates collected in 2013-2015. Int J Med Microbiol. 2016;306(7):479-485.

Příloha 6

Krutova M, Nyc O, Matejkova J, Kuijper E, Jalava J, Mentula S. The recognition and characterisation of *Finnish Clostridium difficile* isolates resembling PCR-ribotype 027. Přijato k publikaci v Journal of Microbiology, Immunology and Infection dne 14.3. 2017.

Příloha 7

Nyc O, Krutova M, Kriz J, Matejkova J, Bebrova E, Hysperska V, Kuijper EJ. *Clostridium difficile* ribotype 078 cultured from post-surgical non-healing wound in a patient carrying ribotype 014 in the intestinal tract. Folia Microbiol (Praha). 2015;60(6):541-4.

Příloha 8

Nyc O, Krutova M, Liskova A, Matejkova J, Drabek J, Kuijper EJ. The emergence of *Clostridium difficile* PCR-ribotype 001 in Slovakia. Eur J Clin Microbiol Infect Dis. 2015;34(8):1701-8.

Příloha 9

Nyc O, Tejkalova R, Kriz Z, Ruzicka F, Kubicek L, Matejkova J, Kuijper E, Krutova M. Two Clusters of Fluoroquinolone and Clindamycin-Resistant *Clostridium difficile* PCR Ribotype 001 Strain Recognized by Capillary Electrophoresis Ribotyping and Multilocus Variable Tandem Repeat Analysis. Microb Drug Resist. 2016 Nov 18.

Příloha 10

Drabek J, Nyc O, Krutova M, Stovicek J, Matejkova J, Keil R. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study. Ann Clin Microbiol Antimicrob. 2015;14:55.

Příloha 11

Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. *Clostridium difficile* ribotype 176 – A predictor for high mortality and risk of nosocomial spread? Anaerobe. 2016;40:35-40.

Příloha 12

Azimirad M; Krutova M; Nyc O; Hasani Z; Afrisham L; Alebouyeh M; Zali MR. Molecular typing of *Clostridium difficile* isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital. V recenzním řízení Anaerobe, po první revizi.

Příloha 1

Krutova M, Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha). 2014;59(6):523-6.

V článku je popsána shoda tří úseků v genomu *C. difficile* ribotypu 176 a ribotypu 027. Jedná se o gen pro produkci toxinu B (*tcdB*), gen pro produkci binárního toxinu (*cdtB*) a jednobodovou deleci v genu *tcdC* (pozice 117). Těchto tří cílových míst v genomu *C. difficile* využívá komerční uzavřený detekční systém Xpert, (Cepheid, USA), který je v ČR nejpoužívanějším přístrojem pro detekci DNA toxigenních kmenů *C. difficile*. Na základě zjištěné shody cílových úseků v genomu *C. difficile* není tento systém schopen od sebe rozlišit C. *difficile* ribotyp 176 a 027. Vzhledem k tomu může docházet k nesprávné interpretaci výsledků.

Folia Microbiol (2014) 59:523–526
DOI 10.1007/s12223-014-0323-5

C. difficile ribotype 027 or 176?

Marcela Krutova & Jana Matejkova & Otakar Nyc

Received: 28 October 2013 / Accepted: 13 May 2014 / Published online: 27 June 2014
Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i. 2014

Abstract *Clostridium difficile* is a major nosocomial pathogen of present times. The analysis of 624 *C. difficile* strains from 11 hospitals in the Czech Republic in 2013 revealed that 40 % of isolates belonged to ribotype 176. These results suggest that the incidence of CDI (*C. difficile* infection) in the Czech Republic has increased probably in connection with *C. difficile* ribotype 176. The molecular systems Xpert *C. difficile* Epi assay (Cepheid Inc., Sunnyvale, CA) diagnoses toxigenic strains and supports *C. difficile* ribotype 027 determination based on three specific target places in the toxigenic *C. difficile* genome. Twenty-nine strains cultivated from stool specimens were evaluated by the Xpert systems as presumed *C. difficile* PCR ribotype 027 were confirmed as a *C. difficile* ribotype 176 based on ribotyping. A further 120 *C. difficile* strains of ribotype 176 were examined for the presence of genes *tcdB*, *cdtB* and deletion in position 117 in the *tcdC* gene. Our experience shows that due to the correspondence of the target places, *C. difficile* ribotype 176 may be interpreted as ribotype 027 by Xpert *C. difficile* Epi assay (Cepheid Inc., Sunnyvale, CA). Further molecular analysis as ribotyping based on capillary electrophoresis is needed to differentiate between *C. difficile* ribotypes 027 and 176 for appropriate epidemiological situation control on local and national levels.

M. Krutova (✉) · J. Matejkova · O. Nyc
Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, V Uvalu 84, 150 06 Prague 5, Czech Republic
e-mail: marcela.krutova@seznam.cz

M. Krutova
DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, V Uvalu 84, 150 06 Prague 5, Czech Republic

Introduction

Clostridium difficile is the most significant causal agent of hospital-acquired diarrhoea (Bauer et al. 2011; He et al. 2013). Early identification of toxigenic strains is necessary for taking measures against the spread of infection and the correct timing of adequate therapy (Rupnik et al. 2009). Methods of nucleic acid amplification based on real-time PCR show high sensitivity and specificity and decrease the turnaround time (Belanger et al. 2003; Peterson et al. 2007; Barbut et al. 2009; Stamper et al. 2009; Terhes et al. 2009; Goldenberg et al. 2010; de Jong et al. 2012). Fifteen Xpert (Cepheid, USA) devices are present in the Czech Republic (firm data), and these molecular systems are the most used for the direct detection of the toxigenic *C. difficile* from stool specimens. Continuous surveillance of CDI (*C. difficile* Infection) has not been systematically organized in the Czech Republic yet. Based on ECDIS-net (The European *C. difficile* infection surveillance network) activity to enhance laboratory capacity for CDI detection and surveillance in Europe, our laboratory currently provides ribotyping of *C. difficile* strains from the cooperating hospitals and collects comprehensive data of its distribution in the Czech Republic. Ribotyping of 624 *C. difficile* strains from 11 participating microbiological departments of hospitals in the Czech Republic was performed in 2013. Forty percent of the *C. difficile* isolates belonged to ribotype 176 (Krutova et al. 2013).

Material and methods

Twenty-nine strains cultivated from stool specimens were evaluated by the Xpert system as presumed *C. difficile* PCR ribotype 027 were sent for confirmation from five different hospitals in the Czech Republic to our central laboratory during 2013. DNA extraction from verified colonies of

C. difficile strains by MALDI- TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry (Bruker Daltonics, USA) (Coltella et al. 2013) was performed using Chelex 100 resin (Valiente et al. 2012). PCR ribotyping based on capillary electrophoresis was performed in accordance with the Standard Operation Protocol ECDIS-net (available at restricted website: http://www.ecdisnet.eu/). PCR fragments were analysed in the ABI Prism 3110 genetic analyser (Applied Biosystems). Separation was performed in the POP7 polymer (Applied Biosystems). A size standard 1200 LIZ (Applied Biosystems) was used as an internal marker for each sample. Data from fragment analysis was analysed using Gene Mapper software 4.1 (Applied Biosystems). The acquired electrophoreograms were compared with the Austrian Agency for Health and Food Webribo database available on the restricted website http://www.webribo.ages.at/ (Indra et al. 2008). The multiplex PCR reaction for detecting genes for toxin A, B and binary toxin production was performed with primers and under conditions described by Persson et al. (2008). The *TcdC* gene was amplified with primers C1 and C2 published by Spigaglia and Mastrantonio (2002) using the following PCR protocol: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s and 72 °C for 10 min. The PCR products were cleaned with Exo/Sap (Fermentas) and sequenced using Sanger's sequencing with the Big Dye terminator Kit v3.1 (Applied Biosystems). The acquired sequences were compared with the NCBI reference sequence NC_009089.1 and with the sequence acquired from a *C. difficile* PCR ribotype 027 strain.

Results

All 29 strains were evaluated by the Xpert systems as presumed *C. difficile* PCR ribotype 027 had one peak less in the electrophoreogram (size 576 bp). This is the main difference between *C. difficile* ribotypes 027 and 176 electrophoreograms (Fig. 1). After uploading the data to the Webribo database, we got the result "existing ribotype 176". In all 29 samples, we obtained positive results for the presence of genes for the production of toxins (A, B and binary) by using the multiplex PCR reaction. All 29 strains had a deletion of one base in position 117 in the *tcdC* gene using Sanger's sequencing.

An additional 120 strains of *C. difficile* that belonged to PCR ribotype 176 taken from 11 microbiological departments in the Czech Republic were analysed for confirmation of our results of the above 29 samples. All 120 samples proved positive for the presence of genes *tcdB*, *cdtB* and the deletion in position 117 in the *tcdC* gene.

Discussion

The principle of molecular detection in the Xpert *C. difficile*/Epi test is based on the determination of three specific target areas present in the toxigenic *C. difficile* genome: the *tcdB* gene for toxin B production, deletion of one base in position 117 in the regulatory *tcdC* gene and the *cdtB* gene for binary toxin production (Babady et al. 2010; Zidaric et al. 2011; Viala et al. 2012; Kok et al. 2011; Pancholi et al. 2012; Shin et al. 2012).

The results show that *C. difficile* PCR ribotype 176 has all three specific target sites same and may be incorrectly interpreted by Xpert *C. difficile*/Epi as presumed 027/NAP1/B1. Our findings are limited by a small amount of data, because the molecular detection of toxigenic strains of *C. difficile* in the Czech Republic is used as the second diagnostic step for confirmation of GDH (glutamate dehydrogenase)-positive and toxin-negative stool samples or in emergency situation.

To confirm our observations, we analysed an additional 120 strains of *C. difficile* belonging to *C. difficile* ribotype 176 from 11 different hospitals in the Czech Republic. All 120 samples gave positive results for the presence of the *tcdB* and *cdtB* genes in the multiplex PCR reaction and also deletion in position 117 in the *tcdC* gene using Sanger's sequencing.

C. difficile PCR ribotype 176 is a common ribotype in the Czech Republic according to our results from ribotyping in 2013, where 40 % of 624 strains *C. difficile* belonged to the PCR ribotype 176 (Krutova et al. 2013).

Beran et al. (2014), who studied the sensitivity profile of toxigenic strains *C. difficile* in the Czech Republic to clindamycin, metronidazol, vancomycin and amoxicillin with clavulanic acid, worked with 62 isolates of *C. difficile* in the Pardubice region (Eastern Bohemia, Czech Republic). Thirty-three strains were evaluated as a hypertoxigenic according to the results of the Xpert system analysis. The higher percentage of hypertoxigenic strains corresponded to our recently published results of molecular typisation of *C. difficile* strains in the Czech Republic (Krutova et al. 2013). We believe that the collection of hypertoxigenic *C. difficile* strains of Pardubice was with high probability composing of *C. difficile* ribotype 176.

The presence of ribotype 176 in the Czech Republic and Poland was published by Nyc et al. (2011). Eleven Polish and ten Czech *C. difficile* strains belonging to ribotype 176 were compared with 59 European *C. difficile* strains belonging to ribotype 027 by using MLVA (multi-locus variable tandem repeats analysis). A sum of 14 tandem repeat differences in three of seven loci was found.

On the 23rd ECCMID 2013, in Berlin, Pituch et al. presented the first results of a Polish surveillance programme of *C. difficile* infections (abstract P2495). This data showed the

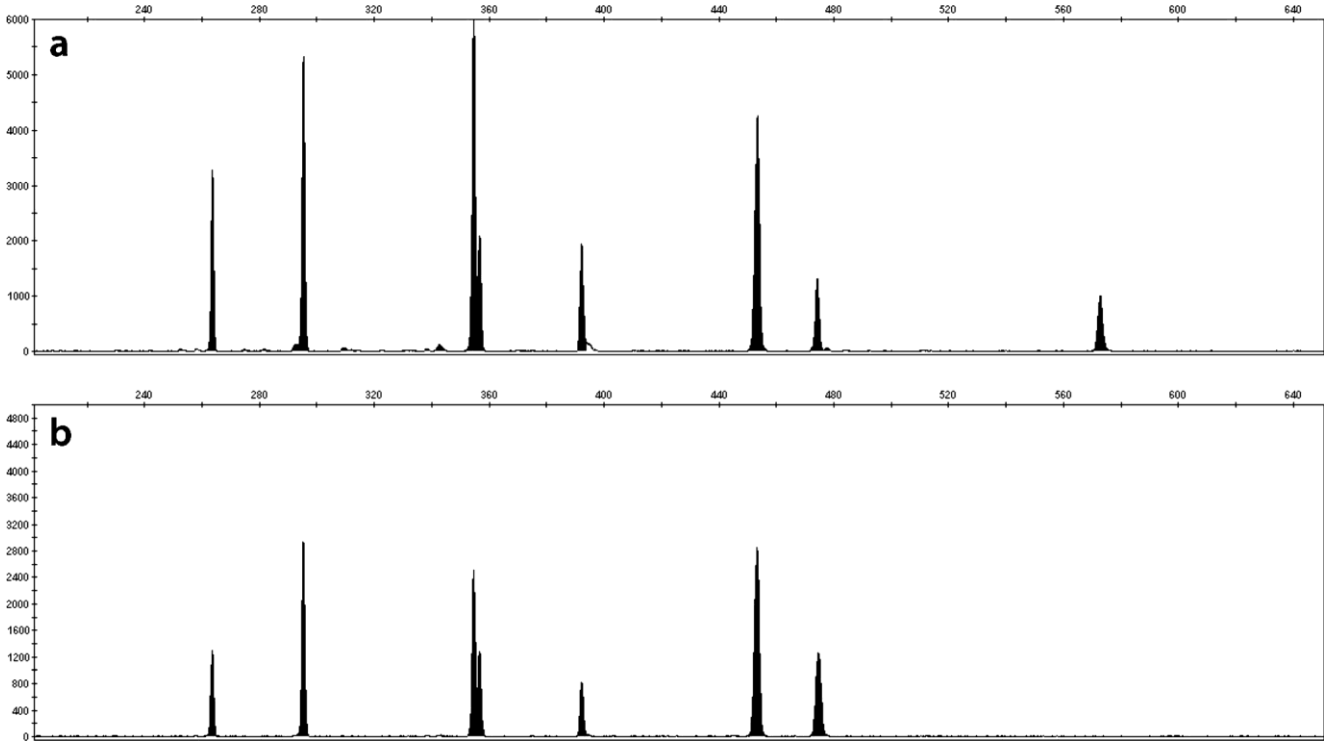


Fig. 1 PCR ribotyping based on capillary electrophoresis. Electrophoreograms of a *C. difficile* PCR ribotype 027 and b *C. difficile* PCR ribotype 176

high prevalence of PCR ribotype 027 and the coexistence of ribotype 176 in Poland.

Emergency and epidemiological connections of PCR ribotype 176 in a Czech faculty hospital were presented by Krutova et al. on the 23rd ECCMID 2013, in Berlin (abstract P2502). Twenty-six of the 50 isolates belonged to the *C. difficile* ribotype 176, and MLVA of these 26 strains revealed 3 clonal complexes and 3 genetically related clusters between them.

Valiente et al. (2012) published in their study the emergence of PCR ribotypes 176, 198 and 244 from the hypervirulent *C. difficile* 027 lineage due to a slight variation in banding pattern. The authors suggest that *C. difficile* ribotype 176 has evolved recently from the 027 lineage and may be as problematic as the *C. difficile* ribotype 027 strains. The high percentage of *C. difficile* 176 in our collection in comparison to the representation of other ribotypes confirms the seriousness of the risk of this ribotype.

C. difficile ribotype 176 was mentioned in the work of Indra et al. (2010) which described the mechanism of variant development in *C. difficile* 16S-23S rRNA intergenic spacer region.

In the recent work of He et al. (2013), the global population structure of *C. difficile* 027 based on whole-genome sequencing was studied, and various strains of *C. difficile* ribotype 176 were included in the study as ribotype 027 (He et al. 2013).

Close genetical relatedness between *C. difficile* ribotypes 027 and 176 based on the presence of the same gene insertion was published by Knetsch et al. (2011).

The question is: how important is it to recognize the difference between *C. difficile* ribotypes 027 and 176? We are able to recognize *C. difficile* ribotypes 027 and 176 by ribotyping based on capillary electrophoresis according to different electrophoreograms, and we believe that the ability to differentiate between 176 and 027 by the ribotyping gives us an important tool for epidemiological control of the spread of CDI independently of their genetic relatedness. We suggest that this is mainly true for the regions where the presence of *C. difficile* PCR ribotype 176 or the presence of simultaneous ribotypes 027 and 176 is known. Interestingly, the presence of *C. difficile* ribotype 176 was reported in isolation in the Czech Republic and Poland, while the presence of *C. difficile* ribotype 027 was reported worldwide (Warny et al. 2005; Smith 2005; Kuijper et al. 2006; Bauer et al. 2011; He et al. 2013). For the understanding of the role and importance of ribotype 176 and comparison with ribotype 027, further investigation is needed in the clinical manifestation of infection, distribution and spread.

Our experience confirms and we agree with Kok et al. (2011) that the identification of *C. difficile* PCR ribotype 027 by the Xpert system should be confirmed using molecular methods, mainly for epidemiology purposes. Even if the Xpert system is not able to differentiate between *C. difficile* PCR ribotypes 176 and 027, according to our results, it is still a very helpful tool for CDI epidemiology situation control.

Acknowledgments We would like to thank the European *C. difficile* infection surveillance network for their professional support. This work was supported by Ministry of Health, Czech Republic - conceptual development of research organization, University Hospital in Motol, Prague, Czech Republic 00064203 and MH CZ IGA NT/14209 – 3. We would like to thank Jamie Byrne for the English revision of this text.

References

Babady NE, Stiles J, Ruggiero P, Khosa P, Huang D, Shuptar S, Kamboj M, Kiehn TE (2010) Evaluation of the Cepheid Xpert *Clostridium difficile* Epi assay for diagnosis of *Clostridium difficile* infection and typing of the NAP1 strain at a cancer hospital. J Clin Microbiol. doi:10.1128/JCM.01648-10

Barbut F, Braun M, Burghoffer B, Lalande V, Eckert C (2009) Rapid detection of toxigenic strains of *Clostridium difficile* in diarrheal stools by real-time PCR. J Clin Microbiol. doi:10.1128/JCM.00309-09

Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ, ECDIS Study Group (2011) *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. doi:10.1016/S0140-6736(10)61266-4

Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG (2003) Rapid detection of *Clostridium difficile* in feces by real-time PCR. J Clin Microbiol 41(2):730–734

Beran V, Chmelar D, Vobejdova J, Konigova A, Nemec J, Tvrdik J (2014) Sensitivity to antibiotics of *Clostridium difficile* toxigenic nosocomial strains. Folia Microbiol (Praha). doi:10.1007/s12223-013-0283-1

Coltella L, Mancinelli L, Onori M, Lucignano B, Menichella D, Sorge R, Raponi M, Mancini R, Russo C (2013) Advancement in the routine identification of anaerobic bacteria by MALDI-TOF mass spectrometry. Eur J Clin Microbiol Infect Dis. doi:10.1007/s10096-013-1865-1

de Jong E, de Jong AS, Bartels CJ, van der Rijt-van den Biggelaar C, Melchers WJ, Sturm PD (2012) Clinical and laboratory evaluation of a real-time PCR for *Clostridium difficile* toxin A and B genes. Eur J Clin Microbiol Infect. doi:10.1007/s10096-012-1558-1

Goldenberg SD, Dieringer T, French GL (2010) Detection of toxigenic *Clostridium difficile* in diarrheal stools by rapid real-time polymerase chain reaction. Diagn Microbiol Infect. doi:10.1016/j.diagmicrobio.2010.02.019

He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD (2013) Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet. doi:10.1038/ng.2478

Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ (2008) Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. doi:10.1099/jmm.0.47714-0

Indra A, Blaschitz M, Kernbichler S, Reischl U, Wewalka G, Allerberger F (2010) Mechanisms behind variation in the *Clostridium difficile* 16S-23S rRNA intergenic spacer region. J Med Microbiol. doi:10.1099/jmm.0.020792-0

Knetsch CW, Hensgens MP, Harmanus C, van der Bijl MW, Savelkoul PH, Kuijper EJ, Corver J, van Leeuwen HC (2011) Genetic markers for *Clostridium difficile* lineages linked to hypervirulence. Microbiology. doi:10.1099/mic.0.051953-0

Kok J, Wang Q, Thomas LC, Gilbert GL (2011) Presumptive identification of *Clostridium difficile* strain 027/NAP1/BI on Cepheid Xpert: interpret with caution. J Clin Microbiol. doi:10.1128/JCM.00752-11

Krutova M, Matejkova J, Nyc O (2013) First results of molecular typing of *Clostridium difficile* in the Czech Republic. Zprávy CEM (SZÚ Praha) 22(12):399–401

Kuijper EJ, Coignard B, Tüll P, ESCMID Study Group for Clostridium difficile; EU Member States; European Centre for Disease Prevention and Control (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect 12(Suppl 6):2–18

Nyc O, Pituch H, Matejkova J, Obuch-Woszczatynski P, Kuijper EJ (2011) *Clostridium difficile* PCR ribotype 176 in the Czech Republic and Poland. Lancet. doi:10.1016/S0140-6736(11)60575-8

Pancholi P, Kelly C, Raczkowski M, Balada-Llasat JM (2012) Detection of toxigenic *Clostridium difficile*: comparison of the cell culture neutralization, Xpert *C. difficile*, Xpert *C. difficile*/Epi, and Illumigene *C. difficile* assays. J Clin Microbiol. doi:10.1128/JCM.06597-11

Persson S, Torpdahl M, Olsen KE (2008) New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect. doi:10.1111/j.1469-0691.2008.02092.x

Peterson LR, Manson RU, Paule SM, Hacek DM, Robicsek A, Thomson RB Jr, Kaul KL (2007) Detection of toxigenic *Clostridium difficile* in stool samples by real-time polymerase chain reaction for the diagnosis of *C. difficile*-associated diarrhea. Clin Infect Dis 45(9):1152–1160

Rupnik M, Wilcox MH, Gerding DN (2009) *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol. doi:10.1038/nrmicro2164

Shin S, Kim M, Kim M, Lim H, Kim H, Lee K, Chong Y (2012) Evaluation of the Xpert *Clostridium difficile* assay for the diagnosis of *Clostridium difficile* infection. Ann Lab Med. doi:10.3343/alm.2012.32.5.355

Smith A (2005) Outbreak of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. Euro Surveill 10(6):E050630.2

Spigaglia P, Mastrantonio P (2002) Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (*TcdC*) among *Clostridium difficile* clinical isolates. J Clin Microbiol 40(9):3470–3475

Stamper PD, Babiker W, Alcabasa R, Aird D, Wehrlin J, Ikpeama I, Gluck L, Carroll KC (2009) Evaluation of a new commercial TaqMan PCR assay for direct detection of the *Clostridium difficile* toxin B gene in clinical stool specimens. J Clin Microbiol. doi:10.1128/JCM.01490-09

Terhes G, Urban E, Soki J, Nacsa E, Nagy E (2009) Comparison of a rapid molecular method, the BD GeneOhm *Cdiff* assay, to the most frequently used laboratory tests for detection of toxin-producing *Clostridium difficile* in diarrheal feces. J Clin Microbiol. doi:10.1128/JCM.01133-09

Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW (2012) Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol. doi:10.1099/jmm.0.036194-0

Viala C, Le Monnier A, Maataoui N, Rousseau C, Collignon A, Poilane I (2012) Comparison of commercial molecular assays for toxigenic *Clostridium difficile* detection in stools: BD GeneOhm Cdiff, XPert *C. difficile* and Illumigene *C. difficile*. J Microbiol Methods. doi:10.1016/j.mimet.2012.04.017

Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet 366(9491):1079–1084

Zidaric V, Kevorkijan BK, Oresic N, Janezic S, Rupnik M (2011) Comparison of two commercial molecular tests for the detection of *Clostridium difficile* in the routine diagnostic laboratory. J Med Microbiol. doi:10.1099/jmm.0.030163-0

Příloha 2

Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, Arvand M. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 2014;30:153-5.

V roce 2013 jsme při plošné ribotypizaci českých izolátů *C. difficile* prvně na území ČR identifikovali *C. difficile* ribotyp 027. Podle anamnézy pacientky se jednalo o importovaný kmen z Německa. Pomocí MLVA jsme potvrdili bližší genetickou příbuznost mezi českým izolátem a německými izoláty z nemocnice, kde byla pacientka v minulosti hospitalizována.

Anaerobe 30 (2014) 153e155

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Note

A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176

Marcela Krutova [a, b, *], Otakar Nyc [a], Ed J. Kuijper [d], Lenka Geigerova [e], Jana Matejkova [a], Tamara Bergerova [e], Mardjan Arvand [c]

[a] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic
[b] Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic
[c] Hesse State Health Office, Centre for Health Protection, Dillenburg, Germany
[d] Leiden University Medical Center, Leiden, The Netherlands
[e] Department of Microbiology, Faculty of Medicine, Charles University in Prague and University Hospital in Plzen, Czech Republic

a r t i c l e i n f o

Article history:
Received 4 September 2014
Received in revised form
23 September 2014
Accepted 29 September 2014
Available online 7 October 2014

Keywords:
Clostridium difficile
PCR ribotype 027
PCR ribotype 176
MLVA
Capillary electrophoresis
C. difficile infection

a b s t r a c t

The first case of *Clostridium difficile* RT027 infection in the Czech Republic (CZ) was identified. The patient had been hospitalised in Germany prior to moving to CZ. Multiple-Locus Variable number tandem repeat Analysis revealed a genetic relatedness between the patient's isolate and RT027 isolate collected in the German hospital.

The first case of *Clostridium difficile* RT027 infection in the Czech Republic (CZ) was identified. The patient had been hospitalised in Germany prior to moving to CZ. Multiple-Locus Variable number tandem repeat Analysis revealed a genetic relatedness between the patient's isolate and RT027 isolate collected in the German hospital.

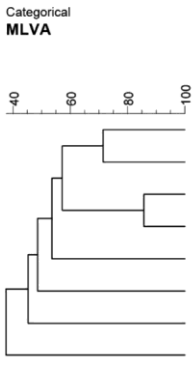
C. difficile is an important cause of hospital acquired diarrhoea worldwide [1]. *C. difficile* PCR ribotype (RT) 027 is considered as a hyper virulent type with capacity to rapidly spread within healthcare facilities [2]. The prevalence of *C. difficile* infection (CDI) by the epidemic RT027 strain varies considerably between European countries. In the Czech Republic reporting of CDI cases is mandatory to the Czech reporting system of infectious diseases (EPIDAT). In 2013 the total number of patients reported with CDI was 5797, representing an incidence rate of 55.1 per 100,000 inhabitants.

In 2012 the Department of Microbiology at the Faculty Hospital Motol participated in the ECDC supported "European *C. difficile* infection surveillance network (ECDIS-net)". The laboratory has provided PCR ribotyping of *C. difficile* isolates for national surveillance purposes. In 2013 a total of 624 *C. difficile* isolates from 11 different healthcare facilities in the Czech Republic were sent to the central laboratory. Of these *C. difficile* isolates, 251 (40%) belonged to RT176. Surprisingly, only one *C. difficile* isolate (287CZ) corresponded to the epidemic RT027. The index patient (88 years old, female) was treated for long term diarrhoea in a hospital in Pilsen in the Czech Republic in November 2013. Interestingly, the patient had lived in Hesse, Germany, for 40 years prior to her actual hospitalisation in Pilsen (CZ). She had a history of several hospitalisations in Germany because of chronic intestinal disease and other co-morbidities. Her last hospital admission in Germany was in February 2013, where she developed CDI after being treated with antibiotics for urinary tract infection. Unfortunately, characterisation of *C. difficile* was not performed at that time.

The aim of this study was to determine the genetic relationship between the index patient's isolate and RT027 isolates collected in

* Corresponding author. Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, V Uvalu 84, 150 06 Prague 5, Czech Republic. Tel.: þ420 732 532 499.
E-mail address: marcela.krutova@seznam.cz (M. Krutova).

http://dx.doi.org/10.1016/j.anaerobe.2014.09.020
1075-9964/



MLVA

A6Cd	B7Cd	C6Cd	E7Cd	F3Cd	G8Cd	H9Cd		
20	10	32	10	5	16	2	383DE	A
16	10	32	10	5	15	2	441DE	A
31	9	33	10	5	15	2	418DE	A
31	9	32	10	5	15	2	440DE	A
23	14	32	10	5	14	2	287CZ	
31	11	39	10	5	17	2	869DE	B
29	8	37	10	5	16	2	439DE	A
15	9	39	11	5	16	2	870DE	B

Fig. 1. A cluster analysis by using the categorical distance and UPGMA (unweighted pair group method with arithmetic mean) algorithms (Bionumerics v5.0, Applied Maths).

the German hospital, where the patient had been hospitalised prior to moving to CZ, in order to evaluate the possible route of transmission of the RT027 strain within Europe.

PCR ribotyping was performed with capillary electrophoresis according to the Standard Operation Protocol of ECDIS-net (http://www.ecdisnet.eu/). Electrophoreograms were evaluated by using the Webribo database as described by Indra et al. (http://www.webribo.ages.at) [3].

MLVA of 7VNTR (Variable Number Tandem Repeats) loci was performed on the index patient's isolate and on 7 RT027 isolates from Hesse, Germany. Five of the German isolates were collected in hospital A (383DE, 418DE, 439DE, 440DE, 441DE), where the patient had been treated prior to moving to the Czech Republic. Two other German RT027 isolates (869DE, 870DE) that were collected in another hospital (hospital B), from the same district in Hesse, were also included for comparison.

DNA was extracted from one colony using Chelex 100 resin [4]. Sanger sequencing of 7VNTR loci (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd) described by van den Berg et al. was performed [5].A Minimum Spanning Tree was created by Bionumerics v5.0 (Applied Maths) by using a Manhattan coefficient to calculate the summed tandem repeat difference (STRD). A cluster analysis using the categorical distance and UPGMA (unweighted pair group method with arithmetic mean) algorithms was also applied. MLVA and PCR ribotyping were performed twice using two separate cultivations of *C. difficile* isolates.

The number of repeats for each of the seven loci is shown on Fig. 1. A sum of 9 tandem repeat differences in 3 loci was found between the index patient's isolate (287CZ) and the German isolate (383DE) from hospital A (Fig. 2). One clonal complex between two German strains (440DE, 418DE) was found. According to the result of the cluster analysis the degree of genetic relatedness between CZ 287 and 383DE was calculated to be 70% (Fig. 1).

We propose that the RT027 isolate was most probably acquired in hospital A in Germany, where the patient had received antimicrobial therapy and developed CDI prior to travelling to the CZ. In contrast to CZ, the prevalence of RT027 is high in some regions of Germany, especially in the region where hospital A is located [6]. Using MLVA, we could not confirm a clonal relatedness of the Czech RT027 with German RT027 isolates, though the strains were genetically related with STD¼9. Interestingly, only 2 out of 5 German RT027 isolates (440DE and 418DE) were highly related to each other with STRD¼1 and formed a clonal complex. Closer genetic relatedness between other German strains (418DE and 439DE; 439DE and 869DE; 383DE and 441DE) was also observed. These results confirm the ability of RT027 to spread within

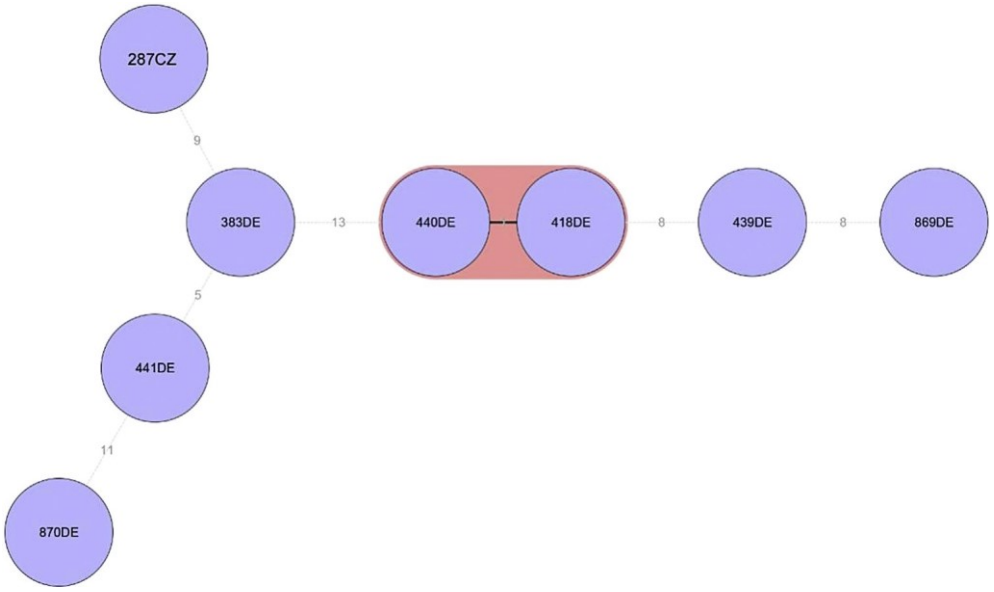


Fig. 2. A Minimum Spanning Tree by using a Manhattan coefficient (Bionumerics v5.0, Applied Maths).

hospitals and suggest that different RT027 clones are circulating in hospital A and between hospitals.

Ribotyping data from 2013 show, that RT176 is the major epidemic *C. difficile* strain in the Czech Republic [7]. RT176 was recently reported to be very common in some hospitals in Poland [8]. It is to be noted that the epidemic RT027 strain was also found to be common in Poland in the latter study. The presence of *C. difficile* RT176 in 2008 in the Czech Republic and Poland was published by Nyc et al. [9]. The inability to distinguish between *C. difficile* ribotypes 176 and 027 due to a high similarity of specific gene loci was discussed by Krutova et al. [10]. Both ribotypes possess the genes for binary toxin (*cdtA*, *cdtB*) and display a mutation at position 117 and 18 bp deletion at position 330e347 in *tcdC* gene.

This case report shows how epidemic *C. difficile* RT027 could potentially spread in Europe, even between different countries. Ribotyping based on capillary electrophoresis is a powerful tool which allows the discrimination between the closely related RT027 and RT176. It also helps us to better understand the epidemiology of epidemic *C. difficile* strains.

Acknowledgement

We would like to thank ECDIS-net for their professional support. We would like to thank Raj Kang for his proofreading.

Supported by: MH CZ IGA NT14209 e 3 and DRO, University Hospital Motol, Prague, Czech Republic 00064203.

References

[1] Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 2011;377(9759):63e73. http://dx.doi.org/10.1016/S0140-6736(10)61266-4.

[2] He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet 2013;45(1):109e13. http://dx.doi.org/10.1038/ng.2478.

[3] Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 2008;57(Pt 11): 1377e82. http://dx.doi.org/10.1099/jmm.0.47714-0.

[4] Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol 2012;61(Pt 1):49e56. http://dx.doi.org/10.1099/jmm.0.036194-0.

[5] van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol 2007;45(3):1024e8.

[6] M1 Arvand, Vollandt D, Bettge-Weller G, Harmanus C, Kuijper EJ, study group Hesse. Increased incidence of *Clostridium difficile* PCR ribotype 027 in Hesse, Germany, 2011 to 2013. Euro Surveill 2014;19(10). pii: 20732.

[7] Krutova M, Matejkova J, Nyc O. First results of molecular typing of *Clostridium difficile* in the Czech Republic. Zprávy CEM (SZÚ, Praha) 2013;22(12): 399e401.

[8] Obuch-Woszczatyński P, Lachowicz D, Schneider A, Mól A, Pawłowska J, Ozdżeńska-Milke E, et al. Occurrence of *Clostridium difficile* PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010. Anaerobe 2014;28C:13e7. http://dx.doi.org/10.1016/j.anaerobe.2014.04.007.

[9] Nyc O, Pituch H, Matejkova J, Obuch-Woszczatynski P, Kuijper EJ. *Clostridium difficile* PCR ribotype 176 in the Czech Republic and Poland. Lancet 2011;377(9775):1407. http://dx.doi.org/10.1016/S0140-6736(11)60575-8.

[10] Krutova M, Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha) 2014. http://dx.doi.org/10.1007/s12223-014-0323-5.

Příloha 3

Krutova M, Matejkova J, Tkadlec J, Nyc O. Antibiotic profiling of *Clostridium difficile* ribotype 176--A multidrug resistant relative to *C. difficile* ribotype 027. Anaerobe. 2015;36:88-90.

V článku jsou popsány výsledky testovaní dvaceti izolátů *C. difficile* ribotypu 176 z deseti různých nemocnic v ČR k citlivosti k deseti antimikrobním látkám. U všech izolátů byla zjištěna získaná rezistence k erytromycinu, ciprofloxacinu a moxifloxacinu. K rifampicinu bylo rezistentních 13 izolátů. Molekulární mechanismus rezistence k fluorochinolonům a rifampicinu byl potvrzen přítomností aminokyselinové záměny Thr82Ile v GyrA a His502Asn společně s Arg505Lys v RpoB. Zjištěná akumulace mechanismů rezistence u českých izolátů *C. difficile* ribotypu 176 podtrhuje závažnost výskytu tohoto ribotypu v ČR.

Anaerobe 36 (2015) 88e90

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Antibiotic profiling of *Clostridium difficile* ribotype 176 e A multidrug resistant relative to *C. difficile* ribotype 027

Marcela Krutova [a, b, *], Jana Matejkova [a], Jan Tkadlec [a], Otakar Nyc [a]

[a] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech Republic
[b] DNA laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech Republic

a r t i c l e i n f o

Article history:
Received 5 May 2015
Received in revised form
27 July 2015
Accepted 30 July 2015
Available online 6 August 2015

Keywords:
Clostridium difficile
PCR-ribotype 176
Resistance
Thr82Ile
Erythromycin
Moxifloxacin
Rifampicin

a b s t r a c t

Antibiotic profiling of twenty Czech *Clostridium difficile* PCR-ribotype 176 isolates revealed a high level of resistance to erythromycin, ciprofloxacin and moxifloxacin (n ¼ 20) and to rifampicin (n ¼ 13). Accumulation of resistance mechanisms to multiple antibiotics highlight that PCR-ribotype 176 belong to problematic epidemic strains.

Clostridium difficile is the global leading pathogen of hospital-acquired diarrhoea. The previous or present use of antibiotics is the major risk factor for developing of CDI (*C. difficile* infection) [1]. Antibiotic resistance in European prevalent *C. difficile* PCR-ribotypes were recently published by Freeman et al. and the Czech Republic was referred to as a country with a high cumulative resistance score (4e5) [2]. PCR-ribotype 176, closely related to 027 [3], is the ribotype that has been contributed significantly to epidemiological situation of CDI in the Czech Republic [4], and its occurrence was also reported from Poland [5].

We investigated twenty *C. difficile* PCR-ribotype 176 isolates from ten different hospitals distributed around the Czech Republic. Ten isolates originated from the year 2012 and ten isolates were cultured in 2014e2015.

MLVA (Multiple-Locus Variable Tandem Repeats analysis) of seven loci [6] was applied for exclusion of isolate clonal relatedness. Minimum spanning tree was created by Bionumerics v5.0 (Applied Maths) (Fig. 1).

The minimum inhibitory concentrations (MICs) for ten different antibiotics were determined by Etest strips (Liofilchem, Italy) on Wilkins Chalgren agar.

Molecular mechanisms of resistance were investigated by PCR amplification and Sanger sequencing.

All isolates (n ¼ 20) revealed a high level of resistance to erythromycin, ciprofloxacin and moxifloxacin. All isolates (n ¼ 20) were sensitive to metronidazole, vancomycin, imipenem, tetracycline and tigecycline. Differences in susceptibilities between isolates were observed among clindamycin and rifampicin. Clindamycin resistance (16 mg/L) was only shown by two isolates (years 2012 and 2014 from the same hospital) and the other eighteen isolates were clindamycin sensitive. A high level of rifampicin resistance was shown by thirteen isolates (four isolates from 2012 and nine isolates from 2014 to 2015) and seven isolates (2012) were rifampicin sensitive. MICs are summarized in Table 1.

All ciprofloxacin and moxifloxacin resistant isolates (n ¼ 20) carried amino acid substitution Thr82Ile in the *gyrA* gene [7e10]. Rifampicin resistant isolates revealed two substitutions in the *rpoB* gene: His502Asn and Arg505Lys [7,11,12] (Fig. 2). Two isolates were *ermB* positive [13], both were clindamycin resistant. The mechanism of erythromycin resistance among *ermB* negative *C. difficile* isolates has not been yet elucidated.

* Corresponding author. Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital in Motol, V Uvalu 84, 150 06 Prague 5, Czech Republic.
E-mail address: marcela.krutova@seznam.cz (M. Krutova).

http://dx.doi.org/10.1016/j.anaerobe.2015.07.009
1075-9964/

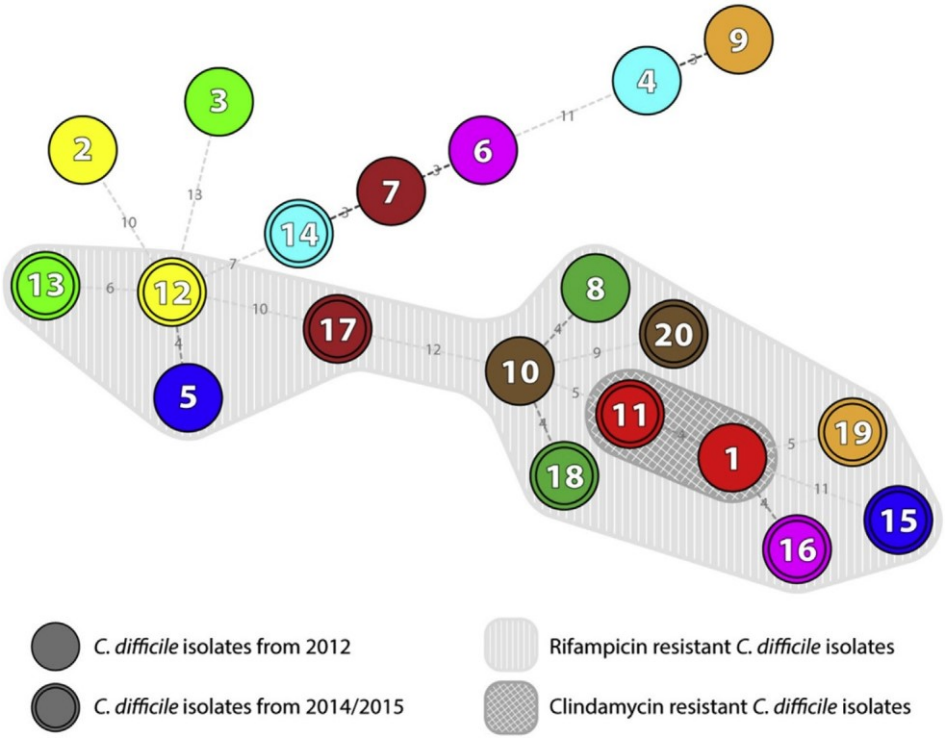


Fig. 1. A Minimum Spanning Tree by using a Manhattan coefficient (Bionumerics v5.0, Applied Maths). Each hospital has a separate colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The investigation of *C. difficile* PCR-ribotype 176 isolates from two time periods showed an unchanging resistance to 2nd and 3rd generation fluoroquinolones and to erythromycin, whereas resistance to rifampicin was detected in four isolates (40%) from 2012 and nine isolates (90%) from 2014 to 2015.

If we compare our results with recently published data on twenty-one PCR-ribotype 176 isolates originating from ten Polish hospitals in 2012, there is some evidence of a difference in variability in the antibiotic resistance spectrum between Czech and Polish *C. difficile* PCR-ribotype 176 isolates. In addition to full resistance to erythromycin, ciprofloxacin and moxifloxacin, the Polish isolates also revealed complete resistance to imipenem and 95.2% of isolates were resistant to clindamycin. In contrast to our result, all Polish isolates were sensitive to rifampicin [14].

The data on Polish PCR-ribotype 027 isolates show a certain degree of similarity in its antibiotic resistance spectrum to that of Czech and Polish PCR-ribotype 176 isolates. Shared resistance to fluoroquinolones, in the case of Czech PCR-ribotype 176 isolates is confirmed by the presence of Thr82Ile in the *gyrA* gene, illustrates their genetic link, probably to the FQR2 lineage [15].

In both studies, the Etest was used for antibiotic susceptibility testing. The differences between MICs for metronidazole and vancomycin obtained by the Etest and agar incorporation methods was reported [16] and reduced susceptibility to metronidazole by agar incorporation was detected among Czech as well as Polish isolates in Freeman et al. study [2].

Although Czech and Polish *C. difficile* PCR-ribotype 176 strains are probably genetically related because they only occur significantly in these two neighbouring countries, the cumulating of resistance to different antibiotics was observed. The accumulation

Table 1
MICs of Czech and Polish PCR-ribotype 176 isolates.

Antimicrobials	Breakpoints mg/L	Range of Etest gradient mg/L	Czech PCR-ribotype 176 isolates (n ¼ 20) MIC mg/L		Polish PCR-ribotype 176 isolates (n ¼ 21) [14] MIC mg/L	
			50%	90%	50%	90%
Metronidazole	2 (EUCAST 2014)	0.016e256	0.5	1	0.094	0.5
Vancomycin	2 (EUCAST 2014)	0.016e256	0.5	1	0.75	1
Clindamycin	8 (CLSI 2007)	0.016e256	0.5	2.5	256	256
Erythromycin	8 (CLSI 2007)	0.016e256	256	256	256	256
Moxifloxacin	4 (EUCAST 2014)	0.002e32	32	32	32	32
Rifampicin	0.004 (EUCAST 2014)	0.016e256[a]	256	256	0.002	0.003
Imipenem	8 (CLSI 2007)	0.002e32	4	4	32	32
Tetracycline	8 (CLSI 2007)	0.016e256	0.016	0.032	0.19	0.38
Tigecycline	0.25 (EUCAST 2014)	0.016e256	0.023	0.023	0.064	0.19
Ciprofloxacin	4 (CLSI 2007)	0.002e32	32	32	32	32

MIC: minimum inhibitory concentration; CLSI: U.S. Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing.
[a] 0.002e32 in Polish study.

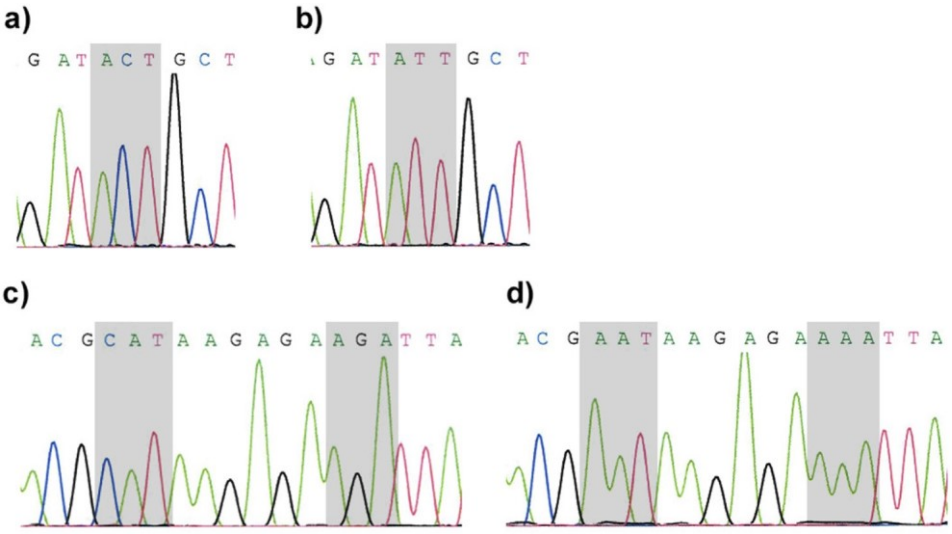


Fig. 2. Missense mutations identified in the study. a) *gyrA* wild-typ e ciprofloxacin and moxifloxacin susceptible *C. difficile* isolate b) *gyrA* Thr82Ile e ciprofloxacin and moxifloxacin resistant *C. difficile* isolate c)*rpoB* wild-type e rifampicin susceptible *C. difficile* isolate d)*rpoB* His502Asn, Arg505Lys e rifampicin resistant *C. difficile* isolate.

of antibiotic resistance among epidemic strains, such as PCR-ribotype 176, probably plays a role in the spread within the hospital environment and may be dependent on local or national antibiotic policy. Based on our results, antibiotic resistance cannot be related to the PCR-ribotype in general, but can be assessed within the national or local epidemiological situation, particularly with respect to the prevailing ribotype in the region or country.

The results of European studies stress the importance of implementing a CDI surveillance system, particularly in countries where the monitoring of CDI is not mandatory. Detailed knowledge about the current CDI epidemiological situation, together with antibiotic resistance data on prevailing PCR-ribotypes, could move us one step ahead the in the fight with the global enemy of hospitalised patients.

Acknowledgements

We are grateful for European activities, namely European Study group for *C. difficile* (ESCMID-ESGCD) and European *Clostridium difficile* infection surveillance network (ECDIS e net), which have helped to monitor the CDI epidemiological situation in Europe.

We would like to thank Dr. James Partridge for proofreading the text.

This study was financially supported by the Internal Grant Agency, Ministry of Health, Czech Republic (NT 14209-3).

References

[1] M.P. Bauer, D.W. Notermans, B.H. van Benthem, J.S. Brazier, M.H. Wilcox, M. Rupnik, et al., *Clostridium difficile* infection in Europe: a hospital-based survey, Lancet 377 (9759) (2011) 63e73, http://dx.doi.org/10.1016/S0140-6736(10)61266-4.

[2] J. Freeman, J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw, et al., Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes' study group, Clin. Microbiol. Infect. 21 (3) (2015) 248.e9e248.e16, http://dx.doi.org/10.1016/j.cmi.2014.09.017.

[3] E. Valiente, L.F. Dawson, M.D. Cairns, R.A. Stabler, B.W. Wren, Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage, J. Med. Microbiol. 61 (Pt 1) (2012) 49e56, http://dx.doi.org/10.1099/jmm.0.036194-0.

[4] M. Krutova, O. Nyc, E.J. Kuijper, L. Geigerova, J. Matejkova, T. Bergerova, et al., A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176, Anaerobe 30 (2014) 153e155, http://dx.doi.org/10.1016/j.anaerobe.2014.09.020.

[5] P. Obuch-Woszczatyński, D. Lachowicz, A. Schneider, A. Mól, J. Pawłowska, E. Ozdzeńska-Milke, et al., Occurrence of *Clostridium difficile* PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010, Anaerobe 28 (2014) 13e17, http://dx.doi.org/10.1016/j.anaerobe.2014.04.007.

[6] R.J. Van den Berg, I. Schaap, K.E. Templeton, C.H. Klaassen, E.J. Kuijper, Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable e number tandem-repeat analysis, J. Clin. Microbiol. 45 (3) (2007) 1024e1028.

[7] P. Spigaglia, F. Barbanti, P. Mastrantonio, European Study Group on *Clostridium difficile* (ESGCD), Multidrug resistance in European *Clostridium difficile* clinical isolates, J. Antimicrob. Chemother. 66 (10) (2011) 2227e2234, http://dx.doi.org/10.1093/jac/dkr292.

[8] P. Spigaglia, F. Barbanti, P. Mastrantonio, J.S. Brazier, F. Barbut, M. Delmee, et al., Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of C. difficile infections in Europe, J. Med. Microbiol. 57 (Pt 6) (2008) 784e789, http://dx.doi.org/10.1099/jmm.0.47738-0.

[9] L. Dridi, J. Tankovic, B. Burghoffer, F. Barbut, J.C. Petit, gyrA and gyrB mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*, Antimicrob. Agents Chemother. 46 (11) (2002) 3418e3421.

[10] D. Drudy, L. Kyne, R. O'Mahony, S. Fanning, gyrA mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027, Emerg. Infect. Dis. 13 (3) (2007) 504e505.

[11] S.R. Curry, J.W. Marsh, K.A. Shutt, C.A. Muto, M.M. O'Leary, M.I. Saul, et al., High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital, Clin. Infect. Dis. 48 (4) (2009) 425e429, http://dx.doi.org/10.1086/596315.

[12] J.R. O'Connor, M.A. Galang, S.P. Sambol, D.W. Hecht, G. Vedantam, D.N. Gerding, et al., Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*, Antimicrob. Agents Chemother. 52 (8) (2008) 2813e2817, http://dx.doi.org/10.1128/AAC.00342-08.

[13] P. Spigaglia, P. Mastrantonio, Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods, J. Med. Microbiol. 53 (Pt 11) (2004) 1129e1136.

[14] D. Lachowicz, H. Pituch, P. Obuch-Woszczatyński, Antimicrobial susceptibility patterns of *Clostridium difficile* strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012, Anaerobe 31 (2015) 37e41, http://dx.doi.org/10.1016/j.anaerobe.2014.09.004.

[15] M. He, F. Miyajima, P. Roberts, L. Ellison, D.J. Pickard, M.J. Martin, T.R. Connor, S.R. Harris, D. Fairley, K.B. Bamford, S. D'Arc, J. Brazier, D. Brown, J.E. Coia, G. Douce, D. Gerding, H.J. Kim, T.H. Koh, H. Kato, M. Senoh, T. Louie, S. Michell, E. Butt, S.J. Peacock, N.M. Brown, T. Riley, G. Songer, M. Wilcox, M. Pirmohamed, E. Kuijper, P. Hawkey, B.W. Wren, G. Dougan, J. Parkhill, T.D. Lawley, Emergence and global spread of epidemic healthcare-associated Clostridium difficile, Nat. Genet. 45 (1) (2013) 109e113, http://dx.doi.org/10.1038/ng.2478.

[16] S.D. Baines, R. O'Connor, J. Freeman, W.N. Fawley, C. Harmanus, P. Mastrantonio, et al., Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*, J. Antimicrob. Chemother. 62 (5) (2008) 1046e1052, http://dx.doi.org/10.1093/jac/dkn313.

Příloha 4

Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc O; Czech *Clostridium difficile* study group. *Clostridium difficile* PCR ribotypes 001 and 176 - the common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014. Euro Surveill. 2016;21(29).

V článku jsou uvedeny výsledky studie změřená na výskyt infekcí vyvolaných *C. difficile*, která proběhla v 18 českých nemocnicích v roce 2014. Celkem bylo typováno 774 izolátů, 225 z nich (29,1 %) příslušelo k ribotypu 176 a 184 (23,8 %) k ribotypu 001. MLVA příbuzenská analýza izolátů ribotypu 176 odhalila 27 klonálních komplexů tvořených 84,5 % izolátů. Izoláty ribotypu 001 (76,6 %) tvořily 14 klonálních komplexů. Data získaná molekulární analýzou izolátů *C. difficile* demonstrují alarmující rozšíření dvou ribotypů v 18 nemocnicích v ČR.

Clostridium difficile PCR ribotypes 001 and 176 – the common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014

M Krutova [1,2], J Matejkova [1], EJ Kuijper [3], P Drevinek [1], O Nyc [1], Czech Clostridium difficile study group [4]

1. Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, Czech Republic
2. DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, Czech Republic
3. Department of Medical Microbiology, Leiden University Medical Centre, Leiden, the Netherlands
4. Members of the study group are listed at the end of the article

Correspondence: Marcela Krutova (marcela.krutova@seznam.cz)

Citation style for this article:
Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc O, Czech Clostridium difficile study group. Clostridium difficile PCR ribotypes 001 and 176 – the common denominator of C. difficile infection epidemiology in the Czech Republic, 2014. Euro Surveill. 2016;21(29):pii=30296. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.29.30296

Article submitted on 27 July 2015 / accepted on 27 November 2015 / published on 21 July 2016

In 2014, 18 hospitals in the Czech Republic participated in a survey of the incidence of *Clostridium difficile* infections (CDI) in the country. The mean CDI incidence was 6.1 (standard deviation (SD):7.2) cases per 10,000 patient bed-days and 37.8 cases (SD: 41.4) per 10,000 admissions. The mean CDI testing frequency was 39.5 tests (SD: 25.4) per 10,000 patient bed-days and 255.8 tests (SD: 164.0) per 10,000 admissions. A total of 774 *C. difficile* isolates were investigated, of which 225 (29%) belonged to PCR ribotype 176, and 184 isolates (24%) belonged to PCR ribotype 001. Multilocus variable-number tandem repeat analysis (MLVA) revealed 27 clonal complexes formed by 84% (190/225) of PCR ribotype 176 isolates, and 14 clonal complexes formed by 77% (141/184) of PCR ribotype 001 isolates. Clonal clusters of PCR ribotypes 176 and 001 were observed in 11 and 7 hospitals, respectively. Our data demonstrate the spread of two *C. difficile* PCR ribotypes within 18 hospitals in the Czech Republic, stressing the importance of standardising CDI testing protocols and implementing mandatory CDI surveillance in the country.

Introduction

Clostridium difficile is the most important bacterial cause of hospital-acquired diarrhoea. Two large studies have been carried out to map and update data on *C. difficile* infection (CDI) in Europe [1,2]. CDI incidence showed an increasing trend: in the first study in 2008, the mean incidence in the participating countries was 4.1 cases per 10,000 patient bed-days [1], while in the second, in 2011–13, it was 7.0 CDI cases per 10, 000 patient bed-days in the countries involved [2].

Results of the 2008 study – a hospital-based survey involving 34 European countries – showed that the Czech Republic had a low incidence of CDI (1.1/10,000 patient bed-days), without the presence of *C. difficile* PCR ribotypes 027 and 176 [1]. Spread of PCR ribotype 027 has been seen worldwide [3] and is known to be associated with hospital CDI outbreaks [4] and severe course of disease and increased mortality [5].

Ribotype 176 is closely related to 027 [6,7] and can be misidentified by commercial tests targeting a single-base-pair deletion at nucleotide 117 in the *C. difficile tcdC* gene [8]. In 2009, shortly afterward the 2008 study, the occurrence of ribotype 176 was reported in certain areas of the country (Eastern Bohemia and Moravia) [9]. This ribotype has persisted in the Czech Republic [8] and was also reported in Poland in 2008–13, which borders the country [10,11].

Results from the second study – involving 20 European countries – revealed an increasing CDI incidence rate in the Czech Republic (4.4 cases in 2011–12/10,000 patient bed-days and 6.2 cases/10,000 patient bed-days in 2012–13) [2].

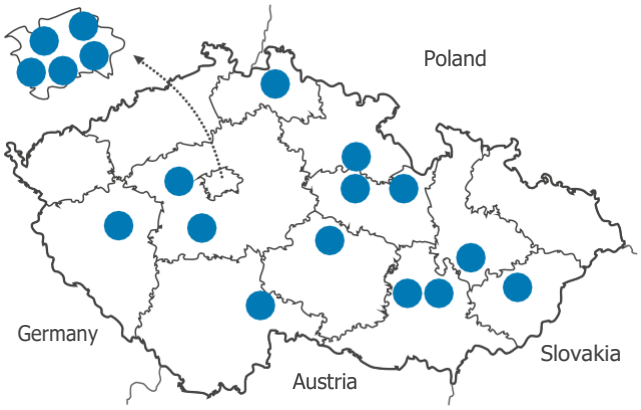
This observation prompted us to determine the CDI incidence in 2014 in a number of hospitals distributed across the Czech Republic (n = 18) and to gain an insight into the prevailing *C. difficile* ribotypes.

Methods

A CDI case was defined as a hospitalised patient (more thantwo years-old) with both diarrhoea and laboratory confirmation of CDI by a positive test result for the presence of GDH and toxin A/B and/or the detection

Figure 1

Location of hospitals participating in survey of incidence of *Clostridium difficile* infection, Czech Republic, 2014 (n = 18)



of a toxin-producing *C. difficile* strain using toxigenic culture or nucleic acid amplification test (NAAT) in the stool sample.

Testing for CDI was requested by the attending physician based on clinical symptoms indicating CDI (primarily diarrhoea). Hospital-associated and community-associated CDI cases were included in the analysis.

During 2014, hospital microbiology departments of the 18 selected hospitals were asked to send *C. difficile* isolates cultured from stool samples from hospitalised CDI patients to the Department of Medical Microbiology of the University Hospital Motol in Prague.

Mean CDI incidence and CDI testing frequency for all participating hospitals was calculated using the total number of admissions, total number of patient bed-days, number of non-duplicated glutamate dehydrogenase (GDH) and toxin A/B positive tests performed in 2014, using information obtained from the participating hospitals. The hospitals also provided information about their CDI laboratory diagnostic algorithms.

C. difficile isolates were further characterised using PCR ribotyping, detection of the presence of genes for toxin production (*tcdA* (A), *tcdB* (B), *cdtA* and *cdtB* (binary)) by a multiplex PCR [12] and multilocus variable-number tandem repeat analysis (MLVA).

PCR ribotyping based on capillary electrophoresis was performed according to the method described by Stubbs et al. [13]. The results were compared with data in WEBRIBO, a web-based database containing a broad spectrum of uploaded capillary electrophoresis-ribotyping profiles [14], and profiles from an international capillary electrophoresis-ribotyping validation study [15]. The diversity of ribotypes for each hospital was calculated using the Shannon index [16], for which a higher value is an indicator of greater diversity.

For MLVA, five regions with short tandem repeats were sequenced: A6Cd, B7Cd, C6Cd, G8Cd [17] and CDR60 [18], with a change of reverse primer for G8Cd, as described elsewhere [19]. The number of tandem repeats was counted manually after software processing (Sequencing Analysis Software, Applied Biosystems). The sum of tandem repeat differences (STRD) in five loci determines the genetic relatedness of isolates. Minimum spanning trees were created using Bionumerics v5.1 (Applied Maths). A clonal complex was defined as an STRD≤ 2, a genetically related cluster as an STRD≥ 3 to≤ 10 [17].

Results

Participating hospitals

A total of 18 hospitals, covering the country's major regions, voluntarily participated in the survey: seven tertiary care institutions, 10 secondary care facilities and one specialised centre. The size of hospital is indicated by the number of beds in 2014 (Table 1). These 18 hospitals represented about 30% of hospital-bed capacity in the Czech Republic in 2013 [20] (2014 data unavailable). Their location is shown in Figure 1.

Incidence of *C. difficile* infection and testing frequency

The incidence of CDI in 2014 varied from 1.5 to 34.7 (median: 3.9) cases per 10,000 patient bed-days (mean: 6.1 cases (standard deviation (SD): 7.2)/10,000 patient bed-days), and from 11.8 to 201.2 (median: 26.5) cases per 10,000 admissions (mean: 37.8 cases (SD: 41.4)/10,000 admissions).

The frequency of testing for CDI in the hospital laboratories varied from 6.0 to 116.3 tests (median: 28.9) per 10,000 patient bed-days (mean: 39.5 (SD: 25.4) tests per 10,000 patient bed-days), and from 36.4 to 673.5 tests (median: 216.7) per 10,000 admissions (mean: 255.8 tests (SD: 164.0)/10,000 admissions) (Table 1).

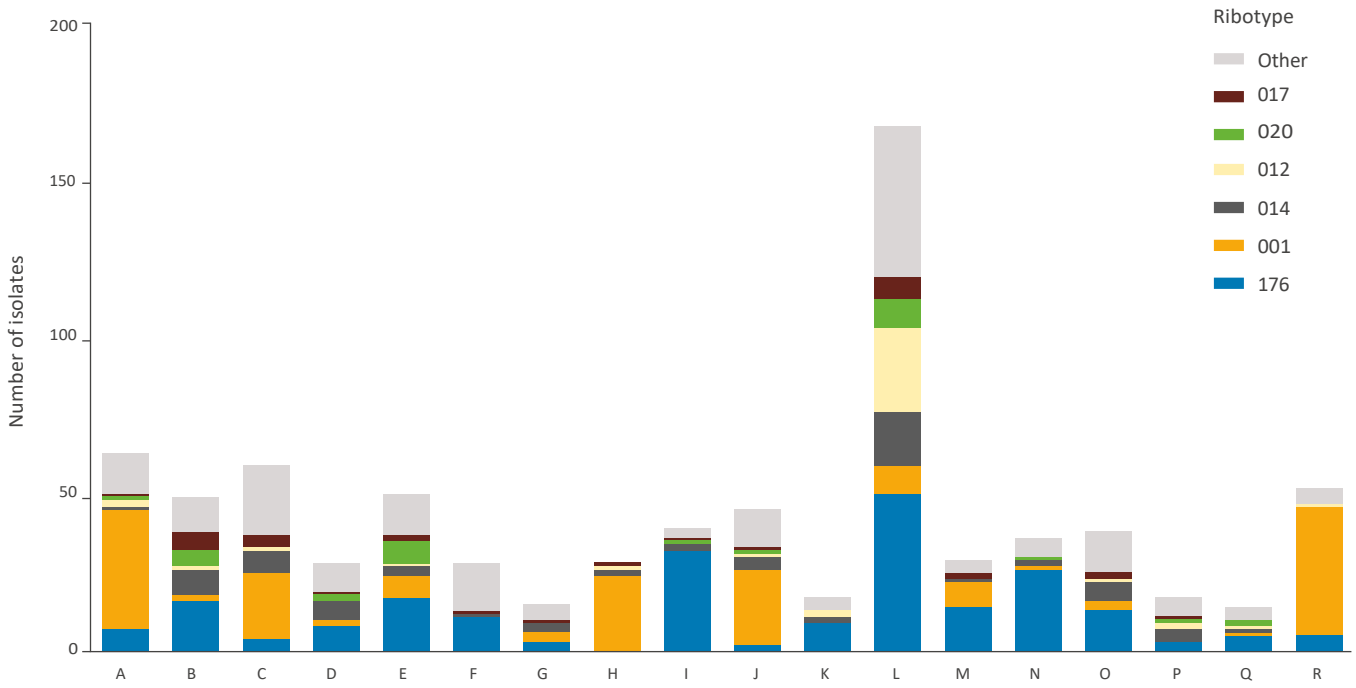
C. difficile infection testing algorithms

Four different CDI testing algorithms were used during the study period (Table 1). All hospitals in the study used the detection of GDH and toxins A/B as the first (screening) part of their testing algorithm: 14 used lateral flow immunoassay (LFIA), three used a chemiluminescent immunoassay (CLIA) and one a chromatographic immunoassay (CIA).

A total of 16 hospitals performed anaerobic culture of GDH-positive and toxin-positive or toxin-negative samples, but only two of these tested toxin production or detected the presence of genes for toxin production of isolated *C. difficile* strains (one by LFIA and one by toxin gene multiplex PCR). The remaining two hospitals, which did not routinely perform anaerobic culture, used PCR detection of the presence of *C. difficile* toxin

Figure 2

Distribution of the six most prevalent PCR ribotypes of *Clostridium difficile* in 18 hospitals participating in survey of incidence of *C. difficile* infection, Czech Republic, 2014 (n = 774)



genes in GDH-positive and toxin A/B-negative stool samples.

Of the 18 hospitals, 10 used a commercial PCR test, eight for rapid diagnosis if requested by the physician. In total, 774 *C. difficile* isolates were available for further analysis in our study: 378 were from male patients (49%) and 396 from female patients (51%). The mean age was 68 years (SD: 20); the median was 72 years (range: 2–101). Of the 774 patients, 537 (69%) were aged 65 years or older.

PCR ribotypes of *C. difficile* isolates

Of the 774 *C. difficile* isolates, 737 (95%) belonged to 33 different ribotypes, and 37 (5%) were defined as new ribotypes, as their electrophoretic profiles differed from each other and did not match any in the WEBRIBO database.

The most frequent PCR ribotype, 176, was found in 225 isolates (29%) in 17 hospitals. The second most frequent, PCR ribotype 001, was identified in 184 isolates (24%) in 14 hospitals. Other frequently found PCR ribotypes were: 014 (n = 70 (9%); 16 hospitals), 012 (n = 41 (5%); 12 hospitals), 020 (n = 31 (4%); 14 hospitals), 017 (n = 30 (4%); 10 hospitals). The distribution of the six most prevalent PCR ribotypes (581 isolates, 75%) within the participating hospitals is shown in Figure 2. Other less frequent ribotypes found were as follows, with the number of isolates per ribotype shown in parentheses: 002 (n=20), 005 (n=14), 081 (n=11), 029 (n=10), 015 (n=10), 070 (n = 9), 023 (n = 8), 078 (n = 7), 003 (n = 6), 503 (n = 5), 449 (n = 5), 046 (n = 5), 018 (n = 5), 087 (n = 5), 049 (n = 5), 126 (n = 4), AI-75 (n = 4), AI-9–1 (n = 4), 054 (n = 4), 446 (n = 3), AI-82/1 (n = 3), 053 (n = 2), 027 (n = 2), AI-60 (n = 2), 043 (n = 1), 236 (n = 1) and AI-12 (n = 1).

The Shannon index, used to determine the diversity of the ribotypes, varied from 0.54 to 2.56. The Shannon index of all the *C. difficile* ribotypes in the study was 2.58, indicating a highly diverse set of *C. difficile* isolates.

Further characterisation of *C. difficile* isolates

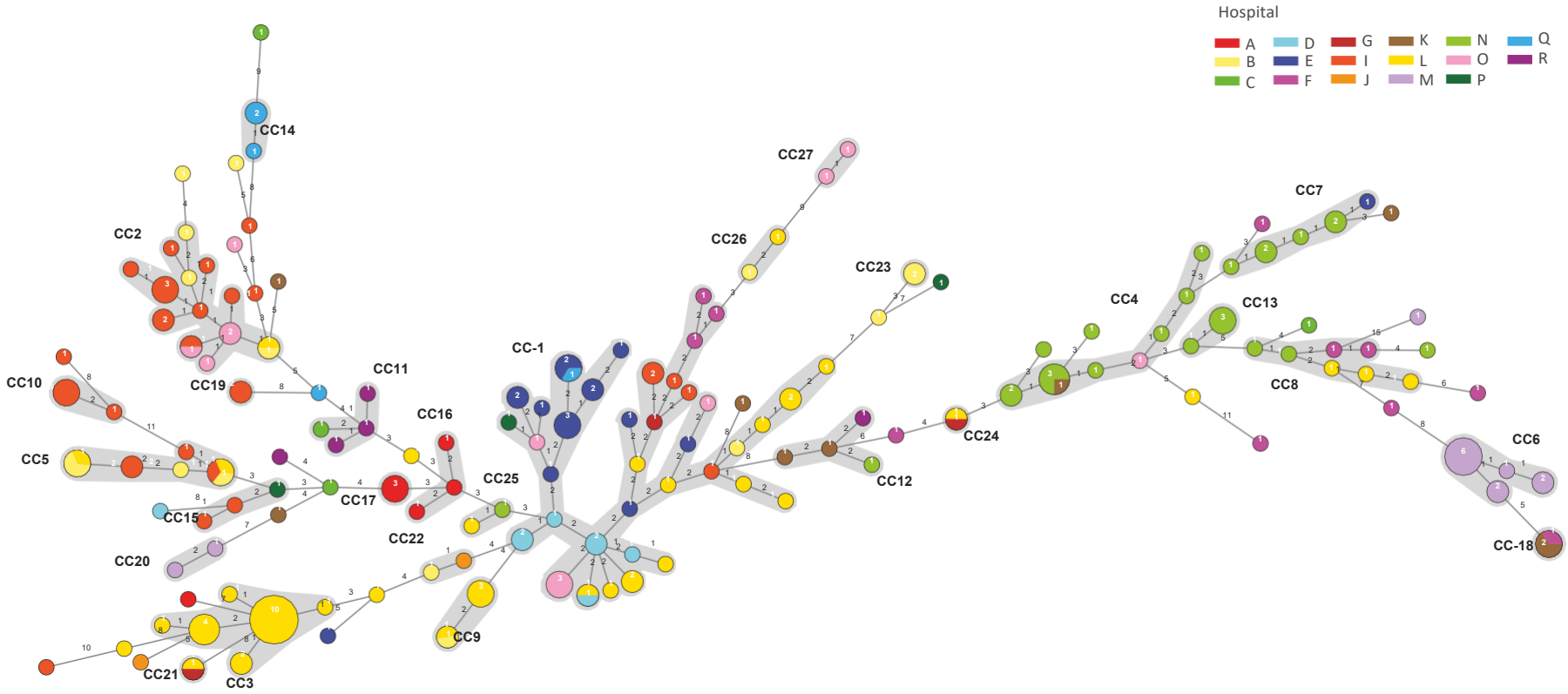
Genes for production of three *C. difficile* toxins (A, B and binary) were detected in 246 (32%) of the isolates belonging to the following PCR ribotypes: 176 (n = 225 (29%)), 023 (n = 8 (1%)), 078 (n = 7 (0.9%)), 126 (n = 4 (0.5%)) and 027 (n = 2 (0.3%)). For the other 528 isolates (68%), only genes for production of toxins A and B were detected.

MLVA of five variable-number tandem repeat loci was performed for the 225 isolates of ribotype 176 and 184 isolates of ribotype 001, and two minimum spanning trees were generated.

In total, 27 clonal complexes comprising 190 isolates (84%) were found in the minimum spanning tree of PCR ribotype 176 isolates (Table 2). For each clonal complex, the number of isolates/number of hospitals in which they were found are shown in parentheses: CC1 (52/10); CC2 (19/4); CC3 (19/1); CC4 (11/3); CC5 (10/3); CC6 (11/1);

Figure 3

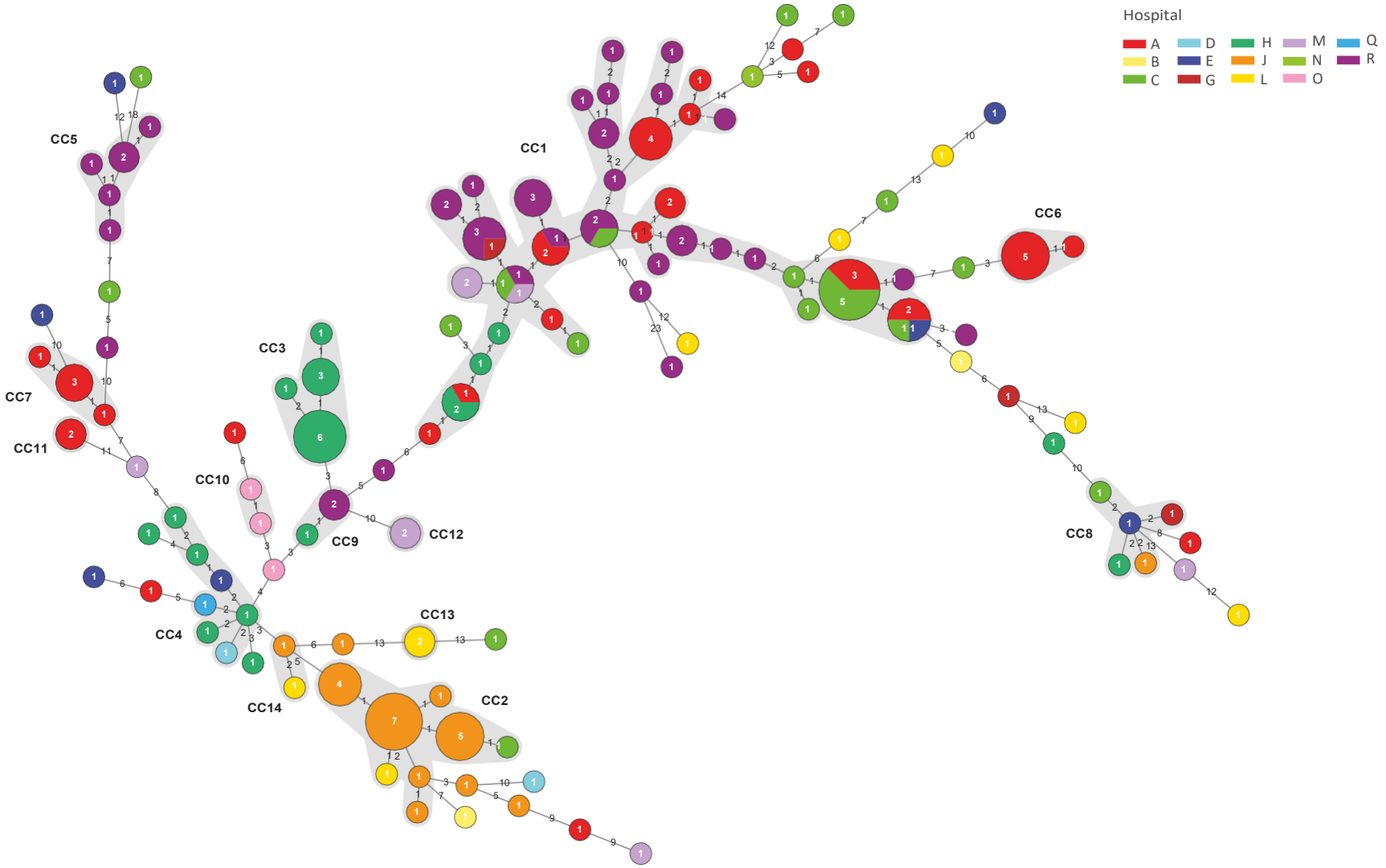
Minimum spanning tree of *Clostridium difficile* PCR ribotype 176 isolates from 17 of 18 hospitals participating in survey of incidence of *C. difficile* infection, Czech Republic, 2014 (n = 225)



Each hospital is represented by a different colour (see key). The numbers in the circles represent the number of *C. difficile* PCR ribotype 176 isolates. If the number is greater than one, it represents the number of isolates with a sum of tandem repeat differences (STRD) = 0 (i.e. 100% identical in five variable-number tandem repeat loci). The numbers on the lines represent the STRD between isolates.

Figure 4

Minimum spanning tree of *Clostridium difficile* PCR ribotype 001 isolates from 14 of 18 hospitals participating in survey of incidence of *C. difficile* infection, Czech Republic, 2014 (n = 184)



Each hospital is represented by a different colour (see key). The numbers in the circles represent the number of *C. difficile* PCR ribotype 001 isolates. If the number is greater than one, it represents the number of isolates with a sum of tandem repeat differences (STRD) = 0 (i.e. 100% identical in five variable-number tandem repeat loci). The numbers on the lines represent the STRD between isolates.

Table 1

Characteristics of hospitals participating in survey of the incidence of *Clostridium difficile* infection, Czech Republic, 2014 (n = 18)

Hospital	Number of beds	Care type	CDI testing algorithm	CDI incidence		Testing frequency per 10,000 bed-days	Testing frequency per 10,000 admissions	Number of isolates (n = 774)	Ribotype diversity[a]
				CDI cases per 10,000 patient bed-days	CDI cases per 10,000 admissions				
A	913	T	LFIA, ANAE	2.7	23.1	29.1	251.3	63	1.60
B	1,001	S	LFIA, TC, NAAT	8.9	53.0	52.5	312.1	49	2.11
C	1,913	T	LFIA, ANAE, NAAT	7.5	45.9	66.3	403.7	59	2.45
D	1,063	T	LFIA, NAAT	1.5	11.8	18.3	139.7	28	2.26
E	550	S	LFIA, ANAE, NAAT	3.4	21.9	6.0	36.4	50	2.22
F	1,368	T	LFIA, ANAE, NAAT	3.5	31.4	55.6	494.2	28	2.31
G	305	SC	LFIA, NAAT	6.2	45.6	71.1	519.4	15	1.96
H	342	S	CLIA, ANAE	6.8	39.3	27.7	159.2	28	0.54
I	531	S	LFIA, ANAE	5.6	29.8	25.8	137.2	39	0.78
J	950	S	LFIA, ANAE	4.1	22.2	28.7	154.6	45	1.90
K	247	S	CLIA, ANAE, NAAT	34.7	201.2	116.3	673.5	17	1.43
L	2,189	T	CLIA, TC, NAAT	2.6	15.7	18.3	111.0	167	2.56
M	1,184	T	LFIA, ANAE	2.5	14.9	17.8	106.6	29	1.47
N	938	S	LFIA, ANAE, NAAT	6.2	39.2	45.4	287.7	36	1.15
O	1,689	T	LFIA, ANAE, NAAT	2.2	14.7	28.7	194.8	38	2.27
P	455	S	CIA, ANAE	2.6	16.5	20.9	134.4	17	2.15
Q	664	S	LFIA, ANAE	3.6	22.3	40.6	250.2	14	1.97
R	962	S	LFIA, ANAE	5.5	31.2	42.0	238.6	52	0.87
Mean (SD)	–	–	–	6.1 (7.2)	37.8 (41.4)	39.5 (25.4)	255.8 (164.0)	–	–
Median (range)	–	–	–	3.9 (1.5–34.7)	26.5 (11.8–201.2)	28.9 (6.0–116.3)	216.7 (36.4–673.5)	–	–

ANAE: anaerobic culture on selective media; CDI: Clostridium difficile infection; CIA: chromatographic immunoassay (two separate tests for GDH and toxins A/B); CLIA: chemiluminescent immunoassay (two separate tests for GDH and toxins A/B); GDH: glutamate dehydrogenase; LFIA: lateral flow immunoassay (simultaneous tests to detect GDH and toxins A/B; NAAT: nucleic acid amplification test; S: secondary care hospital; SC: specialised centre; SD: standard deviation; T: tertiary care hospital; TC: anaerobic culture on selective media followed by LFIA (Hospital B) or NAAT (Hospital L).

[a] Calculated using the Shannon index [16].

CC7 (7/2); CC8 (7/3); CC9 (5/2); CC10 (4/1); CC11 (4/2); CC12 (4/3); CC13 (4/1); CC14, 16 and 17 (3/1); CC15 and 18 (3/2); CC19, 20, 23 and 27 (2/1); CC21, 22, 24, 25 and 26 (2/2) (Figure 3).

MLVA showed an STRD≥ 3 to≤ 10 in 33 isolates and an STRD> 10 in three isolates (Figure 3).

The minimum spanning tree of ribotype 001 isolates revealed 14 clonal complexes of 141 isolates (76.6%) (Table 3). The clonal complexes, with the number of isolates/number of hospitals in which they were found shown in parentheses, were as follows: CC1 (67/7); CC2 (21/3); CC3 (11/1); CC4 (7/4); CC5, 6 (6/1); CC7 (5/1); CC8 (5/5); CC9 (3/2); CC10, 11, 12 and 13 (2/1); CC14 (2/2). MLVA showed an STRD≥ 3 to≤ 10 in 32 isolates, and an STRD> 10 in 15 isolates, including isolates from CC11 and CC13 (Figure 4).

Discussion

In 2008, three Czech tertiary care hospitals participated in the European *C. difficile* infection study (ECDIS) [1]. In 2012–13, 10 Czech hospitals (nine tertiary care, three of which had participated in the 2008 study, and one secondary care) took part in the European, multicentre, prospective, biannual, point-prevalence study

Table 2

MLVA characteristics of *Clostridium difficile* PCR ribotype 176 isolates (n=225) from 17 of 18 hospitals participating in survey of incidence of *C. difficile* infection, Czech Republic, 2014

Hospital	Total number of isolates	Number of ribotype 176 isolates	Number of ribotype 176 isolates in clonal complexes	Clonal complex number/ number of ribotype 176 isolates in the clonal complex	Presence of 100% identical[a] ribotype 176 isolates within a hospital	Presence of 100% identical[a] ribotype 176 isolates between hospitals
A	63	7	6	16/3; 17/3	Yes	No
B	49	16	13	1/1; 2/3; 5/4; 9/1; 22/1; 23/2; 26/1	Yes	Yes (Hospitals L, I)
C	59	4	1	11/1	No	No
D	28	8	7	1/7	Yes	Yes (Hospital L)
E	50	17	16	1/15; 7/1	Yes	Yes (Hospital Q)
F	28	11	6	1/3; 8/2; 18/1	No	Yes (Hospital K)
G	15	3	3	1/1; 21/1; 24/1	No	Yes (Hospital L)
H	28	0	0	–	–	–
I	39	32	28	1/5; 2/11; 5/4; 10/4; 15/2; 19/2	Yes	Yes (Hospitals L, O, B)
J	45	2	1	22/1	No	No
K	17	9	5	4/1; 12/2; 18/2	Yes	Yes (Hospitals F, N)
L	167	50	46	1/13; 2/1; 3/19; 5/2; 8/3; 9/4; 21/1; 25/1; 24/1; 26/1	Yes	Yes (Hospitals B, D, G, I)
M	29	14	13	6/11; 20/2	Yes	No
N	36	26	23	4/9; 7/6; 8/2; 12/1; 13/4; 25/1	Yes	Yes (Hospital K)
O	38	13	12	1/5; 2/4; 4/1; 27/2	Yes	Yes (Hospital I)
P	17	3	2	1/1; 15/1	No	No
Q	14	5	4	1/1; 14/3	Yes	Yes (Hospital E)
R	52	5	4	11/3; 12/1	No	No
Total	**774**	**225**	**190**	–	**11 hospitals**	**11 hospitals**

MLVA: multilocus variable-number tandem repeat analysis.

[a] Sum of tandem repeat differences (STRD) = 0.

of CDI in hospitalised patients with diarrhoea (EUCLID) [2]. Our current study, which involved 18 hospitals distributed across the Czech Republic (including seven tertiary, 10 secondary and one specialised healthcare facility) reflects better the CDI epidemiological situation in the country. Of these 18 hospitals, eight (seven tertiary care and one secondary care) also participated in EUCLID.

In the Czech Republic, it is mandatory to report cases of CDI to EPI-DAT, the Czech reporting system for infectious diseases, but CDI is reported as 'other bacterial intestinal infections'. Colonisation by *C. difficile* is not mandatorily reportable. An increasing incidence of other bacterial intestinal infections was observed, from 26.4 per 100,000 habitants in 2005 to 64.3 per 100,000 habitants in 2014 [21]. As it is impossible to determine which of these infections are CDIs, however, CDI incidence data among hospitalised patients can only be derived from our study and the European studies mentioned above.

The results of our study showed a mean incidence of CDI per hospital of 6.1 cases per 10,000 patient bed-days and 37.8 cases per 10,000 admissions. Compared with incidence data for the Czech Republic in the 2008 European study [1], the incidence of CDI in the country has dramatically increased. Our findings are similar to those of EUCLID, which reported an incidence rate of 6.2 CDI cases per 10,000 patient bed-days in 2012–13 for the Czech Republic [2].

Our study also showed that the mean reported testing frequency was 39.5 tests per 10,000 patient bed-days, which is 1.7 times less than the mean testing frequency reported in EUCLID (65.8 tests per 10,000 patient bed-days) and almost three times less than the mean testing frequency reported for the United Kingdom (139 tests per 10,000 patient bed days) [2]. This indicates that CDI in the Czech Republic is most likely underdiagnosed and highlights the need for improvement of clinical awareness and laboratory algorithms (by adding a confirmatory test for GDH positive and toxin A/B-negative stool samples from patients with clinical symptoms of CDI).

It should be noted that considerable variation in CDI incidence was seen between the 18 participating hospitals. The highest incidence seen in Hospital K is probably due to the fact that this hospital also had

Table 3

MLVA characteristics of *Clostridium difficile* PCR ribotype 001 isolates (n=184) from 14 of 18 hospitals participating in survey of incidence of *C. difficile* infection, Czech Republic, 2014

Hospital	Total number of isolates	Number of ribotype 001 isolates	Number of ribotype 001 isolates in clonal complexes	Clonal complex number/ number of ribotype 001 isolates in the clonal complex	Presence of 100% identical[a] ribotype 001 isolates within a hospital	Presence of 100% identical[a] ribotype 001 isolates between hospitals
A	63	38	32	1/19; 6/6; 7/5; 11/2	Yes	Yes (Hospitals C, E, H, R)
B	49	2	0	–	No	No
C	59	21	13	1/11; 2/1; 8/1	Yes	Yes (Hospitals A, E, M, R)
D	28	2	1	4/1	No	No
E	50	7	3	1/1; 4/1; 8/1	No	A, C
F	28	0	0	–	–	–
G	15	3	2	1/1; 8/1	No	Yes (Hospital R)
H	28	24	21	1/4; 3/11; 4/4; 8/1; 9/1	Yes	Yes (Hospital A)
I	39	0	0	–	–	–
J	45	24	21	2/19; 8/1; 14/1	Yes	No
K	17	0	0	–	–	–
L	167	9	4	2/1; 13/2; 14/1	Yes	No
M	29	8	5	1/3; 12/2	Yes	Yes (Hospitals C, R)
N	36	1	0	–	–	–
O	38	3	2	10/2	No	No
P	17	0	0	–	–	–
Q	14	1	1	4/1	No	No
R	52	41	36	1/28; 5/6; 9/2	Yes	Yes (Hospitals A, C, G, M)
Total	**774**	**184**	**141**	–	**7 hospitals**	**7 hospitals**

MLVA: multiocus variable-number tandem repeat analysis.

[a] Sum of tandem repeat differences (STRD) = 0.

the highest testing frequency, as there were sufficient local financial sources for extensive CDI testing. The high incidence in this hospital had a considerable impact on the SD of the mean incidence for all 18 hospitals. Despite the high incidence, the number of isolates submitted during the study was small. The sending of strains was voluntary and this hospital was unable to send a representative number of isolates.

All 18 participating hospitals used GDH testing, as a recommended screening step [22]. A total of 14 used a lateral flow immunoassay for a single GDH and toxin A/B test as the first step of their testing algorithm; the other four used these tests separately. The use of two separate tests is more economical because testing can be stopped when samples are GDH negative, as a GDH-negative result has a high predictive value for the absence of CDI [23].

Of the 18 hospitals, eight did not confirm toxin production in GDH-positive and toxin A/B-negative stool samples, although they performed anaerobic culture; thus their testing algorithm were suboptimal. Diagnostic uncertainty of diarrhoeal patients with a positive GDH test and negative toxin A/B tests because of a lack of a confirmatory test may have also contributed to the spread of CDI in the Czech Republic.

It is clear that CDI diagnostic testing in the Czech Republic is very variable. A web-questionnaire, completed by 61 laboratories in 2014 showed that 21% (n = 13) used only GDH and toxin A/B test, and 8% (n = 5) used toxin A/B test as a screening test [24].

Ribotyping of *C. difficile* isolates in our study revealed the presence of PCR ribotype 176 in 29% and PCR ribotype 001 in 24% of isolates. The frequent occurrence of PCR ribotype 176 simultaneously with PCR ribotype 027 was reported in Poland in 2008–13 [10,11]. In 2013, the first sporadic case of an imported infection caused by PCR ribotype 027 was found in the Czech Republic [25]. In the study presented here, we diagnosed two new CDI cases due to PCR ribotype 027 infection: one was a man in his 30s, the other a man in his 70s.

Whereas PCR ribotype 176 has been only reported from two countries (Czech Republic and Poland) [10,11,25],

which neighbour each other, PCR ribotype 001 has been problematic for a long time in many European countries [1,2,26]. It has dominated, as in Slovakia in 2012 [27], or has occurred together with PCR ribotype 027, as reported from Germany (Hesse region) in 2011–13 [28], the north-east of England in July 2009 to December 2010 [29] and Scotland in November 2007 to December 2009 [30]. It has also occurred together with other PCR ribotypes, such as 014/020 and 126/078, in a single-day study in Spain [31].

MLVA of the two predominant ribotypes identified in our study revealed close genetic relatedness between isolates of each ribotype. The occurrence of 100%-identical (STRD = 0) PCR ribotype 176 isolates in 11 hospitals and PCR ribotype 001 isolates in seven hospitals, suggests clonal clusters within and between healthcare facilities, probably due to ineffective hospital infection control measures and transfer of patients between healthcare facilities who were in fact CDI cases but had not been diagnosed. This is supported by the observation of clonal complexes in tertiary and secondary hospitals in the same region. The question remains as to which specific molecular characteristics of PCR ribotypes 176 and 001 allow them to spread rapidly within healthcare facilities in contrast to the other less frequent PCR ribotypes identified in the study.

Antibiotic susceptibility testing of *C. difficile* isolates was not performed in this study but multiresistance of PCR ribotype 176 isolates [32,33], as well as PCR ribotype 001 isolates, has been reported [26,34]. The results of a recently published European study on antibiotic resistance among prevalent *C. difficile* ribotypes showed the Czech Republic as a country with a high cumulative resistance score (4–5), calculated based on susceptibility to nine antimicrobials tested [26].

An important limitation of our study is the lack of clinical patient data. The Czech national reference centre for healthcare-associated infections is currently organising the implementation of CDI surveillance based on the recent CDI surveillance protocol from the European Centre for Disease Prevention and Control [35]. The first national CDI incidence data, including clinical data on CDI patients and data on antibiotic susceptibility to metronidazole, vancomycin and moxifloxacin of *C. difficile* isolates, should be available in 2016 (CDI surveillance started in April 2016 in the Czech Republic).

Conclusion

The results of our study showed an unfavourable CDI epidemiological situation in the Czech Republic in 2014 caused by the occurrence of epidemic PCR ribotypes 176 and 001. The absence of national surveillance at that time, the low frequency of testing and variability in testing algorithms probably contributed to the spread of these PCR ribotypes.

A Czech standardised CDI testing protocol and the implementation of CDI surveillance in a large number of hospitals is urgently needed for monitoring, management and reduction of these infections in the Czech Republic.

Members of the Czech Clostridium difficile study group (in no particular order)

Renata Tejkalova, Department of Medical Microbiology, Faculty of Medicine, Masaryk University and St. Anne's University Hospital, Brno, Czech Republic; Katarina Petrlova, Department of Medical Microbiology, Hospital Bulovka, Prague, Czech Republic; Marketa Hanslianova, Department of Medical Microbiology, University Hospital Brno, Czech Republic; Vera Paleckova, Department of Medical Microbiology Thomayer's University Hospital, Prague, Czech Republic; Antonin Melichar, Department Hospital Laboratory, Hospital Havlickuv Brod, Czech Republic; Lenka Ryskova, Department of Clinical Microbiology, University Hospital Hradec Kralove, Czech Republic; Vladimir Petkov, Department of Medical Microbiology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; Denisa Vesela, Department of Medical Microbiology, Hospital Jindrichuv Hradec, Czech Republic; Miloslava Rumlerova, Department of Medical Microbiology, Hospital Kladno, Czech Republic; Lenka Havlinova, Department of Medical Microbiology and Immunology, Hospital Liberec, Czech Republic; Jana Janeckova, Department of Medical Microbiology, Hospital Litomyšl, Czech Republic; Otakar Nyc, Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, Czech Republic; Iva Vagnerova, Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University Olomouc, University Hospital Olomouc, Czech Republic; Lucie Barekova, Department of Microbiology Pardubice Hospital, Department of Epidemiology University of Defence, Hradec Kralove, Czech Republic; Lenka Geigerova, Department of Microbiology, Faculty of Medicine and University Hospital Plzen, Charles University in Prague, Plzen, Czech Republic; Petr Jezek, Department of Medical Microbiology, Hospital Pribram, Czech Republic; Martina Curdova, Department of Medical Microbiology, Military University Hospital Prague, Czech Republic; Natasa Bartonikova, Department of Medical Microbiology, Tomas Bata's Hospital Zlin, Czech Republic.

Acknowledgements

We would like to thank the ESCMID Study Group for Clostridium difficile (ESGCD) for professional support. We would like to thank Dr James Partridge for proofreading the text.

Molecular analysis of C. difficile isolates was supported by the Ministry of Health, Czech Republic Internal Grant Agency NT/14209 – 3.

Conflict of interest

None declared.

Authors' contributions

ON, PD, MK, JM designed the study. ON, JM supervised CDI diagnostics. MK performed molecular characterisation of C. difficile isolates. Study group members provided C. difficile isolates, data on diagnostics algorithm and annual incidence and testing data. MK, ON, EK analysed the collected data. MK wrote the first draft of the manuscript. EK, ON, PD critically revised the subsequent drafts of the manuscript. All

authors approved the final version of the manuscript and agreed with its submission.

References

1. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. , ECDIS Study Group. Clostridium difficile infection in Europe: a hospital-based survey.Lancet. 2011;377(9759):63-73. DOI: 10.1016/S0140-6736(10)61266-4 PMID: 21084111
2. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis. 2014;14(12):1208-19. DOI: 10.1016/S1473-3099(14)70991-0 PMID: 25455988
3. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated Clostridium difficile. Nat Genet. 2013;45(1):109-13. DOI: 10.1038/ng.2478 PMID: 23222960
4. Kuijper EJ, Coignard B, Tüll P, ESCMID Study Group for Clostridium difficile, EU Member States, European Centre for Disease Prevention and Control. Emergence of Clostridium difficile-associated disease in North America and Europe.Clin Microbiol Infect. 2006;12(Suppl 6):2-18. DOI: 10.1111/j.1469-0691.2006.01580.x PMID: 16965399
5. Rao K, Micic D, Natarajan M, Winters S, Kiel MJ, Walk ST, et al. Clostridium difficile ribotype 027: relationship to age, detectability of toxins A or B in stool with rapid testing, severe infection, and mortality. Clin Infect Dis. 2015;61(2):233-41. DOI: 10.1093/cid/civ254 PMID: 25828993
6. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent Clostridium difficile 027 lineage.J Med Microbiol. 2012;61(Pt 1):49-56. DOI: 10.1099/jmm.0.036194-0 PMID: 21903827
7. Knetsch CW, Hensgens MP, Harmanus C, van der Bijl MW, Savelkoul PH, Kuijper EJ, et al. Genetic markers for Clostridium difficile lineages linked to hypervirulence. Microbiology. 2011;157(Pt 11):3113-23. DOI: 10.1099/mic.0.051953-0 PMID: 21873406
8. Krutova M, Matejkova J, Nyc O. C. difficile ribotype 027 or 176?Folia Microbiol (Praha). 2014;59(6):523-6. DOI: 10.1007/s12223-014-0323-5 PMID: 24970104
9. Nyč O, Pituch H, Matějková J, Obuch-Woszczatynski P, Kuijper EJ. Clostridium difficile PCR ribotype 176 in the Czech Republic and Poland.Lancet. 2011;377(9775):1407. DOI: 10.1016/S0140-6736(11)60575-8 PMID: 21515161
10. Obuch-Woszczatyński P, Lachowicz D, Schneider A, Mól A, Pawłowska J, Ożdżeńska-Milke E, et al. Occurrence of Clostridium difficile PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010. Anaerobe. 2014;28:13-7. DOI: 10.1016/j.anaerobe.2014.04.007 PMID: 24799338
11. Pituch H, Obuch-Woszczatyński P, Lachowicz D, Wultańska D, Karpiński P, Młynarczyk G, et al. Hospital-based Clostridium difficile infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro Surveill. 2015;20(38):30025. DOI: 10.2807/1560-7917.ES.2015.20.38.30025 PMID: 26536049
12. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection.Clin Microbiol Infect. 2008;14(11):1057-64. DOI: 10.1111/j.1469-0691.2008.02092.x PMID: 19040478
13. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of Clostridium difficile and construction of a library consisting of 116 different PCR ribotypes.J Clin Microbiol. 1999;37(2):461-3.PMID: 9889244
14. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of Clostridium difficile isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. 2008;57(Pt 11):1377-82. DOI: 10.1099/jmm.0.47714-0 PMID: 18927415
15. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et al. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for Clostridium difficile. PLoS One. 2015;10(2):e0118150. DOI: 10.1371/journal.pone.0118150 PMID: 25679978
16. Shannon CE, Claude E. A mathematical theory of communication. The Bell System Technical Journal. 1948;27(3):379-423. doi:10.1002/j.1538-7305.1948.tb01338.x
17. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of Clostridium difficile isolates by using multiple-locus variable-number tandem-repeat analysis.J Clin Microbiol. 2007;45(3):1024-8. DOI: 10.1128/JCM.02023-06 PMID: 17166961
18. Marsh JW, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN, et al. Multilocus variable-number tandem-repeat analysis for investigation of Clostridium difficile transmission in Hospitals. J Clin Microbiol. 2006;44(7):2558-66. DOI: 10.1128/JCM.02364-05 PMID: 16825380
19. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, et al. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative Clostridium difficile in a general hospital in Buenos Aires, Argentina. Clin Microbiol Infect. 2009;15(12):1080-6. DOI: 10.1111/j.1469-0691.2009.02759.x PMID: 19438624
20. Institute of health information and statistics of the Czech Republic (UZIS). [Inpatient care]. Prague: UZIS. [Accessed 26 Jul 2015]. Czech. Available from: http://www.uzis.cz/en/publications/patient-care-2013
21. National Institute of Public Health (SZU). [Selected infectious diseases in the Czech Republic in the years 2006-2015 - relatively]. Prague: SZU. Czech. [Accessed 26 Jul 2015]. Available from: http://www.szu.cz/publikace/data/vybrane-infekcni-nemoci-v-cr-v-letech-2006-2015-relativne
22. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for Clostridium difficile infection.Clin Microbiol Infect. 2014;20(Suppl 2):1-26. DOI: 10.1111/1469-0691.12418 PMID: 24118601
23. Planche TD, Davies KA, Coen PG, Finney JM, Monahan IM, Morris KA, et al. Differences in outcome according to Clostridium difficile testing method: a prospective multicentre diagnostic validation study of C difficile infection. Lancet Infect Dis. 2013;13(11):936-45. DOI: 10.1016/S1473-3099(13)70200-7 PMID: 24007915
24. Krůtová M, Nyč O. [Diagnosis of infections caused by Clostridium difficile in the Czech Republic: availability, possibilities, and interpretation of laboratory results]. Epidemiol Mikrobiol Imunol. 2015;64(2):92-7. Czech.PMID: 26099613
25. Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, et al. A case of imported Clostridium difficile PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of C. difficile ribotype 176. Anaerobe. 2014;30:153-5. DOI: 10.1016/j.anaerobe.2014.09.020 PMID: 25300750
26. Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, et al. , Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study Group. Pan-European longitudinal surveillance of antibiotic resistance among prevalent Clostridium difficile ribotypes.Clin Microbiol Infect. 2015;21(3):248.e9-16. DOI: 10.1016/j.cmi.2014.09.017 PMID: 25701178
27. Nyc O, Krutova M, Liskova A, Matejkova J, Drabek J, Kuijper EJ. The emergence of Clostridium difficile PCR-ribotype 001 in Slovakia.Eur J Clin Microbiol Infect Dis. 2015;34(8):1701-8. DOI: 10.1007/s10096-015-2407-9 PMID: 25981433
28. Arvand M, Vollandt D, Bettge-Weller G, Harmanus C, Kuijper EJ, Clostridium difficile study group Hesse. Increased incidence of Clostridium difficile PCR ribotype 027 in Hesse, Germany, 2011 to 2013.Euro Surveill. 2014;19(10):20732. DOI: 10.2807/1560-7917.ES2014.19.10.20732 PMID: 24650866
29. Vanek J, Hill K, Collins J, Berrington A, Perry J, Inns T, et al. Epidemiological survey of Clostridium difficile ribotypes in the North East of England during an 18-month period. J Hosp Infect. 2012;81(3):209-12. DOI: 10.1016/j.jhin.2012.04.017 PMID: 22633277
30. Wiuff C, Brown DJ, Mather H, Banks AL, Eastaway A, Coia JE. The epidemiology of Clostridium difficile in Scotland.J Infect. 2011;62(4):271-9. DOI: 10.1016/j.jinf.2011.01.015 PMID: 21300104
31. Alcalá L, Martín A, Marín M, Sánchez-Somolinos M, Catalán P, Peláez T, et al. , Spanish Clostridium difficile Study Group. The undiagnosed cases of Clostridium difficile infection in a whole nation: where is the problem?Clin Microbiol Infect. 2012;18(7):E204-13. DOI: 10.1111/j.1469-0691.2012.03883.x PMID: 22563775
32. Lachowicz D, Pituch H, Obuch-Woszczatyński P. Antimicrobial susceptibility patterns of Clostridium difficile strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012.Anaerobe. 2015;31:37-41. DOI: 10.1016/j.anaerobe.2014.09.004 PMID: 25242196

33. Krutova M, Matejkova J, Tkadlec J, Nyc O. Antibiotic profiling of Clostridium difficile ribotype 176--A multidrug resistant relative to C. difficile ribotype 027.Anaerobe. 2015;36:88-90. DOI: 10.1016/j.anaerobe.2015.07.009 PMID: 26256807

34. Spigaglia P, Barbanti F, Mastrantonio P, European Study Group on Clostridium difficile (ESGCD). Multidrug resistance in European Clostridium difficile clinical isolates.J Antimicrob Chemother. 2011;66(10):2227-34. DOI: 10.1093/jac/dkr292 PMID: 21771851

35. European Centre for Disease Prevention and Control (ECDC). European Surveillance of Clostridium difficile infections. Surveillance protocol version 2.2. Stockholm: ECDC; 2015. Available from http:// http://ecdc.europa.eu/en/publications/Publications/European-surveillance-clostridium-difficile.v2FINAL.pdf

License and copyright

Příloha 5

Krutova M, Nyc O, Matejkova J, Allerberger F, Wilcox MH, Kuijper EJ. Molecular characterisation of Czech *Clostridium difficile* isolates collected in 2013-2015. Int J Med Microbiol. 2016;306(7):479-485.

V článku jsou popsány výsledky typizace 2201 českých izolátů *C. difficile* pomocí PCR-ribotypizace a multiplexové PCR reakce na průkaz genů pro produkci toxinů (A, B, binární). Vybrané izoláty různých ribotypizačních profilů byly následně typovány pomocí multilokusové sekvenační analýzy.

International Journal of Medical Microbiology 306 (2016) 479–485

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

j ournal homepage: www.elsevier.com/locate/ijmm

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

M. Krutova[a,b,*], O. Nyc[a], J. Matejkova[a], F. Allerberger[c], M.H. Wilcox[d], E.J. Kuijper[e]

[a] *Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic*
[b] *DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic*
[c] *Austrian Agency for Health and Food Safety (AGES), Wien, Austria*
[d] *Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom*
[e] *Leiden University Medical Centre, Leiden, The Netherlands*

a r t i c l e i n f o

Article history:
Received 6 June 2016
Received in revised form 27 July 2016
Accepted 31 July 2016

Keywords:
Clostridium difficile
Capillary electrophoresis ribotyping
Molecular typing
Toxin genes
MLST
tcdC

a b s t r a c t

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 *C. difficile* isolates from 32 Czech hospitals were collected for molecular characterisation. Of 2201 *C. difficile* isolates, 177 (8%) were non-toxigenic, 2024 (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 2201 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%).

1. 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), 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 2201 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 to 2015.

2. Material and methods

2.1. C. difficile *strain collection*

Microbiology laboratories in 32 Czech healthcare facilities (7 tertiary care hospitals, 24 secondary care hospitals and 1 special-

* Corresponding author at: Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital in Motol, V Uvalu 84, 150 06 Prague 5, Czech Republic.
E-mail address: marcela.krutova@seznam.cz (M. Krutova).

http://dx.doi.org/10.1016/j.ijmm.2016.07.003
1438-4221/

ized 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 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 2201 *C. difficile* isolates was received or cultured at the department of Medical Microbiology of Motol University Hospital and characterised by molecular methods.

2.2. Molecular characterisation of C. difficile strain collection

2.2.1. 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).

2.2.2. 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 = 2201) 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).

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

2.3.1. 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).

2.3.2. 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).

2.3.3. 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.

3. Results

A total of 2201*C. difficile* isolates was collected from 32 hospitals from 2013 to 2015. The geographical distribution of participating hospitals and the number of *C. difficile* isolates available for molecular characterization is shown in Fig. 1. The mean age of patients was 65.7 years (range 30 days – 97 years). Of 2201 isolates, 82 (3.7%), 103 (4.7%), 509 (23.1%) and 1507 (68.5%) were from patients aged ≤2, 3–18, 19–64 and ≥65 years, respectively.

3.1. Ribotyping and the presence of toxin genes

Of 2201*C. difficile* isolates, CE-ribotyping revealed 53 profiles in 2088 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 1841 (83.7%) of all isolates (n = 2201). 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 1507 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 2201*C. difficile* isolates, 2024 (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: 1454 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 the group of patients two years old and younger was 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 patients 19–64 years was 35.5% (14.7%, 20.8%) and in the 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 Fig. 1, and its distribution according to patient age in Fig. 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

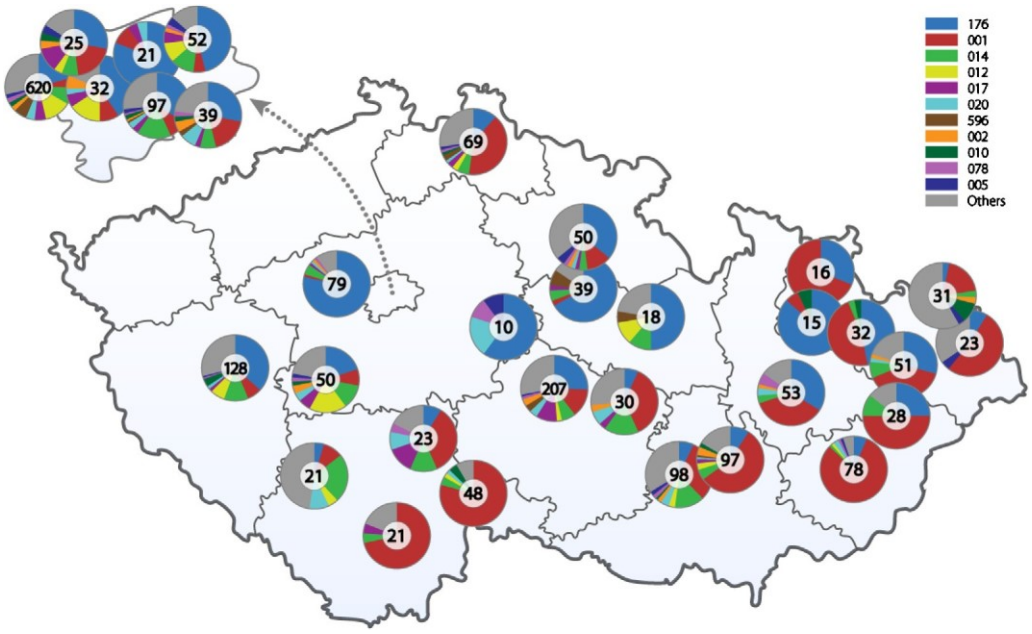


Fig. 1. Distribution of participating hospitals in the study. Pie charts show the most common CE-ribotyping profiles identified per hospital. The numbers in the centre represent number of *C. difficile* isolates sent for molecular characterisation.

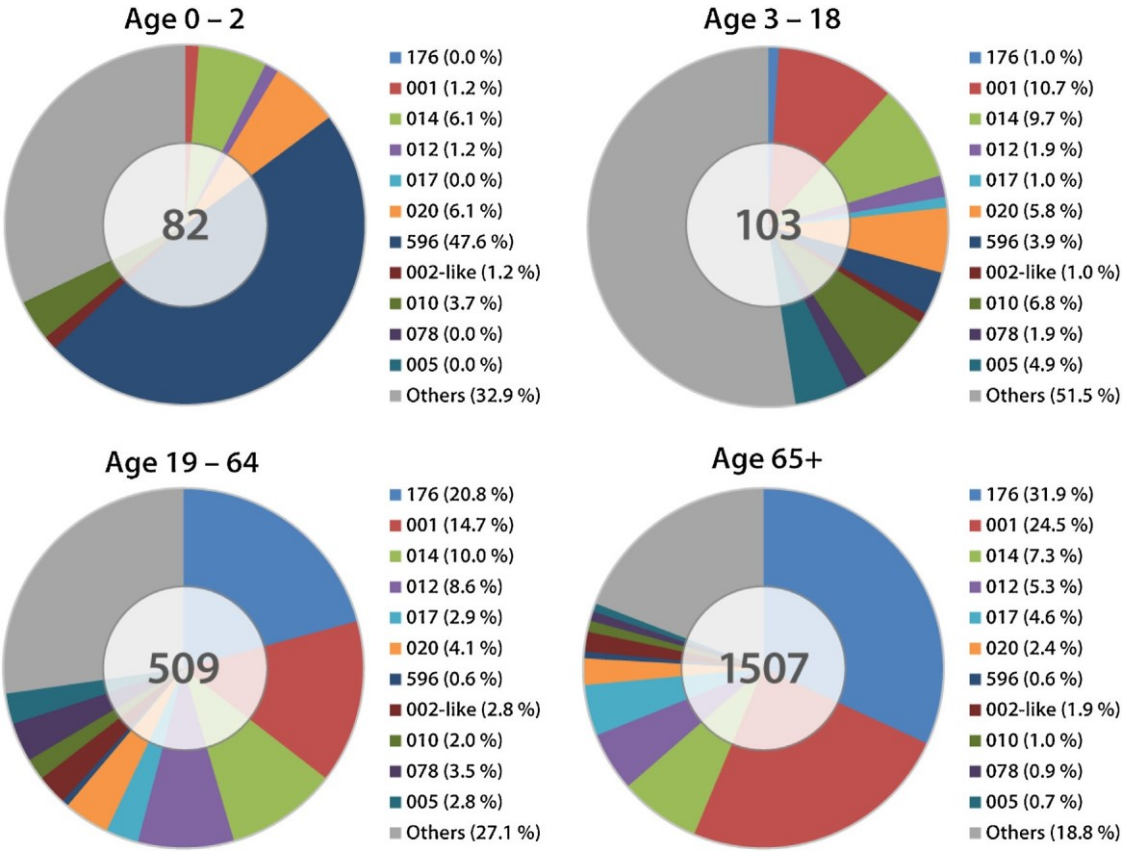


Fig. 2. Distribution of commonest CE-ribotyping profiles depending on the age of patients.

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

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[a]	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[b](2)	588 (26.7)	30	0	1 (0.1)	106 (4.8)	481 (21.9)
001	A, B	3[b](1)	456 (20.7)	30	1 (0.1)	11 (0.5)	75 (3.4)	369 (16.8)
014	A, B	2[b](1)	176 (8.0)	26	5 (0.2)	10 (0.5)	51 (2.3)	110 (5.0)
012	A, B	54[b](1)	127 (5.8)	14	1 (0.1)	2 (0.1)	44 (2.0)	80 (3.6)
017	A, B	37[b](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[b](1)	35 (1.6)	13	3 (0.1)	7 (0.3)	10 (0.5)	15 (0.7)
078	A, B, Bin	11[b](5)	34 (1.6)	15	0	2 (0.1)	18 (0.8)	14 (0.7)
005	A, B	6[b] (1)	30 (1.4)	14	0	5 (0.2)	14 (0.7)	11 (0.5)
029	A, B	16[b] (1)	27 (1.2)	13	0	4 (0.2)	7 (0.3)	16 (0.7)
070	A, B	55[b] (1)	26 (1.2)	14	1 (0.1)	3 (0.1)	5 (0.2)	17 (0.8)
023	A, B, Bin	5[b] (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[b] (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[b] (1)	15 (0.7)	6	0	1 (0.1)	4 (0.2)	10 (0.5)
018	A, B	17[b] (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[b] (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[b] (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[b] (1)	8 (0.4)	5	1 (0.1)	1 (0.1)	2 (0.1)	4 (0.2)
031	non-toxigenic	29[b] (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[b] (1)	5 (0.2)	4	0	0	2 (0.1)	3 (0.1)
027	A, B, Bin	1[b] (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[b] (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[b] (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[b] (1)	2 (0.1)	1	0	0	0	2 (0.1)
053	A, B	63[b] (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)

(ST: sequence type; tcdA/B: genes for toxin A/B production; cdtA/B: genes for binary toxin production; tcdC: toxin gene expression negative regulator).
MLST and *tcdC* sequencing were performed in representative isolates of each CE-ribotyping profile (n = 53). Knetsch et al. identified STs-RTs are marked with b.
[a] 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 (Persson et al., 2008, 2009).

the Supplementary material (UPGMA, Supplementary material – Molecular data on Czech *C. difficile* strain collection).

3.2. 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, Fig. 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

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

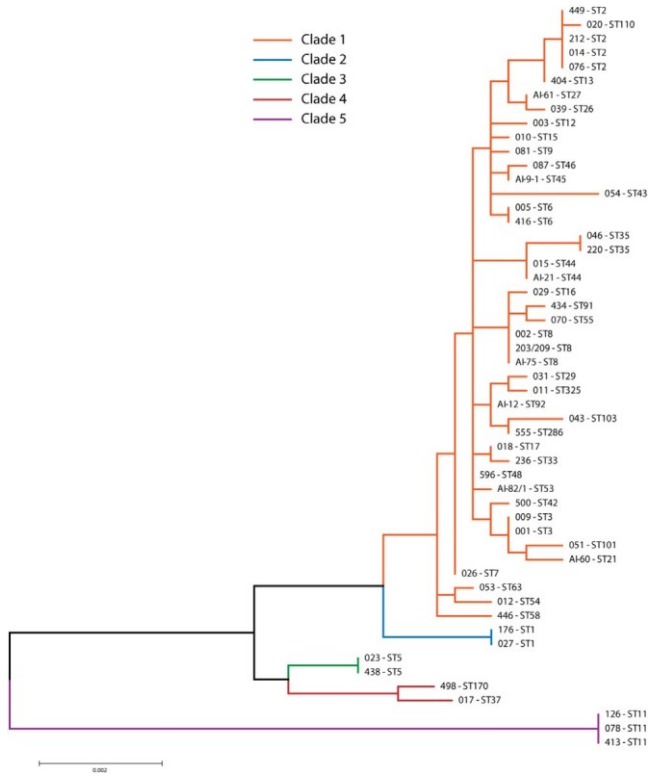


Fig. 3. Maximum likelihood tree generated using alignment of concatenated DNA sequences of seven housekeeping loci (ribotype or WEBRIBO type-sequence type).

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.

4. 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 2201 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 identi- fied only five isolates in four different hospitals over three years; however, hospitals from border areas with Germany and Poland (Fig. 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; Wiuff 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 al., 2013; Indra et al., 2009; Goldová 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 of 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 years), the non-toxigenic and toxigenic isolates ratio decreases (27:76, 33:476 and 53: 1454), 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 of authors von Müller et al., where RT 027 was not present and RT 001 was present in 9.6% in the group of patients 0–17 years and these ratios increased to 30.7% for RT 027 and 38.6% for RT 001 in the 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 b) (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 18bp deletions in the *tcdC* gene. RT 023 and WRT 438 revealed ST5 (clade 3) and had 54bp deletions in the *tcdC* gene. RTs 078, 126 and WRT 413 showed ST11 and 39bp deletions in the *tcdC* gene. Isolates harbouring 54bp and 39bp 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 2008–4.4 cases in 2011–2012 and 6.2 cases per 10,000 patient bed-days in 2012–2013) (Bauer et al., 2011; 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.

5. Conclusion

The molecular characterisation of 2201 Czech clinical *C. difficile* isolates revealed 53 different CE-ribotyping profiles and 40 multi-locus sequence types. Of 2201 *C. difficile* isolates, 2024 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.

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.

Funding

Supported by the MH CZ – DRO, University Hospital Motol, Prague, Czech Republic 00064203.

Acknowledgements

We thank the ESCMID Study Group for *Clostridium difficile* (ESGCD) for their professional support.

We would like to thank Dr James Partridge for proofreading the text.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2016.07.003.

References

Arvand, M., Vollandt, D., Bettge-Weller, G., Harmanus, C., Kuijper, E.J., Clostridium difficile study group Hesse, 2014. Increased incidence of *Clostridium difficile* PCR ribotype 027 in Hesse, Germany, 2011–2013. Euro Surveill. 19 (10), pii: 20732.

Bauer, M.P., Notermans, D.W., van Benthem, B.H., Brazier, J.S., Wilcox, M.H., Rupnik, M., et al., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377, 63–73.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B., Petit, J.C., 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. 175, 261–266.

Curry, S.R., Marsh, J.W., Muto, C.A., O'Leary, M.M., Pasculle, A.W., Harrison, L.H., 2007. tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J. Clin. Microbiol. 45, 215–221.

Davies, K.A., Longshaw, C.M., Davis, G.L., Bouza, E., Barbut, F., Barna, Z., Delmée, M., Fitzpatrick, F., Ivanova, K., Kuijper, E., Macovei, I.S., Mentula, S., Mastrantonio, P., von Müller, L., Oleastro, M., Petinaki, E., Pituch, H., Norén, T., Nováková, E., Nyč, O., Rupnik, M., Schmid, D., Wilcox, M.H., 2014. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect. Dis. 14 (12), 1208–1219.

Debast, S.B., Bauer, M.P., Kuijper, E.J., European Society of Clinical Microbiology and Infectious Diseases, 2014. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. Clin. Microbiol. Infect. 20 (Suppl. 2), 1–26.

Dingle, K.E., Griffiths, D., Didelot, X., Evans, J., Vaughan, A., Kachrimanidou, M., et al., 2011. Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity. PLoS One 6, e19993.

Drabek, J., Nyc, O., Krutova, M., Stovicek, J., Matejkova, J., Keil, R., 2015. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study. Ann. Clin. Microbiol. Antimicrob. 14, 55.

European Centre for Disease Prevention and Control. 2015. European Surveillance of *Clostridium difficile* infections. Surveillance protocol version 2.2 Stockholm: ECDC.

Eyre, D.W., Fawley, W.N., Best, E.L., Griffiths, D., Stoesser, N.E., Crook, D.W., Peto, T.E., Walker, A.S., Wilcox, M.H., 2013. Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. J. Clin. Microbiol. 51 (12), 4141–4149.

Fang, W.J., Jing, D.Z., Luo, Y., Fu, C.Y., Zhao, P., Qian, J., Tian, B.R., Chen, X.G., Zheng, Y.L., Zheng, Y., Deng, J., Zou, W.H., Feng, X.R., Liu, F.L., Mou, X.Z., Zheng, S.S., 2014. *Clostridium difficile* carriage in hospitalized cancer patients: a prospective investigation in eastern China. BMC Infect. Dis. 14, 523.

Fawley, W.N., Knetsch, C.W., MacCannell, D.R., Harmanus, C., Du, T., Mulvey, M.R., Paulick, A., Anderson, L., Kuijper, E.J., Wilcox, M.H., 2015. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One 10, e0118150.

Freeman, J., Vernon, J., Morris, K., Nicholson, S., Todhunter, S., Longshaw, C., Wilcox, M.H., Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study Group, 2015. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin. Microbiol. Infect. 21 (3), 248.e9–248.e16.

Gerding, D.N., Johnson, S., Rupnik, M., Aktories, K., 2014. *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance. Gut Microbes 5 (1), 15–27.

Goldová, J., Malinová, A., Indra, A., Vítek, L., Branny, P., Jirásková, A., 2012. *Clostridium difficile* in piglets in the Czech Republic. Folia Microbiol. 57, 159–161.

Griffiths, D., Fawley, W., Kachrimanidou, M., Bowden, R., Crook, D.W., Fung, R., Golubchik, T., Harding, R.M., Jeffery, K.J., Jolley, K.A., Kirton, R., Peto, T.E., Rees, G., Stoesser, N., Vaughan, A., Walker, A.S., Young, B.C., Wilcox, M., Dingle, K.E., 2010. Multilocus sequence typing of *Clostridium difficile*. J. Clin. Microbiol. 48, 770–778.

He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D.J., Martin, M.J., Connor, T.R., Harris, S.R., Fairley, D., Bamford, K.B., D'Arc, S., Brazier, J., Brown, D., Coia, J.E., Douce, G., Gerding, D., Kim, H.J., Koh, T.H., Kato, H., Senoh, M., Louie, T., Michell, S., Butt, E., Peacock, S.J., Brown, N.M., Riley, T., Songer, G., Wilcox, M., Pirmohamed, M., Kuijper, E., Hawkey, P., Wren, B.W., Dougan, G., Parkhill, J., Lawley, T.D., 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat. Genet. 45 (1), 109–113.

Hell, M., Permoser, M., Chmelizek, G., Kern, J.M., Maass, M., Huhulescu, S., Indra, A., Allerberger, F., 2011. *Clostridium difficile* infection: monoclonal or polyclonal genesis? Infection 39, 461–465.

Indra, A., Huhulescu, S., Schneeweis, M., Hasenberger, P., Kernbichler, S., Fiedler, A., Wewalka, G., Allerberger, F., Kuijper, E.J., 2008. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J. Med. Microbiol. 57, 1377–1382.

Indra, A., Lassnig, H., Baliko, N., Much, P., Fiedler, A., Huhulescu, S., Allerberger, F., 2009. *Clostridium difficile*: a new zoonotic agent? Wien. Klin. Wochenschr. 121, 91–95.

Indra, A., Schmid, D., Huhulescu, S., Simons, E., Hell, M., Stickler, K., Allerberger, F., Austrian C. difficile Study Group, 2015. *Clostridium difficile* ribotypes in Austria: a multicenter, hospital-based survey. Wien. Klin. Wochenschr. 127, 587–593.

Janezic, S., Zidaric, V., Pardon, B., Indra, A., Kokotovic, B., Blanco, J.L., Seyboldt, C., Diaz, C.R., Poxton, I.R., Perreten, V., Drigo, I., Jiraskova, A., Ocepek, M., Weese, J.S., Songer, J.G., Wilcox, M.H., Rupnik, M., 2014. International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. BMC Microbiol. 14, 173.

Knetsch, C.W., Terveer, E.M., Lauber, C., Gorbalenya, A.E., Harmanus, C., Kuijper, E.J., Corver, J., van Leeuwen, H.C., 2012. Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages. Infect. Genet. Evol. 12, 1577–1585.

Knetsch, C.W., Lawley, T.D., Hensgens, M.P., Corver, J., Wilcox, M.W., Kuijper, E.J., 2013. Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections. Euro Surveill. 18, 20381.

Krutova, M., Matejkova, J., Nyc, O., 2014a. *C. difficile* ribotype 027 or 176? Folia Microbiol. 59, 523–526.

Krutova, M., Nyc, O., Kuijper, E.J., Geigerova, L., Matejkova, J., Bergerova, T., Arvand, M., 2014b. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe 30, 153–155.

Mentula, S., Laakso, S., Lyytikäinen, O., Kirveskari, J., 2015. Differentiating virulent 027 and non-027 *Clostridium difficile* strains by molecular methods. Expert Rev. Mol. Diagn. 15 (9), 1225–1229.

Novak, A., Rubic, Z., Dogas, V., Goic-Barisic, I., Radic, M., Tonkic, M., 2015. Antimicrobial susceptibility of clinically isolated anaerobic bacteria in a University Hospital Centre Split, Croatia in 2013. Anaerobe 31, 31–36.

Nyc, O., Krutova, M., Liskova, A., Matejkova, J., Drabek, J., Kuijper, E.J., 2015. The emergence of *Clostridium difficile* PCR-ribotype 001 in Slovakia. Eur. J. Clin. Microbiol. Infect. Dis. 34 (8), 1701–1708.

Persson, S., Torpdahl, M., Olsen, K.E., 2008 and 2009. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect. 14: 1057-64. Erratum in: Clin Microbiol Infect. 15: 296.

Pituch, H., Obuch-Woszczatyński, P., Lachowicz, D., Wultańska, D., Karpiński, P., Młynarczyk, G., van Dorp, S.M., Kuijper, E.J., Polish Clostridium difficile Study Group, 2015. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011–2013. Euro Surveill. 20, 38.

Polivkova, S., Krutova, M., Petrlova, K., Benes, J., Nyc, O., 2016. *Clostridium difficile* ribotype 176 – a predictor for high mortality and risk of nosocomial spread? Anaerobe 40, 35–40.

Rafila, A., Indra, A., Popescu, G.A., Wewalka, G., Allerberger, F., Benea, S., Badicut, I., Aschbacher, R., Huhulescu, S., 2014. Occurrence of *Clostridium difficile* infections due to PCR ribotype 027 in Bucharest, Romania. J. Infect. Dev. Ctries. 8, 694–698.

Rupnik, M., 2010. *Clostridium difficile* toxinotyping. Methods Mol. Biol. 646, 67–76.

Schneeberg, A., Neubauer, H., Schmoock, G., Baier, S., Harlizius, J., Nienhoff, H., Brase, K., Zimmermann, S., Seyboldt, C., 2013. *Clostridium difficile* genotypes in piglet populations in Germany. J. Clin. Microbiol. 51, 3796–3803.

Spigaglia, P., Mastrantonio, P., 2002. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J. Clin. Microbiol. 40, 3470–3475.

Stabler, R.A., Dawson, L.F., Valiente, E., Cairns, M.D., Martin, M.J., Donahue, E.H., Riley, T.V., Songer, J.G., Kuijper, E.J., Dingle, K.E., Wren, B.W., 2012. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS One 7 (3), e31559.

Stubbs, S.L., Brazier, J.S., O'Neill, G.L., Duerden, B.I., 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J. Clin. Microbiol. 37, 461–463.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Taori, S.K., Wroe, A., Hardie, A., Gibb, A.P., Poxton, I.R., 2014. A prospective study of community-associated *Clostridium difficile* infections: the role of antibiotics and co-infections. J. Infect. 69 (2), 134–144.

Valiente, E., Dawson, L.F., Cairns, M.D., Stabler, R.A., Wren, B.W., 2012. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J. Med. Microbiol. 61 (Pt 1), 49–56.

van den Berg, R.J., Schaap, I., Templeton, K.E., Klaassen, C.H., Kuijper, E.J., 2007. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J. Clin. Microbiol. 45 (3), 1024–1028.

von Müller, L., Mock, M., Halfmann, A., Stahlmann, J., Simon, A., Herrmann, M., 2015. Epidemiology of *Clostridium difficile* in Germany based on a single center long-term surveillance and German-wide genotyping of recent isolates provided to the advisory laboratory for diagnostic reasons. Int. J. Med. Microbiol. 305 (7), 807–813.

Wiuff, C., Brown, D.J., Mather, H., Banks, A.L., Eastaway, A., Coia, J.E., 2011. The epidemiology of *Clostridium difficile* in Scotland. J. Infect. 62 (4), 271–279.

Příloha 6

Krutova M, Nyc O, Matejkova J, Kuijper E, Jalava J, Mentula S. The recognition and characterisation of *Finnish Clostridium difficile* isolates resembling PCR-ribotype 027. Přijato k publikaci v Journal of Microbiology, Immunology and Infection dne 14.3. 2017.

Ve spolupráci s National Institute for Health and Welfare, Finland, Helsinki jsme provedli detailní analýzu 28 finských izolátů *C. difficile*, které byly vybrány z finské národní sbírky pro svoji genetickou podobnost s epidemickým ribotypem 027 (přítomnost genů pro binární toxin a 18bp delece v regulačním genu *tcdC*). Mezi 28 izoláty jsme identifikovali 12 různých ribotypizačních profilů a 11 sekvenačních typů. Analýza variabilních oblastí genomu potvrdila regionální klonální šíření u izolátů třech ribotypů a také u izolátů ribotypu 027, které byly zařazeny jako kontrolní kmeny. Navíc 22 izolátů osmi různých ribotypů neslo deleci v pozici 117 genu *tcdC*, která je cílovým místem některých komerčních systémů pro odlišení ribotypu 027 od ostatních ribotypů.

Original article

The recognition and characterisation of Finnish *Clostridium difficile* isolates resembling PCR-ribotype 027

Marcela Krutova[1, 2], Otakar Nyc[1], Jana Matejkova[1], Ed J. Kuijper[3], Jari Jalava[4], Silja Mentula[4]

1. Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic

2. DNA laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Czech Republic

3. Leiden University Medical Centre, Leiden, the Netherlands

4. Bacterial Infections Unit, Department of Infectious Diseases, National institute for Health and Welfare, Helsinki, Finland

Running title: *C. difficile* isolates resembling PCR-ribotype 027

Address of corresponding author:

Marcela Krutova, Department of Medical Microbiology, Charles University, 2nd Faculty of Medicine and Motol University Hospital, V Uvalu 84, Prague 5, 150 06, Czech Republic, Tel: +420 732 532 499, email: marcela.krutova@seznam.cz

Abstract

Purpose: To characterise and compare twenty-eight Finnish *C. difficile* RT027-like isolates, selected based on the presence of 18bp deletion in the *tcdC* gene and toxin gene profile (A, B, binary), with eleven RT027 isolates from different Finnish geographical areas and time periods.

Methods: Twenty-eight *C. difficile* RT027-like isolates and 11 RT027 comparative strains were characterised by capillary-electrophoresis (CE) ribotyping, multi-locus variable tandem-repeats analysis (MLVA), multi-locus sequence typing (MLST), and sequencing of *tcdC* and *gyrA* gene fragments. Susceptibility to moxifloxacin was determined by E-test.

Results: Of 28 RT027-like isolates, seven RTs (016, 034, 075, 080, 153, 176 and 328), three WEBRIBO types (411, 475, AI-78) and three new profiles (F1-F3) were identified. MLVA revealed six clonal complexes (RTs 016, 027, 176 and F3). MLST showed eleven sequence types (1, 41, 47, 67, 95, 191,192, 223, 229, 264 and new ST). Twenty-two isolates (RTs 016, 080, 176, 328, F1, F2, F3 and WRTAI-78) carried Δ117 in the *tcdC* gene. Isolates of RTs 016, 027 and 176 were moxifloxacin resistant and harboured Thr82Ile in the GyrA.

Conclusion: Our results show a high diversity within 28 Finnish RT027-like *C. difficile* isolates, with twelve CE-ribotyping profiles and eleven STs. MLVA revealed the regional spread of RTs 016, 027, 176 and F3. The presence of Δ117 in the *tcdC* gene in eight non-027 RTs highlights the importance of careful interpretation of the results from molecular systems targeting this site in the genome of *C. difficile* and the need of strain typing for epidemiological purposes.

Keywords: Capillary-electrophoresis ribotyping; *Clostridium difficile*; MLST; MLVA; moxifloxacin resistance.

Introduction

Clostridium difficile is the leading pathogen of hospital acquired diarrhoea and PCR-ribotype 027/NAP1/B1 is the most notorious "hypervirulent" ribotype.[1] Its route of global spread was traced by phylogeographic analysis of whole genome sequencing data indicating that two distinct epidemic lineages of *C. difficile* RT027 (FQR1 and FQR2) emerged in North America and separately acquired fluoroquinolone resistance and a conjugative transposon. The FQR2 lineage spread to the UK, continental Europe and Australia.[2]

CE-ribotyping is currently the recommended standard for characterization of *C. difficile* isolates.[3] Based on slight variations in ribotyping banding patterns, three RTs (176, 198 and 244) have been determined as closely related to RT027. [4] Interestingly, RT176 is associated with outbreaks in the Czech Republic[5] and Poland[6], whereas RT244 emerged in Australia.[7] Recently, other RT027-like ribotypes (016, 036) have been reported that belong to the same multi-locus sequence type (ST1) as RT027.[8]

Finland is a country with a high CDI testing frequency (124.3 tests in 2011 - 2012 and 223.3 tests per 10,000 patient bed-days in 2012 - 2013) and corresponding high CDI incidence (14.9 cases in 2011 - 2012 and 28.7 cases per 10,000 patient bed-days in 2012 - 2013).[9] In Finland, a national free of charge ribotyping service has been available for clinical microbiology laboratories since 2008. This service is mainly for *C. difficile* strains from patients with severe course of *C. difficile* infection (CDI) and for supporting the management of local CDI outbreaks. During 2008 - 2015, a total of 1771 isolates, representing 4.1% of notified CDI cases (0.2 - 5.9% range by year) were sent to the Finnish national reference laboratory for molecular characterisation. Of 1771 typed *C. difficile* isolates representing 146 different RTs by gel-based ribotyping[10], 662 (37%) tested positive for binary toxin genes belonged to 28 different RTs. Of these 662 isolates, 344 were RT027 (harboured 18bp deletion), 253 had 39/54bp deletion, 37 had no deletion, and 28 isolates showed similar molecular characteristics to RT027 (contained genes for toxins A, B and binary toxin and had an 18bp deletion in the *tcdC* gene).

The aim of the study was to characterise and compare twenty-eight Finnish RT027-like *C. difficile* isolates with eleven RT027 isolates from different Finnish geographical areas and time periods.

Material and Methods

Strain collection

Twenty-eight *tcdA* (toxin A), *tcdB* (toxin B) and *cdtA, cdtB* (binary toxin) positive *C. difficile* isolates also containing 18bp deletion in the *tcdC* gene were identified at the Finnish national reference laboratory. Eleven Finnish *C. difficile* RT027 isolates from different Finnish geographical areas and time periods were included as comparative strains. CE-ribotyping, confirmation of toxin gene profiles, MLVA, MLST and susceptibility testing were performed at the Department of Medical Microbiology, Motol University Hospital, Czech Republic.

Capillary electrophoresis-ribotyping

DNA was extracted using Chelex-100 resin (Bio-Rad). Amplification of the 16S-23S intergenic spacer regions was performed according to the consensus CE-ribotyping protocol[3] with Bidet primers.[11] Fragment analysis was carried out using ABI 3130 with default settings for POP7 and 36cm capillary length. LIZ 1200 was used as a size standard. The electrophoretic profiles were compared with the ECDC-Leeds-Leiden *C. difficile* reference dataset

in the Leiden University Medical Centre, the Netherlands. The raw data obtained (*fsa files) were also uploaded to the freely available WEBRIBO database (https://webribo.ages.at/)[12] to compare with profiles present in the database.

The presence of toxin genes and *tcdC* gene fragment sequencing

The presence of genes (*tcdA, tcdB, cdtA, cdtB*) for toxin production (A, B and binary) was investigated by multiplex PCR[13] with visualization of amplified products by agarose-gel electrophoresis. The *tcdC* gene fragment was amplified and sequenced with primers C1 and C2[14], and obtained sequences were compared with NCBI reference sequence NC_009089.1.

Multi-locus variable tandem-repeats analysis

MLVA was performed by sequencing of seven regions with short tandem repeats (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd)[15] with a change of reverse primer for G8Cd loci, as described elsewhere.[16] Minimum spanning tree was created using Bionumerics v5.1 (Applied Maths) by using a Manhattan coefficient to calculate the summed tandem repeat difference (STRD). A clonal complex was defined as an STRD $\leq$ 2. [15]

Multi-locus sequence typing

MLST was performed in 22 non-clonal related isolates (based on results of MLVA) by amplification and sequencing of seven housekeeping genes (*adk, atpA, dxr, glyA, recA, sodA* and *tpi*) previously described.[17] The sequence type (ST) was determined as a combination of alleles identified by comparing obtained sequences with sequences available in the *C. difficile* MLST database available at: http://pubmlst.org/cdifficile/.[17]

Testing of susceptibility to moxifloxacin

Susceptibility to moxifloxacin was determined by E-test strips (Liofilchem, Italy) with gradient antibiotic range from 0.016-32 mg/L on Wilkins Chalgren agar. A breakpoint 4 mg/L for moxifloxacin[18] was applied. The fragment of the *gyrA* gene was amplified and sequenced with primers *gyrA*1 and *gyrA*2[19] and obtained sequences were compared with NCBI reference sequence NC_009089.1.

Results

The summary of results of molecular characterisation of 39 Finnish *C. difficile* isolates (28 RT027-like and 11 RT027 is shown in Table 1.

Capillary electrophoresis-ribotyping

Twelve different CE-ribotyping profiles in twenty-eight RT027-like isolates were observed. Four of these, RTs 016 (n=3), 075 (n=3), 080 (n=1), 176 (n=5), were identically identified by the WEBRIBO database and by the Leeds-Leiden reference *C. difficile* strain dataset. In contrast, one CE-ribotyping profile was recognized by the WEBRIBO database as a WRT475, and by the Leeds-Leiden database as RT034. Two CE-ribotyping profile were only recognized by the Leeds-Leiden database as RT153 and 328. Two CE-ribotyping profiles were only identified by the WEBRIBO database as WRTs AI-78 and 411. The remaining three CE-ribotyping profiles did not match any type in both databases and were designated as F1 (n=1), F2 (n=1), and F3 (n=9). CE-ribotyping profiles and

fragment sizes of new CE-ribotyping profiles are shown in Figure 1. Geographical distribution of isolates with specific CE-ribotyping profiles are depicted in Figure 2.

The presence of toxin genes and *tcdC* sequencing

All 39 isolates (twenty-eight RT027-like isolates and eleven RT027 controls) contained *tcdA, tcdB* and *cdtA, cdtB* genes for production of toxins A, B and binary, respectively. Sequencing of the *tcdC* gene fragment confirmed the presence of 18bp deletion (at position 330-347) in all 39 *C. difficile* isolates. The presence of the single base deletion at position 117 in the *tcdC* gene was observed in 33 isolates (RTs 016, 176, 027, 080, 328, WRTAI-78 and F1, F2, F3). In contrast, RTs 034, 075, 153 and WRT411 carried substitution A>G at position 117 in the *tcdC* gene.

Multi-locus variable tandem-repeats analysis (MLVA)

MLVA revealed six clonal complexes in RTs 016, 027, 176 and F3 (Figure 3). No clonal complexes were found between isolates from different RTs. CC1 included nine isolates of F3 profile (three hospitals, the same region and the same year of culture (2015)). Nine control isolates belonging to RT027 formed three clonal complexes (CC2, CC4 and CC6). CC2 included four isolates from three hospitals collected in the time period 2008-2011. CC4 included three isolates from two hospitals cultured in 2013 and CC6 included two RT027 isolates from two hospitals collected in the time period 2011 and 2012. CC3 included two RT016 isolates from the same hospital and different year of culture (2011, 2012). CC5 included 3 isolates of RT176 from two hospitals and the same year of culture (2008).

Genetic relatedness (defined as STRD≤10), between isolates belonging to the same RT where observed between isolates of RT027 in CC2 (n=4) and CC4 (n=3) STRD=6, between isolates of RT176 (isolate no. 2688 and three isolates in CC5, STRD=3) and between isolates of RT016 (isolate no. 2296 and two isolates in CC3, STRD=6).

Multi-locus sequence typing (MLST)

MLST of seven housekeeping genes, performed in 22 non-clonal related *C. difficile* isolates belonging to thirteen different CE-ribotyping profiles, revealed eleven different sequence types (STs), of which, ten clustered to clade 2 and one (ST191) to clade 1. RTs 016 (n=2), 027 (n=5) and 176 (n=3) belonged to ST1. Other found STs were: ST41 (F1, F3), ST47 (WRT411), ST67 (RT153), ST95 (RT075), ST191 (WRTAI-78), ST192 (RT080), ST223 (RT034), ST229 (RT328) and ST264 (F2) and new ST (RT075, n=2). The alleles profile of new ST in both RT075 isolates was: *adk*=1, *atpA*=5, *dxr*=11, *glyA*=17, *recA*=1, *sodA*=22 *and tpi*=2).

Testing of susceptibility to moxifloxacin

Nineteen *C. difficile* isolates belonging to RTs 016 (n=3), 027 (n=11), 176 (n=5) were moxifloxacin resistant (≥32 mg/L) and also carried amino acid substitution Thr82Ile in the GyrA. Other *C. difficile* isolates (n=20) were moxifloxacin susceptible (MICs=0.016-1.5 mg/L) and were wild types in the sequenced *gyrA* gene fragment.

Discussion

Of 1771 genotyped Finnish *C. difficile* isolates, 662 (37.3%) carried binary toxin genes. Of these, 372 (21.0%) also had an 18bp deletion in the *tcdC* gene. A majority of the isolates (n=344, 19.4%) were RT027. The remaining

28 isolates were considered as RT027-like and represented 1.6% of the Finnish *C. difficile* collection. These twenty-eight RT027-like *C. difficile* isolates belonged to twelve different ribotyping profiles. Among these twelve CE-ribotyping profiles, one was incorrectly identified (RT034 as WRT475) in the WEBRIBO database, two were only typed by Leeds-Leiden database (RT328 and 153) two were identified only in WEBRIBO database (WRTs 411 and AI-78) and three CE-ribotyping profiles were completely new (F1-F3), not present in both databases.

Several CE-ribotyping profiles in the study revealed closer fragment peaks similarity to ribotypes present in the Leeds-Leiden database. The strains F3 has a closest match to RT016, WRT411 to RT375 and WRTAI-78 to RT046. The minor differences of the CE-ribotyping profiles with split of peaks suggest genetic relatedness, but additional differences were also found, such as a different ST (RT016=ST1 and F3=ST41) or different toxin genes profile (RT046 harbour only *tcdA* and *tcdB*) indicating more rearrangements in the *C. difficile* genomes. RTs 080, 034, 153, 328; WRT AI-78, 411 and new CE-ribotyping profiles F1, F2 are represented by only one isolate in the collection and their distribution in Finland and Europe remains unclear.

The occurrence of RT176 has been reported from Czech Republic where it belongs to the prevailing ribotypes[20] and from Poland where it persists with RT027.[6, 21] The clinical relevance of RT176 has been studied in both countries.[21, 22, 23] Interestingly, four out of five Finnish RT176 isolates in this study were sent for molecular typing because of a severe course of CDI. Three RT027-like isolates in the study belonged to RT016. The increased occurrence of RT016 was identified in North East England in 2009-2010.[24]

Twenty-two (78.6%) *C. difficile* RT027-like isolates (RTs 016, 176, 080, 328, WRTAI-78 and F1, F2, F3) had Δ117 in the *tcdC* gene. The Δ117 is used as a target site for differentiating RT027 from other ribotypes, and the presence of Δ117 in the *tcdC* gene in non-RT027 isolates leads to the incorrect RT027 identification by molecular methods.[25, 26, 27] This highlights the need of molecular characterisation of *C. difficile* isolates by ribotyping for CDI surveillance purposes. Interestingly, the Leeds-Leiden reference strain RT080 included for confirmation of MLST result also revealed substitution A>G at position 117 in the *tcdC* gene, which differs from Finnish RT080 *C. difficile* isolate.

As was recently published by Eyre et al., RT244 isolates (n=25) also harboured Δ117 but in the absence of other deletion in the *tcdC* gene[7]. Because the presence of 18bp deletion was one of inclusion criteria for isolates in this study, we might not have recognized isolates with only Δ117 in the *tcdC* gene in the Finnish strain collection.

MLVA revealed six clonal complexes in RTs 016, 027, 176 and F3. Isolates revealing STRD≤10 but belonging to different RTs were not considered as genetically related because the MLVA is suitable as subtyping molecular method in isolates belonging to the same RT.[15, 28] MLVA was firstly used for subtyping of *C. difficile* RT176 isolates in the study Nyc *et al*. [29] where genetically as well as clonal relatedness in ten Czech (n=10) and in eleven Polish isolates was confirmed. Clonal spread of RT176 was also found in two Czech single center studies in 2013[22, 23] and in eleven Czech hospitals from eighteen hospitals involved in the study in 2014.[20] In *C. difficile* RT027 isolates, the MLVA was applied in several studies to determine the genetic relatedness of isolates.[5,28-34]

MLST of seven housekeeping genes revealed eleven different STs. Three RTs (016, 027 and 176) had an identical ST1 as reported earlier.[8] Likewise, RT075 was reported as ST95 and clade 2.[8] Surprisingly, two other RT075 isolates in our study revealed new ST, with nearest match with STs 47, 61, and 95. Both isolates derived from the same hospital but were identified in the different years (2008 and 2010). ST41 and clade 2, identified in one isolate

with new CE-ribotyping profile F1, has also been reported in RTs 156, 208[8] and in RTs 106, 194, 321 together with presence of similar deletions (18bp and Δ117) in the *tcdC* gene.[35] ST67 identified in isolate with new CE-ribotyping profile F4 has been reported in RT019[8, 35] and also with wild type genotype at position 117 in the *tcdC* gene.[35] Additionally, we performed MLST in the ECDC-Leeds-Leiden reference strain RT080 and the same ST192 was observed in both the Leeds-Leiden and Finnish RT080 *C. difficile* isolates.

Only isolates of RTs 027, 016 and 176 were moxifloxacin resistant and carried amino acid substitution Thr82Ile in the GyrA, which has been associated with fluoroquinolone resistance.[19, 36, 37]

In conclusion, the molecular characterisation of twenty-eight *C. difficile* 027-like isolates revealed seven known ribotypes, three WEBRIBO types, three new CE-ribotyping profiles and eleven different sequence types. MLVA revealed outbreaks and regional spread of RTs 016, 027, 176 and F3. Twenty-two non-RT027 *C. difficile* isolates of eight ribotypes showed the presence of Δ117 in the *tcdC* gene, a target site for detection of RT027 by commercial molecular methods that could result in an incorrect identification. These results highlight the importance of careful interpretation of the results from commercial systems targeting this site in the genome of *C. difficile* and the need of strain typing for epidemiological purposes.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Funding

MH CZ – DRO, University Hospital Motol, Prague, Czech Republic 00064203, ECDC-framework service contract ECDC/2016/016 (OJ/05/11/2015-PROC/2015/029).

Acknowledgement

The authors thank the ESCMID Study Group for *Clostridium difficile* (ESGCD) for their professional support. We would also like to thank Céline Harmanus and Arja Kanervo-Nordström for technical assistance, Dr. Warren Fawley for CE-ribotyping profiles evaluation and Dr. Partridge for proofreading of the manuscript.

References:

1. Kuijper EJ, Coignard B, Brazier JS, Suetens C, Drudy D, Wiuff C, et al. Update of *Clostridium difficile*-associated disease due to PCR ribotype 027 in Europe. Euro Surveill 2007; 12(6): E1-2.

2. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet 2013; 45(1):109-13.

3. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et al. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One 2015; 10(2): e0118150.

4. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol 2012; 61(Pt 1):49-56.

5. Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, et al. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe 2014; 30:153-5.

6. Pituch H, Obuch-Woszczatyński P, Lachowicz D, Wultańska D, Karpiński P, Młynarczyk G, et al. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro Surveill 2015; 20(38).

7. Eyre DW, Tracey L, Elliott B, Slimings C, Huntington PG, Stuart RL, et al. Emergence and spread of predominantly community-onset *Clostridium difficile* PCR ribotype 244 infection in Australia, 2010 to 2012. Euro Surveill 2015; 20(10):21059

8. Knetsch CW, Terveer EM, Lauber C, Gorbalenya AE, Harmanus C, Kuijper EJ, et al. Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages. Infect Genet Evol 2012; 12(7):1577-85.

9. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis 2014; 14(12):1208-19.

10. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol 1999; 37(2):461-3.

11. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett 1999; 175(2):261-6.

12. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 2008; 57(Pt 11):1377-82.

13. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect 2008; 14(11):1057-64. Erratum in: Clin Microbiol Infect. 2009;15(3):296

14. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol 2002; 40(9):3470-5.

15. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol 2007; 45(3):1024-8.

16. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, et al. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. Clin Microbiol Infect 2009; 15(12):1080-6.

17. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, et al. Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol 2010; 48(3):770-8.

18. European Committee on Antimicrobial Susceptibility Testing, EUCAST. Clinical breakpoints for bacteria. Available from: http://www.eucast.org/clinical_breakpoints/. Accessed 31 March 2016

19. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC. *gyrA* and *gyrB* mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother 2002; 46(11):3418-21.

20. Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc O; Czech *Clostridium difficile* study group. *Clostridium difficile* PCR ribotypes 001 and 176 - the common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014. Euro Surveill 2016; 21(29).

21. Obuch-Woszczatyński P, Lachowicz D, Schneider A, Mól A, Pawłowska J, Ożdżeńska-Milke E, Pruszczyk P, Wultańska D, Młynarczyk G, Harmanus C, Kuijper EJ, van Belkum A, Pituch H. Occurrence of *Clostridium difficile* PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010. Anaerobe 2014; 28:13-7.

22. Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. *Clostridium difficile* ribotype 176 - A predictor for high mortality and risk of nosocomial spread? Anaerobe 2016; 40:35-40.

23. Drabek J, Nyc O, Krutova M, Stovicek J, Matejkova J, Keil R. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study. Ann Clin Microbiol Antimicrob 2015; 14:55.

24. Vanek J, Hill K, Collins J, Berrington A, Perry J, Inns T, Gorton R, Magee J, Sails A, Mullan A, Gould FK. Epidemiological survey of *Clostridium difficile* ribotypes in the North East of England during an 18-month period. J Hosp Infect 2012; 81(3):209-12.

25. Mentula S, Laakso S, Lyytikäinen O, Kirveskari J. Differentiating virulent 027 and non-027 *Clostridium difficile* strains by molecular methods. Expert Rev Mol Diagn 2015; 15(9):1225-9.

26. Kok J, Wang Q, Thomas LC, Gilbert GL. Presumptive Identification of *Clostridium difficile* Strain 027/NAP1/BI on Cepheid Xpert: Interpret with Caution. *Journal of Clinical Microbiology* 2011; 49(10):3719-3721.

27. Krutova M, Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha) 2014; 59(6):523-6.

28. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, et al. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol 2008; 46(2):431-7.

29. Nyč O, Pituch H, Matějková J, Obuch-Woszczatynski P, Kuijper EJ. *Clostridium difficile* PCR ribotype 176 in the Czech Republic and Poland. Lancet 2011; 377(9775):1407.

30. Fawley WN, Freeman J, Smith C, Harmanus C, van den Berg RJ, Kuijper EJ, et al. Use of highly discriminatory fingerprinting to analyze clusters of *Clostridium difficile* infection cases due to epidemic ribotype 027 strains. J Clin Microbiol 2008; 46(3):954-60.

31. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, et al. Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J Antimicrob Chemother 2008; 62(5):1046-52.

32. Eckert C, Vromman F, Halkovich A, Barbut F. Multilocus variable-number tandem repeat analysis: a helpful tool for subtyping French *Clostridium difficile* PCR ribotype 027 isolates. J Med Microbiol 2011; 60(Pt 8):1088-94.

33. Goorhuis A, Debast SB, Dutilh JC, van Kinschot CM, Harmanus C, Cannegieter SC, et al. Type-specific risk factors and outcome in an outbreak with 2 different *Clostridium difficile* types simultaneously in 1 hospital. Clin Infect Dis 2011; 53(9):860-9.

34. Manzoor SE, Tanner HE, Marriott CL, Brazier JS, Hardy KJ, Platt S, et al. Extended multilocus variable-number tandem-repeat analysis of *Clostridium difficile* correlates exactly with ribotyping and enables identification of hospital transmission. J Clin Microbiol 2011; 49(10):3523-30.

35. Dingle KE, Griffiths D, Didelot X, Evans J, Vaughan A, Kachrimanidou M, et al. *Clinical Clostridium difficile*: clonality and pathogenicity locus diversity. PLoS One 2011; 6(5):e19993.

36. Drudy D, Kyne L, O'Mahony R, Fanning S. gyrA mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027. Emerg Infect Dis 2007; 13(3):504-5.

37. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmée M, et al. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. J Med Microbiol 2008; 57(Pt 6):784-9.

DNA	Year	Sex	Age	City	CE-ribotyping profile	*tcdC p.* 117	GyrA	MOX mg/L	MLVA							MLST
									A6Cd	B7Cd	C6Cd	E7Cd	F3Cd	G8Cd	H9Cd	ST/clade
2656	2011	M	81	Hamina	016	del A	Thr82Ile	≥32	50	19	40	10	5	13	2	1/2
2657	2012	M	77	Hamina	016	del A	Thr82Ile	≥32	50	19	39	10	5	14	2	-
2296	2011	F	70	Hamina	016	del A	Thr82Ile	≥32	51	18	35	10	5	14	2	1/2
2689	2008	F	93	Karhula	027	del A	Thr82Ile	≥32	41	19	37	10	5	14	2	1/2
2692	2009	M	88	Kotka	027	del A	Thr82Ile	≥32	41	19	38	10	5	14	2	-
2694	2010	M	81	Pyhtää	027	del A	Thr82Ile	≥32	42	19	39	10	5	14	2	-
2695	2011	M	72	Jokela	027	del A	Thr82Ile	≥32	29	8	33	10	5	14	2	1/2
2697	2012	M	77	Hyvinkää	027	del A	Thr82Ile	≥32	29	9	33	10	5	14	2	-
2298	2013	M	87	Hamina	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	1/2
2690	2008	M	84	Pori	027	del A	Thr82Ile	≥32	30	8	40	10	5	14	2	1/2
2696	2011	M	65	Kotka	027	del A	Thr82Ile	≥32	42	20	38	10	5	14	2	-
2698	2012	M	77	Seinäjoki	027	del A	Thr82Ile	≥32	19	12	25	10	5	15	2	1/2
2699	2013	N	83	Hamina	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	-
2700	2013	M	72	Kotka	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	-
2307	2015	F	67	Helsinki	034=WRT475	A>G	WT	0.016	31	11	22	7	6	15	2	223/2
2669	2008	N	20	Oulu	075	A>G	WT	1	28	8	40	7	6	24	2	New ST
2670	2010	N	66	Oulu	075	A>G	WT	1.5	29	9	19	7	6	13	2	New ST
2301	2012	M	64	Kemi	075	A>G	WT	0.016	29	21	30	9	5	9	2	95/2
2302	2013	F	52	Muhos	080	del A	WT	0.016	18	29	36	12	6	10	2	192/2
2682	2009	M	82	Seinäjoki	153	A>G	WT	1.5	33	17	31	8	6	8	2	67/2
2686	2008	M	64	Uusikaupunki	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	1/2
2687	2008	F	87	Salo	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	-
2688	2008	N	96	Turku	176	del A	Thr82Ile	≥32	29	8	34	10	5	12	2	1/2
2701	2008	F	87	Salo	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	-
2305	2009	M	70	Helsinki	176	del A	Thr82Ile	≥32	29	9	36	10	5	14	2	1/2
2299	2008	F	88	Turku	328	del A	WT	0.016	47	19	43	10	5	15	2	229/2
2658	2015	N	90	Espoo	New F3	del A	WT	1.5	32	14	27	8	6	13	2	41/2
2659	2015	M	77	Espoo	New F3	del A	WT	1	32	14	27	8	6	13	2	-
2660	2015	N	94	Helsinki	New F3	del A	WT	1	32	14	27	8	5	13	2	-
2661	2015	M	40	Helsinki	New F3	del A	WT	1.5	33	14	27	8	5	13	2	-
2662	2015	N	62	Helsinki	New F3	del A	WT	1	31	14	27	8	6	13	2	-
2663	2015	M	81	Vantaa	New F3	del A	WT	1	32	15	27	8	6	13	2	-
2665	2015	N	90	Espoo	New F3	del A	WT	1.5	33	14	27	8	6	13	2	-
2666	2015	N	85	Espoo	New F3	del A	WT	1.5	32	14	27	8	6	13	2	-
2667	2015	N	90	Espoo	New F3	del A	WT	1	32	14	27	8	6	13	2	-
2297	2013	M	74	Seinäjoki	New F1	del A	WT	0.016	29	9	29	10	5	14	2	41/2
2306	2009	M	82	Kempele	New F2	del A	WT	0.016	21	15	27	2	5	16	2	264/2
2668	2008	N	25	Oulu	WRT411	A>G	WT	1.5	33	17	11	5	6	13	2	47/2
2671	2008	M	60	Helsinki	WRTAI-78	del A	WT	1	32	16	21	7	6	12	2	191/1

Table 1: Molecular characteristic of Finnish C. difficile isolates in the study. WT - wild type, ST - sequence type, MOX - moxifloxacin, MLVA - Multi-Locus Variable Tandem-Repeats analysis, MLST- Multi-Locus Sequence Typing, WRT – WEBRIBO type.

Figure 1: new CE-ribotyping profiles identified in the study. Band sizes in base pairs are following: F1– 232, 322, 324, 383, 444, 482, 541, 545; F2 - 232, 284, 324, 444, 536, 543, 545; F3 – 262, 323, 362, 422, 441, 443, 446, 543.

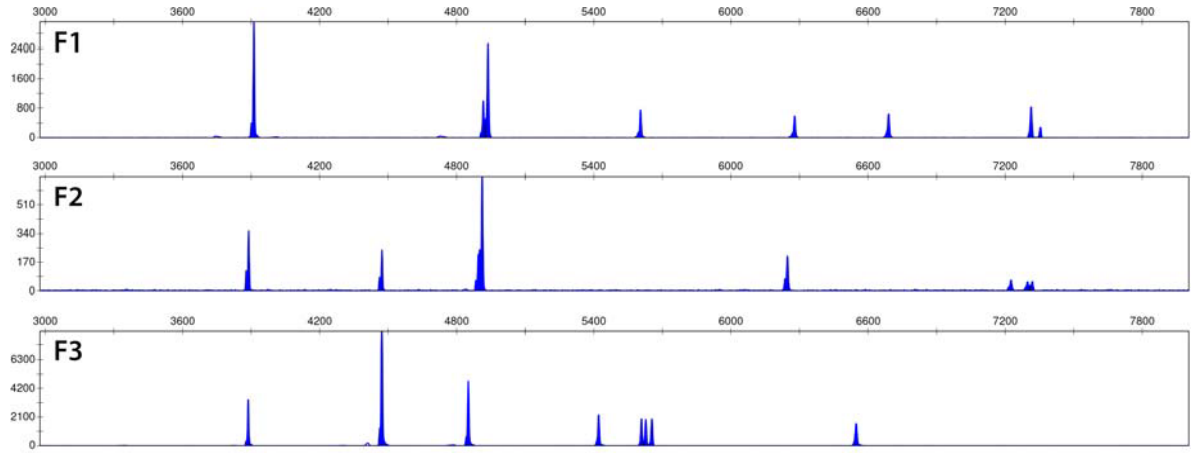


Figure 2: Geographic distribution of *C. difficile* isolates in the study. Each CE-ribotyping profile is represented by different colour.

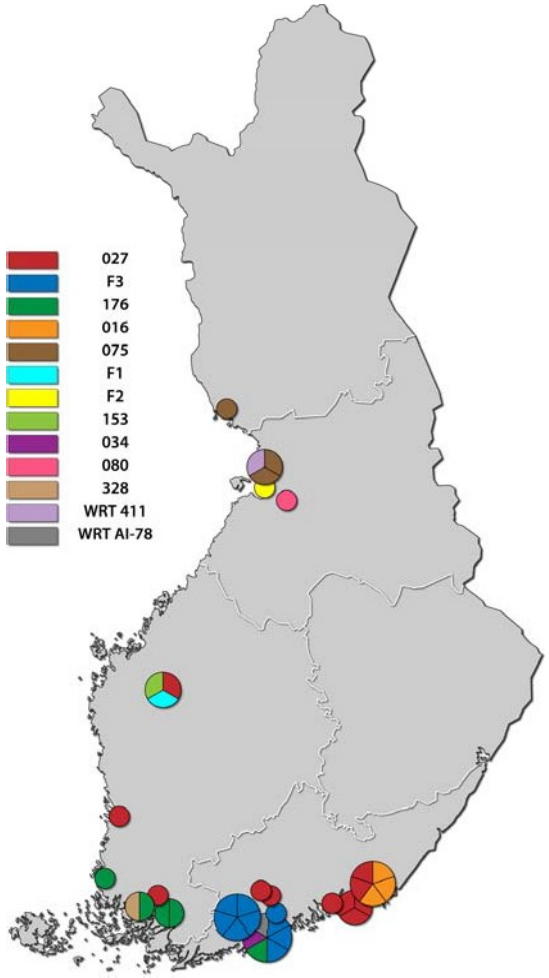
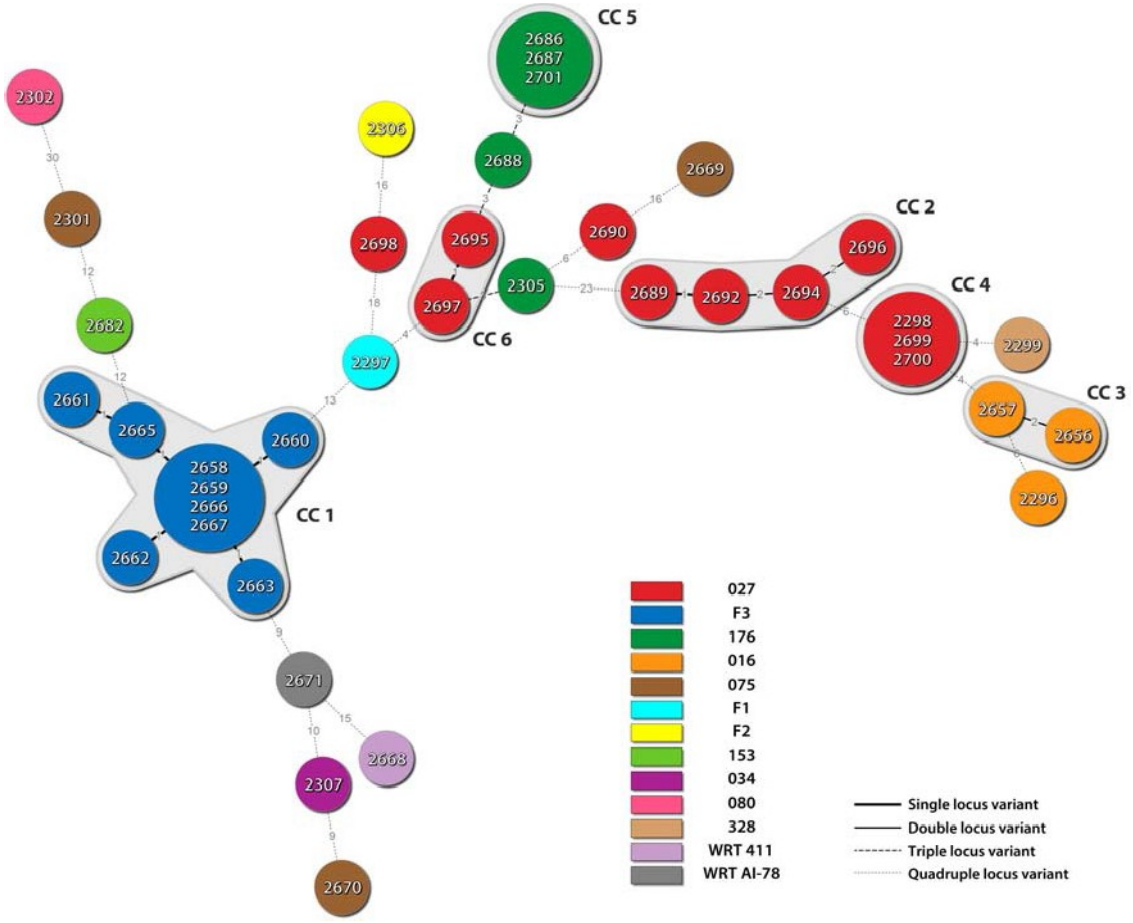


Figure 3: Minimum spanning tree of Finnish *C. difficile* isolates in the study. Each CE-ribotyping profile is represented by different colour. The numbers in the circles represent DNA number of isolate. If the more than one number is present in one circle, it represents isolates with STRD=0 (i.e. 100% identical in seven variable-number tandem repeat loci). The numbers on the lines represent STRD between isolates. CC - clonal complex, STRD - sum of tandem repeat differences, WRT – WEBRIBO type.



Příloha 7

Nyc O, Krutova M, Kriz J, Matejkova J, Bebrova E, Hysperska V, Kuijper EJ. *Clostridium difficile* ribotype 078 cultured from post-surgical non-healing wound in a patient carrying ribotype 014 in the intestinal tract. Folia Microbiol (Praha). 2015;60(6):541-4.

V článku je popsána kazuistika pacienta hospitalizovaného na oddělení Spinální jednotky FN v Motole. Tento pacient byl přijat s mnohočetnými dekubity, které byly chirurgicky řešeny plastikou. Z jedné dlouhodobě se nehojící pooperační rány bylo vykultivováno *C. difficile*. Tento kmen příslušel k ribotypu 078. Po nasazení adekvátní terapie, došlo ke zklidnění a zhojení rány. K určení možného zdroje infekce byla vyšetřena i stolice pacienta. Vykultivovaný kmen byl určen jako ribotyp 014. Pacient byl tedy pravděpodobně kolonizován odlišným ribotypem. Tato kazuistika upozorňuje na uplatnění *C. difficile* v etiologii infekce kůže a přilehlých struktur a na nebezpečí environmentální kontaminace rány.

Folia Microbiol (2015) 60:541–544
DOI 10.1007/s12223-015-0392-0

Clostridium difficile ribotype 078 cultured from post-surgical non-healing wound in a patient carrying ribotype 014 in the intestinal tract

Otakar Nyc [1] & Marcela Krutova[1,2] & Jiri Kriz[3] & Jana Matejkova[1] & Eliska Bebrova[1] & Veronika Hysperska[3] & Ed J. Kuijper[4]

Received: 1 February 2015 / Accepted: 2 April 2015 / Published online: 3 May 2015

Abstract Extra-intestinal infections caused by *Clostridium difficile* are rare. The risk of extra-intestinal infections associated with *C. difficile* may be particularly relevant in environments contaminated with *C. difficile* spores. This paper describes the case of a non-diarrheic patient colonized with *C. difficile* ribotype 014 in the intestinal tract who developed a post-surgical wound infection by *C. difficile* ribotype 078. The infection responded to metronidazole administered first intravenously and then orally. This case indicates that *C. difficile* may not only be related to diarrheic diseases, but also to infections of non-healing wounds, especially in situations when *C. difficile* is the only isolated pathogen.

Case report

This 26-year-old paraplegic patient suffered a fracture of the T12 with a complete spinal cord lesion (Neurological Level of Injury T10, Impairment Scale A) after a car accident in 2010. After several months of rehabilitation at the Spinal Cord Unit (SCU), he was released to home care, where decubitus ulcers developed. He was then readmitted to the SCU at the end of 2012 as the multiple progressing decubitus ulcers required surgical intervention.

The pressure sores (sacral, trochanteric bilateral, and left ischiadic) have already been revised several times and cultures revealed MRSA, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*, and *Alcaligenes faecalis*. Anaerobic cultures were negative for strict anaerobic pathogens. The first phase of the surgical treatment consisted of plastic surgery in the ischiadic and sacral regions that was carried out on day 14 of hospitalization under prolonged antibiotic coverage by vancomycin and piperacillin/tazobactam. After 12 days, the antibiotics were changed to cotrimoxazole.

On day 37 of hospitalization, before the second phase of surgical intervention, *Klebsiella pneumoniae* (ESBL positive) were cultured from urine and *P. aeruginosa* were cultured from the right trochanter decubitus (decubitus d). As a result of these bacteriological findings, therapy was changed again to piperacillin/tazobactam. Four days later, piperacillin/tazobactam was changed to imipenem/cilastatin because ESBL-positive *E. coli* was isolated from the drain (decubitus b). On day 43 of hospitalization, decubitus ulcers in the trochanteric region were surgically treated with the administration of vancomycin and imipenem/cilastatin.

On day 54 of hospitalization, post-surgical wound dehiscence appeared in the area of the right trochanter. *P. aeruginosa* resistant to imipenem/cilastatin was cultured, and a combination of cefoperazone/sulbactam and amikacin was administered. Due to persistent non-purulent dehiscence of the wound, samples for additional bacteriological assessments were collected again on day 69 of hospitalization.

Gram staining of fluid from the drain showed gram-positive rods sporadically with leukocytes. A wound smear and the fluid

* Marcela Krutova
marcela.krutova@seznam.cz

[1] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Uvalu 84, 150 06 Prague 5, Czech Republic

[2] DNA Laboratory, Department of Child Neurology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic

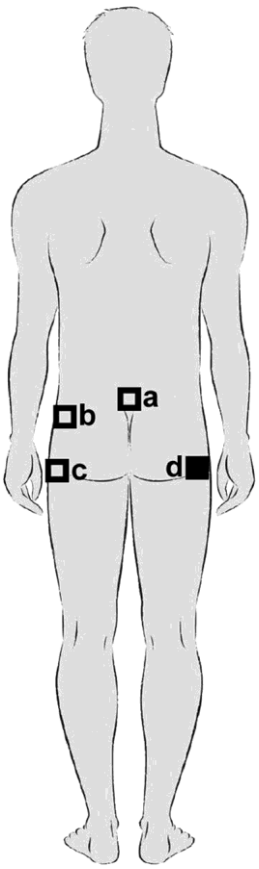
[3] Spinal Cord Unit, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic

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

from the drain were cultured on a MacConkey agar (Oxoid) at 37 °C under aerobic conditions, chocolate agar (Oxoid) at 37 °C under aerobic conditions with 5 % CO_2, and blood agar (Oxoid) incubated at 37 °C, both under aerobic and anaerobic conditions. The aerobic cultures were negative. Massive growth of colonies was observed on the anaerobically cultured blood agar after 48 h of culture. The suspected colony was confirmed as *Clostridium difficile* using MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) mass spectrometry (Bruker, Daltonics, USA). Susceptibility to vancomycin and metronidazole was assessed using the E-test (BioMérieux) and revealed MIC values of metronidazole 1 mg/L and vancomycin 0.25 mg/L, respectively. Antibiotic therapy was changed to metronidazole 500 mg three times daily at 8-h intervals (the first 6 days intravenously and then orally for the next 8 days). Ten days later, local findings in the trochanteric region showed improvement and a surgical revision of the trochanteric area was performed. The antibiotic coverage was supplemented by piperacillin/tazobactam and vancomycin. After negative bacteriological findings, vancomycin was discontinued after 16 days and piperacillin/tazobactam after 20 days of the therapy (Fig. 1).

Based on the positive culture of the wound, the patient's stool sample was cultured for the presence of *C. difficile* on day 72 of the hospitalization. The stool was solid and the patient had no symptoms of diarrhoea. The stool contained *C. difficile.* Subsequent typing of the cultured strains was performed using molecular biological methods.

Three different *C. difficile* colonies growing from the wound, and three different colonies growing from the stools were investigated further by PCR-ribotyping. PCR-ribotyping was performed according to the standard operating procedure using capillary electrophoresis issued by ECDIS-net (European *Clostridium difficile* infection surveillance network)



MICROBIOLOGICAL FINDINGS AND TREATMENT

2nd day

Decubitus ***a-d***: polymicrobial findings: MRSA, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*, and *Alcaligenes faecalis.*

14th day

Decubitus ulcers ***a, b*** – plastic surgery

Antibiotic coverage: vancomycin and piperacillin/tazobactam

26th day

Change to cotrimoxazol

37th day

Decubitus ***d***: *Pseudomonas aeruginosa*, decubitus ***a-c***: normal microflora

Urine: *Klebsiella pneumoniae* (ESBL positive)

Change to piperacillin/tazobactam

41st day

Drain decubitus ***b***: ESBL-positive *E. coli*

Change to imipenem/cilastatin

43th day

Decubitus ulcers ***c, d*** – surgical intervention

Antibiotic coverage: imipenem/cilastatin was supplemented by vancomycin

54th day

Decubitus ***d***: *Pseudomonas aeruginosa* resistant to imipenem/cilastatin

Change to: cefoperazone/sulbactam and amikacin

71st day

Decubitus ***d***: *Clostridium difficile*

Change to: metronidazole (the first six days intravenously and then orally for the next eight days)

81st day

Decubitus ***d:*** surgical revision

Antibiotic coverage: piperacillin/tazobactam (20 days) and vancomycin (16 days)

Fig. 1 Localization of decubitus ulcers (a–d) and timeline of microbiological findings and antibiotic treatment

available on the http://www.ecdisnet.eu website (access restricted). The profiles that were obtained were compared to the Austrian Agency for Health and Food Safety Webribo database available on the http://www.webribo.ages.at/ website (access restricted) (Indra et al. 2008).

C. difficile strains cultured from the wound (smear and drain) were assigned to PCR-ribotype 078, while the *C. difficile* strain cultured from the patient's stool was assigned to PCR-ribotype 014.

The presence of genes for encoding the toxins *tcdA* (toxin A), *tcdB* (toxin B), and *cdtA/cdtB* (binary toxin genes) was determined using the multiplex PCR described by Persson et al. (2008). The positivity of genes encoding the toxins A and B and the binary toxin was confirmed for the *C. difficile* PCR-ribotype 078. Positive evidence of the genes for encoding toxins A and B was found for the *C. difficile* PCR-ribotype 014.

Discussion

Extra-intestinal infections caused by *C. difficile* are rare. In a recent review encompassing 59 patients with extra-intestinal *C. difficile* infection (CDI) (Bedimo and Weinsten 2003), three major forms of clinical manifestation were described: bacteraemia with or without focal infection (Bedimo and Weinsten 2003; Feldman et al. 1995; Libby and Bearman 2009; Lee et al. 2010; Gerard et al. 1989; Hemminger et al. 2011; Choi et al. 2013), intra-abdominal infection, and extra-abdominal abscesses (Bedimo and Weinsten 2003), such as a splenic abscess (Stieglbauer et al. 1995; Studemeister et al. 1987; Saginur et al. 1983) and a brain abscess (Gravisse et al. 2003). *C. difficile* has also been associated with reactive arthritis (Loffler et al. 2004; Birnbaum et al. 2008; Prati et al. 2010), osteomyelitis (Al-Najjar et al. 2013; Pron et al. 1995; Riley and Karthigasu 1982), and prosthetic shoulder and knee joint infection (Pron et al. 1995; Ranganath and Midturi 2013; McCarthy and Stingemore 1999). Necrotizing fasciitis (Bhargava et al. 2000; Duburcq et al. 2013) and post-traumatic wound infections with *C. difficile* have been reported in two case reports (Deptula et al. 2009; Urbán et al. 2010). The review of Mattila et al. (2013) included 31 patients, of whom 13 had wound infections (Mattila et al. 2013). Similar to our observations, decubitus ulcers infected with *C. difficile* were found in four of the 13 wound infections (Mattila et al. 2013).

The source of extra-intestinal *C. difficile* infections is most likely the environment. Risk of environmental contamination with *C. difficile* spores at a spinal cord unit in a medical centre in Cleveland, USA, was recently described by Dumford et al. (2011). Of the 22 patients involved in the survey, 50 % were asymptomatic carriers of the toxigenic *C. difficile*. Interestingly, 12 skin isolates from six asymptomatic carriers, and nine environmental isolates from five asymptomatic carriers had an identical PCR-ribotype to the *C. difficile* strains cultured from the stool samples (Dumford et al. 2011).

We cultured *C. difficile* from both the patient's wound and stool, but the isolates belonged to different PCR-ribotypes. Despite the fact that we investigated several colonies of *C. difficile*, we cannot exclude the coexistence of various PCR-ribotypes. Interestingly, no case of CDI caused by PCR-ribotype 078 was registered at the Spinal Cord Unit before or during the hospitalization of the patient. Before the index patient was hospitalized at Spinal Cord Unit, we identified two cases of CDI: one case of CDI due to PCR-ribotype 176 occurred 1 month previous to the index patient's admission and the second case due to PCR-ribotype 002 was diagnosed 1 day before admission of the index patient. No other cases of CDI were identified while the index patient was hospitalized. Although the source of the wound infection could not be determined, it remains possible that the patient harboured two different PCR-ribotypes in the intestinal tract, of which only PCR-ribotype 014 was cultured (van den Berg et al. 2005). *C. difficile* PCR-ribotype 078 is considered as a more virulent type, as described by Goorhuis et al. (2008). *C. difficile* PCR-ribotype 078 has been reported frequently as a community-derived PCR-ribotype with genetic relationship to *C. difficile* strains of swine origin (Bakker et al. 2010).

We isolated *C. difficile* as the only pathogen, but the prolonged broad spectrum antibiotic therapy probably contributed to the suppression of other microflora. Mattila et al. (2013) described 13 wound infections with culture positivity of *C. difficile*. In 12 cases, *C. difficile* was isolated together with other microbes (Mattila et al. 2013), indicating that *C. difficile* is rarely found as a solitary pathogen of wound infections.

The standards for treating soft tissue infection caused by *C. difficile* have not been determined (Kikkawa et al. 2008). The clinical outcome for our patient after the administration of intravenous, then oral metronidazole was positive, similarly to a case report of surgical site infection described by Kikkawa et al. (2008) where only oral metronidazole was administered (Kikkawa et al. 2008). In contrast to our observation, Urbán et al. (2010) found empirical treatment by metronidazole combined with cefazolin to be ineffective, and this therapy changed to imipenem/cilastatin on the basis of antibiotic susceptibility (Urbán et al. 2010).

In conclusion, a patient admitted to the Spinal Cord Unit developed post-surgical wound infection by the toxigenic *C. difficile* PCR-ribotype 078. The infection responded to the administration of intravenous, then oral metronidazole. The risk of extra-intestinal infections associated with *C. difficile* may be particularly relevant in environments contaminated with the spores of *C. difficile*.

Acknowledgments We would like to thank James Partridge for proofreading the text. This study is supported by the Ministry of Health, Czech Republic-conceptual development of research organization, University Hospital Motol, Prague, Czech Republic, 00064203 and MH CZ IGA NT/14209-3.

References

Al-Najjar A, Al-Rawahi GN, Hoang LM, Kollmann TR (2013) *Clostridium difficile* vertebral osteomyelitis. Pediatr Infect Dis J 32(9):1030–2

Bakker D, Corver J, Harmanus C, Goorhuis A, Keessen EC, Fawley WN et al (2010) Relatedness of human and animal *Clostridium difficile* PCR ribotype 078 isolates determined on the basis of multilocus variable-number tandem-repeat analysis and tetracycline resistance. Clin Microbiol 48(10):3744–9

Bedimo R, Weinsten J (2003) Recurrent extraintestinal *Clostridium difficile* infection. Am J Med 114(9):770–1

Bhargava A, Sen P, Swaminathan A, Ogbolu C, Chechko S, Stone F (2000) Rapidly progressive necrotizing fasciitis and gangrene due to *Clostridium difficile*: case report. Clin Infect Dis 30(6):954–5

Birnbaum J, Bartlett JG, Gelber AC (2008) *Clostridium difficile*: an under-recognized cause of reactive arthritis? Clin Rheumatol 27(2):253–5

Choi JL, Kim BR, Kim JE, Woo KS, Kim KH, Kim JM et al (2013) A case of *Clostridium difficile* bacteremia in a patient with loop ileostomy. Ann Lab Med 33(3):200–2

Deptula A, Kruszynska E, Mikucka A, Gospodarek E, Olszewski K, Kruczynski J et al (2009) Toxin A-producing *Clostridium difficile* as an aetiological factor of post-traumatic wound infection. J Med Microbiol 58(Pt 7):963–4

Duburcq T, Parmentier-Decrucq E, Poissy J, Mathieu D (2013) Pseudomembranous colitis due to *Clostridium difficile* as a cause of perineal necrotising fasciitis. BMJ Case Rep 2013

Dumford DM 3rd, Nerandzic M, Chang S, Richmond MA, Donskey C (2011) Epidemiology of *Clostridium difficile* and vancomycin-resistant *Enterococcus* colonisation in patients on a spinal cord injury unit. J Spinal Cord Med 34(1):22–7

Feldman RJ, Kallich M, Weinstein MP (1995) Bacteremia due to *Clostridium difficile*: case report and review of extraintestinal *C. difficile* infections. Clin Infect Dis 20(6):1560–2

Gerard M, Defresne N, van der Auwera P, Meunier F (1989) Polymicrobial septicemia with *Clostridium difficile* in acute diverticulitis. Eur J Clin Microbiol Infect Dis 8(4):300–2

Goorhuis A, Bakker D, Cover J, Debast SB, Harmanus C, Notermans DW et al (2008) Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis 47(9):1162–70

Gravisse J, Barnaud G, Hanau-Berçot B, Raskine L, Riahi J, Gaillard JL et al (2003) *Clostridium difficile* brain empyema after prolonged intestinal carriage. J Clin Microbiol 41(1):509–11

Hemminger J, Balada-Llasat JM, Raczkowski M, Buckosh M, Pancholi P (2011) Two case reports of *Clostridium difficile* bacteremia, one with the epidemic NAP-1 strain. Infection 39(4):371–3

Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A et al (2008) Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 57(Pt 11):1377–82

Kikkawa H, Miyamoto K, Takiguchi N, Kondo T, Hitomi S (2008) Surgical-site infection with toxin A-nonproducing and toxin B-producing *Clostridium difficile*. J Infect Chemother 14(1):59–61

Lee NY, Huang YT, Hsueh PR, Ko WC (2010) *Clostridium difficile* bacteremia, Taiwan. Emerg Infect Dis 16(8):1204–10

Libby DB, Bearman G (2009) Bacteremia due to *Clostridium difficile* review of the literature. Int J Infect Dis 13(5):e305–9

Loffler HA, Pron B, Mouy R, Wulffraat NM, Prieur AM (2004) *Clostridium difficile* associated reactive arthritis in two children. Joint Bone Spine 71(1):60–2

Mattila E, Arkkila P, Mattila PS, Tarkka E, Tissari P, Anttila VJ (2013) Extraintestinal *Clostridium difficile* infections. Clin Infect Dis 57(6): e148–53

McCarthy J, Stingemore N (1999) *Clostridium difficile* infection of a prosthetic joint presenting 12 months after antibiotic-associated diarrhoea. J Infect 39(1):94–6

Persson S, Torpdahl M, Olsen KEP (2008) New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect 14(11):1057–64

Prati C, Bertolini E, Toussirot E, Wendling D (2010) Reactive arthritis due to *Clostridium difficile*. Joint Bone Spine 77(2):190–2

Pron B, Merckx J, Touzet P, Ferroni A, Poyart C, Berche P et al (1995) Chronic septic arthritis and osteomyelitis in a prosthetic knee joint due to *Clostridium difficile*. Eur J Clin Microbiol Infect Dis 14(7): 599–601

Ranganath S, Midturi JK (2013) Unusual case of prosthetic shoulder joint infection due to *Clostridium difficile*. Am J Med Sci 346(5):422–3

Riley TV, Karthigasu KT (1982) Chronic osteomyelitis due to *Clostridium difficile*. Br Med J (Clin Res Ed) 284(6324):1217–8

Saginur R, Fogel R, Begin L, Cohen B, Mendelson J (1983) Splenic abscess due to *Clostridium difficile*. J Infect Dis 147(6):1105

Stieglbauer KT, Gruber SA, Johnson S (1995) Elevated serum antibody response to toxin A following splenic abscess due to *Clostridium difficile*. Clin Infect Dis 20(1):160–2

Studemeister AE, Beilke MA, Kirmani N (1987) Splenic abscess due to *Clostridium difficile* and *Pseudomonas paucimobilis*. Am J Gastroenterol 82(4):389–90

Urbán E, Terhes G, Markotics A, Sóki J, Nagy E (2010) Rare extraintestinal infection caused by toxin-producing *Clostridium difficile*. Anaerobe 16(3):301–3

van den Berg RJ, Ameen HA, Furusawa T, Claas EC, van der Vorm ER, Kuijper EJ (2005) Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies. J Med Microbiol 54(Pt 2):173–9

Příloha 8

Nyc O, Krutova M, Liskova A, Matejkova J, Drabek J, Kuijper EJ. The emergence of *Clostridium difficile* PCR-ribotype 001 in Slovakia. Eur J Clin Microbiol Infect Dis. 2015;34(8):1701-8.

V článku jsou uvedeny výsledky pilotní měsíční surveillanční studie infekcí vyvolaných *C. difficile* na Slovensku. V rámci této pilotní studie bylo testováno 194 stolic od hospitalizovaných pacientů s podezřením na klostridiovou kolitidu z 10 univerzitních nemocnic na Slovensku. Z 20 izolátů *C. difficile* 17 příslušelo k ribotypu 001. Těchto 17 izolátů jsme podrobili bližší příbuzenské analýze pomocí MLVA, která odhalila dva klonální komplexy a blízkou genetickou příbuznost mezi izoláty z šesti různých nemocnic. Molekulární analýza genů a mutací asociovaných s rezistencí k antibiotikům ukázala, že 85 % izolátů neslo Thr82Ile v GyrA záměnu asociovanou s rezistencí k fluorochinolonům a 90 % izolátů bylo pozitivních pro *ermB* gen associovaný s rezistencí k MLS_B skupině antibiotik. Tyto výsledky upozorňují na závažnou epidemiologickou situaci infekcí vyvolaných *C. difficile* na Slovensku, a na významný potenciál ribotypu 001 ke klonálnímu šíření a akumulaci rezistence k několika antibiotikům.

Eur J Clin Microbiol Infect Dis (2015) 34:1701–1708
DOI 10.1007/s10096-015-2407-9

ARTICLE

The emergence of *Clostridium difficile* PCR-ribotype 001 in Slovakia

O. Nyc[1] & M. Krutova[1,2] & A. Liskova[3] & J. Matejkova[1] & J. Drabek[4] & E. J. Kuijper[5]

Received: 26 March 2015 / Accepted: 6 May 2015 / Published online: 17 May 2015

Abstract

Purpose The purpose of this study was to determine the incidence of *Clostridium difficile* infections (CDI) and to characterise the isolates in 14 departments of ten academic hospitals in Slovakia.

Methods During a one-month study (September 2012) all unformed stool samples were investigated using a rapid test to detect the presence of GDH and toxins A/B. Positive samples were cultured anaerobically and *C. difficile* isolates were characterised by ribotyping, multiple-locus variable-number tandem repeats analysis, and *gyrA*, *rpoB* and *ermB* investigation.

Results A total of 194 unformed stool samples were investigated and 38 (19.6 %) had a positive rapid test. Of 38 samples, 27 revealed a positive result for GDH and free toxins A/B in the stool, and 11 samples only for the presence of GDH. The mean CDI incidence in 2012 was 5.2 cases per 10,000 patient bed-days. Twenty *C. difficile* isolates were available for molecular analysis; seventeen belonged to PCR-ribotype 001 (85 %) whereas the remaining three isolates were identified as PCR-ribotypes 017, 078 and 449. MLVA of the PCR-ribotype 001 isolates identified two clonal complexes and a close genetic relatedness between isolates from six different hospitals. Molecular analysis of antibiotic-resistance determinants revealed the presence of *ermB* gene encoding resistance to the MLS_B group of antibiotics in 90 % of isolates, and Thr82Ile amino acid substitution in the *gyrA* gene associated with resistance to fluoroquinolones in 85 % of isolates.

Conclusions We conclude that *C. difficile* PCR-ribotype 001 is the predominant PCR-ribotype in Slovakia with a strong potential for clonal spread and development of multidrug resistance.

Electronic supplementary material The online version of this article (doi:10.1007/s10096-015-2407-9) contains supplementary material, which is available to authorized users.

* M. Krutova
marcela.krutova@seznam.cz

[1] Department of Medical Microbiology, Charles University, 2nd Faculty of Medicine and University Hospital Motol, V Uvalu 84, Prague 5 150 06, Czech Republic

[2] DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czech Republic

[3] Department of Medical Microbiology, Faculty Hospital Nitra, Nitra, Slovak Republic

[4] Department of Internal Medicine, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czech Republic

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

Introduction

Infections caused by *Clostridium difficile* are a worldwide problem with increasing incidence, considerable mortality and a significant economic burden [1]. A European study performed in 2008 showed Slovakia as a country with a low incidence of CDI (1.4 per 10,000 patient bed-days) [2], but a recently published survey performed in 2012 and 2013 reported an incidence rate of 5.3 per 10,000 patient bed-days in the period 2011–2012, and 1.2 per 10,000 patient bed-days in the period 2012–2013 [3].

Slovakia has been referred as a country with a high cumulative resistance score (4–5) in a recently published Pan-European longitudinal survey of antibiotic resistance. Twenty-five Slovak isolates were investigated and 72 % of epidemic PCR-ribotypes belonged to PCR-ribotype 001 [4].

In Slovakia, national guidelines for diagnosis and treatment of *C. difficile* infections are available (http://www.infektologia.sk), but there is no mandatory reporting of CDI.

The aim of this study was to determine the incidence of CDI in 14 departments of ten large hospitals and to investigate the distribution of PCR-ribotypes with further molecular analysis of isolates.

Material and methods

Study design

The study was carried out in September 2012. Fourteen departments (12 departments of internal medicine, one gastroenterology and one department of infectious diseases) in ten academic hospitals distributed equally across Slovakia participated (Fig. 1). The stool samples from all adult patients who presented or developed diarrhoea at admission or during the hospitalisation were tested for the presence of toxin-producing *C. difficile* using GDH (glutamate dehydrogenase) and toxins A/B (*C. diff.* Quik Chek Complete®, Alere, USA) as part of routine bacteriological testing at the relevant hospital microbiology department, or at the outsourcing laboratory. GDH positive samples were cultured anaerobically, after an alcohol shock treatment, on selective agar (Oxoid) to isolate *C. difficile*. *C. difficile* isolates were sent to the Department of Medical Microbiology of Faculty Hospital Motol in Prague, Czech Republic for further molecular testing.

The clinical data were collected retrospectively, using a specific web-based questionnaire developed for the purposes of this survey, from patients whose stool samples tested positive by a rapid test. Data on risk factors preceding the infection (age, comorbidity, previous hospitalisation and use of antibiotics), clinical symptoms (stool count, fever, white blood cell count and the presence of pseudomembranes), hospitalisation at ICU and treatment of CDI were recorded.

CDI was divided into health care-associated (HA-CDI) (patients who developed diarrhoea after 48 hours (>48 h) of hospitalisation) or community-associated (CA-CDI) (patients who developed diarrhoea<48 h or who were admitted with diarrhoea and had no previous hospitalisation in their medical records in the preceding 3 months) [2, 5, 6]. Patients with previous hospitalisation in the preceding 3 months were evaluated as an indeterminate group because the records about hospitalisation over the previous 4 weeks were not the part of the questionnaire.

The severity of CDI was defined according to ECDC definition [7, 8], and patient records on stool counts, fever, white blood cell count and the presence of pseudomembranes were collected.

The mean incidence of CDI was calculated using the ECDC definitions of CDI [7, 8] and the collected data on number of performed tests, number of toxin A/B positive tests, number of admitted patients and number of patient bed-days in ten hospitals from January to December in 2012.

Molecular characterisation of isolates

PCR-Ribotyping was performed according the SOP (Standard Operating Protocol) of ECDIS-net (the European *C. difficile* Infection Surveillance Network) (http://www.ecdisnet.eu/) with primers described by Stubbs et al. [9], and fragment analysis using capillary electrophoresis. The Webribo database (http://www.webribo.ages.at) was used for evaluation of acquired electrophoreograms [10].

MLVA (multiple-locus variable-number tandem repeats analysis) of seven previously published VNTR (variable-number tandem repeats) loci (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd) was applied to isolates belonging to

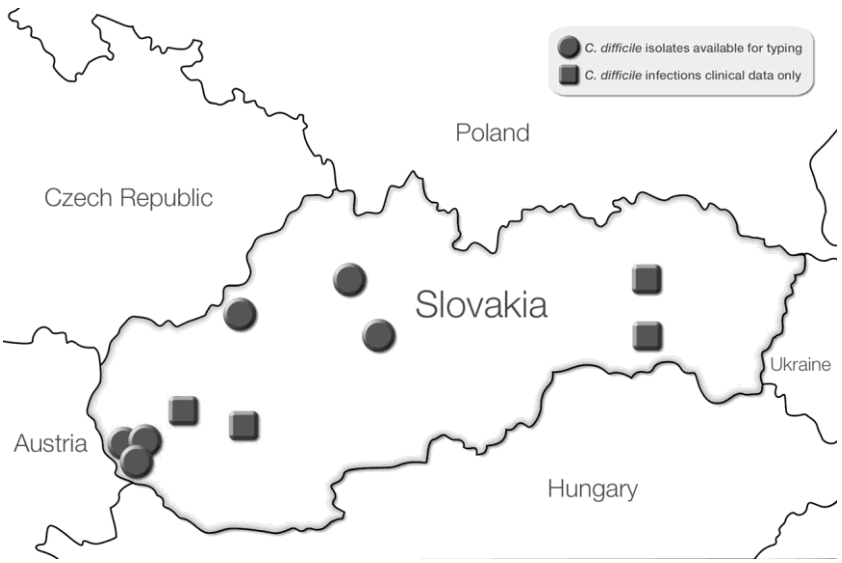


Fig. 1 Distribution of participating hospitals in the study

the predominant PCR-ribotype in order to determine the mutual genetic relatedness [11, 12]. The number of repeats was calculated manually after Sanger's sequencing of each locus. The summed tandem repeat differences (STRD) between isolates was determined by Bionumerics v5.0 (Applied Maths) using a Manhattan coefficient.

The antibiotic-resistance determinants were investigated by the presence of the *ermB* gene as a marker of resistance to the MLS_B (macrolide/lincosamide/streptogramin B) group of antibiotics using primers published by Spigaglia et al. [13]. Quinolone resistance was determined using the method described by Dridi et al. using amplification and sequencing of *gyrA* and *gyrB* genes [14]. The resistance to rifamycin and its derivatives was investigated by determining point mutations in the *rpoB* gene region, as published by Curry et al. [15].

Results

Microbiological data

During the one-month period of the study, 194 unformed stool samples were investigated for the presence of GDH and toxins A/B. A total of 27 samples (13.9 %) tested positive for both GDH and toxins A/B, and 20 *C. difficile* were cultured from these samples. Eleven stool samples (5.7 %) were GDH positive only and all 11 samples subsequently tested positive by an anaerobic culture for the presence of *C. difficile*. Of 31 positive cultures, 20 *C. difficile* isolates (14 samples were GDH and toxin A/B positive, six samples were GDH positive only) were available for molecular characterisation. Of these 20 *C. difficile* isolates, 17 belonged to PCR- ribotype 001 (85 %). Three remaining isolates were PCR-ribotypes: 017, 078 and 449 (Fig. 2).

MLVA was performed on all 17 *C. difficile* isolates belonging to PCR-ribotype 001. The minimum spanning tree (Fig. 3) revealed two clonal complexes defined as STDR≤ 1. The first clonal complex consisted of two isolates (SK11, SK7) from hospital B. Interestingly, three isolates (SK12, SK1, SK4,) from hospital A, one isolate (SK17) from hospital D and one isolate (SK14) from hospital C formed the second clonal complex. A cluster with eight genetically related isolates (defined as STDR≤ 10) was found (SK2, SK5, SK6, SK9, SK10, SK18, SK19, SK21); isolates were derived from hospitals A–F.

The presence of the *ermB* gene was found in 18 isolates (90 %) belonging to PCR-ribotype 001 (n= 17) and PCR-ribotype 078 (*n*=1).

The sequence analysis of the *gyrA* fragment (protein position 47-149) revealed that the amino-acid substitution Thr82Ile was present in 17 isolates (85 %). The majority of these isolates belonged to the PCR-ribotype 001 (*n*=16/17) and one isolate was PCR-ribotype 017.

The missense mutation Ser416Ala in the *gyrB* fragment (protein position 376-478) was identified in one *C. difficile* isolate belonging to PCR-ribotype 078.

Two missense mutations in two isolates (10 %) were observed in *rpoB* sequence (protein position 481-611); Arg505Lys was found in PCR-ribotype 001 (*n* =1/17) and PCR-ribotype 017 (*n* = 1) isolate. This latter isolate also contained amino-acid substitution His502Asn.

Clinical data of 27 patients with a positive GDH and toxin A/B test

Twenty-seven CDI cases were laboratory confirmed by a positive test for both GDH and toxins. The group comprised 15 male and 12 female patients with an average age of 62 years

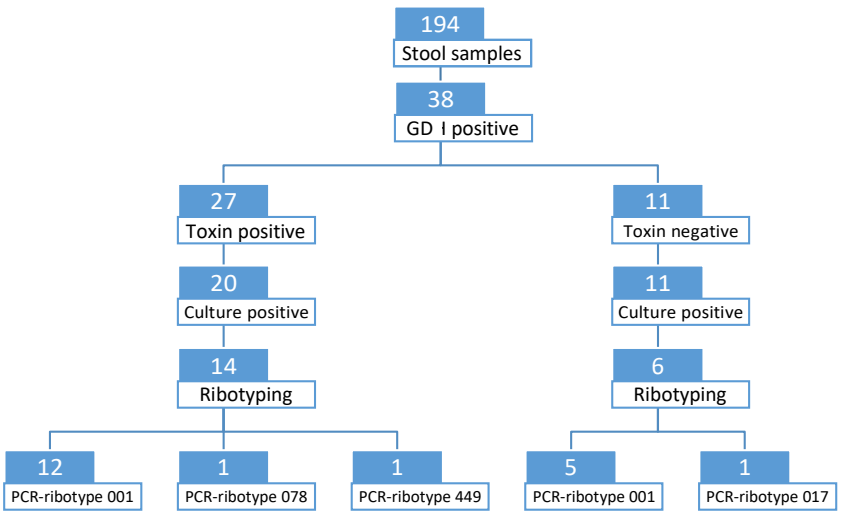


Fig. 2 Summary of *C. difficile* GDH, toxins A/B, anaerobic culture and ribotyping results

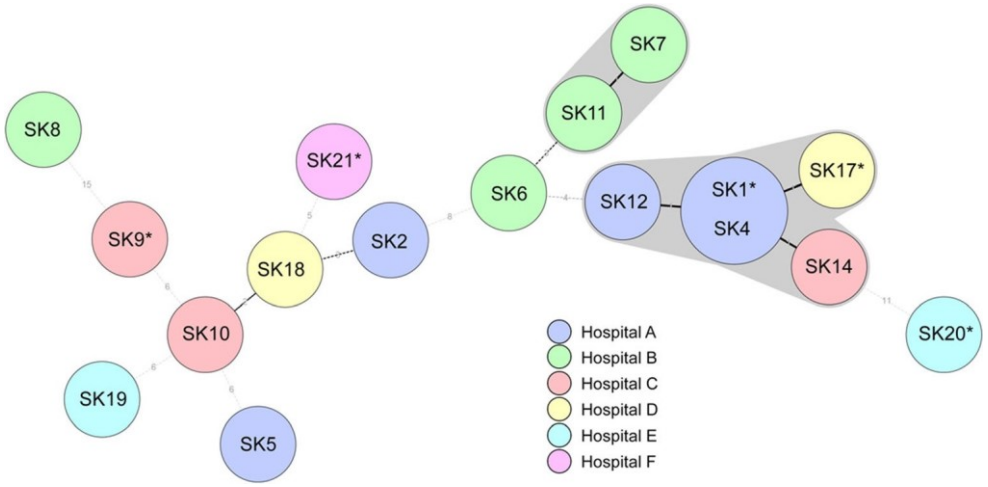


Fig. 3 A minimum spanning tree (Bionumerics v5.0) *C. difficile* PCR ribotype 001, *GDH positive, toxins A/B negative *C. difficile* isolates.

(the youngest being 23 years and the oldest 88 years). Clinical data were collected from 22 cases of CDI.

HA-CDI was diagnosed in 68.2 % of cases, CA-CDI in 13.6 % and 18.2 % of cases were considered as indeterminate. Two patients (9.1 %) had recurrent CDI and 13 patients (59.1 %) were hospitalised in the previous three months. Fifteen patients (68.2 %) had received antibiotic treatment in the previous month and three patients (13.7 %) in the three months before an actual hospitalisation. The spectrum of used antibiotics in the monotherapy or in the combination was as follows: 13.7 % aminopenicillin, 9.1 % second-generation cephalosporines, 27.3 % third-generation cephalosporines, 13.7 % carbapenems, 4.6 % colistin, 45.5 % second-generation fluoroquinolones, 13.7 % lincosamides, 4.6 % macrolides. Of the 22 patients, 13.6 % died. No death was directly related to *C. difficile* infection, but CDI had contributed to all.

CDI was considered as mild in 59.1 % and severe in 40.9 %. An antibiotic treatment was initiated in all 22 cases of CDI: 86.5 % of patients were treated by metronidazole, 9 % by metronidazole in combination with vancomycin and 4.5 % with fidaxomicin.

Clinical data of 11 patients with GDH positive and toxin A/B negative test

Of the 11 patients, four patients were male, seven were female and the average age was 71.6 years (the youngest being 21 years and the oldest 90 years). Clinical data were available for ten cases. All patients had symptoms of diarrhoea suspected for CDI and eight patients (80 %) developed diarrhoea more than 48 hours after admission. Five patients (50 %) had symptoms indicating a severe course of CDI (fever or leucocytosis). One patient (10 %) had a previous episode of CDI in their medical record. Two patients (20 %) were hospitalised at ICU, six patients (60 %) had comorbidities, eight patients (80 %) were hospitalised in the previous three months, and six patients (60 %) had received antimicrobial treatment in the previous month and two patients (20 %) in the previous 3 months before CDI was diagnosed. The spectrum of used antibiotics as monotherapy or in combination with other antibiotics was: 10 % aminopenicillin, 10 % aminopenicillin/beta-lactamase inhibitors, 10 % second-generation cephalosporines, 20 % third-generation cephalosporines, 50 % second-generation fluoroquinolone, 10 % glycopeptides, 10 % rifamycin.

Of the ten patients included, five patients (50 %) were considered as CDI as physicians initiated a specific anti-CDI treatment. Three patients (30 %) were treated by metronidazole, two patients (20 %) were treated by vancomycin in combination with metronidazole. Other causes of diarrhoea were excluded by routine bacteriological testing. The remaining five patients (50 %) were considered as patients who carried *C. difficile* strain and these patients received symptomatic anti-diarrhoeal therapy. One patient (10 %) died, unrelated to CDI.

A summary of epidemiological characteristics of patients with available clinical data is shown in Table 1.

The mean incidence of CDI

The mean incidence was calculated as 5.2 CDI cases per 10,000 patient bed-days (varying from 2.2 to 11.3) and 40 CDI cases per 10,000 admissions (varying from 14.7 to 82.4) in 2012.

Discussion

The first data on incidence of CDI in Slovakia were obtained in 2008 and showed a CDI incidence of 1.4 per 10,000 patient bed-days. However, of the three participating hospitals only two provided incidence data [2]. Recently published data from the EUCLID study revealed a high difference in the six participating hospitals between reported CDI incidence rates and

Table 1 Epidemiological characteristics of patients with available clinical data

Characteristic	GDH, toxin A/B positive (*n*=22)	GDH positive only (*n*=10)
Male	13 (59.1 %)	4 (40 %)
Age 65 years	12 (54.5 %)	9 (90 %)
HA-CDI	15 (68.2 %)	8 (80 %)
CA-CDI	3 (13.6 %)	2 (20 %)
Indeterminate	4 (18.2 %)	_
Recurrent CDI	2 (9.1 %)	1 (10 %)
Severe CDI	9 (40.9 %)	5 (50 %)
Death - CDI contributed	3 (13.6 %)	_
Previous hospitalisation (3 months)	13 (59.1 %)	8 (80 %)
Previous antibiotic use (1 month)	15 (68.2 %)	6 (60 %)
Previous antibiotic use (3 months)	3 (13.7 %)	2 (20 %)
Aminopenicillin	3 (13.7 %)	1 (10 %)
Aminopenicillin/beta-lactamase inhibitors	_	1 (10 %)
Second-generation cephalosporines	2 (9.1 %)	1 (10 %)
Third-generation cephalosporines	6 (27.3 %)	2 (20 %)
Carbapenems	3 (13.7 %)	_
Colistin	1 (4.6 %)	_
Second-generation fluoroquinolones	10 (45.5 %)	5 (50 %)
Lincosamides	3 (13.7 %)	_
Macrolides	1 (4.6 %)	_
Glycopeptides	_	1 (10 %)
Rifamycin	_	1 (10 %)

GDH glutamate dehydrogenase

rates measured during the survey. The reported rate in the period 2011–2012 was 5.3 CDI cases, whereas the measured rate was 9.6 CDI cases per 10,000 patients bed-days. In the period 2012–2013, the reported rate was only 1.2 and the measured rate was 24.1 CDI per 10,000 patient bed-days [3]. The mean incidence calculated in the current study (5.2 CDI cases per 10,000 patient bed-days) corresponds with EU-CLID study data (2011–2012 period) [3].

Ribotyping of *C. difficile* isolates in our study revealed a high percentage of PCR-ribotype 001 (85 %). This PCR-ribotype was identified as the second most frequent PCR-ribotype (37 isolates from a total of 389) in a European multicentre survey performed in 2008 [2]. In the same year, a study including 84 German hospitals revealed that out of 670 *C. difficile* isolates, 312 (47 %) belonged to PCR-ribotype 001 [16]. A Scottish survey performed in the period November 2007 to December 2009, showed that PCR-ribotype 001 accounted for 22 % of 1,623 isolates [17]. Recently published work from the Croatian University Hospital also shows a high prevalence of PCR-ribotype 001 (27.8 %) and its association with multidrug resistance during the period January 2010 to December 2011 [18].

C. difficile ribotypes 001, 106 and 027 were the most prevalent in the North East of England [19]. Spanish data from a survey among 118 laboratories covering 75.4 % of the Spanish population revealed that from a total of 807 specimens PCR-ribotype 001 accounted for 18.2 % of isolates [20]. Data from a survey performed in the Czech Republic in 2013 showed the presence of PCR-ribotype 001 among 24 *C. difficile* isolates, 3.9 % of 624 investigated isolates [21]. The latest published data from Hesse, Germany showed that PCR-ribotype 001 was the most prevalent type (31.8 %) after analysing 214 toxigenic *C. difficile* isolates in the period 2011–2013 [22].

The data from all these studies show that PCR-ribotype 001 is a long-term problematic epidemic strain in some European countries.

The association of the PCR-ribotype 001, as well as PCR-ribotypes 017 and 027, with the lethal course of CDI in Hesse, Germany was reported by Arvand et al., where case fatality rate was 19 % of severe cases of CDI [23]. A European study revealed CDI contributed to death in 7 % [2] of cases. In our study, three CDI patients died, of whom two were infected by PCR-ribotype 001.

Three other different PCR-ribotypes were found in our survey, identified as 017, 078 and 449. PCR-ribotypes 017 and 078 belonged to the most frequent PCR-ribotypes in the Bauer et al. study, when PCR-ribotype 078 was represented in 8 % of cases (the third after PCR-ribotype 001) and PCR-ribotype 017 in 4 % [2]. PCR-ribotype 078 is referred to as a

hypervirulent strain [24] and genetic relatedness between *C. difficile* PCR-ribotype 078 isolates of human and swine origin was determined by MLVA and tetracycline resistance [25] and next generation sequencing [26]. Recently published studies showed a high prevalence of *C. difficile* PCR-ribotype 078 in swine [27–29] and piglets [30]. *C. difficile* PCR-ribotype 078 was identified as the most common (19.0 %) PCR-ribotype causing community-acquired CDI [31].

Two clonal complexes and the closer genetic relatedness between other *C. difficile* isolates PCR-ribotype 001 from different hospitals, suggest nosocomial spread and circulation of several clones between participating hospitals in Slovakia. Two patients from hospital A and two patients from hospital B, whose *C. difficile* isolates (SK1, 12 – hospital A and SK 7, 11 – hospital B) formed two clonal complexes, were hospitalised in the same department with an overlapping date of hospitalisation, which suggests that there is a possibility of spreading a particular *C. difficile* strain between patients. All isolates from the second clonal complex came from three different hospitals, where hospitals A and C are localised in the same city and hospital D is geographically close, and transfers of patients between these hospitals may occur.

Two groups of patients were studied and only the group of 27 patients with positive tests for both GDH and toxins A/B were considered as CDI. The second group was made up of 11 patients with only a positive GDH test and probably represent *C. difficile* carriers, though five patients were treated for presumed CDI by their physicians. A second more sensitive test for toxin detection in stools would have resulted in a better discrimination between CDI patients and carriers since the reported sensitivity of *C. diff* Quik Chek Complete® (Alere, USA) varies between 59.6 % and 74.4 % [32, 33]. However, five isolates from GDH positive stool samples belonged to the PCR-ribotype 001 and were included in MLVA analysis. Surprisingly, two isolates (SK1, SK17) were part of the second clonal complex. This observation confirms that patients with a GDH positive, toxin negative stool sample could contribute to the transmission of *C. difficile* to other patients [34, 35].

Unfortunately, *in vitro* antibiotic sensitivity testing was not performed in this study and antibiotic resistance was examined only on a molecular level. The *ermB* gene positivity in our study was 90 %. The frequency of *C. difficile ermB* carriers performed on 316 European *C. difficile* isolates was 28 % [36], 69 % in 133 Irish *C. difficile* isolates [37] and 85 % in 33 of 39 clindamycin-resistant toxin A negative and toxin B positive isolates from Europe, the USA and Japan [38]. The Polish survey that included 79 isolates observed 39 with high resistance to clindamycin and erythromycin and the carrying of *ermB* in 37 of these isolates [39]; in the German study, the *ermB* gene was detected in 34 of 83 MLS_B resistant isolates [40].

The amino acid substitutions Thr82Ile in the *gyrA* gene and Ser416Ala in *gyrB* identified in this study were previously described by Spigaglia et al. [41]. Thr82Ile in *gyrA* was found in *C. difficile* clinical isolates resistant to moxifloxacin, ciprofloxacin, gatifloxacin, levofloxacin [41] and also to ofloxacin [42], whereas the amino acid substitution Ser416Ala in the *gyrB* gene was observed in two moxifloxacin susceptible isolates belonging to toxinotype V [41].

Two missense mutations (Arg505Lys and His502Asn) in *rpoB* were previously described in *C. difficile* isolates resistant to rifampicin [15, 36, 43].

Conclusion

The results of a one-month survey to CDI revealed that *C. difficile* PCR-ribotype 001 was the predominant PCR-ribotype in Slovakia. Two clonal complexes determined by MLVA demonstrated the capacity of *C. difficile* PCR-ribotype 001 to spread between various hospitals. The presence of the Thr82Ile mutation in the *gyrA* gene in 85 % of the isolates and *ermB* in 90 % of the isolates suggests multidrug resistance of *C. difficile* strains circulating in Slovakia. This strengthens the need for continuous CDI surveillance, including ribotyping and antibiotic susceptible testing.

Acknowledgments We would like to thank ECDIS-net and the European Study Group of *Clostridium difficile* infections (ESGCD, ESCMID) for their professional support.

We would like to thank Dr. James Partridge for proofreading the text.

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.

Funding Supported by the Ministry of Health, Czech Republic Internal Grant Agency NT/14209-3 and MH CZ – DRO, University Hospital Motol, Prague, Czech Republic 00064203.

Conflict of interest The authors declare that they have no competing interests.

References

1. Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH (2010) The changing epidemiology of *Clostridium difficile* infections. Clin Microbiol Rev 23(3):529–49
2. Bauer MP1, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ, ECDIS Study Group (2011) *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377(9759):63–73. doi:10.1016/S0140-6736(10)61266-4
3. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, Delmée M, Fitzpatrick F, Ivanova K, Kuijper E, Macovei IS, Mentula S, Mastrantonio P, von Müller L, Oleastro M, Petinaki E, Pituch H, Norén T, Nováková E, Nyč O, Rupnik M, Schmid D, Wilcox MH (2014) Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-

prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis 4(12):1208–19. doi:10.1016/S1473-3099(14)70991-0

4. Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C et al (2015) Pan-European Longitudinal Surveillance of antibiotic resistance among prevalent *Clostridium difficile* Ribotypes' study group. Clin Microbiol Infect 21(3):248, e9-248.e16
5. Kuijper EJ, Coignard B, Tüll P, ESCMID Study Group for Clostridium difficile; EU Member States; European Centre for Disease Prevention and Control (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect 12(6):2–18, Review
6. McDonald LC, Coignard B, Dubberke E, Song X, Horan T, Kutty PK; Ad Hoc Clostridium Difficile Surveillance Working Group (2007) Recommendations for surveillance of *Clostridium difficile*-associated disease. Infect Control Hosp Epidemiol 28(2):140–5
7. Bauer MP, Kuijper EJ, van Dissel JT, European Society of Clinical Microbiology and Infectious Diseases (2009) European society of clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for *Clostridium difficile* infection (CDI). Clin Microbiol Infect 15(12):1067–79. doi:10.1111/j.1469-0691.2009.03099.x
8. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical Microbiology and Infectious Diseases (2014) European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. Clin Microbiol Infect 20(2):1–26. doi:10.1111/1469-0691.12418
9. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol 37(2):461–3
10. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ (2008) Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 57:1377–82. doi:10.1099/jmm.0.47714-0
11. Van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ (2007) Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol 45(3):1024–8
12. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, Lumelsky G, Kuijper EJ (2009) Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. Clin Microbiol Infect 15(12):1080–6. doi:10.1111/j.1469-0691.2009.02759.x
13. Spigaglia P, Mastrantonio P (2004) Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. J Med Microbiol 53(11):1129–36
14. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC (2002) *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother 46(11):3418–21
15. Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, Pasculle AW, Harrison LH (2009) High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital. Clin Infect Dis 48(4):425–9. doi:10.1086/596315
16. Zaiß NH, Witte W, Nübel U (2010) Fluoroquinolone resistance and *Clostridium difficile*, Germany. Emerg Infect Dis 16(4):675–677. doi:10.3201/eid1604.090859
17. Wiuff C, Brown DJ, Mather H, Banks AL, Eastaway A, Coia JE (2011) The epidemiology of *Clostridium difficile* in Scotland. J Infect 62(4):271–9. doi:10.1016/j.jinf.2011.01.015
18. Novak A, Spigaglia P, Barbanti F, Goic-Barisic I, Tonkic M (2014) First clinical and microbiological characterization of *Clostridium difficile* infection in a Croatian University Hospital. Anaerobe 30:18–23. doi:10.1016/j.anaerobe.2014.07.007
19. Vanek J, Hill K, Collins J, Berrington A, Perry J, Inns T, Gorton R, Magee J, Sails A, Mullan A, Gould FK (2012) Epidemiological survey of *Clostridium difficile* ribotypes in the North East of England during an 18-month period. J Hosp Infect 81(3):209–12. doi:10.1016/j.jhin.2012.04.017
20. Alcalá L, Martín A, Marín M, Sánchez-Somolinos M, Catalán P, Peláez T, Bouza E, Spanish Clostridium Difficile Study Group (2012) The undiagnosed cases of *Clostridium difficile* infection in a whole nation: where is the problem? Clin Microbiol Infect 18(7):E204–13. doi:10.1111/j.1469-0691.2012.03883.x
21. Krutova M, Matejkova J, Nyc O (2013) First results of molecular typing of *Clostridium difficile* in the Czech Republic. Zprávy CEM (SZÚ, Praha) 22(12):399–401
22. Arvand M, Vollandt D, Bettge-Weller G, Harmanus C, Kuijper EJ, Clostridium difficile study group Hesse (2014) Increased incidence of *Clostridium difficile* PCR ribotype 027 in Hesse, vol 19. Euro Surveill, Germany, 2011 to 2013
23. Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G (2009) *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal *C. difficile* infection in Hesse, vol 14. Euro Surveill, Germany, 45
24. Goorhuis A, Bakker D, Cover J, Debast SB, Harmanus C, Notermans DW, Bergwerff AA, Dekker FW, Kuijper EJ (2008) Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis 47:1162–70
25. Bakker D, Corver J, Harmanus C, Goorhuis A, Keessen EC, Fawley WN, Wilcox MH, Kuijper EJ (2010) Relatedness of human and animal *Clostridium difficile* PCR ribotype 078 isolates determined on the basis of multilocus variable number tandem-repeat analysis and tetracycline resistance. J Clin Microbiol 48:3744–3749
26. Knetsch C, Connor T, Mutreja A, van Dorp S, Sanders I, Browne H, Harris D, Lipman L, Keessen E, Corver J, Kuijper E, Lawley T (2014) Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. Euro Surveill 19(45):20954
27. Peláez T, Alcalá L, Blanco JL, Álvarez-Pérez S, Marín M, Martín-López A, Catalán P, Reigadas E, García ME, Bouza E (2013) Characterization of swine isolates of *Clostridium difficile* in Spain: a potential source of epidemic multidrug resistant strains? Anaerobe 22:45–9. doi:10.1016/j.anaerobe.2013.05.009
28. Spigaglia P, Drigo I, Barbanti F, Mastrantonio P, Bano L, Bacchin C, Puiatti C, Tonon E, Berto G, Agnoletti F (2015) Antibiotic resistance patterns and PCR-ribotyping of *Clostridium difficile* strains isolated from swine and dogs in Italy. Anaerobe 31:42–6. doi:10.1016/j.anaerobe.2014.10.003
29. Janezic S, Zidaric V, Pardon B, Indra A, Kokotovic B, Blanco JL, Seyboldt C, Diaz CR, Poxton IR, Perreten V, Drigo I, Jiraskova A, Ocepek M, Weese JS, Songer JG, Wilcox MH, Rupnik M (2014) International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. BMC Microbiol 14:173. doi:10.1186/1471-2180-14-173
30. Schneeberg A, Neubauer H, Schmoock G, Baier S, Harlizius J, Nienhoff H, Brase K, Zimmermann S, Seyboldt C (2013) *Clostridium difficile* genotypes in piglet populations in Germany. J Clin Microbiol 51(11):3796–803. doi:10.1128/JCM.01440-13
31. Taori SK, Wroe A, Hardie A, Gibb AP, Poxton IR (2014) A prospective study of community-associated *Clostridium difficile* infections: the role of antibiotics and co-infections. J Infect 69(2):134–44. doi:10.1016/j.jinf.2014.04.002

32. Sharp SE, Ruden LO, Pohl JC, Hatcher PA, Jayne LM, Ivie WM (2010) Evaluation of the *C.Diff* Quik Chek Complete Assay, a new glutamate dehydrogenase and A/B toxin combination lateral flow assay for use in rapid, simple diagnosis of *Clostridium difficile* disease. J Clin Microbiol 48(6):2082–6. doi:10.1128/JCM.00129-10
33. Eastwood K, Else P, Charlett A, Wilcox M (2009) Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay for *C. difficile tcdB*, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. J Clin Microbiol 47(10):3211–7. doi:10.1128/JCM.01082-09
34. Planche TD, Davies KA, Coen PG, Finney JM, Monahan IM, Morris KA, O'Connor L, Oakley SJ, Pope CF, Wren MW, Shetty NP, Crook DW, Wilcox MH (2013) Differences in outcome according to *Clostridium difficile* testing method: a prospective multicentre diagnostic validation study of *C. difficile* infection. Lancet Infect Dis 13(11):936–45. doi:10.1016/S1473-3099(13)70200-7
35. Wilcox MH (2012) Overcoming barriers to effective recognition and diagnosis of *Clostridium difficile* infection. Clin Microbiol Infect 18(Suppl 6):13–20. doi:10.1111/1469-0691.12057
36. Spigaglia P, Barbanti F, Mastrantonio P, European Study Group on Clostridium difficile (ESGCD) (2011) Multidrug resistance in European *Clostridium difficile* clinical isolates. Antimicrob Chemother 66(10):2227–34. doi:10.1093/jac/dkr292
37. Solomon K, Fanning S, McDermott S, Murray S, Scott L, Martin A, Skally M, Burns K, Kuijper E, Fitzpatrick F, Fenelon L, Kyne L (2011) PCR ribotype prevalence and molecular basis of macrolide-lincosamide-streptogramin B (MLSB) and fluoroquinolone resistance in Irish clinical *Clostridium difficile* isolates. J Antimicrob Chemother 66(9):1976–82. doi:10.1093/jac/dkr275
38. van den Berg RJ, Claas EC, Oyib DH, Klaassen CH, Dijkshoorn L, Brazier JS, Kuijper EJ (2004) Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J Clin Microbiol 42(3):1035–41
39. Pituch H, Brazier JS, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M (2006) Prevalence and association of PCR ribotypes of *Clostridium difficile* isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLSB) type resistance. J Med Microbiol 55(Pt 2):207–13
40. Ackermann G, Degner A, Cohen SH, Silva J Jr, Rodloff AC (2003) Prevalence and association of macrolide-lincosamide-streptogramin B (MLS(B)) resistance with resistance to moxifloxacin in *Clostridium difficile*. J Antimicrob Chemother 51(3):599–603
41. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmée M, Kuijper E, Poxton IR, European Study Group on Clostridium difficile (ESGCD) (2008) Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. J Med Microbiol 57(Pt 6):784–9. doi:10.1099/jmm.0.47738-0
42. Drudy D, Kyne L, O'Mahony R, Fanning S (2007) gyrA mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027. Emerg Infect Dis 13(3):504–5
43. O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S (2008) Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. Antimicrob Agents Chemother 52(8):2813–7. doi:10.1128/AAC.00342-08

Příloha 9

Nyc O, Tejkalova R, Kriz Z, Ruzicka F, Kubicek L, Matejkova J, Kuijper E, Krutova M. Two Clusters of Fluoroquinolone and Clindamycin-Resistant *Clostridium difficile* PCR Ribotype 001 Strain Recognized by Capillary Electrophoresis Ribotyping and Multilocus Variable Tandem Repeat Analysis. Microb Drug Resist. 2016 Nov 18.

V článku jsou popsány výsledky mikrobiologicko-epidemiologické studie pětinásobně zvýšeného výskytu CDI na chirurgickém oddělení Fakultní nemocnice u svaté Anny v Brně v roce 2014. Studie zahrnovala jedenáct případů CDI vyvolaných shodným ribotypem 001. MLVA těchto jedenácti izolátů odhalila výskyt dvou nepříbuzných MLVA profilů (pět a šest izolátů). Klonální šíření MLVA profilu A zahrnovalo i případ CDI s měsíčním odstupem od posledního výskytu CDI na oddělení, kdy bylo oddělení na několik dní zavřeno a byla provedena kompletní sanitace.

MICROBIAL DRUG RESISTANCE
Volume 00, Number 00, 2016
Mary Ann Liebert, Inc.
DOI: 10.1089/mdr.2016.0159

Two Clusters of Fluoroquinolone and Clindamycin-Resistant *Clostridium difficile* PCR Ribotype 001 Strain Recognized by Capillary Electrophoresis Ribotyping and Multilocus Variable Tandem Repeat Analysis

Otakar Nyc,[1] Renata Tejkalova,[2] Zdenek Kriz,[3] Filip Ruzicka,[2] Lubos Kubicek,[3] Jana Matejkova,[1] Ed Kuijper,[4] and Marcela Krutova[1,5]

Aim: To perform a retrospective analysis of the high occurrence of *Clostridium difficile* infection in the surgical department of a Czech tertiary care hospital and to identify weaknesses in *C. difficile* infection (CDI) prevention and control policies. *Methods:* Clinical and epidemiological data on eleven CDI cases were collected. *C. difficile* isolates were characterized by capillary electrophoresis ribotyping, multilocus variable tandem repeat analysis (MLVA), *gyrA* gene fragment sequencing, and *erm*(B) fragment PCR amplification. Antibiotic susceptibility to metronidazole, vancomycin, ciprofloxacin, moxifloxacin, and clindamycin was tested. *Findings:* Eleven CDI cases were caused by *C. difficile* PCR ribotype 001 strains. These strains revealed two different MLVA profiles with 11 tandem repeat differences. All isolates were susceptible to metronidazole and vancomycin and resistant to ciprofloxacin (MIC ‡32 mg/L), moxifloxacin (MIC ‡32 mg/L), and clindamycin (MIC ‡256 mg/L). All isolates revealed amino acid substitution Thr82Ile, in the GyrA and were *erm*(B) negative. *Conclusion:* Two fluoroquinolone and clindamycin-resistant *C. difficile* PCR ribotype 001 strain clusters occurred at one of the surgical departments of a tertiary care hospital. Ineffective decontamination with suboptimal concentration and time of exposure of sporicidal disinfectants may have resulted in *C. difficile* transmission.

Keywords: *Clostridium difficile*, PCR-ribotype 001, capillary electrophoresis ribotyping, MLVA, antimicrobial drug resistance, Thr82Ile

Introduction

Clostridium difficile has become the leading causative agent of hospital-acquired diarrhea due to the spread of epidemic strains.[1] Toxin production, spore forming, and antimicrobial resistance are its most important virulence and spread factors. Toxin production is associated with *C. difficile* infection development[2] and spore formation plays a key role in *C. difficile* hospital transmission.[3] *C. difficile* spore transmission takes place through the hands of healthcare workers contaminated after carrying an infected patient or after touching a contaminated environment.[4]

C. difficile infection (CDI) incidence and CDI testing frequency vary widely in European countries (0.7–28.7 cases per 10,000 patient bed days; 4.6–223.3 tests per 10,000 bed days).[5] Underdiagnosed CDI cases due to suboptimal CDI laboratory algorithms and/or a low CDI testing frequency have undoubtedly contributed to the increasing CDI incidence trend.

Molecular typing allows differentiation of individual *C. difficile* isolates from each other. PCR ribotyping is the recommended typing method for CDI surveillance.[6] Application of capillary electrophoresis for detection of amplified 16S and 23S intergenic region fragments provides better discrimination power than gel-based ribotyping and, moreover, allows laboratory data exchange.[7,8]

Some *C. difficile* genotypes, such as PCR ribotype 027, appear to be more epidemically successful, with a large number of reported national outbreaks[9] and tracking of worldwide clonal spread by whole-genome sequencing.[10] Whereas PCR ribotype 027 has spread worldwide, other PCR ribotypes have been

[1]Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Praha, Czech Republic.
[2]Department of Medical Microbiology, St. Anne's University Hospital, and Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[3]2nd Department of Surgery, St. Anne's University Hospital, and Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[4]Leiden University Medical Centre, Leiden, the Netherlands.
[5]DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Praha, Czech Republic.

restricted to specific geographic areas; for example, PCR ribotype 244 in Australia[11] and PCR ribotype 176 in Poland[12] and the neighboring Czech Republic.[13] An outbreak association of other *C. difficile* types not from the PCR ribotype 027 family was reported, *e.g.*, toxin A-negative PCR ribotype 017[14,15] and PCR ribotype 106.[16]

To detect the clonal spread of *C. difficile* belonging to the same ribotype requires the use of another subtyping technique, such as multilocus variable tandem repeat analysis (MLVA)[17,18] or whole-genome sequencing.[19,20] A concordance of 95% in potential outbreak investigation was found between MLVA and whole-genome sequencing.[21]

In 2014, a fivefold higher CDI incidence at the surgical department was observed in comparison with the overall CDI incidence in the hospital. In response, we performed retrospective analysis to identify potential CDI outbreak risks and weaknesses in departmental CDI prevention and control policies.

Methods

Infection prevention control measures before and during increased CDI incidence

Appropriate hand hygiene and use of protective clothing. When contact with a CDI case occurred, healthcare workers, patients, and visitors washed their hands using soap and water, and after drying their hands, they used alcohol-based hand cleanser to avoid transmission of other nosocomial pathogens. The department staff used disposable gloves for every contact with patients and they also had to wear the disposable coat provided at every entrance to the department.

Environmental cleaning. Over the duration of the multiple CDI occurrences, admission of new patients was suspended and daily deep cleaning with sporicidal disinfectants (Persteril and Oxiper) was ordered. After the last patient was discharged, the surgical ward was closed and a complete environmental decontamination was performed.

When CDI occurred, cleaning was performed three times daily for frequently touched surfaces and twice daily for other surfaces (floor), and two sporicidal disinfectants (Persteril and Oxiper) were used alternately. Persteril, based on hydrogen peroxide in combination with peracetic acid, was used at the manufacturer's recommended concentration of 5,000 ppm and 30-minute exposure. Oxiper, based on hydrogen peroxide in combination with quarternary ammonium, was used at the manufacturer's recommended concentration of 2,500 ppm and 30-minute exposure.

Microbiological methods

Hospital CDI testing algorithm. Diarrheal stools are tested only at the physician's request. As a first diagnostic step, a dual lateral flow immunoenzymatic assay (Quik Chek complete, Alere) was used, which detects the presence of glutamate dehydrogenase and toxin A/B simultaneously in the stool sample. Stool samples that tested positive for GDH and toxin A/B or GDH only were cultured for the presence of *C. difficile* on a selective agar medium (Oxoid; *C. difficile* Selective Brazier's Medium, PB5191A) after an alcohol shock treatment. Selected *C. difficile* isolates were sent to the Department of Medical Microbiology of Motol University Hospital for molecular typing.

Ribotyping, toxin gene multiplex PCR, MLVA. Capillary electrophoresis ribotyping was performed according to the standard operation protocol (www.ecdisnet.eu) using primers published by Stubbs *et al.*[22] The Leeds-Leiden *C. difficile* reference strain collection was used to determine the ribotype.

The presence of genes for toxin (A, B, binary) production was investigated by multiplex PCR.[23]

MLVA was performed by amplification and sequencing of seven previously published loci (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, and H9Cd)[17] with change of reverse primer for locus G8Cd.[24] The sum of tandem repeat difference (STRD) was counted manually after sequence software processing (Sequencing Analysis Software, Applied Biosystems).

Antibiotic susceptibility testing and molecular resistance mechanism investigation. Susceptibility to metronidazole, vancomycin, ciprofloxacin, moxifloxacin, and clindamycin was determined by E-test (Liofilchem) on Wilkins-Chalgren agar. Clinical breakpoints of 8 mg/L for clindamycin, 4 mg/L for ciprofloxacin,[25] 4 mg/L for moxifloxacin, and 2 mg/L for metronidazole and vancomycin[26] were assessed. The molecular mechanism of resistance to fluoroquinolones was investigated by amplification and sequencing of the *gyrA* gene[27] and to clindamycin by amplification of the *erm*(B) gene fragment.[28]

Results

CDI case history

Four CDI cases occurred from January 2014 to the end of February 2014 (patients 1–4) and six CDI cases occurred from November 2014 to the end of December 2014 (patients 6–11) in the surgical department (17 beds) of the tertiary care hospital (900 beds). Between these two multiple CDI case occurrences, one CDI case, ostensibly unrelated, was observed (patient 5, March 2014). All eleven CDI cases were considered as healthcare-associated infections. Ten patients (Fig. 1, patients 2–11) developed symptoms of CDI more than 48 hours after admission and one patient (Fig. 1, patient 1) showed the onset of CDI at the time of admission, but she had been transferred from another healthcare facility. Detailed CDI timelines are depicted in Fig. 1.

Three patients had a recurrent episode of CDI (patients 1–3). CDI incidence in the surgical ward was 13.7 cases per 10,000 patient bed days or 94.9 cases per 10,000 admissions. The overall hospital CDI incidence was 2.7 cases per 10,000 patient bed days or 23.1 cases per 10,000 admissions.

Of 11 patients, 9 were males and 2 females. Average age was 70.7 years. All patients had used antibiotic therapy in previous months. The spectrum of antibiotics in combination or monotherapy was as follows: lincosamides (n = 5), cephalosporins (1st generation n = 3 and 2nd and 3rd generation n = 1), 2nd generation of fluoroquinolones (n = 3), piperacillin/tazobactam (n = 3), oral penicillins (n = 2), aminopenicillins (n = 1), and carbapenems (n = 1). CDI-specific antimicrobial therapy was initiated in all 11 CDI cases. Metronidazole was used together with vancomycin in all 11 cases, and, in one patient, in combination with fidaxomicin (fulminant CDI, leucocytosis 40.1 · 10^9/L). All three recurrences were treated with fidaxomicin. Characteristics of patients are summarized in the Table 1. The timeline of individual CDI cases is shown in Fig. 1.

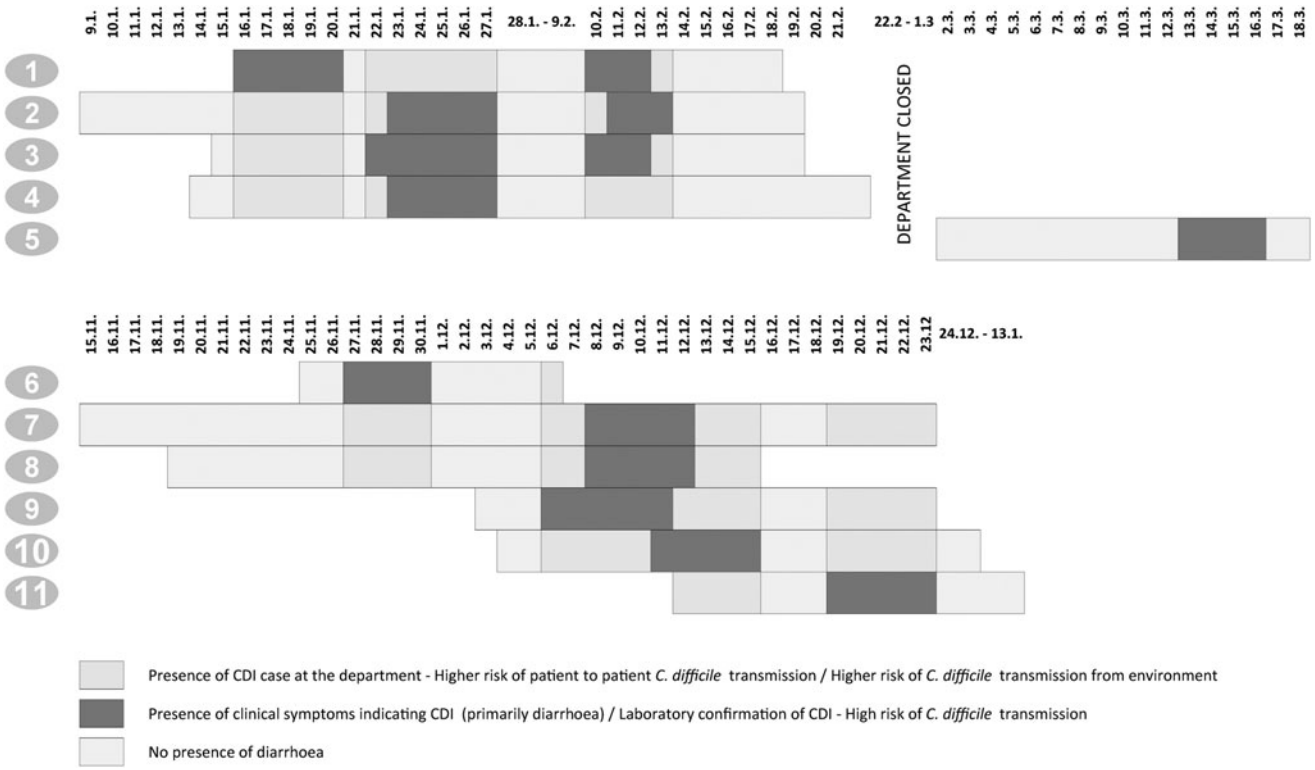


FIG. 1. Each *line* represents the timeline of hospitalization of the patient. The presence and no presence of diarrhea are marked in *black* and *gray* color. *Dark gray color* indicates the higher risk for *Clostridium difficile* transmission for other patients due to the presence of CDI at the department. CDI, *C. difficile* infection.

Microbiological data

A total of 11 *C. difficile* isolates were sent for molecular typing. CE ribotyping revealed the same electrophoretic profile belonging to PCR ribotype 001.

Subtyping by MLVA divided the isolates into two groups. The first MLVA profile A (5 isolates) was A6Cd = 41, B7Cd = 18, C6Cd = 33, E7Cd = 4, F3Cd = 6, G8Cd = 6, and H9Cd = 1 and the second MLVA profile B (6 isolates) was A6Cd = 35, B7Cd = 21, C6Cd = 35, E7Cd = 4, F3Cd = 6, G8Cd = 6, and H9Cd = 1. The sum of the tandem repeat differences between these two MLVA profiles was 11.

MLVA profiles corresponded with multiple CDI occurrence timelines. The causative *C. difficile* strain of one solitary CDI case (Fig. 1, patient 5, CDI case in March 2014) had the identical MLVA profile to the *C. difficile* strain A cluster.

All isolates were susceptible to metronidazole (ranged from 1.5 to 2 mg/L) and vancomycin (ranged 0.5–1 mg/L) and resistant to ciprofloxacin (MIC ‡32 mg/L), moxifloxacin (MIC ‡32 mg/L), and clindamycin (MIC ‡256 mg/L). All isolates (n = 11) revealed amino acid substitution, Thr82Ile, in the GyrA and they were *erm*(B) negative.

Discussion

In the period January to December 2014, a fivefold higher CDI incidence was observed at the surgical ward (13.7 cases per 10,000 patient bed days or 94.9 cases per 10,000 admissions) compared with the hospital CDI incidence (2.7 cases per 10,000 patient bed days or 23.1 cases per 10,000 admissions).

The hospital CDI incidence showed the constant rates (2.8 cases per 10,000 patient bed days or 20.6 cases per 10,000 admissions in 2013 and 2.8 cases per 10,000 patient bed days or 23.9 cases per 10,000 admissions in 2015) lower than was reported for the Czech Republic (6.2 CDI cases/10,000 patient bed days or 35.1 CDI cases per 10,000 admissions in 2012–13).[4] CDI incidence at the surgical ward, in comparison with the hospital CDI figures, revealed higher changing rates (11.5 CDI cases/10,000 patient bed days in 2012–2013 or 80.0 CDI cases per 10,000 admissions and 4.9 CDI cases/10,000 patient bed days or 34.0 CDI cases per 10,000 admissions in 2015).

The causative *C. difficile* strains were typed as PCR ribotype 001. This ribotype is one of the successful epidemic ribotypes in Europe[29] with a reported association with the lethal course of CDI.[30]

In the Czech Republic, PCR ribotype 001 (20.7%) and PCR ribotype 176 (26.7%) belong to the prevailing RTs in the *C. difficile* strain collection (n = 2,201).[31] During the period 2013–2015, a total of 97 *C. difficile* isolates (2, 71, and 24, respectively) were sent for molecular typing from St. Anne's University Hospital. Of these, 54 isolates (55.7%) belonged to PCR ribotype 001 and nine isolates (9.3%) to PCR ribotype 176, which reflects the current unfavorable epidemiological situation in the Czech Republic.[31] Subtyping by MLVA divided the isolates into two groups. Eleven STRDs were found between these two MLVA profiles, which is very close to the MLVA threshold for genetically related strains (‡2 to £10 STRD),[17] raising the question of the possibility of their genetic relatedness.

Table 1. Characterization of Patients Infected by Two Different PCR Ribotype 001 *Clostridium difficile* Strains

				First CDI episode			*Recurrent CDI episode*		
	Number/ sex/age	*Underlying disease*	*Antibiotic usage in previous month*	*WBC 10^9/L*	*CRP*	*CDI treatment*	*WBC 10^9/L*	*CRP*	*CDI treatment*
Cluster of C. difficile PCR ribotype 001 strain A	1/F/65	Implantation of femoropopliteal bypass	2nd generation of fluoroquinolones, piperacillin/tazobactam	6.1	NA	V, MTZ	6.8	73	FDX
	2/M/74	Implantation of pedal bypass	3rd generation of cephalosporins	12.2	124	V, MTZ	14.7	93	FDX
	3/M/68	Peripheral angioplasty of lower limb	2nd generation of fluoroquinolones, aminopenicillins, lincosamides	13.6	79	V, MTZ	15.9	196	FDX
	4/M/68	Above-knee amputation	2nd generation of cephalosporins, piperacillin/tazobactam, vancomycin	14.0	98	V, MTZ			
	5/F/78	Peripheral angiography	Lincosamides	12	131	V, MTZ			
Cluster of C. difficile PCR ribotype 001 strain B	6/M/69	Above-knee amputation	Lincosamides, 2nd generation of fluoroquinolones, piperacillin/ tazobactam	40.1	216	V, MTZ, FDX			
	7/M/84	Femoral prosthesis infection	Penicillins, carbapenems, 1st generation of cephalosporins	NA	NA	V, MTZ			
	8/M/77	Implantation of femoropopliteal bypass	Penicillins, lincosamides	11.6	80	V, MTZ			
	9/M/70	Implantation of femoropopliteal bypass	1st generation of cephalosporins	13.6	130	V, MTZ			
	10/M/59	Implantation of femoropopliteal bypass	Lincosamides	13.6	112	V, MTZ			
	11/M/66	Angioplasty of lower limb, finger amputation	1st generation of cephalosporins	13.8	133	V, MTZ			

CDI, *C. difficile* infection.

In the Czech Republic, no recommendations for sending *C. difficile* isolates for molecular characterization are available. The Ontario Ministry of Health has established the threshold number of cases when CDI preventive measures are triggered: for wards of <20 beds, if two HA-CDI cases occur in 1 ward within a 7-day period or four cases of HA-CDI within a 4-week period, and for wards of ‡20 beds, if three cases of HA-CDI occur in 1 ward within a 7-day period or five cases of HA-CDI within a 4-week period.[32] Adopting these thresholds for triggering CDI preventive measures and for sending *C. difficile* isolates for molecular characterization to a reference laboratory could markedly improve CDI management and control.

Several factors contributed simultaneously to CDI development in patients in the study. Patients hospitalized in the surgical ward belonged to an older age group (>65 years), had chronic underlying disease, and used repeated prolonged antimicrobial therapy with majority representation of cephalosporins, clindamycin, and quinolones. These antimicrobial drugs belong to the group of high-risk CDI-associated antibiotics.[33] The restricted use of these specific groups of antibiotics (cephalosporins, clindamycin, and quinolones) to minimize patient susceptibility was one of the key factors that led to decreasing CDI hospital incidence in several studies.[33]

All *C. difficile* isolates in this study revealed resistance to ciprofloxacin, moxifloxacin, and clindamycin. Resistance of *C. difficile* PCR ribotype 001 to clindamycin and moxifloxacin was reported in European *C. difficile* isolates.[34–36] All isolates in the study revealed the amino acid substitution Thr82Ile, in the GyrA, which is associated with resistance to fluoroquinolones[27] and was also reported in *C. difficile* PCR ribotype 001 moxifloxacin-resistant isolates.[35] All isolates resistant to clindamycin were *erm*(B) negative. The *erm*(B) negativity in high-level clindamycin-resistant isolates is a well-known phenomenon[35,37] and the molecular mechanism of resistance remains unexplained.

In both clusters of CDI, hydrogen peroxide in combination with peracetic acid at a concentration of 5,000 ppm or hydrogen peroxide in combination with quarternary ammonium at a concentration of 2,500 ppm with 30-minute exposure was used for daily cleaning as well as for terminal decontamination of the ward. These concentrations and time of exposure were probably not sufficient since Perez *et al.* reported that the concentration of hydrogen peroxide required for inactivation of all *C. difficile* spores in 10 minutes was above 7,000 ppm.[38] The inclusion of chlorine products in the disinfectant scheme would be beneficial.[39,40]

Implementation of Czech national guidance for infection prevention and control practices and participation in the CDI surveillance organized by the ECDC are the two most important steps for improving management and control of CDI in the Czech Republic.

Conclusion

Two *C. difficile* PCR ribotype 001 fluoroquinolones and clindamycin-resistant strain clusters were identified at a surgical department of a tertiary care hospital. Ineffective decontamination with suboptimal concentration and time of exposure of sporicidal disinfectants may have resulted in *C. difficile* spore transmission. The findings of our study stress the need for the implementation of Czech national guidance for CDI prevention and control to standardize environmental decontamination procedures as well as to define the CDI occurrence threshold as to when to send *C. difficile* isolates for molecular typing.

Acknowledgments

The authors would like to thank the ESCMID Study Group for *C. difficile* (ESGCD) for their professional support. This study was supported by the MH CZ–DRO, University Hospital Motol, Prague, Czech Republic 00064203.

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.

Disclosure Statement

The authors declare that they have no conflicts of interest. O.N. received a foundation grant from the Ministry of Health Czech Republic in 2013–2015.

References

1. Freeman, J., M.P. Bauer, S.D. Baines, J. Corver, W.N. Fawley, B. Goorhuis, E.J. Kuijper, and M.H. Wilcox. 2010. The changing epidemiology of *Clostridium difficile* infections. Clin. Microbiol. Rev. 23:529–549.
2. Shen, A. 2012. *Clostridium difficile* toxins: mediators of inflammation. J. Innate. Immun. 4:149–158.
3. Barra-Carrasco, J., and D. Paredes-Sabja. 2014. *Clostridium difficile* spores: a major threat to the hospital environment. Future Microbiol. 9:475–486.
4. Barbut, F. 2015. How to eradicate *Clostridium difficile* from the environment? J. Hosp. Infect. 89:287–295.
5. Davies, K.A., C.M. Longshaw, G.L. Davis, E. Bouza, F. Barbut, Z. Barna, M. Delmée, F. Fitzpatrick, K. Ivanova, E. Kuijper, I.S. Macovei, S. Mentula, P. Mastrantonio, L. von Müller, M. Oleastro, E. Petinaki, H. Pituch, T. Norén, E. Nováková, O. Nye, M. Rupnik, D. Schmid, and M.H. Wilcox. 2014. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect. Dis. 14:1208–1219.
6. European Centre for Disease Prevention and Control. 2015. European Surveillance of *Clostridium difficile* infections. Surveillance protocol version 2.2. ECDC, Stockholm.
7. Indra, A., S. Huhulescu, M. Schneeweis, P. Hasenberger, S. Kernbichler, A. Fiedler, G. Wewalka, F. Allerberger, and E.J. Kuijper. 2008. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J. Med. Microbiol. 57(Pt 11):1377–1382.
8. Fawley, W.N., C.W. Knetsch, D.R. MacCannell, C. Harmanus, T. Du, M.R. Mulvey, A. Paulick, L. Anderson, E.J. Kuijper, and M.H. Wilcox. 2015. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One. 10:e0118150.

9. Kuijper, E.J., F. Barbut, J.S. Brazier, N. Kleinkauf, T. Eckmanns, M.L. Lambert, D. Drudy, F. Fitzpatrick, C. Wiuff, D.J. Brown, J.E. Coia, H. Pituch, P. Reichert, J. Even, J. Mossong, A.F. Widmer, K.E. Olsen, F. Allerberger, D.W. Notermans, M. Delmée, B. Coignard, M. Wilcox, B. Patel, R. Frei, E. Nagy, E. Bouza, M. Marin, T. Akerlund, A. Virolainen-Julkunen, O. Lyytikäinen, S. Kotila, A. Ingebretsen, B. Smyth, P. Rooney, I.R. Poxton, and D.L. Monnet. 2008. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Euro. Surveill. 13: pii: 18942.
10. He, M., F. Miyajima, P. Roberts, L. Ellison, D.J. Pickard, M.J. Martin, T.R. Connor, S.R. Harris, D. Fairley, K.B. Bamford, S. D'Arc, J. Brazier, D. Brown, J.E. Coia, G. Douce, D. Gerding, H.J. Kim, T.H. Koh, H. Kato, M. Senoh, T. Louie, S. Michell, E. Butt, S.J. Peacock, N.M. Brown, T. Riley, G. Songer, M. Wilcox, M. Pirmohamed, E. Kuijper, P. Hawkey, B.W. Wren, G. Dougan, J. Parkhill, and T.D. Lawley. 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat. Genet. 45:109–113.
11. Eyre, D.W., L. Tracey, B. Elliott, C. Slimings, P.G. Huntington, R.L. Stuart, T.M. Korman, G. Kotsiou, R. McCann, D. Griffiths, W.N. Fawley, P. Armstrong, K.E. Dingle, A.S. Walker, T.E. Peto, D.W. Crook, M.H. Wilcox, and T.V. Riley. 2015. Emergence and spread of predominantly community-onset *Clostridium difficile* PCR ribotype 244 infection in Australia, 2010 to 2012. Euro. Surveill. 20:21059.
12. Pituch, H., P. Obuch-Woszczatyński, D. Lachowicz, D. Wultańska, P. Karpiński, G. Mıynarczyk, S.M. van Dorp, E.J. Kuijper, and Polish *Clostridium difficile* Study Group. 2015. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro. Surveill. 20:38.
13. Krutova, M., O. Nyc, E.J. Kuijper, L. Geigerova, J. Matejkova, T. Bergerova, and M. Arvand. 2014. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 30:153–155.
14. Du, P., B. Cao, J. Wang, W. Li, H. Jia, W. Zhang, J. Lu, Z. Li, H. Yu, C. Chen, and Y. Cheng. 2014. Sequence variation in tcdA and tcdB of *Clostridium difficile*: ST37 with truncated tcdA is a potential epidemic strain in China. J. Clin. Microbiol. 52:3264–3270.
15. Goorhuis, A., S.B. Debast, J.C. Dutilh, C.M. van Kinschot, C. Harmanus, S.C. Cannegieter, E.C. Hagen, and E.J. Kuijper. 2011. Type-specific risk factors and outcome in an outbreak with 2 different *Clostridium difficile* types simultaneously in 1 hospital. Clin. Infect. Dis. 53:860–869.
16. Ratnayake, L., J. McEwen, N. Henderson, D. Nathwani, G. Phillips, D. Brown, and J. Coia. 2011. Control of an outbreak of diarrhoea in a vascular surgery unit caused by a high-level clindamycin-resistant *Clostridium difficile* PCR ribotype 106. J. Hosp. Infect. 79:242–247.
17. van den Berg, R.J., I. Schaap, K.E. Templeton, C.H. Klaassen, and E.J. Kuijper. 2007. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J. Clin. Microbiol. 45:1024–1028.
18. Eckert, C., F. Vromman, A. Halkovich, and F. Barbut. 2011. Multilocus variable-number tandem repeat analysis: a helpful tool for subtyping French *Clostridium difficile* PCR ribotype 027 isolates. J. Med. Microbiol. 60(Pt 8): 1088–1094.
19. Mac Aogáin, M., G. Moloney, S. Kilkenny, M. Kelleher, M. Kelleghan, B. Boyle, and T.R. Rogers. 2015. Whole-genome sequencing improves discrimination of relapse from reinfection and identifies transmission events among patients with recurrent *Clostridium difficile* infections. J. Hosp. Infect. 90:108–116.
20. Steglich, M., A. Nitsche, L. von Müller, M. Herrmann, T.A. Kohl, S. Niemann, and U. Nübel. 2015. Tracing the Spread of *Clostridium difficile* Ribotype 027 in Germany Based on Bacterial Genome Sequences. PLoS One. 10:e0139811.
21. Eyre, D.W., W.N. Fawley, E.L. Best, D. Griffiths, N.E. Stoesser, D.W. Crook, T.E. Peto, A.S. Walker, and M.H. Wilcox. 2013. Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. J. Clin. Microbiol. 51:4141–4149.
22. Stubbs, S.L., J.S. Brazier, G.L. O'Neill, and B.I. Duerden. 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J. Clin. Microbiol. 37:461–463.
23. Persson, S., M. Torpdahl, and K.E. Olsen. 2008, 2009. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. Clin. Microbiol. Infect. 14:1057–1064. Erratum in: Clin. Microbiol. Infect. 15:296.
24. Goorhuis, A., M.C. Legaria, R.J. van den Berg, C. Harmanus, C.H. Klaassen, J.S. Brazier, G. Lumelsky, and E.J. Kuijper. 2009. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina. Clin. Microbiol. Infect. 15:1080–1086.
25. Clinical and Laboratory Standards Institute. 2007. Methods for antimicrobial susceptibility testing of anaerobic bacteria. Approved standard M11-A7. (7th ed.) CLSI, Wayne, PA.
26. European Committee on Antimicrobial Susceptibility Testing, EUCAST. Clinical breakpoints for bacteria. Available at www.eucast.org/clinical_breakpoints/
27. Dridi, L., J. Tankovic, B. Burghoffer, F. Barbut, and J.C. Petit. 2002. *gyrA* and *gyrB* mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob. Agents Chemother. 46: 3418–3421.
28. Spigaglia, P., P. Mastrantonio. 2004. Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. J. Med. Microbiol. 53(Pt 11):1129–1136.
29. Bauer, M.P., D.W. Notermans, B.H. van Benthem, J.S. Brazier, M.H. Wilcox, M. Rupnik, D.L. Monnet, J.T. van Dissel, E.J. Kuijper, and ECDIS Study Group. 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. 377:63–73.
30. Arvand, M., A.M. Hauri, N.H. Zaiss, W. Witte, and G. Bettge-Weller. 2009. *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal *C. difficile* infection in Hesse, Germany. Euro. Surveill. 14: pii: 19403.
31. Krutova, M., O. Nyc, J. Matejkova, F. Allerberger, M.H. Wilcox, and E.J. Kuijper. 2016. Molecular characterisation of Czech *Clostridium difficile* isolates collected in

2013–2015. Int. J. Med. Microbiol. [Epub ahead of print]; DOI:10.1016/j.ijmm.2016.07.003.
32. Goldstein, E.J., S. Johnson, P.J. Maziade, L.V. McFarland, W. Trick, L. Dresser, M. Millette, H. Mazloum, and D.E. Low. 2015. Pathway to Prevention of Nosocomial *Clostridium difficile* Infection. Clin. Infect. Dis. 60 Suppl 2: S148–S158.
33. Khanafer, N., N. Voirin, F. Barbut, E. Kuijper, and P. Vanhems. 2015. Hospital management of *Clostridium difficile* infection: a review of the literature. J. Hosp. Infect. 90:91–101.
34. Barbut, F., P. Mastrantonio, M. Delmée, J. Brazier, E. Kuijper, I. Poxton, and European Study Group on *Clostridium difficile* (ESGCD). 2007. Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. Clin. Microbiol. Infect. 13:1048–1057.
35. Spigaglia, P., F. Barbanti, P. Mastrantonio, and European Study Group on *Clostridium difficile* (ESGCD). 2011. Multidrug resistance in European *Clostridium difficile* clinical isolates. J. Antimicrob. Chemother. 66:2227–2234.
36. Freeman, J., J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw, M.H. Wilcox, and Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent *Clostridium difficile* Ribotypes' Study Group. 2015. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin. Microbiol. Infect. 21:248.e9–248.e16.
37. Krutova, M., J. Matejkova, J. Tkadlec, and O. Nyc. 2015. Antibiotic profiling of *Clostridium difficile* ribotype 176- A multidrug resistant relative to *C. difficile* ribotype 027. Anaerobe. 36:88–90.
38. Perez, J., V.S. Springthorpe, and S.A. Sattar. 2005. Activity of selected oxidizing microbicides against the spores of *Clostridium difficile*: relevance to environmental control. Am. J. Infect. Control. 33:320–325.
39. Speight, S., A. Moy, S. Macken, R. Chitnis, P.N. Hoffman, A. Davies, A. Bennett, and J.T. Walker. 2011. Evaluation of the sporicidal activity of different chemical disinfectants used in hospitals against *Clostridium difficile*. J. Hosp. Infect. 79:18–22.
40. Martin, M., W. Zingg, E. Knoll, C. Wilson, M. Dettenkofer, and PROHIBIT Study Group. 2014. National European guidelines for the prevention of *Clostridium difficile* infection: a systematic qualitative review. J. Hosp. Infect. 87:212–219.

Address correspondence to:
Marcela Krutova, MSc
Department of Medical Microbiology
2nd Faculty of Medicine
Charles University in Prague
and Motol University Hospital
Praha 15006
Czech Republic

E-mail: marcela.krutova@seznam.cz

Příloha 10

Drabek J, Nyc O, Krutova M, Stovicek J, Matejkova J, Keil R. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study. Ann Clin Microbiol Antimicrob. 2015;14:55.

V článku jsou popsány výsledky roční studie výskytu onemocnění vyvolaných *C. difficile* na Interním oddělení ve FN Motole. Práce je zaměřena na korelaci závažnosti klinického průběhu onemocnění s molekulární charakteristikou izolátů. U pacientů s infekcí *C. difficile* ribotypem 176 byla zjištěna vyšší hodnota ATLAS skóre a Hornova indexu svědčící pro závažnější průběh infekce. Epidemiologicky významné je i zjištění dvou klonálních komplexů, které zahrnovaly 10 ze 14 izolátů PCR-ribotypu 176. Daná skutečnost svědčí pro šíření toho ribotypu v rámci oddělení.

Drabek et al. Ann Clin Microbiol Antimicrob (2015) 14:55
DOI 10.1186/s12941-015-0114-0

Annals of Clinical Microbiology and Antimicrobials

SHORT REPORT Open Access

Clinical features and characteristics of *Clostridium difficile* PCR-ribotype 176 infection: results from a 1-year university hospital internal ward study

Jiri Drabek[1], Otakar Nyc[2], Marcela Krutova[2,3], Jan Stovicek[1], Jana Matejkova[2] and Radan Keil[1*]

Abstract

Background: *Clostridium difficile* infection (CDI) is a major cause of antibiotic-associated diarrhoea. Given an increasing CDI incidence and global spread of epidemic ribotypes, a 1-year study was performed to analyse the molecular characteristics of *C. difficile* isolates and associated clinical outcomes from patients diagnosed with CDI in the Internal Medicine department at University Hospital Motol, Prague from February 2013 to February 2014.

Results: A total of 85 unformed stool samples were analysed and CDI was laboratory confirmed in 30 patients (6.8 CDI cases per 10,000 patient bed days and 50.6 CDI cases per 10,000 admissions). The CDI recurrence rate within 3 months of treatment discontinuation was 13.3% (4/30). Mortality within 3 months after first CDI episode was 26.7% (8/30), with CDI the cause of death in two cases. 51.9% of *C. difficile* isolates belonged to PCR-ribotype 176. MLVA of ribotype 176 isolates revealed two clonal complexes formed by 10/14 isolates. ATLAS scores and Horn's index were higher in patients with ribotype 176 infections than with non-ribotype 176 infections.

Conclusion: This study highlights the clinical relevance of *C. difficile* PCR-ribotype 176 and its capacity to spread within a healthcare facility.

Keywords: *Clostridium difficile*, PCR-ribotype 176, Horn's index, ATLAS score, Ribotyping, MLVA

Findings

Background

Clostridium difficile infection (CDI) is a major cause of antibiotic-associated diarrhoea and a significant burden to healthcare services worldwide [1]. Results of a pan-European epidemiological study in 2008 indicated that the Czech Republic has a relatively low CDI incidence (1.1 per 10,000 patient bed-days and 7.0 per 10,000 hospital admissions) [2], although a recent epidemiological study suggested a CDI incidence rate of 4.4 and 6.2 cases per 10,000 patient bed-days in 2011–12 and 2012–13, respectively [3].

In 2013, the high prevalence of PCR-ribotype 176 (n = 251; 40 %) was revealed by ribotyping of 624 *C. difficile* isolates from 11 Czech healthcare facilities [4] *C. difficile* ribotype 176 is thought to share many similarities to ribotype 027 [5–7] and it has been suggested that this type may be misdiagnosed as a ribotype 027 infection [8]. The long-term epidemic occurrence of *C. difficile* PCR-ribotype 176 was also reported in Poland [9, 10].

In response to the reported unfavourable global CDI epidemiological situation, including in Czech Republic, a 1-year study was initiated to monitor the incidence of CDI, clinical features and outcomes and to investigate the molecular characteristics of *C. difficile* isolates in patients with CDI hospitalised in the Internal Medicine department of University Hospital Motol, Prague, Czech Republic, from February 2013 to February 2014.

*Correspondence: Radan.Keil@fnmotol.cz
[1] Department of Internal Medicine, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, 150 06, V Uvalu 84, Praha 5, Prague, Czech Republic
Full list of author information is available at the end of the article

Microbiological testing

Stool samples of 85 patients aged ≥18 years with three or more unformed stools per day were investigated at the Department of Medical Microbiology. CDI was laboratory diagnosed using the *C. difficile* Quik Chek Complete® test (Alere) and *C. difficile* Alere and simultaneous toxin A/B positivity was detected in 24 samples (80 %). In six samples that were only GDH-positive but where patients had relevant clinical symptoms, the presence of toxigenic *C. difficile* was confirmed using PCR (GeneXpert®, Cepheid). Positive stool samples (GDH and toxin positive; GDH positive, toxin negative and PCR positive) were cultured anaerobically, after an alcohol shock treatment, on selective media (Oxoid); anaerobic culture was positive for *C. difficile* in 27/30 samples (90 %). Antibiotic susceptibility of *C. difficile* isolates to metronidazole and vancomycin was determined by E-test® (BioMérieux) and minimum inhibitory concentrations for all *C. difficile* isolates ranged from 0.03–2 mg/L for metronidazole and 0.015–1 mg/L for vancomycin (Table 1). No isolates were found to be resistant to either metronidazole or vancomycin.

C. *difficile* isolates molecular characterisation

PCR-ribotyping was performed according to the Standard Operating Protocol of ECDIS-net (http://www.ecdisnet.eu) using capillary electrophoresis after PCR amplification with primers previously described by Stubbs et al. [11]. Electrophoreograms were confirmed using the Webribo database [12]. PCR-ribotypes were identified for all 27 *C. difficile* isolates and 14 (51.9 %) belonged to ribotype 176. Other identified ribotypes were 012 (n = 2; 7.4 %), 014 (n = 2; 7.4 %), 001, 002, 005, 017, 020, 049, 078, 434 and 015 (all n = 1; 3.7 %).

The presence of toxin genes was determined by multiplex PCR with specific primers for *tcdA* (toxin A), *tcdB* (toxin B), *cdtA* and *cdtB* (binary toxin) [13]. All *C. difficile* isolates revealed presence of genes for production of toxins A/B, while genes for production of binary toxin (*cdtA*/*cdtB*), which has been associated with increased attachment to epithelial cells, increased virulence and higher recurrence rates [14–16] were only found in isolates of ribotypes 176 and 078 (15/27; 55.6 %). Summary of microbiological and molecular characteristics of C. difficile isolates is shown in Table 1.

The *tcdC* gene was amplified with primers C1 and C2 [17] and the obtained sequence was compared to NCBI reference sequence NC_009089.1. Two deletions (position 117, which introduces a frame-shift mutation leading to protein truncation [17], and 330–347) in the *tcdC* gene were found in all 14 ribotype 176 isolates. One isolate, ribotype 078, revealed 39-bp deletion from nucleotides 341–379 in the *tcdC* gene. No deletion in other 12 isolates was found. The precise function of the *tcdC* gene is not yet clear [18].

Genetic relatedness among *C. difficile* ribotype 176 isolates was achieved using multi-locus-variable tandem repeats-analysis (MLVA). The number of tandem repeats were determined by Sanger sequencing in five previously published variable tandem repeat (VNTR) loci (A6Cd, B7Cd, C6Cd, G8Cd) [19, 20] and CDR60 [21]. A Minimum Spanning Tree (MST) was created by Bionumerics v5.0 (Applied Maths) using a Manhattan coefficient to calculate the summed tandem repeat difference (STRD). A cluster analysis using the categorical distance and unweighted pair group method with arithmetic mean algorithms was also applied. The number of tandem repeats for each locus is summarised in Fig. 1. MST identified two clonal complexes when STRD ≤1 (Fig. 2). The first clonal complex was formed from eight isolates (55, 269, 351, 263, 273, 279, 294 and 308). The second clonal complex consisted of two isolates (336 and 322). Between clonal complexes one and two, STDR = 6 were found. Isolate 248 revealed STDR = 9 to isolate 294 (CC1), isolate 259 showed STDR = 5 to isolate 336 (CC2), isolate 316 showed STDR = 6 and isolate 303 STDR = 9 to isolate 322 (CC2).

The time intervals of hospitalisation of patients infected by *C. difficile* ribotype 176 did not overlap except for two patients. This finding suggests that the probable source of infection may have come from the hospital environment and, given the high incidence of this ribotype previously reported in the Czech Republic [4], it is possible that ribotype 176 is endemic in the country and this type has

Fig. 1 A Categorical MLVA of *C. difficile* ribotype 176 isolates (Bionumerics v5.0, Applied Maths)

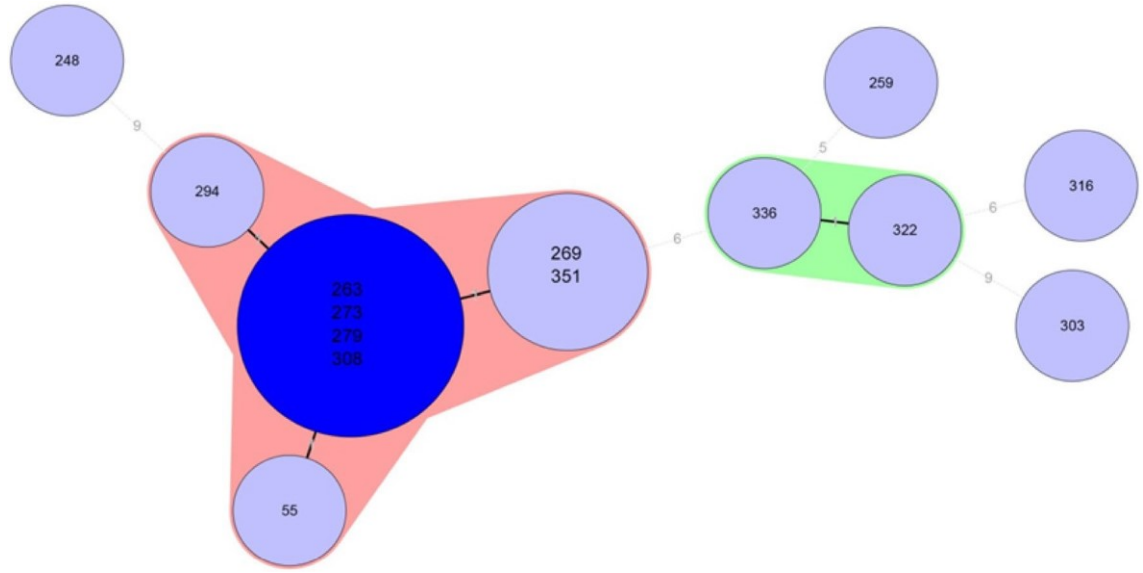


Fig. 2 A Minimum Spanning Tree of *C. difficile* ribotype 176 isolates (Bionumerics v5.0, Applied Maths). The *numbers in the circles* represent *C. difficile* PCR-ribotype 176 isolate number. The *numbers in the lines* represent the sum of tandem repeat differences between isolates

been introduced into the hospital environment on several occasions.

Clinical and epidemiological data analysis

CDI was diagnosed in 30 patients (female n = 13, male n = 17; mean age 69.0 years). The overall CDI incidence in the Internal Medicine ward during the study period was calculated as 6.8 CDI cases per 10,000 patient bed-days and 50.6 CDI cases per 10,000 admissions which indicated a higher CDI incidence compared with recently reported rates [3].

Healthcare-associated CDI (HA-CDI) was diagnosed in 26 CDI cases (86.7 %) and community-associated CDI (CA-CDI) was diagnosed in four CDI cases (13.3 %). Severe CDI was diagnosed in 17 (56.7 %) patients according to the Horn's index [22, 23] and 18 (60 %) according to the ATLAS score [24]. Antibiotic treatment prior to CDI diagnosis was noted for 83.3 % (25/30) of patients. The most commonly used antibiotics were aminopenicillins with beta-lactamase inhibitors (n = 12), fluoroquinolones (n = 12), broad-spectrum cephalosporins (n = 11), carbapenems (n = 4), piperacillin–tazobactam (n = 3) and aminoglycosides (n = 3). Administered CDI treatments, according to valid guidelines at the time of the study [25], were metronidazole (n = 10; 33.3 %), vancomycin (n = 3; 10.0 %), combined metronidazole and vancomycin (n = 13; 43.3 %), and metronidazole with other therapies (n = 1; 3.3 %). Three patients did not receive treatment for CDI. CDI recurrence within 3 months of treatment discontinuation was observed in 13.3 % (4/30) of patients and two received faecal transplant for recurrent disease. Mortality within 3 months after first CDI episode was 26.7 % (8/30); CDI was the cause of death in two cases 6.7 % (2/30) (Table 2).

To assess the association between *C. difficile* ribotype and disease severity, the clinical outcomes of patients with ribotype 176 infections were compared to those with other ribotype infections (Table 3). Analysis of ATLAS scores and the Horn's index found that 11/14 (78.6 %) patients with ribotype 176 infections had an ATLAS score of 6–9 or a Horn's index score of 3 or 4 compared with 6/13 (46.2 %) and 7/13 (53.9 %) of patients with non-ribotype 176 infections. Furthermore, the mortality rate appeared to be higher in patients with ribotype 176 infections compared with non-ribotype 176 infections (35.7 versus 15.4 %). No significant ribotype-associated differences were noted in recurrence rates, ICU admission rates or prior antibiotic use (Table 3).

Clostridium difficile ribotype 027 strains are often thought to be associated with CDI outbreaks of increased disease severity [1, 5], but the clinical severity associated with ribotype 176 infections has not yet been studied in detail with exception of clinical data on ten patients, of whom 50 % had severe form of CDI, reported by Obuch-Woszczatyński et al. [9]. Our finding of a trend towards increased Horn's index and ATLAS scores in patients with

Table 1 Microbiological and molecular characteristics of *C. difficile* isolates

CDI case no.	Isolate no.	GDH	Toxin A/B	Anaerobic culture	PCR-ribotype	Toxin gene presence (A/B/bin)[a,b]	Vancomycin MIC (mg/L)	Metronidazole MIC (mg/L)
1	n/a	+	+	–	n/a	n/a	n/a	n/a
2	259	+	+	+	176	A/B/bin	0.25	0.25
3	263	+	+	+	176	A/B/bin	0.25	0.25
4	269	+	+	+	176	A/B/bin	0.5	0.5
5	205	+	+	–	n/a	n/a	n/a	n/a
6	273	+	+	+	176	A/B/bin	0.6	0.25
7	298	+	–	+	049	A/B	1	0.5
8	280	+	+	–	n/a	n/a	n/a	n/a
9	279	+	+	+	176	A/B/bin	1	0.25
10	294	+	+	+	176	A/B/bin	0.12	1
11	303	+	+	+	176	A/B/bin	0.25	0.5
12	277	+	–	+	001	A/B	0.5	0.5
13	304	+	+	+	014	A/B	0.25	0.5
14	44	+	–	+	002	A/B	0.25	0.5
15	307	+	+	+	017	A/B	0.25	0.25
16	308	+	+	+	176	A/B/bin	0.25	0.25
17	316	+	+	+	176	A/B/bin	0.5	0.25
18	320	+	–	+	012	A/B	0.5	0.5
19	319	+	+	+	012	A/B	0.5	0.12
20	322	+	+	+	176	A/B/bin	0.5	2
21	365	+	–	+	176	A/B/bin	0.5	2
22	323	+	+	+	020	A/B	0.5	0.5
23	325	+	+	+	015	A/B	0.5	0.25
24	331	+	+	+	078	A/B/bin	0.5	0.25
25	336	+	+	+	176	A/B/bin	0.5	1
26	351	+	+	+	176	A/B/bin	0.25	1
27	365	+	–	+	014	A/B	0.5	0.5
28	391	+	+	+	005	A/B	0.25	0.5
29	388	+	+	+	434	A/B	0.25	0.12
30	248	+	+	+	176	A/B/bin	0.12	0.5

GDH glutamate dehydrogenase, *MIC* minimum inhibitory concentration

[a] Toxin A/toxin B/binary toxin

[b] 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 [13]

ribotype 176 infections compared with non-ribotype 176 infections provides some evidence to support the clinical importance of this ribotype. However, the small sample size of patients in this study indicates a need for further studies, incorporating a larger number of patients, to better understand the relative virulence of ribotype 176. The high incidence of epidemic *C. difficile* PCR-ribotype 176 in our study emphasises the importance of implementing continuous surveillance programmes for CDI at national and European level, including PCR ribotyping.

Table 2 Study population and patient demographics (n = 30)

Patient characteristic	N (%)
Male	17 (56.7)
Age ≥ 65 years	22 (73.3)
HA-CDI	26 (86.7)
CA-CDI	4 (13.3)
Recurrent CDI	4 (13.3)
Severe CDI—Horn's index	17 (56.7)
Severe CDI—Atlas score	18 (60)
Mortality within 3 months	8 (26.7)
CDI cause of death	2 (6.7)
Previous hospitalisation	13 (59.1)
Previous antibiotic use	25 (83.3)
Aminopenicillin/beta-lactamase inhibitors	12 (40)
Cephalosporines	11 (36.7)
Fluoroquinolones	12 (40)
Carbapenems	4 (13.3)
Piperacilin/tazobactam	3 (10)
Aminoglycosides	3 (10)

Table 3 Comparison of clinical outcomes in patients grouped by isolated *C. difficile* PCR-ribotype

Clinical outcome	Ribotype 176 (n = 14)		Other ribotypes (n = 13)	
	N	%	N	%
Horn's index				
1			1	7.7
2	3	21.4	6	46.1
3	9	64.3	5	38.5
4	2	14.3	1	7.7
Atlas score				
1–2			2	15.4
3–5	3	21.4	4	30.8
6–7	9	64.3	5	38.5
8–9	2	14.3	2	15.4
Recurrent CDI within 3 months of first episode (Yes)	2	14.3	2	15.4
CDI in 8 weeks prior to admission (Yes)	1	7.1	1	7.7
Admitted to ICU (Yes)	3	21.4	3	23.1
Antibiotic treatment within 1 month prior to admission (Yes)	12	85.7	10	76.9
Mortality within 3 months of first CDI episode (Yes)	5	35.7	2	15.4

Authors' contributions

JD analysed and interpreted of data, drafted manuscript. ON co-designed and coordinated the study, supervised the microbiological part of the study, critical revised manuscript. MK carried out molecular analysis of isolates, drafted a part of the manuscript. JS analysed data, drafted the part of the manuscript. JM was responsible for bacteriological investigation of stool samples. RK designed and coordinated the study, supervised the clinical part of the study, critically revised the manuscript. All authors read and approved the final manuscript.

Author details

[1] Department of Internal Medicine, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, 150 06, V Uvalu 84, Praha 5, Prague, Czech Republic. [2] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czech Republic. [3] DNA Laboratory, Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czech Republic.

Acknowledgements

Medical writing services were provided by David Burns on behalf of Astellas Pharma EMEA to assist with grammar and English translations. Astellas Pharma EMEA had no role in study design, data collection or the decision to publish the manuscript. We would like to thank Bohdana Hola for statistical analysis of data. The authors thank the ESCMID Study Group for *Clostridium difficile* (ESGCD) for their professional support. Molecular and microbiological analysis of *C. difficile* isolates was supported by the Ministry of Health, Czech Republic Internal Grant Agency NT/14209-3 and MH CZ-DRO, University Hospital Motol, Prague, Czech Republic 00064203.

Competing interests

The authors declare that they have no competing interests.

Received: 29 June 2015 Accepted: 22 November 2015
Published online: 23 December 2015

References

1. Kuijper EJ, Coignard B, Tüll P, ESCMID Study Group for Clostridium difficile. EU Member States; European Centre for Disease Prevention and Control. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect. 2006;12(Suppl 6):2–18.
2. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ, ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. 2011;377(9759):63–73. doi:10.1016/S0140-6736(10)61266-4.
3. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, Delmée M, Fitzpatrick F, Ivanova K, Kuijper E, Macovei IS, Mentula S, Mastrantonio P, von Müller L, Oleastro M, Petinaki E, Pituch H, Norén T, Nováková E, Nyč O, Rupnik M, Schmid D, Wilcox MH. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis. 2014;14(12):1208–19. doi:10.1016/S1473-3099(14)70991-0.
4. Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, Arvand M. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 2014;30:153–5. doi:10.1016/j.anaerobe.2014.09.020.
5. Valiente E, Cairns MD, Wren BW. The *Clostridium difficile* PCR ribotype 027 lineage: a pathogen on the move. Clin Microbiol Infect. 2014;20(5):396–404. doi:10.1111/1469-0691.12619.
6. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol. 2012;61(Pt 1):49–56. doi:10.1099/jmm.0.036194-0.
7. Knetsch CW, Hensgens MP, Harmanus C, van der Bijl MW, Savelkoul PH, Kuijper EJ, Corver J, van Leeuwen HC. Genetic markers for *Clostridium difficile* lineages linked to hypervirulence. Microbiology. 2011;157(Pt 11):3113–23. doi:10.1099/mic.0.051953-0.
8. Krutova M, Matejkova J, Nyc O. *C. difficile* ribotype 027 or 176? Folia Microbiol (Praha). 2014;59(6):523–6. doi:10.1007/s12223-014-0323-5.
9. Obuch-Woszczatyński P, Lachowicz D, Schneider A, Mól A, Pawłowska J, Ożdżeńska-Milke E, Pruszczyk P, Wultańska D, Młynarczyk G, Harmanus C, Kuijper EJ, van Belkum A, Pituch H. Occurrence of *Clostridium difficile*

PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008–2010. Anaerobe. 2014;28:13–7. doi:10.1016/j.anaerobe.2014.04.007.
10. Pituch H, Obuch-Woszczatyński P, Lachowicz D, Wultańska D, Karpiński P, Młynarczyk G, van Dorp SM, Kuijper EJ. Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013. Euro Surveill. 2015;. doi:10.2807/1560-7917.ES.2015.20.38.30025.
11. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S–23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 1999;37(2):461–3.
12. Indra A, Schmid D, Huhulescu S, Hell M, Gattringer R, Hasenberger P, Fiedler A, Wewalka G, Allerberger F. Characterization of clinical *Clostridium difficile* isolates by PCR ribotyping and detection of toxin genes in Austria, 2006–2007. J Med Microbiol. 2008;57(Pt 6):702–8. doi:10.1099/jmm.0.47476-0.
13. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect. 2008;4(11):1057–64. doi:10.1111/j.1469-0691.2008.02092.x.
14. Stewart DB, Berg A, Hegarty J. Predicting recurrence of *C. difficile* colitis using bacterial virulence factors: binary toxin is the key. J Gastrointest Surg. 2013;17(1):118–24. doi:10.1007/s11605-012-2056-6 **discussion p.124-5**.
15. Bacci S, Mølbak K, Kjeldsen MK, Olsen KE. Binary toxin and death after *Clostridium difficile* infection. Emerg Infect Dis. 2011;17(6):976–82. doi:10.3201/eid/1706.101483.
16. Schwan C, Stecher B, Tzivelekidis T, van Ham M, Rohde M, Hardt WD, Wehland J, Aktories K. *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. PLoS Pathog. 2009;5(10):e1000626. doi:10.1371/journal.ppat.1000626.
17. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (*TcdC*) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40(9):3470–5.
18. Bakker D, Smits WK, Kuijper EJ, Corver J. TcdC does not significantly repress toxin expression in *Clostridium difficile* 630ΔErm. PLoS One. 2012;7(8):e43247. doi:10.1371/journal.pone.0043247.
19. van den Berg RJ, Schaap I, Templeton KE, Klaassen CHW, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J J Clin Microbiol. 2007;45(3):1024–8.
20. Goorhuis A, Legaria MC, van den Berg RJ, Harmanus C, Klaassen CH, Brazier JS, Lumelsky G, Kuijper EJ. Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires. Argent Clin Microbiol Infect. 2009;15(12):1080–6. doi:10.1111/j.1469-0691.2009.02759.x.
21. Marsh JW, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN, Muto CA, Harrison LH. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. J Clin Microbiol. 2006;44(7):2558–66.
22. Arora V, Kachroo S, Ghantoji SS, DuPont HL, Garey KW. High Horn´s index score predicts poor outcomes in patients with *Clostridium difficile* infection. J Hosp Infect. 2011;79(1):23–6. doi:10.1016/j.jhin.2011.04.027.
23. Horn SD. Measuring severity of illness: comparisons across institutions. Am J Public Health. 1983;73(1):25–31.
24. Miller MA, Louie T, Mullane K, Weiss K, Lentnek A, Golan Y, Kean Y, Sears P. Derivation and validation of a simple clinical bedside score (ATLAS) for *Clostridium difficile* infection which predicts response to therapy. BMC Infect Dis. 2013;13:148. doi:10.1186/1471-2334-13-148.
25. Bauer MP, Kuijper EJ, van Dissel JT. European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for *Clostridium difficile* infection (CDI). Clin Microbiol Infect. 2009;15(12):1067–79. doi:10.1111/j.1469-0691.2009.03099.x.

Příloha 11

Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. *Clostridium difficile* ribotype 176 - A predictor for high mortality and risk of nosocomial spread? Anaerobe. 2016;40:35-40.

V článku jsou uvedeny výsledky roční studie (2013) výskytu *C. difficile* na Infekční oddělení nemocnice Na Bulovce. Dominantním ribotypem ve studii byl ribotyp 176 (57.7%). MLVA odhalila 11 klonálních komplexů. U pacientů s infekcí vyvolanou ribotypem 176 bylo zjištěno více případů těžkého průběhu infekce, rekurencí a mortality v porovnání s pacienty s infekcí vyvolanou jinými ribotypy *C. difficile*.

Anaerobe 40 (2016) 35e40

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clostridium difficile ribotype 176 e A predictor for high mortality and risk of nosocomial spread?

Sylvia Polivkova [a], Marcela Krutova [b, c, *], Katarina Petrlova [d], Jiri Benes [a], Otakar Nyc [b]

[a] Department of Infectious Diseases, 3rd Faculty of Medicine, Bulovka Teaching Hospital, Prague, Czech Republic
[b] Department of Medical Microbiology, Charles University, 2nd Faculty of Medicine and Motol University Hospital, Prague, Czech Republic
[c] DNA Laboratory, Department of Paediatric Neurology, Charles University, 2nd Faculty of Medicine and Motol University Hospital, Prague, Czech Republic
[d] Department of Clinical Microbiology, Bulovka Teaching Hospital, Prague, Czech Republic

a r t i c l e i n f o

Article history:
Received 26 November 2015
Received in revised form
1 May 2016
Accepted 2 May 2016
Available online 4 May 2016

Handling Editor: Elisabeth Nagy

Keywords:
Clostridium difficile
PCR-Ribotype 176
ATLAS score
Ribotyping
MLVA
Thr82Ile

a b s t r a c t

Purpose: The objective of this survey was to determine the incidence of *Clostridium difficile* infections (CDI) at the Department of Infectious Diseases, Bulovka Hospital, and to evaluate clinical and epidemiological data on CDI patients together with a detailed molecular characterisation of *C. difficile* isolates. The patient outcomes were correlated to causative *C. difficile* PCR-ribotype.

Methods: The twelve-month study (2013) comprised patients two years of age and older with CDI. CDI severity was estimated using ESCMID criteria and ATLAS scoring. *C. difficile* isolates were further characterized using ribotyping, Multiple-Locus Variable Tandem-Repeats analysis (MLVA) and investigation of antibiotic-resistance determinants (*gyrA*, *gyrB*, *rpoB*, *ermB*).

Results: A total of 619 diarrhoeal stools were investigated. Seventy-two stool samples were GDH and toxin A/B positive, and 39 samples were GDH positive only and subsequently toxigenic *C. difficile* was cultured. In total, 111 *C. difficile* isolates were characterized, of which 64 (57.7%) belonged to PCR-ribotype 176. MLVA analysis of PCR-ribotype 176 isolates revealed 11 clonal complexes. Seventy-two isolates (64.9%) showed amino acid substitution Thr82Ile in the *GyrA*, and sixty-two isolates (55.9%) showed amino acid substitutions Arg505Lys together with His502Asn, or Asp492Glu together with Arg505Lys in the *RpoB*. Twelve isolates (10.8%) were *ermB* positive.

Severe CDI according to the ESCMID criteria was recorded in forty-two patients (37.8%), and sixteen patients (14.4%) had ATLAS score 2: 6. Twenty-nine patients (26.1%) had recurrent CDI and twenty-four patients (21.6%) died during the study period.

Conclusions: A higher rate of severe CDI, recurrences and mortality in association with PCR-ribotype 176 infections were observed. The high incidence of PCR-ribotype 176 in the study, and the presence of clonal relatedness between PCR-ribotype 176 isolates, indicate its higher capacity to spread in a hospital setting, which in turn highlights the need to implement strict epidemic measures when PCR-ribotype 176 occurs.

1. Introduction

Clostridium difficile is the major pathogen of hospital-acquired diarrhoea. The increasing incidence of *C. difficile* infections (CDI) represents a significant economic burden for health-care facilities and a high risk for hospitalised patients [1]. In the Czech Republic, a national CDI surveillance system has not yet been implemented and Czech CDI incidence data, together with *C. difficile* isolate characterisation, are available from time-limited European studies [2e4]. CDI incidence is increasing in many European countries including the Czech Republic, where CDI incidence in 2008 was 1.1 per 10,000 patient bed-days [2] and in 2012e2013 increased to 6.2 cases per 10,000 patient bed-days [3]. The rising CDI incidence in the Czech Republic is undoubtedly contributed to by the epidemiologically significant occurrence of *C. difficile* PCR-ribotype 176 [5], which is closely related to PCR-ribotype 027 [6] e the most monitored *C. difficile* strain due to its hospital outbreaks and the higher mortality and morbidity of the disease worldwide [7].

In order to study CDI incidence, the risk factors preceding

* Corresponding author. Department of Medical Microbiology, Charles University, 2nd Faculty of Medicine and Motol University Hospital, V Uvalu 84, Prague 5, 150 06, Czech Republic.
E-mail address: marcela.krutova@seznam.cz (M. Krutova).

http://dx.doi.org/10.1016/j.anaerobe.2016.05.002
1075-9964/

infection, CDI clinical features and outcomes, together with the detailed molecular characterisation of *C. difficile* isolates, we performed a twelve-month study at the Department of Infectious Diseases, Bulovka Hospital, Prague, Czech Republic. The patient outcomes were correlated to causative *C. difficile* PCR-ribotypes.

2. Material and methods

A year-long study was carried out in 2013 (from January to December) at the Department of Infectious Diseases, Bulovka Hospital in Prague, Czech Republic. This hospital is a secondary health-care facility with 1100 beds and 264,989 patient bed-days, and 44,600 patients were admitted in 2013. The Department of Infectious Diseases at the hospital has 168 beds, and 5159 patients were admitted in 2013.

Our study included patients two years of age and older who were admitted with or who developed diarrhoea during hospitalization at the Department of Infectious Diseases.

Diarrhoeal stool samples were tested at the hospital's Department of Microbiology for the presence of glutamate dehydrogenase (GDH) and free toxins A/B using combined lateral flow assay (C.diff Quik Chek Complete®, Alere), according to the manufacturer's instructions. All GDH positive samples were cultured anaerobically on selective media for *C. difficile* (Oxoid) after an alcohol shock. Toxin production of *C. difficile* isolates, cultured from GDH-positive and toxin-negative samples, was detected using the same lateral flow assay, when a bacterial culture solution was investigated as a stool sample.

DNA from *C. difficile* isolates was extracted by Qiagen isolation kit using the automated QIAcube (Qiagen) purification system, and sent to the Department of Medical Microbiology at Motol University Hospital, Prague for further molecular analysis.

PCR-ribotyping was performed in accordance with the European *C. difficile* Surveillance network (ECDIS-net) Standard Operation Protocol with primers previously described by Stubbs et al. [8], and by capillary electrophoresis fragment analysis using POP 7 polymer and LIZ 1200 (Applied Biosystems) as a size standard. Electrophoretic profiles were uploaded to the Webribo database [9], for the determination of the specific ribotype.

The presence of genes for toxin production: *tcdA* (A), *tcdB* (B), *cdtA* and *cdtB* (binary) was investigated by a multiplex PCR [10].

The molecular mechanism of antibiotic resistance to fluoroquinolones was investigated by sequencing of the quinolone resistance-determining region (QRDR) in the *gyrA* and the *gyrB* genes [11], and to rifampicin by sequencing of the *rpoB* gene region [12]. Obtained sequences were compared with a NCBI reference sequence (*Peptoclostridium difficile* 630, complete genome NC_009089.1) after software processing (Sequencing Analysis Software v5.4, Applied Biosystems). The molecular mechanism of resistance to the macrolide/lincosamide/streptogramin B (MLS_B) group of antibiotics was examined by amplification of the *ermB* gene fragment [13] and PCR products were visualized by agarose gel electrophoresis.

MLVA of eight previously published variable number tandem-repeats (VNTR) loci was applied to PCR-ribotype 176 isolates. A total of 8 VNTR loci were amplified and sequenced, specifically A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd [14] and CDR 60 [15], with change of reverse primer for locus G8Cd [16]. The number of tandem repeats was counted manually after software processing (Sequencing Analysis Software v5.4, Applied Biosystems). A Minimum Spanning Tree (MST) was created by Bionumerics v5.0 using the Manhattan coefficient. The total sum of tandem repeat differences (STRD) in eight VNTR loci determines the genetic relatedness of isolates. A clonal complex (CC) was defined as STRD ≤ 2 and a genetically related cluster was defined as STRD ≤ 10 [14].

CDI incidence was calculated on the departmental level using data on the number of admitted patients, the number of patient bed-days and the number of toxin A/B-positive tests, together with the number of GDH positive, toxin A/B negative, toxigenic *C. difficile* positive results in 2013 [17]. A CDI case was defined as a patient with diarrhoea and positive laboratory confirmation of CDI by positivity of GHD and toxin A/B test, or positivity of GDH and detection of toxigenic *C. difficile* by toxigenic culture. Recurrence was defined as a CDI episode in the period of 2–8 weeks after the previous episode [18].

CDI severity was estimated using a bedside ATLAS score [19] and ESCMID criteria [20].

CDI were divided into health-care associate infections (HA-CDI), and community-associated (CA-CDI) [7]. An unknown association of CDI (UA-CDI) was defined as patients who had onset of symptoms within 48 h after admission and were also discharged from the health-care facility in the period of 4 weeks to 3 months before the onset of symptoms [21].

3. Results

3.1. CDI testing

A total of 619 diarrhoeal stool samples were investigated by a rapid test. Positivity for both GDH and the toxin A/B test was revealed in 72 samples, and 41 samples were GDH positive only. *C. difficile* was cultured from all 113 stool samples. *C. difficile* isolates cultured from only GDH positive stool samples were tested for toxin production, 39 isolates were toxin-producing and two isolates were non-toxigenic. A total of 111 CDI were laboratory confirmed.

3.2. C. difficile isolate molecular characterisation

A total of 111 toxigenic *C. difficile* isolates were ribotyped and eighteen different ribotypes were identified. Five electrophoretic profiles did not match any in the database and they were identified as new ribotypes. The most common ribotype in our study was PCR ribotype 176, which formed 57.7% (n=64) of isolates. Other PCR-ribotypes found were: 001 (n = 8; 7.2%), 002 (n = 7; 6.3%), 003, 012, 017, 023 (n = 3; 2.7%), 014, 015, 020, 049 (n = 2; 1.8%) and 018, 029, 070, 078, 087, 449, AI-9-1 (n = 1; 0.9%) (Table 1).

The presence of binary toxin was revealed in 69 isolates (62.2%): PCR-ribotype 176 (n = 64), 023 (n 3), 078 (n 1) and new ribotype (n = 1) (Table 1).

A total of 111 isolates were investigated for point mutations in the *gyrA* and *rpoB* gene fragments, and for the presence of the *ermB* gene fragment. The *gyrB* gene region was sequenced among 39 isolates without point mutation in the sequenced fragment of the *gyrA* gene.

The amino acid substitution Thr82Ile in the *GyrA* was found in 72 isolates (64.9%): PCR-ribotypes 176 (n = 64), 001 (n 3), new ribotype (n = 2) and 012, 017, 049 (n = 1). No amino acid substitutions were found in the *GyrB* (Table 1).

The three different amino acid substitutions were identified in 62 isolates (55.9%) in the *RpoB*. His502Asn together with Arg505Lys were found in 57 isolates (51.4%): PCR-ribotypes 176 (n = 53), 049 (n = 1), 012 (n = 1), 017 (n = 1), new ribotype (n = 1), and Asp492Glu together with Arg505Lys in 5 isolates: PCR-ribotype 176 (n = 5; 4.5%) (Table 1).

The presence of the *ermB* internal fragment was observed in 12 isolates (10.8%): PCR-ribotypes 012 (n = 3), 001 (n = 2), 017 (n = 2), 176 (n = 2) and 002, 014, 015 (n = 1) (Table 1).

MLVA identified a total of 11 clonal complexes (CC1 = 28 isolates, CC2 and CC3 = 4 isolates, CC4 and CC5 = 3 isolates and CC6-CC11 = 2 isolates). The number of 10 STRDs was only exceeded

Table 1
Molecular characteristics of *C. difficile* isolates.

Ribotyping (N; %)	Binary toxin genes (*cdtB and cdtA*)	*GyrA* Thr82Ile	*RpoB* Arg505Lys and His502Asn	*RpoB* Arg505Lys and Asp492Glu	*ermB*
176 (64; 57.7%)	yes (64)	64 (57.7%)	53 (47.8%)	5 (4.5%)	2 (1.8%)
001 (8; 7.2%)		3 (2.7%)			2 (1.8%)
002 (7; 6.3%)					1 (0.9%)
003 (3; 2.7%)					
012 (3; 2.7%)		1 (0.9%)	1 (0.9%)		3 (2.7%)
017 (3; 2.7%)		1 (0.9%)	1 (0.9%)		2 (1.8%)
023 (3; 2.7%)	yes (3)				
014 (2; 1.8%)					1 (0.9%)
015 (2; 1.8%)					1 (0.9%)
020 (2; 1.8%)					
049 (2; 1.8%)		1 (0.9%)	1 (0.9%)		
018 (1; 0.9%)					
029 (1; 0.9%)					
070 (1; 0.9%)					
078 (1; 0.9%)	yes (1)				
087 (1; 0.9%)					
449 (1; 0.9%)					
AI-9-1 (1; 0.9%)					
New (5; 4.5%)	yes (1)	2 (1.8%)	1 (0.9%)		
111		72 (64.9%)	62 (55.9%)		12 (10.8%)

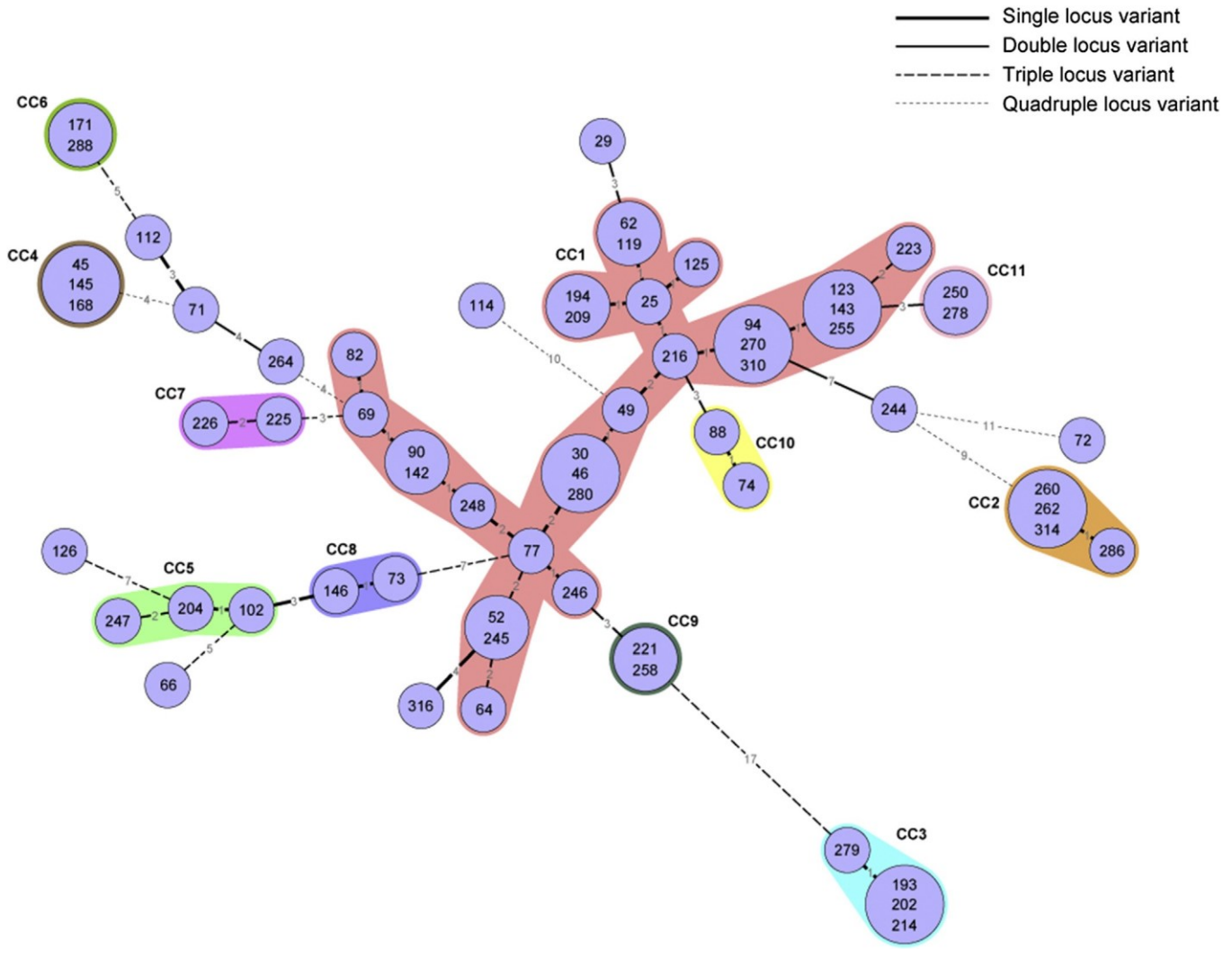


Fig. 1. A Minimum Spanning Tree of *C. difficile* PCR-ribotype 176 isolates. The numbers in the circle represent isolate number. The numbers in the lines represent the sum of tandem repeat differences between isolates. Type of dashed line represents the number of loci where tandem repeats differences were found.

between CC9-CC3 (STRD¼17) and isolates 244 and 72 (STRD ¼ 11) (Fig. 1).

3.3. Clinical data analysis

CDI was diagnosed in 111 patients based on the test results and the presence of clinical symptoms. The average age was 71 years (ranging from 2 to 96 years), the median was 76 years; 57% of patients were females.

CDI incidence at the Department of Infectious diseases, Bulovka Hospital in 2013 was 31.7 cases per 10,000 patient bed-days and 215 cases per 10,000 admissions. CDI testing frequency was 174.4 tests per 10,000 patient bed-days and 1199 tests per 10,000 admissions.

Patient demographic data are shown in Table 2.

HA-CDI was diagnosed in 63 cases (56.8%), CA-CDI in 17 cases (15.3%) and 28 cases (25.2%) were of unknown association. Eighty patients (72.1%) had been hospitalised in the previous three months and 29 of them were hospitalised in the long-term care facility.

Previous antibiotic treatment was administered in 101 patients (91%). The spectrum of most frequently used antibiotics in monotherapy or in combination was as follows: aminopenicillins with beta lactamase inhibitors (36.9%), fluoroquinolones (29.7%), cephalosporins (26.1%) and lincosamides (8.1%). Proton pump inhibitors were used in 44.1% of patients. Seventy-two patients (64.8%) had Charlson comorbidity index 52: Immobile were 60 patients (54.1%): full immobile in 34 cases (30.6%) and partial immobile in 26 cases (23.4%).

Using ESCMID criteria, CDI was considered as mild in 69 patients (62.2%) and severe in 42 patients (37.8%). Using the bedside score system, 42 patients (37.8%) had an ATLAS score of 0e2, 53 patients (47.7%) had an ATLAS score of 3e5, and 16 patients (14.4%) had an ATLAS score2:6.

Recurrent CDI was recorded in 29 patients (26.1%). Mortality rate in the study period was 21.6% (n ¼ 24). Thirty-day mortality was 14.2% (n ¼ 16), when in 9 cases CDI was the primary cause of death and in 7 cases CDI contributed.

CDI-specific antibiotics were administered to 103 patients. Monotherapy with oral antibiotic was used in 51 patients (metronidazole n¼44, fidaxomicin n ¼ and vancomycin n¼3). The combination therapy oral metronidazole and vancomycin was used in 50 patients. Two patients did not respond to the treatment and metronidazole with vancomycin was switched to fidaxomicin with a good clinical response.

To evaluate the association between *C. difficile* ribotypes and patient clinical outcomes, data on patients infected by PCR-ribotype 176 were compared with data on patients infected by non-PCR-ribotype 176 in the study (Table 2).

Higher rates of patients with severe CDI were observed in the group of patients with PCR-ribotype 176 infections compared to the non-PCR-ribotype 176 patient group: 26.1%e11.7% according to ESCMID criteria and 11.7%e2.7% of patients with ATLAS score 6; recurrent CDI (18.9%e7.2%) and deaths (14.4%, 7.2%) showed a similar trend.

4. Discussion

A total of 111 CDI cases were laboratory confirmed. Thirty-nine stool samples (35%) were toxin A/B negative and the presence of toxigenic *C. difficile* had to be confirmed by toxigenic culture. Observed lower rapid test toxin A/B sensitivity (65%), also reported by Eastwood et al. (59.6%) [22], emphasizes the need to use an additional test in the CDI diagnostics algorithm [20].

We identified eighteen different ribotypes in the set of 111 *C. difficile* isolates. PCR ribotype 176 was the predominant ribotype (57.7%). The emergence of PCR-ribotype 176 in the Czech Republic and Poland was reported in 2009 [23]. Whilst PCR-ribotype 176 occurs together with PCR-ribotype 027 in Poland [24], the occurrence of CDI caused by PCR-ribotype 027 in the Czech Republic is rare [5]. The spectrum of other PCR-ribotypes identified in the study corresponds with the spectrum of most frequent PCR-ribotypes from the European survey performed in 2008 [2].

In vitro antibiotic susceptibility of *C. difficile* isolates was not

Table 2
Epidemiological and clinical characteristics of patients in the study (n ¼ 111).

	Total		176		Non-176	
	N	%	N	%	N	%
Man: woman ratio	49:62	e	27:37	e	22:25	e
Average age; median	71; 76	e	77; 80	e	65; 73	e
HA-CDI	63	56.8	49	44.2	14	12.6
CA- CDI	17	15.3	0	e	17	15.3
Unknown CDI	31	27.9	15	13.5	16	14.4
Hospitalization in previous 3 months	80	72.1	57	51.4	23	20.7
Previous stay in a long-term care facility	29	26.1	20	18	9	8.1
Previous antibiotic use (2 months)	101	91.0	60	54.1	41	36.9
Fluoroquinolones	33	29.7	24	21.6	9	8.1
Aminopenicillins	41	36.9	22	19.8	19	17.1
Cephalosporins	29	26.1	17	15.3	12	10.8
Lincosamides	9	8.1	6	5.4	3	2.7
Proton pump inhibitors use	49	44.1	30	27	19	17.1
Charlson comorb. index average; median	6.2; 7	e	7.3; 7	e	4.8; 5	e
Immobility (full)	34	30.6	23	20.7	11	9.9
Immobility (partial)	26	23.4	20	18.0	6	5.4
Severe CDI	42	37.8	29	26.1	13	11.3
ATLAS score 0-2	42	37.8	19	17.1	23	20.7
ATLAS score 3-5	53	47.7	32	28.8	21	18.9
ATLAS score 2: 6	16	14.4	13	11.7	3	2.7
Leukocytes > 15.10^9	26	23.4	19	17.1	7	6.3
Serum CRP > 100 mg/L	39	35.1	25	22.5	14	12.6
Gut perforation, toxic megacolon	2	1.8	2	1.8	0	e
Recurrence	29	26.1	21	18.9	8	7.2
Mortality	24	21.6	16	14.4	8	7.2

tested in this study, but multidrug resistance in PCR-ribotype 176 isolates was recently reported [4,25,26].

The amino acid substitution Thr82Ile in the *Gyr A* corresponds to codon 83 in *E. coli* and has been described several times in isolates resistant to moxifloxacin [27,28], to moxifloxacin and ciprofloxacin [26,29], as well as to other quinolones: gatifloxacin, levofloxacin [30] and mofloxacin [31]. This amino acid substitution was identified in 64.9% of isolates in our study, of which 57.7% were PCR-ribotype 176 isolates (all in the study). Fluoroquinolones were identified as the second most commonly used antibiotic before a CDI episode and interestingly it was used 2.7 times more often in the group of patients infected by *C. difficile* PCR-ribotype 176. The uniform presence of Thr82Ile in PCR-ribotype 176 isolates illustrates the genetic link of PCR-ribotype 176 to the FQR2 lineage of PCR-ribotype 027 [32] and suggests a possible advantage for its spread within healthcare facilities, which is supported by the presence of 100% identical PCR-ribotype 176 isolates detected by MLVA in our study.

The high number of clonal clusters (n¼11) suggests that PCR-ribotype 176 was introduced several times by patient transfer from another health care facility. The close genetic relatedness between PCR-ribotype 176 isolates, together with high prevalence of PCR-ribotype 176 in the Czech Republic [5], shows that this type is a long-term persisting ribotype in a hospital environment.

The three amino acid substitutions in *RpoB* identified in this study were previously observed in *C. difficile* strains resistant to rifampin and its derivatives [26,27,33,34]. The carrying of these detected amino acid substitutions were observed mainly in PCR-ribotype 176 isolates (58/64). Interestingly, the occurrence of these particular amino acid substitutions corresponds with clonal complexes identified by MLVA (CC2¼ Asp492Glu þ Arg505Lys; isolates in CC5, CC8 and isolate 126 were wild types).

The CDI incidence during the 12-month study period (31.7 cases per 10,000 bed days and 215 cases per 10,000 admissions), and the testing frequency (174 tests per 10,000 bed days and 1199 tests per 10,000 admissions), were fivefold higher than recently published European reported rates in 2012e2013 [3]. The reason for the high CDI incidence associated with more frequent testing at the departmental level is the transferring of patients with diarrhoea for isolation purposes or with a complicated chronic and recurrent course of CDI to our department, as a specialised centre, from other hospital departments and other hospitals.

Recurrence rate in our study (26.1%) was higher than the European mean recurrence rate (18%) [2], and recurrence rates observed in studies performed in the Eastern Europe (11.3%, 16.4% and 11.8%) [35,36,37]. The higher recurrence rate could be affected by above mentioned transferring of CDI patients.

The mortality rate in our study (21.6%) is similar to the mean European mortality rate (22%) [2] and higher than the 19.7% rate given in the study by Balihar et al. [36]. Thirty-day mortality (14.2%) was lower than that observed by Kurti et al. (21.9%) [35].

There is some evidence supporting the genetic link between PCR-ribotypes 176 and 027 [6,32], which has been associated with CDI outbreaks [7] and increased disease severity [38], but there are relatively few data on the clinical relevance of PCR-ribotype 176 [24]. We observed a higher rate of severe CDI, recurrences and mortality rate in association with PCR-ribotype 176 infections compared with data on patients infected by other ribotypes (26.1%, 18.9%, 14.4% to 11.3%, 7.2%, 7.2%, respectively), which provides some evidence of the clinical relevance PCR-ribotype 176 as previously suggested [24]. The group of patients infected by PCR-ribotype 176 was accounted for by the higher age group of patients as was also observed in patients with PCR-ribotype 027 infection [38].

5. Conclusions

A higher rate of severe CDI, recurrences and mortality rate in association with PCR-ribotype 176 infections was observed. The presence of amino acid substitution Thr82Ile in the *GyrA*, associated with resistance to fluoroquinolones in all PCR-ribotype 176 isolates in the study stresses the need for the introduction of antibiotic stewardship focused on reduction and monitoring of fluoroquinolone administration.

The high incidence of PCR-ribotype 176 in the study, and the presence of clonal relatedness between PCR-ribotype 176 isolates, indicate its higher capacity to spread in a hospital setting, which in turn highlights the need to implement Czech national guidelines for infection prevention and control, and to intensify CDI surveillance on a local and national level.

Acknowledgements

We would like to thank ECDIS-net and the European Study Group for *Clostridium difficile* (ESGCD, ESCMID) for their professional support.

We would like to thank Dr. James Partridge for proofreading the text.

Molecular analysis of isolates was supported by MH CZ e DRO, University Hospital Motol, Prague, Czech Republic 00064203.

References

[1] J. Freeman, M.P. Bauer, S.D. Baines, J. Corver, W.N. Fawley, B. Goorhuis, et al., The changing epidemiology of Clostridium difficile infections, Clin. Microbiol. Rev. 23 (3) (2010) 529e549.

[2] M.P. Bauer, D.W. Notermans, B.H. van Benthem, J.S. Brazier, M.H. Wilcox, M. Rupnik, et al., Clostridium difficile infection in Europe: a hospital-based survey, Lancet 377 (9759) (2011) 63e73.

[3] K.A. Davies, C.M. Longshaw, G.L. Davis, E. Bouza, F. Barbut, Z. Barna, et al., Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual,point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID), Lancet Infect. Dis. 14 (12) (2014) 1208e1219.

[4] J. Freeman, J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw, et al., Pan-European longitudinal surveillance of antibiotic resistance among prevalent Clostridium difficile ribotypes, Clin. Microbiol. Infect. 21 (3) (2015) 248 e9-248.e16.

[5] M. Krutova, O. Nyc, E.J. Kuijper, L. Geigerova, J. Matejkova, T. Bergerova, et al., A case of imported Clostridium difficile PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of C. difficile ribotype 176, Anaerobe 30 (2014) 153e155.

[6] E. Valiente, L.F. Dawson, M.D. Cairns, R.A. Stabler, B.W. Wren, Emergence of new PCR ribotypes from the hypervirulent Clostridium difficile 027 lineage, J. Med. Microbiol. 61 (Pt 1) (2012) 49e56.

[7] E.J. Kuijper, B. Coignard, P. Tüll, ESCMID Study Group for Clostridium difficile, EU Member States, European Centre for Disease Prevention and Control, Emergence of Clostridium difficile-associated disease in North America and Europe, Clin. Microbiol. Infect. 12 (6) (2006) 2e18. Review.

[8] S.L. Stubbs, J.S. Brazier, G.L. O'Neill, B.I. Duerden, PCR targeted to the 16S-23S rRNA gene intergenic spacer region of Clostridium difficile and construction of a library consisting of 116 different PCR ribotypes, J. Clin. Microbiol. 37 (2) (1999) 461e463.

[9] A. Indra, S. Huhulescu, M. Schneeweis, P. Hasenberger, S. Kernbichler, A. Fiedler, et al., Characterization of Clostridium difficile isolates using capillary gel electrophoresis-based PCR ribotyping, J. Med. Microbiol. 57 (Pt 11) (2008) 1377e1382.

[10] S. Persson, M. Torpdahl, K.E. Olsen, New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection, Clin. Microbiol. Infect. 14 (11) (2008) 1057e1064. Erratum in: Clin Microbiol Infect. 2009; 15 (3); 296.

[11] L. Dridi, J. Tankovic, B. Burghoffer, F. Barbut, J.C. Petit, gyrA and gyrB mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in Clostridium difficile, Antimicrob. Agents Chemother. 46 (11) (2002) 3418e3421.

[12] S.R. Curry, J.W. Marsh, K.A. Shutt, C.A. Muto, M.M. O'Leary, M.I. Saul, et al., High frequency of rifampin resistance identified in an epidemic Clostridium difficile clone from a large teaching hospital, Clin. Infect. Dis. 48 (4) (2009) 425e429.

[13] P. Spigaglia, P. Mastrantonio, Comparative analysis of Clostridium difficile clinical isolates belonging to different genetic lineages and time periods,

J. Med. Microbiol. 53 (Pt 11) (2004) 1129e1136.
[14] R.J. van den Berg, I. Schaap, K.E. Templeton, C.H. Klaassen, E.J. Kuijper, Typing and subtyping of Clostridium difficile isolates by using multiple-locus vari- able-number tandem-repeat analysis, J. Clin. Microbiol. 45 (3) (2007) 1024e1028.
[15] J.W. Marsh, M.M. O'Leary, K.A. Shutt, A.W. Pasculle, S. Johnson, D.N. Gerding, et al., Multilocus variable-number tandem-repeat analysis for investigation of Clostridium difficile transmission in Hospitals, J. Clin. Microbiol. 44 (7) (2006) 2558e2566.
[16] A. Goorhuis, M.C. Legaria, R.J. van den Berg, C. Harmanus, C.H. Klaassen, J.S. Brazier, et al., Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative Clostridium difficile in a general hospital in Buenos Aires, Argentina, Clin. Microbiol. Infect. 15 (12) (2009) 1080e1086.
[17] European Centre for Disease Prevention and Control, European Surveillance of Clostridium difficile Infections. Surveillance Protocol Version 2.1, ECDC, Stockholm, 2015.
[18] M.P. Bauer, E.J. Kuijper, J.T. van Dissel, European Society of Clinical Microbiology and Infectious Diseases, European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for Clostridium difficile infection (CDI), Clin. Microbiol. Infect. 15 (12) (2009) 1067e1079.
[19] M.A. Miller, T. Louie, K. Mullane, K. Weiss, A. Lentnek, Y. Golan, et al., Derivation and validation of a simple clinical bedside score (ATLAS) for Clostridium difficile infection which predicts response to therapy, BMC Infect. Dis. 13 (2013) 148.
[20] S.B. Debast, M.P. Bauer, E.J. Kuijper, European Society of Clinical Microbiology and Infectious Diseases, European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for Clostridium difficile infection, Clin. Microbiol. Infect. 20 (2) (2014) 1e26.
[21] S.H. Cohen, D.N. Gerding, S. Johnson, C.P. Kelly, V.G. Loo, L.C. McDonald, et al., Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA), Infect. Control Hosp. Epidemiol. 31 (5) (2010) 431e455.
[22] K. Eastwood, P. Else, A. Charlett, M. Wilcox, Comparison of nine commercially available Clostridium difficile toxin detection assays, a real-time PCR assay for C. difficile tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods, J. Clin. Microbiol. 47 (10) (2009) 3211e3217.
[23] O. Nyc, H. Pituch, J. Matejkova, P. Obuch-Woszczatynski, E.J. Kuijper, Clostridium difficile PCR ribotype 176 in the Czech Republic and Poland, Lancet 377 (9775) (2011) 1407.
[24] P. Obuch-Woszczatynski, D. Lachowicz, A. Schneider, A. Mol, J. Pawłowska, E. Ozdzenska-Milke, et al., Occurrence of Clostridium difficile PCR-ribotype 027 and it's closely related PCR-ribotype 176 in hospitals in Poland in 2008-2010, Anaerobe 28 (2014) 13e17.
[25] D. Lachowicz, H. Pituch, P. Obuch-Woszczatynski, Antimicrobial susceptibility patterns of Clostridium difficile strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012, Anaerobe 31 (2015) 37e41.
[26] M. Krutova, J. Matejkova, J. Tkadlec, O. Nyc, Antibiotic profiling of Clostridium difficile ribotype 176-A multidrug resistant relative to C. difficile ribotype 027, Aug 6, Anaerobe S1075e9964 (15) (2015) 30045e30047.
[27] P. Spigaglia, F. Barbanti, P. Mastrantonio, European Study Group on Clostridium difficile (ESGCD). Multidrug resistance in European Clostridium difficile clinical isolates, J. Antimicrob. Chemother. 66 (10) (2011) 2227e2234.
[28] G. Ackermann, Y.J. Tang, R. Kueper, P. Heisig, A.C. Rodloff, J. Silva Jr., et al., Resistance to moxifloxacin in toxigenic Clostridium difficile isolates is associated with mutations in gyrA, Antimicrob. Agents Chemother. 45 (8) (2001) 2348e2353.
[29] L. Dridi, J. Tankovic, B. Burghoffer, F. Barbut, J.C. Petit, gyrA and gyrB mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in Clostridium difficile, Antimicrob. Agents Chemother. 46 (11) (2002) 3418e3421.
[30] P. Spigaglia, F. Barbanti, P. Mastrantonio, J.S. Brazier, F. Barbut, M. Delmee, et al., Fluoroquinolone resistance in Clostridium difficile isolates from a prospective study of C. difficile infections in Europe, J. Med. Microbiol. 57 (6) (2008) 784e789.
[31] D. Drudy, L. Kyne, R. O'Mahony, S. Fanning, gyrA mutations in fluoroquinolone-resistant Clostridium difficile PCR-027, Emerg. Infect. Dis. 13 (3) (2007) 504e505.
[32] M. He, F. Miyajima, P. Roberts, L. Ellison, D.J. Pickard, M.J. Martin, et al., Emergence and global spread of epidemic healthcare-associated Clostridium difficile, Nat. Genet. 45 (1) (2013) 109e113.
[33] J.R. O'Connor, M.A. Galang, S.P. Sambol, D.W. Hecht, G. Vedantam, D.N. Gerding, et al., Rifampin and rifaximin resistance in clinical isolates of Clostridium difficile, Antimicrob. Agents Chemother. 52 (8) (2008) 2813e2817.
[34] S.R. Curry, J.W. Marsh, K.A. Shutt, C.A. Muto, M.M. O'Leary, M.I. Saul, et al., High frequency of rifampin resistance identified in an epidemic Clostridium difficile clone from a large teaching hospital, Clin. Infect. Dis. 48 (4) (2009) 425e429.
[35] Z. Kurti, B.D. Lovasz, M.D. Mandel, Z. Csima, P.A. Golovics, B.D. Csako, et al., Burden of Clostridium difficile infection between 2010 and 2013: trends and outcomes from an academic center in Eastern Europe, World J. Gastroenterol. 21 (21) (2015) 6728e6735.
[36] K. Balihar, F. Kozak, J. Kozeluhova, V. Hejda, L. Fremundova, M. Krcma, et al., Clostridium difficile infection in hospitalized patients at a Czech tertiary center: analysis of epidemiology, clinical features, and risk factors of fulminant course, Eur. J. Gastroenterol. Hepatol. 26 (8) (2014) 880e887.
[37] A. Novak, P. Spigaglia, F. Barbanti, I. Goic-Barisic, M. Tonkic, First clinical and microbiological characterization of Clostridium difficile infection in a Croatian University Hospital, Anaerobe 30 (2014) 18e23.
[38] K. Rao, D. Micic, M. Natarajan, S. Winters, M.J. Kiel, S.T. Walk, et al., Clostridium difficile ribotype 027: relationship to age, detectability of toxins A or B in stool with rapid testing, severe infection, and mortality, Clin. Infect. Dis. 61 (2) (2015) 233e241.

Příloha 12

Azimirad M; Krutova M; Nyc O; Hasani Z; Afrisham L; Alebouyeh M; Zali MR. Molecular typing of *Clostridium difficile* isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital. V recenzním řízení Anaerobe, po první revizi.

Ve spolupráci s Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti a University of Medical Sciences, Tehran, Iran jsme otypovali 23 vzorků DNA extrahované z izolátů *C. difficile* kultivovaných ze stolic hospitalizovaných pacientů s CDI a také ze stěrů z nemocničního prostředí včetně zdravotnických pomůcek a přístrojů.

C. difficile ribotyp 126 byl nejčastějším ribotypem identifikovaným ve studii (21,7 %). Další nalezené ribotypy byly: 001, 003, 014, 017, 029, 039, 081, 103 a 150. *C. difficile* ribotypy 001, 126 a 150 byly identifikovány u izolátů *C. difficile* kultivovaných jak ze vzorků stolic pacientů, tak i ze stěrů z prostředí (pacientské lůžko) a zdravotnických pomůcek (kolonoskop, endoskop), což naznačuje možnou cestu přenosu.

Molecular typing of *Clostridium difficile* isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital

Masoumeh Azimirad [a], Zahra Hasani [a], Marcela Krutova [b], Leili Afrisham [c], Otakar Nyc [b], Masoud Alebouyeh [a], Mohammad RezaZali [c]

[a] Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

[b] Department of Medical Microbiology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czech Republic.

[c] Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Correspondence: Masoud Alebouyeh, PhD of Medical Bacteriology, Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Phone number: +98-2122432518; Fax number: +98-2122432517; E-mail address: masoud.alebouyeh@gmail.com.

Abstract

Purpose: This study aimed to characterize *Clostridium difficile* isolates cultured from stool samples of patients with *C. difficile* infection (CDI) and swabs from a medical environment in a gastroenterology centre in Tehran, Iran.

Methods: A total of 158 samples (105 stool samples from hospitalized patients and 53 swabs from medical devices and the environment) were collected from January 2011 to August 2011 and investigated for the presence of *C. difficile* by direct anaerobic culture on a selective media for *C. difficile*. *C. difficile* isolates were further characterized by capillary electrophoresis (CE) ribotyping and toxin gene multiplex PCR.

Results: Of 158 samples, *C. difficile* was cultured in 19 of 105 stool samples (18%) and in 4 of 53 swabs (7.5%). *C. difficile* PCR ribotype (RT) 126 was the most common RT in the study (21.7%). Further RTs were: 001, 003, 014, 017, 029, 039, 081, 103 and 150. *C. difficile* RTs 001, 126 and 150 were identified in isolates cultured from stool samples of patients and swabs of medical equipment. In conclusion, the predominance of RT 126 was found in Tehran gastroenterology centre. RTs 126, 001, 150 were cultured from both the stool samples and swabs of medical devices and the hospital environment which suggest a possible route of transmission.

Keywords: *Clostridium difficile*, capillary electrophoresis ribotyping, PCR ribotype 126, PCR ribotype 150, Iran.

Running Title: Prevalence of *C. difficile* PCR ribotype 126 in Tehran gastroenterology centre.

Introduction

Clostridium difficile, recently reclassified as *Clostridioides difficile* [1], is a leading world-wide spread nosocomial pathogen [2, 3]. The molecular typing of clinical significant strains is an important part of *C. difficile* infection surveillance. The spectrum of typing methods is wide and, therefore, to compare the data from different studies is difficult. Currently, the most commonly used method for typing of *C. difficile* isolates is polymerase chain reaction (PCR) ribotyping, a method based on the variability of intergenic spacer region (ISR) between *16S* and *23S* rRNA genes [4].

Several studies aimed to map the prevalence of CDI in Middle East were recently published. In Kuwait, the prevalence of CDI was 9.7% in 2003, 7.8% in 2004 and 7.2% in 2005 with the most frequently found RTs 002, 001, 126 and 140 [5]. In contrast, in a study focused on community associated CDI in Kuwait, the prevalence of CDI was 0.62% and the occurrence of RTs 139, 014, 056, 070, 097 and 179 was found [6]. In Lebanon, in a single center study, *C. difficile* was detected in 65.2% of stool samples with a prevalence of toxinotype 0-like [7]. In Israel, the multicenter study, including six general hospitals and ten long-term care facilities, showed a high prevalence of RT027 (31.8%) with a reduced susceptibility to metronidazole [8]. In Qatar, a study of 1,532 patients from two hospitals performed between 2011-2012, showed a CDI prevalence of 7.9% and RT258 was the most frequently found ribotype between 36 RTs identified [9]. In Saudi Arabia, the annual CDI incidence was 2.4 and 1.7 per 10,000 patient days in 2007 and 2008, respectively [10]. A study performed in a

Turkish university hospital gave a CDI incidence of 0.26 per 1,000 hospitalization-days and the prevalence of RT 002 between October 2004 and February 2005 [11].

In the Iranian study performed in 2015, *C. difficile* was detected in 8.75% of pediatric stool samples, both diarrheal and non-diarrheal, with the prevalence of ribotyping profiles R27 and R1 [12]. The high prevalence of RT027 (11.52%) was also revealed in Iranian pediatric patients aged five years and younger [13]. The predominance of RT078/toxinotype V (21%) was reported in an Iranian single center study performed between October 2010 and March 2011 [14]. The occurrence of toxigenic *C. difficile* strains (A(+)B(+), 64 (85.3%), A(+)B(-),5 (6.7%) and A(-)B(+),6 (8%) was found in a Tehran specialized health care center from November 2010 to October 2011 [15] and a 6.1% CDI prevalence was reported in a Tehran tertiary care hospital from December 2002 to February 2006 [16]. In Iran, the occurrence of *C. difficile* from a non-hospital setting was reported in farm animals [17, 18], raw meat [17, 19, 20], hamburgers [21], and ready-to-eat salads [22].

We performed a single center study to investigate the occurrence of *C. difficile* in hospitalized patients with suspected CDI in a hospital environment. *C. difficile* isolates were further characterized by molecular methods recommended for CDI surveillance.

Materials and Methods

The study was carried out in the Research Institute for Gastroenterology and Liver Diseases, in Tehran, Iran, from January 2011 to August 2011. This institute is a specialized health care facility with 44 beds, and 1938 admissions per year.

A total of 105 diarrheal stool samples of hospitalized patients with suspected *C. difficile* infection (CDI), 53 swabs of medical devices (colonoscope, forceps of the endosonographic imaging system, forceps of the ERCP device and patients' bed) and gastroenterology unit environment (washing sink for the imaging device) were investigated by direct anaerobic culture on a selective *C. difficile* medium (Mast, United Kingdom) supplemented with 7% horse blood and selective components at 37 °C. Swab sampling of the imaging devices was performed by wet cotton swab (sterile physiological salt solution) after routinely used surface treatment by 2% glutaraldehyde and 10-minute sterilization.

DNA extraction and PCR

The crude DNA from the several *C. difficile* colonies was extracted by InstaGene matrix extraction kit (Bio-Rad, USA). Capillary electrophoresis (CE) ribotyping was performed

according to the consensus ribotyping protocol [23] in the Department of Medical Microbiology, Motol University hospital, Prague, Czech Republic. The obtained CE-ribotyping profiles were compared with the profiles used in a CE-ribotyping validation study [23]. The *fsa files were also uploaded to the WEBRIBO database [24]. Multiplex toxin gene PCR was performed with primers described by Persson et al. [25].

Results

A total of 158 samples were investigated (105 stools and 53 swabs) and 23 *C. difficile* isolates were cultured (14.5%). Nineteen *C. difficile* isolates (19/105, 18.1%) were derived from stool samples of symptomatic patients, (9 male, 10 female) and four *C. difficile* isolates (4/53, 7.5%) were cultured from swabs of medical devices and the environment in the gastroenterology unit (colonoscope (1/15), forceps of the endosonographic imaging system (1/3), forceps of the ERCP device (1/1) and patient bed (1/17)).

Out of the 23 *C. difficile* isolates, seven following ribotypes were identified: 001 (n=2), 003 (n=1), 014 (n=1), 017 (n=1), 029 (n=1), 039 (n=1), 081 (n=1) and 126 (n=5). Three CE-profiles were recognized by WEBRIBO as WEBRIBO types: AI-12 (n=2), AI-29 (n=1) and AI-82/1 (n=1). Three CE-profiles were not identified and three DNA samples were not repeatedly amplified by the primers used for this assay.

Of 23 *C. difficile* isolates, 22 isolates were toxigenic (*tcdA*, *tcdB*) and five of them were also positive for the presence of binary toxin genes (all isolates of RT126). The remaining single isolate was non-toxigenic.

A summary of the results is shown in Table 1.

Discussion

In Iran, several studies that focused on *C. difficile* infection in human, animals and food were performed [12-22], however, the incoherence or the absence of molecular typing methods do not allow us to compare *C. difficile* molecular patterns with previously published data.

PCR ribotyping was applied in several Iranian studies. Two studies focused on pediatric patients identified the *C. difficile* ribotyping patterns classified as R27 (14.28%), R1 (10.71%), R12 (7.14%), R13 (7.14%) and R18 (7.14%) in the first study [12], and as RT027 (11.52 %), R1 (9.61 %) and R13 (7.68 %) in the second study [13]. Twelve different ribotyping patterns (IR1-10, 078, 014), with a prevalence of RT078, were recognized in Isfahan University of Medical Sciences Teaching Hospital [14].

Among animal isolates, twenty-one RTs were identified and IR12, IR33 and IR37 were more frequently found (14.2% of each profile) in the study of Esfandiari et al. [17]. Eight ribotyping profiles (IR11-18) were identified in the study focused on occurrence of *C. difficile* in beef and mutton meat samples [20]. In contrast, 53.9% prevalence of RT078 was found in raw beef, cow, sheep, goat, camel and buffalo meat [19]. Four different ribotyping profiles (IR21, IR 22, IR 23 and IR24) were identified in hamburgers [21].

Despite the distinct geographic location of this study, the spectrum of ribotyping profiles identified (001, 003, 014, 017, 029, 081, 126) belong to the human RTs occurring in Europe [25]. And moreover, from this study, the same spectra of RTs and WEBRIBO profiles (except WRT AI-29) were also found in the international animal strain collection [26].

The most prevalent RT identified in our study was RT 126 (20.8%). Its clinical significance was reported in three CDI cases [27] and its zoonotic potential in human CDI was recently suggested based on the subtyping of human and animal isolates [28]. This is also supported by the occurrence of RT126 reported in pigs, piglets, raw pork and calves [29-33].

Three CE-profiles were recognized by WEBRIBO database as WEBRIBO types: AI-12 (n=2), AI-29 (n=1) and AI-82/1 (n=1). Two of them, AI-12 and AI-82/1 were recently identified as RTs 150 and 103, respectively in the UK ribotyping reference laboratory, Leeds [27].

Conclusion

The predominance of RT 126 was found in a Tehran gastroenterology centre. RTs 126, 001, 150 were cultured from both stool samples and swabs from medical devices and the hospital environment, which suggest a possible route of transmission. These results indicate the need for a larger multicenter study to confirm our findings.

Acknowledgment

This study was part of proposal number RIGLD 613 from the Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Behehsti University of Medical Sciences, Tehran, Iran. PCR ribotyping was supported by MH CZ – DRO, University Hospital Motol, Prague, Czech Republic 00064203. The authors thank the Department of Medical Microbiology of Charles University and the ESCMID Study Group for *Clostridium difficile* (ESGCD) for their professional support.

Conflicts of interest

All authors declare no conflicts of interest.

References

[1] P.A. Lawson, D.M. Citron, K.L. Tyrrell, S.M. Finegold. Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prévot 1938. Anaerobe. 40 (2016) 95-9.

[2] M. Rupnik, M.H. Wilcox, D.N. Gerding. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nature Reviews Microbiology. 7 (7) (2009) 526-36.

[3] J. Freeman, M. Bauer, S.D. Baines, J. Corver, W. Fawley, B. Goorhuis, et al. The changing epidemiology of *Clostridium difficile* infections. Clinical Microbiology Reviews. 23 (3) (2010) 529-49.

[4] C. Knetsch, T. Lawley, M. Hensgens, J. Corver, M. Wilcox, E. Kuijper. Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections. Euro Surveill. 18 (4) (2013) 20381.

[5] W. Jamal, V. Rotimi, J. Brazier, B. Duerden. Analysis of prevalence, risk factors and molecular epidemiology of *Clostridium difficile* infection in Kuwait over a 3-year period. Anaerobe. 16 (6) (2010) 560-5.

[6] W. Jamal, E. Pauline, V. Rotimi. A prospective study of community-associated *Clostridium difficile* infection in Kuwait: epidemiology and ribotypes. Anaerobe. 35 (Pt B) (2015) 28-32.

[7] R. Moukhaiber, G.F. Araj, K.A.B. Kissoyan, K.A. Cheaito, G.M. Matar. Prevalence of *Clostridium difficile* toxinotypes in infected patients at a tertiary care center in Lebanon. The Journal of Infection in Developing Countries. 9 (7) (2015) 732-5.

[8] A. Adler, T. Miller-Roll, R. Bradenstein, C. Block, B. Mendelson, M. Parizade, et al. A national survey of the molecular epidemiology of *Clostridium difficile* in Israel: the dissemination of the ribotype 027 strain with reduced susceptibility to vancomycin and metronidazole. Diagnostic Microbiology and Infectious Disease. 83 (1) (2015) 21-4.

[9] A.A. Al-Thani, W.S. Hamdi, N.A. Al-Ansari, S.H. Doiphode. Polymerase chain reaction ribotyping of *Clostridium difficile* isolates in Qatar: a hospital-based study. BMC Infectious Diseases. 14 (2014) 502.

[10] J.A. Al-Tawfiq, M.S. Abed. *Clostridium difficile*-associated disease among patients in Dhahran, Saudi Arabia. Travel Medicine and Infectious Disease. 8 (6) (2010) 373-6.

[11] E. Ergen, H. Akalın, E. Yılmaz, M. Sınırtaş, O. Alver, Y. Heper, et al. Nosocomial diarrhea and *Clostridium difficile* associated diarrhea in a Turkish University Hospital. Médecine et Maladies Infectieuses. 39 (6) (2009) 382-7.

[12] S. Kouzegaran, M. Ganjifard, A.S. Tanha. Detection, Ribotyping and Antimicrobial Resistance Properties of *Clostridium difficile* Strains Isolated From The Cases of Diarrhea. Materia Socio-Medica. 28 (5) (2016) 324-328.

[13] A. Khoshdel, R. Habibian, N. Parvin, A. Doosti, F. Famouri, A. Eshraghi, et al. Molecular characterization of nosocomial *Clostridium difficile* infection in pediatric ward in Iran. SpringerPlus. 4 (2015) 627.

[14] M. Jalali, F. Khorvash, K. Warriner, J.S. Weese. *Clostridium difficile* infection in an Iranian hospital. BMC Research Notes. 5 (2012) 159.

[15] M. Goudarzi, H. Goudarzi, M. Alebouyeh, M.A. Rad, F.S.S. Mehr, M.R. Zali, et al. Antimicrobial susceptibility of *Clostridium difficile* clinical isolates in Iran. Iranian Red Crescent Medical Journal. 15 (8) (2013) 704-11.

[16] N. Sadeghifard, M.H. Salari, M.R. Ghassemi, S. Eshraghi, F.A. Harati. The incidence of nosocomial toxigenic *Clostridium difficile* associated diarrhea in Tehran tertiary medical centers. Acta Medica Iranica. 48 (5) (2010) 320-5.

[17] Z. Esfandiari, J.S. Weese, H. Ezzatpanah, M. Chamani, P. Shoaei, M. Yaran, et al. Isolation and Characterization of *Clostridium difficil*e in Farm Animals from Slaughterhouse to Retail Stage in Isfahan, Iran. Foodborne Pathogens and Disease. 12 (10) (2015) 864-6.

[18] A. Doosti, A. Mokhtari-Farsani. Study of the frequency of *Clostridium difficil*e *tcdA*, *tcdB*, *cdtA* and *cdtB* genes in feces of Calves in south west of Iran. Annals of Clinical Microbiology and Antimicrobials. 13 (2014) 21.

[19] E. Rahimi, M. Jalali, J.S. Weese. Prevalence of *Clostridium difficile* in raw beef, cow, sheep, goat, camel and buffalo meat in Iran. BMC Public Health. 14 (2014) 119.

[20] Z. Esfandiari, M. Jalali, H. Ezzatpanah, J.S. Weese, M. Chamani. Prevalence and characterization of *Clostridium difficile* in beef and mutton meats of Isfahan region, Iran. Jundishapur Journal of Microbiology. 7 (8) (2014) e16771.

[21] Z. Esfandiari, S. Weese, H. Ezzatpanah, M. Jalali, M. Chamani. Occurrence of *Clostridium difficile* in seasoned hamburgers and seven processing plants in Iran. BMC Microbiology. 14 (2014) 283.

[22] M. Yamoudy, M. Mirlohi, B.N. Isfahani, M. Jalali, Z. Esfandiari, N.S. Hosseini. Isolation of toxigenic *Clostridium difficile* from ready-to-eat salads by multiplex polymerase chain reaction in Isfahan, Iran. Advanced Biomedical Research. 4 (2015) 87.

220 [23] W.N. Fawley, C. Knetsch, D.R. MacCannell, C. Harmanus, T. Du, M.R. Mulvey, et al.
221 Development and validation of an internationally-standardized, high-resolution capillary gel-
222 based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One. 10 (2)
223 (2015) e0118150.
224 [24] A. Indra, S. Huhulescu, M. Schneeweis, P. Hasenberger, S. Kernbichler, A. Fiedler, et al.
225 Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based
226 PCR ribotyping. Journal of Medical Microbiology. 57 (Pt 11) (2008) 1377-82.
227 [25] S. Persson, M. Torpdahl, K. Olsen. New multiplex PCR method for the detection of
228 *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes
229 applied to a Danish strain collection. Clinical Microbiology and Infection. 14 (11) (2008)
230 1057-64. Erratum in: Clin Microbiol Infect. 15 (3) (2009) 296.
231 [26] M.P. Bauer, D.W. Notermans, B.H. Van Benthem, J.S. Brazier, M.H. Wilcox, M.
232 Rupnik, et al. *Clostridium difficile* infection in Europe: a hospital-based survey. The Lancet
233 377 (9759) (2011) 63-73.
234 [27] S. Janezic, V. Zidaric, B. Pardon, A. Indra, B. Kokotovic, J.L. Blanco, et al. International
235 *Clostridium difficile* animal strain collection and large diversity of animal associated strains.
236 BMC Microbiology. 14 (2014) 173.
237 [28] Y.P. Hung, H.J. Lin, B.Y. Tsai, H.C. Liu, H.C. Liu, J.C. Lee, et al. *Clostridium difficile*
238 ribotype 126 in southern Taiwan: a cluster of three symptomatic cases. Anaerobe. 30 (2014)
239 188-92.
240 [29] S. Álvarez-Pérez, J.L. Blanco, C. Harmanus, E. Kuijper, M.E. García. Subtyping and
241 antimicrobial susceptibility of *Clostridium difficile* PCR ribotype 078/126 isolates of human
242 and animal origin. Veterinary Microbiology. 199 (2017) 15-22.
243 [30] S. Andrés-Lasheras, R. Bolea, R. Mainar-Jaime, E. Kuijper, E. Sevilla, I. Martín-Burriel,
244 et al. Presence of *Clostridium difficile* in pig faecal samples and wild animal species
245 associated with pig farms. Journal of Applied Microbiology. 122 (2) (2017) 462-472.
246 [31] Y.C. Wu, C.M. Chen, C.J. Kuo, J.J. Lee, P.C. Chen, Y.C. Chang, et al. Prevalence and
247 molecular characterization of *Clostridium difficile* isolates from a pig slaughterhouse, pork,
248 and humans in Taiwan. International Journal of Food Microbiology 242 (2017) 37-44.
249 [32] A. Schneeberg, H. Neubauer, G. Schmoock, S. Baier, J. Harlizius, H. Nienhoff, et al.
250 *Clostridium difficile* genotypes in piglet populations in Germany. Journal of Clinical
251 Microbiology 51 (11) (2013) 3796-803.
252 [33] V. Zidaric, B. Pardon, T. dos Vultos, P. Deprez, M.S.M. Brouwer, A.P. Roberts, et al.
253 Different antibiotic resistance and sporulation properties within multiclonal *Clostridium*

difficile PCR ribotypes 078, 126, and 033 in a single calf farm. Applied and Environmental Microbiology 78 (24) (2012) 8515-22.

Table 1: Molecular characteristic of *C. difficile* isolates in the study.

ID code	Source	*tcdA*	*tcdB*	*cdtA*	*cdtB*	Ribotype/WEBRIBO type
32-2	Colonoscope	+	+	-	-	150/AI-12
114-2	Stool sample	+	+	-	-	150/AI-12
184-2	Stool sample	+	+	-	-	001
75-2	ERCP	+	+	-	-	001
54-2	Endoscope	+	+	+	+	126
20-2	stool sample	+	+	+	+	126
141-2	stool sample	+	+	+	+	126
85-2	stool sample	+	+	+	+	126
65-2	stool sample	+	+	+	+	126
105-2	stool sample	+	+	-	-	unknown
90-2	stool sample	+	+	-	-	--------
83-2	stool sample	+	+	-	-	--------
60-2-	stool sample	+	+	-	-	unknown
45-2	stool sample	-	-	-	-	039
100-2	stool sample	+	+	-	-	081
127-2	stool sample	+	+	-	-	103/AI-82/1
40-2	Bed	+	+	-	-	AI-29
142-2	stool sample	+	+	-	-	003
30-2	stool sample	+	+	-	-	unknown
130-2	stool sample	+	+	-	-	---------
134-2	stool sample	+	+	-	-	029
110-2	stool sample	+	+	-	-	017
89-2	stool sample	+	+	-	-	014

ERCP - endoscopic retrograde cholangiopancreatogram, *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 [25].