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IZBOLJŠAVA POSTOPKOV ZA DOKAZOVANJE PRISOTNOSTI BAKTERIJE *CLOSTRIDIUM DIFFICILE* PRI ŽIVALIH

Doktorska disertacija

IMPROVEMENT OF THE PROCEDURES FOR THE DETECTION OF *CLOSTRIDIUM DIFFICILE* IN ANIMALS

Doctoral dissertation

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Izboljšava postopkov za dokazovanje prisotnosti bakterije *Clostridium difficile* pri živalih

Delo je bilo opravljeno na Inštitutu za mikrobiologijo in parazitolgijo ter na Inštitutu za anatomijo, histologijo in embriologijo Veterinarske fakultete v Ljubljani. Toksinotipizacija in PCR-ribotipizacija sevov je bila opravljena na Zavodu za zdravstveno varstvo Maribor.

Javni zagovor je bil opravljen:

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Izjava o delu:

Izjavljam, da je doktorska disertacija rezultat lastnega raziskovalnega dela, da so rezultati korektno navedeni in nisem kršila avtorskih pravic in intelektualne lastnine drugih.

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IZVLEČEK

Ključne besede: klostridijske infekcije – mikrobiologija – diagnostika; *Clostridium difficile* – izolacija in čiščenje; bakteriološke tehnike; polimerazna, verižna reakcija v realnem času – metode; feces – mikrobiologija; občutljivost in specifičnost; primerjalna študija; živali, domače

V preteklosti je bila že večkrat potrjena prisotnost bakterije *Clostridium difficile* (*C. difficile*) pri živalih. Z metodo obogatitvene kulture smo *C. difficile* tudi v okviru naše študije dokazali pri različnih domačih živalih. Metoda je občutljiva, vendar zelo dolgotrajna. Zato smo želeli najti čim hitrejši ter hkrati dovolj občutljiv in specifičen postopek dokazovanja *C. difficile* pri živalih, saj so obstoječe metode v večini validirane za humane vzorce. Razvili smo dve novi metodi PCR v realnem času (rtPCR) za vse tri gene, ki kodirajo toksina A in B ter binarni toksin (TMrtPCR, LC rtPCR), za ugotavljanje variantnih oblik *C. difficile* neposredno v blatu živali. Rezultati TMrtPCR in LC rtPCR so se ujemali pri 97,7 % vzorcev, medtem ko je bilo ujemanje z metodo obogatitvene kulture 75 %. Testa rtPCR nista bila uporabna kot samostojna metoda, saj je bilo 11–12 % vzorcev kulturapozitivnih/rtPCR-negativnih. Zato smo v nadaljevanju študije izboljšali postopek dokazovanja *C. difficile* v vzorcih z nizkim številom te bakterije. Uporabili smo predobogatitev v tekočem selektivnem gojišču pred pomnoževanjem z rtPCR. Že enodnevna inkubacija v bujonu je očitno povečala število kultura- in rtPCR-pozitivnih vzorcev, poleg tega pa ni bilo kulturapozitivnih/LC rtPCR-negativnih vzorcev. Rezultati nakazujejo, da je rtPCR v kombinaciji s predobogatitvijo zanesljiv in hiter presejalni test, s katerim lahko učinkovito ugotavljamo prisotnost *C. difficile* tudi pri zdravih živalih, ki so lahko izločevalci bakterije in bi zato lahko imele pomembno vlogo pri širjenju okužbe.

ABSTRACT

Keywords: clostridium infections – microbiology – diagnosis; *Clostridium difficile* – isolation and purification; bacteriological techniques; real-time polymerase chain reaction – methods; feces – microbiology; sensitivity and specificity; comparative study; animals, domestic

In previous studies *Clostridium difficile* (*C. difficile*) was frequently detected in animals. *C. difficile* was also isolated with the enrichment culture from different domestic animals in our study. The method used is sensitive, but time-consuming. The aim of the doctoral dissertation was to find a fast and reliable procedure for the detection of *C. difficile* in animals, while most of the existing methods are validated for human samples. We developed two real-time PCR (rtPCR) assays targeting the *tcdA*, *tcdB* and *cdtB* toxin genes (TMrtPCR, LC rtPCR) capable of detecting variant strains in animal faeces. The comparison of LC rtPCR with TMrtPCR results showed a complete concordance in 97.7 % samples, while the correlation with the enrichment culture was in 75 % samples. Neither of the rtPCR assays were adequate as a stand-alone test, while assays resulted in 11-12 % culture positive/rtPCR negative samples. Therefore, we improved the procedure for the detection of *C. difficile* in the samples with a low number of bacteria, using a pre-enrichment step followed by the rtPCR amplification. One day of enrichment already evidently increased the number of culture and rtPCR positive samples, and no culture positive/rtPCR negative samples were observed. The results of this study demonstrate that rtPCR in combination with the enrichment culture can be applied as an accurate and rapid screening test. It can be used for reliable detection of *C. difficile* also in healthy animals, which could be the shedders of bacteria and might have an important role in the spread of the infection.

KAZALO VSEBINE

KAZALO SLIK

SEZNAM OKRAJŠAV

AFLP	polimorfizem dolžin pomnoženih fragmentov; angl. amplified fragment lenght polymorphism
bp	bazni par
BrtPCR	predhodno objavljen *tcdB* PCR v realnem času (van den Berg in sod., 2006)
CDT	binarni toksin bakterije *Clostridium difficile*
C. difficile	bakterijska vrsta *Clostridium difficile*
DNA	deoksiribonukleinska kislina; angl. deoxyribonucleic acid
EIA	encimskoimunski test; angl. enzyme immunoassay
FDA	ameriški vladni urad za zdravila in prehrano; angl. U.S. Food and Drug Administration
GDH	glutamat-dehidrogenaza
LAMP	metoda izotermalnega pomnoževanja; angl. loop-mediated isothermal amplification
LC rtPCR	LightCycler PCR v realnem času
LCT	veliki klostridijski toksini; angl. large clostridial toxins
MLST	tipizacija na osnovi zaporedij več lokusov; angl. multilocus sequence typing
MLVA	multilokusno spremenljivo število tandemskih ponovitev; angl. multilocus variable number tandem repeat analysis
NNV	negativna napovedna vrednost
PCR	verižna reakcija s polimerazo; angl. polymerase chain reaction

PCRFast	komercialni komplet PCRFast *Clostridium difficile* A/B test (ifp Institut für Produktqualitet, Nemčija)
PCR-RFLP	PCR-polimorfizem dolžin restrikcijskih fragmentov; angl. PCR-restriction fragment length polymorphism
PFGE	gelska elektroforeza v pulzirajočem električnem polju; angl. pulse-field gel electrophoresis
PNV	pozitivna napovedna vrednost
REA	polimorfizem restrikcijskih fragmentov celotne DNA; angl. restriction endonuclease analysis
RNA	ribonukleinska kislina; angl. ribonucleic acid
rtPCR	PCR v realnem času; angl. real-time PCR
*slpA*ST	alternativna metoda serotipizacije *slpA*; angl. surface layer protein A gene sequnce typing
TcdA	toksin A bakterije *Clostridium difficile*
TcdB	toksin B bakterije *Clostridium difficile*
TMrtPCR	TaqMan PCR v realnem času
TSA	angl. tryptic soy agar

1 UVOD

Bakterija *Clostridium difficile* (*C. difficile*) povzroča bolnišnične črevesne okužbe pri ljudeh, vendar v zadnjem času narašča tudi število doma pridobljenih okužb pri bolnikih brez dejavnikov tveganja za okužbo. V teh primerih so možen rezervoar bakterije živali, pri katerih je lahko *C. difficile* prisoten kot povzročitelj bolezni ali kot komenzalna bakterija v prebavnem traktu. Dobro ujemanje človeških in živalskih genotipov pa nakazuje zoonotski potencial bakterije (Keessen in sod., 2011a).

Trenutno dostopne metode za diagnostiko so hitre in manj občutljive oziroma specifične ali pa občutljive in specifične, vendar dolgotrajne, zato se uveljavljajo molekularne metode (npr. verižna reakcija s polimerazo v realnem času; rtPCR; angl. real-time polymerase chain reaction) (Carroll, 2011; Tenover in sod., 2011; Badger in sod., 2012). Na tržišču je veliko komercialno dostopnih testov za dokazovanje toksinov ali DNA *C. difficile* pri ljudeh, vendar je uporaba teh testov na živalskih vzorcih omejena, saj rezultati niso primerljivi z metodo izolacije bakterije oziroma z njimi ne moremo dokazati vseh variantnih oblik, ki so pri živalih pogoste (Keessen in sod., 2011b). Do sedaj je bil razvit le en test rtPCR za dokazovanje *C. difficile* pri živalih, medtem ko rtPCR s hibridizacijskimi sondami za vse tri toksinske gene še ni bil opisan (Houser in sod., 2010).

Namen doktorskega dela je bil razvoj novih metod rtPCR, s katerimi bi lahko zaznali variantne oblike *C. difficile*, in v nadaljevanju najti najučinkovitejšo kombinacijo klasične gojiščne in molekularne preiskave, tako da bi dosegli zadovoljivo občutljivost in specifičnost metode ter dokazali *C. difficile* hitreje, kot to omogočajo dostopne metode. Z novimi postopki bi tako hitreje dobili podatke o prisotnosti bakterije *C. difficile* v živalski populaciji, kar bi omogočilo določanje preventivnih ukrepov za omejitev širjenja in izbruhov okužb pri živalih ter preprečevanje možnega prenosa na ljudi.

Postavili smo naslednje hipoteze raziskave:

- Metoda rtPCR z uporabo hibridizacijskih sond na aparaturi LightCycler (Roche, Nemčija) bo imela boljšo specifičnost kot metoda z uporabo sond TaqMan na aparaturi ABI Prism 7000 (Applied Biosystems, ZDA). Meja zaznave in občutljivost bosta podobni.

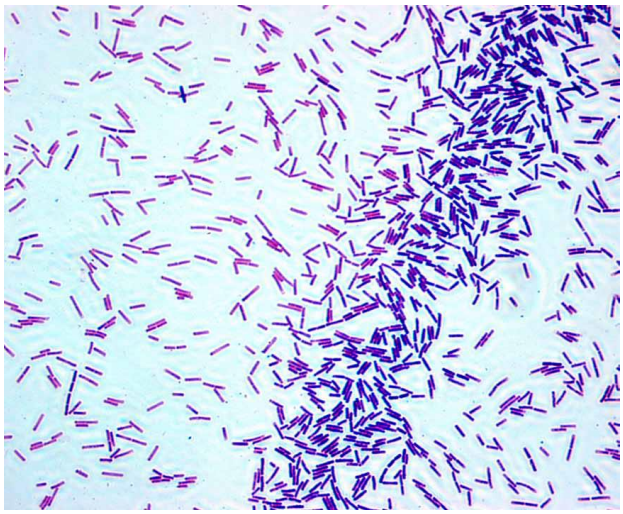
- Izključna uporaba rtPCR za neposredno dokazovanje *C. difficile* v blatu ne bo dovolj občutljiva, da bi jo lahko v diagnostiki *C. difficile* uporabljali samostojno.

- Najbolj optimalna glede na zanesljivost in hitrost bo uporaba metode rtPCR za dokazovanje *C. difficile* pri simptomatskih pujskih v obogatitvenem selektivnem gojišču po enodnevni inkubaciji.

2 PREGLED LITERATURE

2.1 LASTNOSTI BAKTERIJE *CLOSTRIDIUM DIFFICILE*

Bakterija *C. difficile* je po Gramu pozitiven anaeroben sporogen bacil, ki je del črevesne mikrobiote (Gubina, 2002) (Slika 1). Kot za vse patogene predstavnike rodu *Clostridium* je tudi za *C. difficile* značilno, da bolezenske znake povzročajo samo toksigeni sevi. *C. difficile* lahko proizvaja tri različne toksine: toksin A (TcdA; 308 kDa) in toksin B (TcdB; 207 kDa), ki sta glavna virulenčna dejavnika, ter binarni toksin (CDT), ki naj bi bil dodatni virulenčni dejavnik, vendar njegova vloga pri razvoju bolezni še ni povsem pojasnjena (Davies in sod., 2011). Toksigeni sevi lahko proizvajajo samo toksina A in B (A+B+CDT–), vse tri toksine (A+B+CDT+), samo toksin B in binarni toksin (A–B+CDT+) ali pa proizvajajo samo toksin B oziroma binarni toksin (A–B+CDT-, A–B–CDT+) (Rupnik, 2011). Lemire in sod. (2012) pa so opisali tudi prvi primer seva, ki ima samo toksin A in je brez toksina B (A+B–).



Slika 1: Po Gramu obarvane bakterije *Clostridium difficile*
Figure 1: Gram stain of *Clostridium difficile*

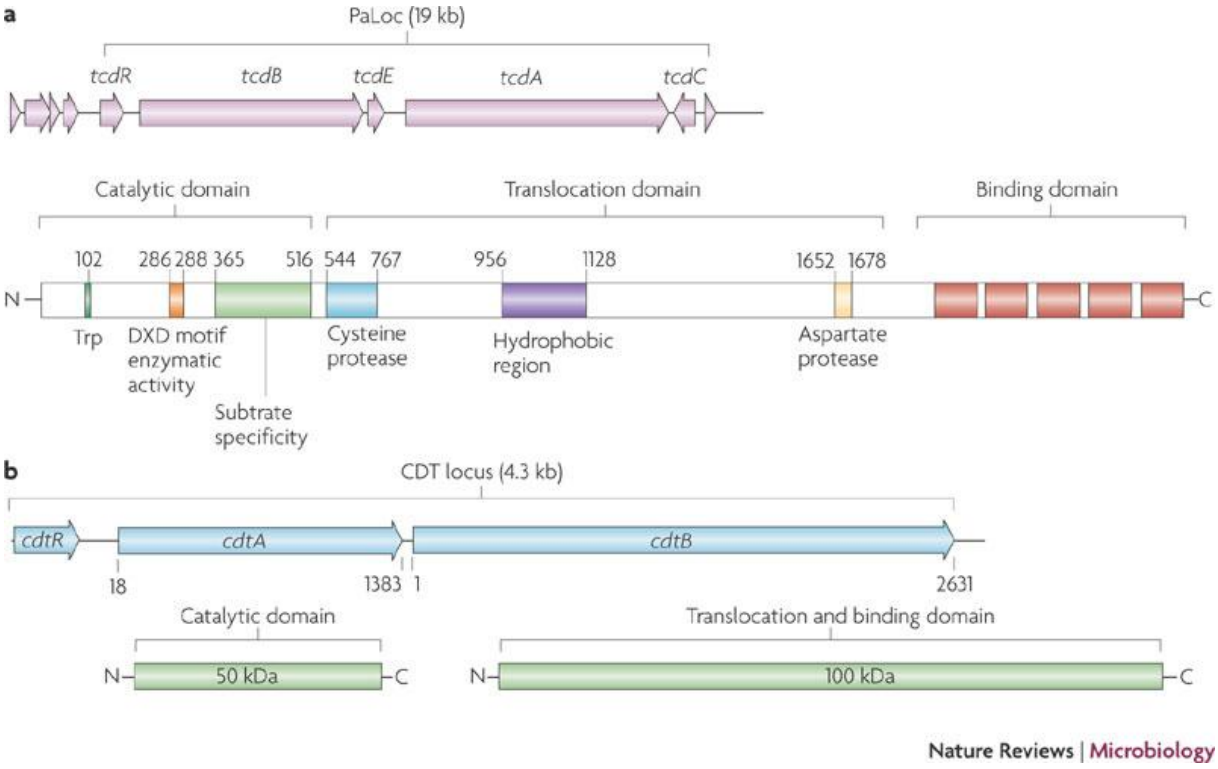
Toksina TcdA in TcdB sodita v družino velikih klostridijskih toksinov (LCTs; angl. large clostridial toxins). Oba delujeta citotoksično, TcdA pa tudi enterotoksično (Just in sod., 2000). Glavna posledica delovanja toksina TcdA so vnetne spremembe v črevesni steni in propad tesnih stikov med celicami, kar se kaže kot driska in hemoragične nekroze, medtem ko je TcdB odgovoren za nastanek razjed in rumeno belih oblog (psevdomembrane) na steni

debelega črevesja (Voth in Ballard, 2005). Tretji toksin, binarni toksin CDT, ki ga uvrščamo v skupino klostridijskih binarnih toksinov; je adenozin-difosfat-riboziltransferaza in deluje citotoksično (Perelle in sod., 1997). CDT so sprva pripisovali živalskim sevom, vendar je vedno več takih izolatov tudi pri ljudeh, pri katerih opisujejo težji potek bolezni in večjo smrtnost (Barbut in sod., 2005; Bacci in sod., 2011).

Gena za toksina A (*tcdA*) in B (*tcdB*) sta še s tremi dodatnimi geni (*tcdR*, *tcdE*, *tcdC*) zapisana na kromosomu v patogenskem lokusu (PaLoc), ki ima lastnosti mobilnega genskega elementa, medtem ko je pri netoksigenih sevih na tem mestu sekvenca velika 115 baznih parov (bp) (Hammond in Johnson, 1995) (Slika 2). Regija PaLoc je velika 19,6 kbp (Braun in sod., 1996). Glede na razlike v tem lokusu razdelimo seve na 31 toksinotipov, ki jih označujemo z rimskimi številkami (Rupnik, 2011). Razlike v lokusu PaLoc najpogosteje nastajajo zaradi delecij, insercij ali točkovnih mutacij (Rupnik in sod., 1998). Gen *tcdE* kodira hidrofobni holinu podoben protein, ki je verjetno odgovoren za sprostitev toksina iz celice, tako da permeabilizira celično steno bakterije (Tan in sod., 2001). Gen *tcdC* kodira negativni regulator prepisa *tcdA* in *tcdB* in je izražen v začetku logaritemske faze rasti celice, nato pa pride do represije v stacionarni fazi rasti (Hundsberger in sod., 1997). Gen *tcdR* pa kodira alternativni faktor sigma RNA-polimeraze in tako deluje kot pozitivni regulator prepisovanja obeh toksinskih genov v stacionarni fazi rasti celice (Mani in Dupuy, 2001). Do izražanja genov *tcdA* in *tcdB* in nastajanja TcdA in TcdB pride v pozni logaritemski in stacionarni fazi rasti celice kot odziv na različne okoljske signale (temperatura, prisotnost glukoze, aminokislin, antibiotikov in maslene kisline), kar vodi do različnih fizioloških sprememb v celici in razvoju bolezni (Voth in Ballard, 2005).

CDT kodirata dva gena: *cdtA* za encimsko podenoto toksina in *cdtB* za transportno podenoto toksina, ki sta zapisana na kromosomu (Perelle in sod., 1997). Sevi *C. difficile* so lahko brez zapisa za CDT, imajo popoln zapis za CDT (4,3 kb) ali skrajšan zapis – psevdogena *cdtA* in *cdtB* (2,3 kb), ki ne vodi v izražanje funkcionalnega CDT (Geric Stare in sod., 2007). Carter in sod. (2007) so odkrili, da funkcionalni lokus *cdt* vsebuje še tretji gen, ki so ga poimenovali *cdtR* in naj bi verjetno kodiral odzivni regulator, pomemben za optimalno izražanje binarnega toksina. Geni *cdtA*, *cdtB* in *cdtR* so genetsko povezani, zato so del lokusa CDT, imenovanega CdtLoc (Slika 2). Gen *cdtR* imajo tako sevi s popolnim zapisom za CDT kot tudi sevi s

skrajšanim zapisom. CDT negativni sevi imajo na mestu CdtLoc specifično sekvenco, veliko 68 bp.



Slika 2: Genski zapisi za toksine in toksini, ki jih proizvaja bakterija *Clostridium difficile* (povzeto po Rupnik in sod., 2009). a) Patogenski lokus PaLoc z zapisom za toksina A in B, pod njim struktura toksina B (TcdB); b) Lokus CdtLoc z zapisom za binarni toksin in struktura toksina

Figure 2: Toxin genes and toxins produced by *Clostridium difficile* (adopted from Rupnik in sod., 2009). a) Pathogenicity locus PaLoc with genes encoding toxin A and B, below the PaLoc is TcdB domain organization; b) Locus CdtLoc encoding binary toxin and its domain organization

2.2 EPIDEMIOLOGIJA IN BOLEZNI, KI JIH POVZROČA BAKTERIJA *CLOSTRIDIUM DIFFICILE* PRI LJUDEH

Bakterija *C. difficile* je pri ljudeh najpogostejši povzročitelj bolnišničnih črevesnih okužb. Zaradi jemanja antibiotikov, citostatičnega zdravljenja ali operativnih posegov se poruši normalna črevesna mikrobiota, kar omogoči germinacijo spor in posledično namnožitev *C. difficile*. Pomembni so še drugi dejavniki tveganja, kot so starost nad 65 let, bivanje v bolnišnici in pridružene bolezni (Johnson in Gerding, 1998). Linsky in sod. (2010) so

dokazali, da terapija z zaviralci protonskih črpalk (za zaviranje izločanja želodčne kisline) za 42 % poveča možnost ponovnega pojava bolezni, povzročene s *C. difficile*, medtem ko se bolezen po zdravljenju običajno ponovi v 20–30 % primerov (Pepin in sod., 2006). Bolnik pride v stik s sporami v bolnišnici (npr. roke bolniškega osebja, asimptomatski prenašalci) – v bolnišnici pridobljena okužba – ali pa je imel spore že prej prisotne v prebavilih (20 %) (Barbut in Petit, 2001). *C. difficile* so izolirali tudi iz zraka v bolnišničnih sobah in straniščih, kar nakazuje možnost aerosolnega prenosa (Best in sod., 2010; Best in sod., 2012) in zahteva uvedbo dodatnih pravil za nadzor širjenja okužb. Bolezen pri ljudeh poteka v različnih oblikah: od blage driske, hemoragičnega kolitisa do hudega psevdomembranoznega kolitisa in predrtja črevesja, ki se lahko konča s smrtjo (Bartlett in sod., 1978). Ljudje so lahko tudi asimptomatski prenašalci bakterije (< 5 %), pri čemer imajo prisoten netoksigen sev *C. difficile* ali pa zadostno količino protiteles IgG proti toksinu TcdA, da ne pride do razvoja bolezni (Shim in sod., 1998). Predvsem pri dojenčkih je ta odstotek lahko zelo visok (tudi do 62 %), vendar pri njih zelo redko pride do razvoja simptomov, kar naj bi bilo povezano s pomanjkanjem receptorjev za toksin A (Gubina, 2002; Enoch in sod., 2011).

Predvsem v humani medicini je zaznati opazno naraščanje števila okužb s *C. difficile* in pojav močno virulentnih sevov s povečano odpornostjo proti antibiotikom – seva 027/NAP1/BI/III, 017 (Kuijper in sod., 2007; Rupnik in sod., 2009). V državah Evropske unije so trenutno s težjimi obolenji povezani drugi PCR-ribotipi (018, 056), medtem ko so najpogostejši PCR-ribotipi 014/020, 001, 078, 018 in 106 (Bauer in sod., 2011). Opazen je porast okužb s *C. difficile* pri ljudeh, ki niso bili hospitalizirani, niso bili zdravljeni z antibiotiki in nimajo drugih dejavnikov tveganja za razvoj bolezni. Gre za t. i. doma pridobljene okužbe (angl. community-acquired *Clostridium difficile* infection) (Pituch, 2009; Otten in sod., 2010; Kuntz in sod., 2011; Khanna in sod., 2012). V nedavni študiji poročajo, da je v ZDA 32 % vseh okužb s *C. difficile* pridobljenih doma in da je kar četrtina teh bolnikov v sedmih dneh po postavljeni diagnozi hospitalizirana (Lessa, 2013). Predvsem so ogroženi otroci in nosečnice (Rouphael in sod., 2008; Enoch in sod., 2011). Pri teh okužbah se pogosto pojavlja PCR-ribotip 078/toksinotip V, ki je pri različnih živalih eden izmed najpogostejših tipov (Hensgens in sod., 2012). Na Nizozemskem opažajo porast okužb s tem PCR-ribotipom predvsem pri mlajši populaciji, poleg tega pa se humani in živalski sevi ne razlikujejo (Goorhuis in sod., 2008a; Debast in sod., 2009).

2.3 BAKTERIJA *CLOSTRIDIUM DIFFICILE* PRI ŽIVALIH, V HRANI IN OKOLJU

Bakterijo *C. difficile* so dokazali pri različnih domačih in divjih živalih (konji, teleta, psi, mačke, noji, laboratorijski glodavci, prašiči, perutnina, slon, drobnica). V prebavnem traktu ima vlogo komenzalne ali patogene bakterije. *C. difficile* povzroča enterokolitis in drisko predvsem v neonatalnem obdobju pri pujskih, teletih in žrebetih, pri starejših živalih pa je bolezen tako kot pri ljudeh najpogosteje povezana z antibiotičnim zdravljenjem (Lowe in sod., 1980; Borriello in sod., 1983; Frazier in sod., 1993; Madewell in sod., 1999; Baverud in sod., 2003; Bojesen in sod., 2006; Simango, 2006; Songer in Anderson, 2006; Keel in sod., 2007; Clooten in sod., 2008; Hammit in sod., 2008; Simango in Mwakurudza, 2008; Weese in sod., 2010a; Medina-Torress in sod., 2011; Koene in sod., 2012; Romano in sod., 2012a; Schneeberg in sod., 2012). Živali so pogosto klinično zdravi prenašalci *C. difficile*. Visok odstotek klinično zdravih prenašalcev je predvsem pri mladih živalih, s starostjo pa odstotek koloniziranih živali upada (Rodriguez-Palacious in sod., 2006; Norman in sod., 2009; Hoffer in sod., 2010; Weese in sod., 2010a; Costa in sod., 2011; Thitaram in sod., 2011; Rodriguez in sod., 2012; Knight in sod., 2013). Dokazano je, da ni razlike v prisotnosti *C. difficile* pri pujskih z drisko in brez nje, medtem ko bolezen povzročena s *C. difficile* pri perutnini še ni opisana in je, kot kaže, perutnina zgolj rezervoar bakterije (Zidarič in sod., 2008; Alvarez-Perez in sod., 2009; Hopman in sod., 2011a; Koene in sod., 2012).

Enakost odkritih genotipov pri živalih in ljudeh nakazuje možnost medvrstnega prenosa *C. difficile* (Arroyo in sod., 2005a; Lefebvre in sod., 2006; Keel in sod., 2007; Jhung in sod., 2008; Debast in sod., 2009; Janežič in sod., 2012; Koene in sod., 2012). Živali bi bile lahko potencialno nov rezervoar bakterije za okužbe ljudi, predvsem v primerih doma pridobljenih okužb. Poleg tega so odkrili *C. difficile* tudi v hrani, in sicer v surovem mesu (svinjina, govedina, piščančje in puranje meso), pripravljenih mesnih izdelkih, školjkah, ribah, kozicah, surovi zelenjavi in pripravljenih solatah (Al Saif in Brazier, 1996; Bakri in sod., 2009; Rodriguez-Palacious in sod., 2009; Songer in sod., 2009; Weese in sod., 2009; Metcalf in sod., 2010a; Weese in sod., 2010b; Harvey in sod., 2011a; Metcalf in sod., 2011; Pasquale in sod., 2012). Odstotek pozitivnih vzorcev mesa se med Severno Ameriko in Evropo močno razlikuje: 1,8–20 % v Kanadi in 8–50 % v ZDA, medtem ko je v Evropi odstotek pozitivnih vzorcev mesa nizek (0–4,3 %) (Rodriguez-Palacios in sod., 2007; Indra in sod., 2009; Songer in sod., 2009; von Abercron in sod., 2009; Bouttier in sod., 2010; Hoffer in sod., 2010; Jöbstl

in sod., 2010; Metcalf in sod., 2010b; de Boer in sod., 2011; Harvey in sod., 2011b; Houser in sod., 2012). Sevi iz hrane in sevi, prisotni pri ljudeh in živalih, pripadajo istim PCR-ribotipom. V Severni Ameriki sta PCR-ribotipa 078 in 027 celo najpogosteje prisotna v hrani (Rodriguez-Palacios in sod., 2007; Songer in sod., 2009; Weese in sod., 2009). Glede na to, da so živali pozitivne tako v Severni Ameriki kot Evropi, gre verjetno za kontaminacijo mesa s sporami iz okolja pri pridelavi in predelavi mesa za prodajo, vendar vloga hrane kot vira okužbe ni poznana, saj ni znana infektivna doza spor za razvoj okužbe s *C. difficile* (Weese, 2010c; Rupnik in Songer, 2010).

Okolje predstavlja vir spor, s katerimi se okužijo živali in ljudje, saj lahko spore v okolju preživijo več mesecev. *C. difficile* so izolirali iz vode (površinske vode, pitna in bazenska voda), zemlje, zraka in prahu na farmah (Al Saif in Brazier, 1996; Simango, 2006; Zidarič in sod., 2010; Hopman in sod., 2011a). Prisotnost bakterije na dotoku in iztoku iz biološke čistilne naprave predstavlja nevarnost za širjenje bakterije v okolje in kaže na potrebo po previdnosti pri uporabi blata iz čistilnih naprav za gnojenje pridelovalnih površin (Hensgens in sod., 2012; Romano in sod., 2012b). Keessen in sod. (2011c) so dokazali širjenje *C. difficile* po zraku na prašičjih farmah in povečano koncentracijo *C. difficile*, kadar je bilo osebje na farmi aktivno (hranjenje, cepljenje, premikanje živali). Na prašičjih farmah bi lahko bili možen vektor insekti (muhe, hrošči), miši in ptiči (vrabci), saj so *C. difficile* pri njih dokazali v 56–100 % (Burt in sod., 2012).

2.4 STANJE V SLOVENIJI

V Sloveniji smo leta 2011 zabeležili prvi izbruh *C. difficile* v eni izmed slovenskih bolnišnic. Dokazan je bil močno virulenten PCR-ribotip 027, povezan s težjim potekom bolezni. Poleg tega število prijavljenih primerov strmo narašča, saj se je od leta 2007 do 2011 povečalo z 0,9 na 6,6 primerov/100.000 prebivalcev, vendar ocenjujejo, da je število močno podcenjeno. Večina prijavljenih primerov je povezana z običajnimi dejavniki tveganja za okužbo s *C. difficile*. Število hospitaliziranih zaradi okužbe s *C. difficile* se je v enem letu (2010–2011) povečalo skoraj za 100 % (Kraigher in sod., 2012).

Pri živalih v Sloveniji je *C. difficile* zelo razširjen pri sesnih pujskih (52 %), manj pri govedu (Pirš in sod., 2008). Pri perutnini je prevalenca koloniziranih živali (62 %) odvisna od starosti živali in se s starostjo zmanjšuje, poleg tega pa so prisotni zelo raznoliki genotipi (Zidarič in

sod., 2008). Pri drugih živalskih vrstah (psi, mačke, konji, ovce, koze) pa je bil *C. difficile* dokazan samo občasno pri posameznih živalih (Avberšek in sod., 2012; Avberšek in Ocepek, neobjavljeni podatki). *C. difficile* pri prostoživečih pticah pevkah ni bil dokazan (Bandelj in sod., 2011). Zidarič in sod. (2010) so dokazali *C. difficile* tudi v 68 % vzorcev, odvzetih iz rek in izolirali 34 različnih PCR-ribotipov (od tega 25 toksigenih), medtem ko bakterije v mesu, mesnih izdelkih in solati niso dokazali. S površine enega od 49 kokošjih jajc pa so izolirali dva seva *C. difficile* (PCR-ribotip 014/020 in 002) (Rupnik in sod., 2012).

Primerjava izolatov (humani, živalski, okoljski), dobljenih med letoma 2008 in 2010, kaže, da je 11 od 90 PCR-ribotipov prisotnih pri živalih, ljudeh in v okolju, 16 PCR-ribotipov je prisotnih pri ljudeh in v okolju, 8 PCR-ribotipov pa je enakih pri ljudeh in živalih (Janežič in sod., 2012). Med njimi ni PCR-ribotipa 078, ki ga pri živalih še nismo izolirali. Najpogostejši PCR-ribotipi, prisotni pri ljudeh, živalih in v okolju, so 014/020, 002 in 001/072, kar se sklada s prevalenco drugod v Evropi (Bauer in sod., 2011; Janežič in sod., 2012). Možnost preživetja posameznega seva v različnih okoljih verjetno omogoča uspešno širjenje in s tem večjo prevalenco določenega PCR-ribotipa.

2.5 LABORATORIJSKO DOKAZOVANJE BAKTERIJE *CLOSTRIDIUM DIFFICLE*

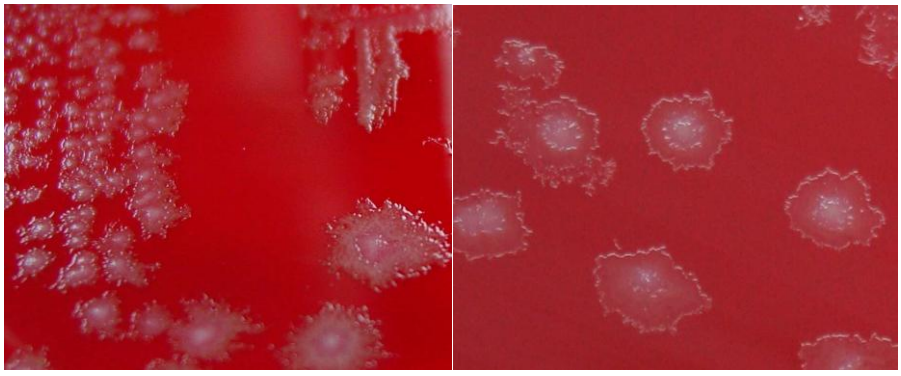
Najprimernejša kužnina za dokazovanje okužb s *C. difficile* je sveže blato. Vsa dosedanja odkritja in sprememba pojavnosti okužb s *C. difficile* zahtevajo izboljšanje diagnostičnih metod in epidemiološke raziskave izbruhov. V nadaljevanju so opisane poznane metode za laboratorijsko dokazovanje *C. difficile*.

2.5.1 Izolacija bakterije *Clostridium difficile* na gojiščih

Laboratorijska diagnostika temelji na t. i. toksigeni kulturi, ki vključuje izolacijo bakterije iz blata in nadaljnje *in vitro* dokazovanje toksinov s testom citotoksičnosti ali z encimskoimunskim testom (EIA; angl. enzyme immunoassay) (Delmee in sod., 2005). Toksigena kultura je najobčutljivejša med opisanimi metodami (94–100 %), vendar zamudna in zahteva izkušeno osebje (Delmee, 2001; Cohen in sod., 2010; Badger in sod., 2012).

Za izolacijo se najpogosteje uporablja selektivno gojišče – fruktozni agar s cikloserinom in cefoksitinom (George in sod., 1979). Na tržišču pa so še druga gojišča: *C. difficile* gojišče z norfloksacinom in moksalaktamom, kromogeno gojišče Chrom ID® *C. difficile* (BioMerieux,

Francija) idr. (Aspinall in Hutchinson, 1992). Gojišča lahko vsebujejo različne dodatke, ki izboljšajo germinacijo spor oziroma obogatijo gojišče (konjska ali ovčja kri, jajčni rumenjak, lizocim, cistein, natrijev tauroholat idr.), ali indikator, ki spremeni barvo, ko *C. difficile* poraste (CDSA agar z nevtral rdečim indikatorjem; Becton Dickinson, ZDA) (Marler in sod., 1992; Wilcox in sod., 2000). Selektivno gojišče se inkubira 48 ur v anaerobni atmosferi pri 37 °C. Občutljivost metode se poveča z uporabo etanolnega šoka pred inokulacijo vzorca (enak volumen blata in etanola zmešamo in inkubiramo 30 minut do ene ure) (Borriello in Honour, 1981). Arroyo in sod. (2005b) so primerjali uporabo različnih postopkov za izolacijo bakterije. Najboljša je bila uporaba obogatitvenega selektivnega tekočega gojišča (fruktozni bujon s cikloserinom in cefoksitinom) z dodatkom 0,1 % natrijevega tauroholata, ki omogoča boljšo germinacijo spor (Wilson in sod., 1982), v kombinaciji z alkoholnim šokom, ki ji sledi izolacija *C. difficile* na trdem selektivnem gojišču. Hink in sod. (2013) so objavili prvo študijo, ki je primerjala več gojišč in postopkov za izolacijo *C. difficile* iz vzorcev blata in rektalnih brisov. V obeh primerih je bila najboljša uporaba toplotnega šoka (segrevanje 10 minut na 80 °C), ki mu sledi inokulacija vzorca v manitolni bujon s cikloserinom, cefoksitinom, tauroholatom, cisteinom in lizocimom in nato po sedmih dneh izolacija *C. difficile* na agarju TSA II (angl. tryptic soy agar) s 5-odstotno ovčjo krvjo. Uporaba obogatitvenega bujona izboljša občutljivost metode za približno 20 %, vendar se postopek podaljša na približno devet dni (Tenover in sod., 2011).



Slika 3: Kolonije *Clostridium difficile* na krvnem agarju
Figure 3: *Clostridium difficile* colonies on blood agar

Identifikacija kolonij *C. difficile* temelji na tipični morfologiji (Slika 3), rumeno-zeleni fluorecsenci pod UV-svetlobo, vendar se le-ta lahko razlikuje glede na to, katero gojišče je

uporabljeno, po tipičnem vonju (konjski gnoj), plinsko tekočinski kromatografiji kolonij (dokaz maslene in izo-kaprojske kisline), dokazu prolinske aminopeptidaze z diski (Levett, 1984; Fedorko in Williams, 1997). Uporaba biokemijskih testov in antigenske lateksne aglutinacije običajno ne da zanesljivih rezultatov (Tenover in sod., 2011).

2.5.2 Test citotoksičnosti

Referenčna metoda (t. i. zlati standard) je še vedno test citotoksičnosti za dokazovanje toksina B na celični kulturi, kjer opazujemo citopatske učinke (zaokrožanje celic), vendar nekateri raziskovalci zaradi boljše občutljivosti navajajo kot referenčno metodo t. i. toksigeno kulturo (Crobach in sod., 2009; Cohen in sod., 2010). Najpogosteje uporabljene so celice Vero, Hep2, CHO ali HeLa, vendar naj bi bile celice Vero najbolj občutljive. Pri testu citotoksičnosti le-to potrdimo z nevtralizacijo s specifičnimi protitelesi (Chang in sod., 1978). Test je učinkovit, vendar ima pomanjkljivosti: ni standardiziran, traja 24–48 ur, zaradi hitrega razpada ali majhne količine toksina v vzorcu ni vidnega citopatskega učinka, kar privede do lažno negativnih rezultatov, zahteva izkušeno osebje in vzdrževanje celičnih kultur (Freeman in Wilcox, 2003; Delmee in sod., 2005). Hkrati obstaja možnost lažno pozitivnih rezultatov, do katerih pride pri nevtralizaciji s protitelesi, ki lahko nevtralizirajo tudi letalni toksin bakterije *Clostridium sordellii* (Knoop in sod., 1993; O'Connor in sod., 2001). Test citotoksičnosti odlikuje visoka specifičnost (99–100 %) in pozitivna napovedna vrednost (PNV) (93–100 %), medtem ko je občutljivost v primerjavi s toksigeno kulturo slabša (50–85 %) (Stamper in sod., 2009a; Peterson in sod., 2011).

2.5.3 Serološke metode

Za dokazovanje toksinov TcdB in/ali TcdA v blatu so na voljo tudi številni komercialni testi EIA, ki pa so manj občutljivi kot test citotoksičnosti (Barbut in sod., 1993; Planche in sod., 2008; Chand in sod., 2011). Podatki o zanesljivosti testov EIA se v literaturi zelo razlikujejo. V nekaterih študijah poročajo o občutljivosti, slabši od 50 %, redko pa dosežejo vrednosti nad 90 %, poleg tega pa je pomanjkljivost tudi nizka PNV testov (predvsem v populaciji z nizko prevalenco), medtem ko sta v večini primerov specifičnost (84–100 %) in negativna napovedna vrednost (NNV) (84–98 %) dobri (Alcala in sod., 2008; Planche in sod., 2008; Sloan in sod., 2008; Crobrach in sod., 2009; Nemat in sod., 2009; Goldenberg in sod., 2010a; Peterson in sod., 2011; de Jong in sod., 2012). V humani medicini se še vedno večinoma

uporabljajo kot presejalni testi, pri čemer je nujna uporaba testov, ki zaznajo prisotnost obeh toksinov (Johnson in sod., 2001). Kljub hitri izvedbi (rezultate odčitamo že po 2–4 urah) pa novejše raziskave odsvetujejo uporabo testov EIA (Eastwood in sod., 2009; Cohen in sod., 2010; Carroll in Loeffelholz, 2011; Boyanton in sod., 2012). Komercialno dostopni testi so na voljo v dveh izvedbah: EIA v vdolbinicah mikrotitrske plošče ali membranski imunokromatografski tip EIA (Vanpoucke in sod., 2001).

Z EIA lahko dokazujemo tudi prisotnost encima glutamat-dehidrogenaza (GDH), ki je specifičen za *C. difficile*, vendar ne razlikuje toksigenih sevov od netoksigenih (Landry in sod., 2001; Zheng in sod., 2004). Teste GDH EIA odlikuje visoka NNV, zato se večinoma uporabljajo kot prvi presejalni test v večstopenjskih algoritmih za diagnostiko okužb s *C. difficile* (Reller in sod., 2007). Občutljivost je boljša kot pri EIA za dokazovanje toksinov, vendar se razlikuje med testi različnih proizvajalcev (83–100 %) (Goldenberg in sod., 2010a; Peterson in sod., 2011). Testi GDH EIA pa imajo tudi nizko PNV (Shetty in sod., 2011). Poleg tega lahko prisotnost določenega PCR-ribotipa zmanjša občutljivost testa, kar poraja dvom o njegovi uporabnosti (Tenover in sod., 2010).

2.5.4 Dokazovanje nukleinske kisline

V diagnostiki so na pomenu začele pridobivati molekularne metode – npr. verižna reakcija s polimerazo (PCR; angl. polymerase chain reaction), s katerimi lahko zaradi hitro pridobljenih rezultatov preprečimo izkustveno zdravljenje z antibiotiki in dolgotrajno hospitalizacijo. V začetni fazi so bile molekularne metode omejene na dokazovanje gena za 16S rRNA, vendar je njihova slabost ta, da ne ločijo med toksigenimi in netoksigenimi sevi, zato so kasneje razvili tudi več protokolov PCR za dokazovanje genov, ki kodirajo toksina A in B (Gumerlock in sod., 1993; Kato in sod., 1993: Kuhl in sod., 1993). Hitrejša in občutljivejša je metoda rtPCR za dokazovanje prisotnosti toksinskih genov, vendar je dražja. Belanger in sod. (2003) so prvi opisali in uporabili metodo rtPCR za dokazovanje genov *tcdA* in *tcdB* z uporabo molekularnih svetil na aparaturi SmartCycler (Cepheid, ZDA), vendar niso zaznali gena *tcdB* pri variantnih toksinotipih (III, IV, VI). Opisane so metode rtPCR za hitro dokazovanje *C. difficile* v blatu, vendar z njimi večinoma dokazujemo gen za 16S rRNA (Rinttilä in sod., 2004; Penders in sod., 2005; Tonooka in sod., 2005, Mutters in sod., 2009) ali samo gen za toksin B (van den Berg in sod., 2006; Peterson in sod., 2007; Larson in sod., 2010). Poleg tega veliko protokolov opisuje dokazovanje genov z barvilom SYBR Green

(Rinttilä in sod., 2004; Tonooka in sod., 2005; Peterson in sod., 2007), ki pa je zaradi vezave na vsako nastalo dvoverižno DNA slabo specifično (Espy in sod., 2006). Sloan in sod. (2008) in Wolff in sod. (2009) so razvili metodo rtPCR z uporabo aparature LightCycler, s katero dokazujemo regulatorni gen *tcdC* – negativni regulator genov *tcdA* in *tcdB*. Wroblewski in sod. (2009) so prvi razvili multipli rtPCR (angl. multiplex rtPCR), s katerim dokazujemo gene *tcdA, tcdB, cdtA* in *cdtB* s sondami TaqMan. Metoda je perspektivna, vendar niso testirali različnih variantnih toksinotipov, ki se vedno pogosteje pojavljajo, poleg tega je bila uporabljena in preizkušena samo na majhnem številu kliničnih humanih vzorcev. V zadnjih treh letih je bilo objavljenih še nekaj novih ″in-house″ protokolov rtPCR za dokazovanje toksinskih genov (*tcdA, tcdB, tcdC, cdtA*) *C. difficile* pri ljudeh (de Boer in sod., 2010; Barbut in sod., 2011; Luna in sod., 2011; Hoegh in sod., 2012; Jayaratne in sod., 2013). Vsi protokoli uporabljajo hidrolizirajoče sonde TaqMan, vendar nekateri nimajo vključene interne kontrole, dokazujejo samo en toksinski gen ali niso preizkušeni na različnih toksinotipih. Občutljivost ″in-house″ testov rtPCR se giblje 83–100 %, specifičnost med 88 in 99 %, PNV 66–94 % in NPV 96–100 % (zlati standard toksigena kultura).

Na tržišču so dostopni številni komercialni testi za dokazovanje toksigenega *C. difficile* neposredno v blatu, ki so namenjeni in preizkušeni za uporabo v humani medicini. Objavljene so študije treh komercialnih testov rtPCR, ki jih je odobril ameriški vladni urad za zdravila in prehrano (FDA; angl. U.S. Food and Drug Administration), za neposredno dokazovanje *C. difficile* v blatu ljudi. Dva temeljita na dokazovanju *tcdB* z rtPCR – BD GeneOhm Cdiff (BD Diagnostics, ZDA) (Stamper in sod., 2009a) in ProGastro Cd (Prodesse, ZDA) (Stamper in sod., 2009b), eden pa na hkratnem dokazovanju gena *tcdB*, gena za binarni toksin in delecije gena *tcdC*, ki napoveduje PCR-ribotip 027 – Cepheid Xpert *C. difficile* (Cepheid, ZDA) (Huang in sod., 2009). Za izvedbo vseh treh testov potrebujemo točno določeno aparaturo za rtPCR, kar predstavlja veliko pomanjkljivost predvsem za laboratorije, ki so že opremljeni za molekularno diagnostiko. Objavljenih je več študij, ki te teste primerjajo z drugimi metodami. Če so kot zlati standard vzeli toksigeno kulturo, so imeli testi občutljivost 77–100 %, specifičnost 94–100 %, PNV 68–100 % in NNV 95–100 % (Barbut in sod., 2009; Eastwood in sod., 2009; Huang in sod., 2009; Stamper in sod., 2009a; Stamper in sod., 2009b; Terhes in sod., 2009; Doing in sod., 2010; Goldenberg in sod., 2010b, Kvach in sod., 2010; Novak-Weekley in sod., 2010; Tenover in sod., 2010; Peterson in sod., 2011; Zidarič in sod., 2011; LaSala in sod., 2012; Shin in sod., 2012).

Najnovejša objavljena molekularna metoda, odobrena s strani FDA, je t. i. metoda z zanko posredovanega izotermičnega pomnoževanja (LAMP; angl. loop-mediated isothermal amplification). Na začetku pride do pomnoževanja 204 bp velike regije gena *tcdA* pri konstanti temperaturi (65 °C), med pomnoževanjem nastaja stranski produkt magnezijev pirofosfat, ki tvori bel percipitat in se ga dokazuje turbidimetrično. Metoda je hitra (1 h) in njena občutljivost, specifičnost in NNV so primerljive s komercialnimi testi rtPCR, medtem ko je PNV slabša (83–92 %) (Boyanton in sod., 2012). Njena pomanjkljivost je, da z njo dokazujemo samo gen *tcdA* in tako lahko dobimo lažno negativen rezultat v primeru variantnih tipov *C. difficile* (Lalande in sod., 2011; Noren in sod., 2011).

Avtorji različnih študij opozarjajo na preveliko občutljivost metode rtPCR, saj pozitiven rezultat ne odraža nujno dejanske bolezni, povzročene s *C. difficile*, ampak samo prisotnost *C. difficile* (Barbut in sod., 2011; de Jong in sod., 2012). Peterson in Robicsek (2009) tako priporočata samo testiranje tistih bolnikov, ki zadostijo kriterijem ponavljajočih epizod driske, hkrati pa je treba pri ovrednotenju rezultatov diagnostične metode upoštevati klinično sliko preiskovanca (Dubberke in sod., 2011). Leslie in sod. (2012) so ugotovili, da imajo na toksin negativni vzorci 10–10000-krat manj *C. difficile* v blatu kot na toksin pozitivni vzorci, kar nakazuje, da je od koncentracije *C. difficile* odvisno proizvajanje toksinov. Kvantifikacija *C. difficile* z metodo rtPCR bi lahko bila velik doprinos obstoječim metodam za ločevanje bolnih in asimptomatskih ljudi, vendar so potrebne nadaljnje študije. Poleg tega z metodo rtPCR ne dokažemo toksina, ampak samo gen, za katerega ne vemo, če je funkcionalen oziroma če dejansko pride do izražanja in tvorbe toksina (t. i. klinična specifičnost) (Carroll in Loeffelholz, 2011).

2.5.5 Večstopenjski algoritmi za dokazovanje bakterije *Clostridium difficile* v humani medicini

Trenutno dostopne metode za diagnostiko so bodisi hitre in manj občutljive/specifične bodisi občutljive/specifične in dolgotrajne (Tenover in sod., 2011), zato je priporočena uporaba dvo- ali tri-stopenjskih algoritmov za diagnostiko okužb s *C. difficile* pri ljudeh (Carroll, 2011). Prvi presejalni test naj bi bil občutljiv in z visoko NNV (GDH EIA, rtPCR), pozitivne vzorce pa nato potrdimo z drugim potrditvenim testom (de Boer in sod., 2010; Knetsch in sod., 2011; Jones in sod., 2013). Prvi algoritmi so kot presejalni test uporabljali GDH EIA, kot drugega pa EIA za dokazovanje toksinov (Fenner in sod., 2008). Številni avtorji so ugotovili, da testi

EIA niso primerni kot potrditveni testi, saj imajo preslabo občutljivost, zato so za potrditveni test predlagali test citotoksičnosti ali toksigeno kulturo, vendar se tako čas preiskave zelo podaljša (Ticehurst in sod., 2006; Reller in sod., 2007; Gilligan, 2008; Planche in sod., 2008; Cohen in sod., 2010). Z razvojem molekularnih metod, ki poleg toksigene kulture edine dosegajo dovolj dobro občutljivost, pa se za potrditveni test priporoča rtPCR oziroma tristopenjski algoritem – GDH-pozitivne vzorce testiramo z EIA za dokaz toksinov in nato na toksin negativne še z molekularno metodo, vendar s tem znižamo občutljivost in NNV testa rtPCR (Goldenberg in sod., 2010a; Carroll in Loeffelholz, 2011; Peterson in sod., 2011). Najnovejše študije pa porajajo vprašanje o uporabnosti algoritmov, saj je tudi občutljivost testov GDH EIA vprašljiva (Larson in sod., 2010; Novak-Weekley in sod., 2010; Tenover in sod., 2010). Istočasno se predlaga test rtPCR kot samostojna metoda, dodatna prednost so predvsem testi rtPCR, s katerimi dokazujemo več genov, saj kljub višji ceni dobimo rezultat hitreje, s čimer se zmanjšajo stroški zdravljenja in možnost prenosa *C. difficile* (Babady in sod., 2010; Kvach in sod., 2010; Wilcox in sod., 2010; Peterson in sod., 2011; Tenover in sod., 2011; Hoegh in sod., 2012).

2.5.6 Uporabnost metod za dokazovanje bakterije *Clostridium difficile* v veterinarski medicini

Vse zgoraj opisane metode so potencialno uporabne tudi v veterinarski medicini, vendar so bile vse razvite in prilagojene uporabi v humani medicini, z izjemo metode izolacije *C. difficile* iz blata. Glede na to, da je v živalskih vzorcih blata velikokrat malo vegetativnih celic oziroma več spor, je priporočljiva predhodna uporaba obogatitvenega selektivnega tekočega gojišča, ki pa podaljša čas izvedbe metode. Thitaram in sod. (2011) so primerjali dva postopka izolacije na vzorcih prašičev in goveda. Uporabili so enojni alkoholni šok po obogatitvi v selektivnem bujonu in dvojni alkoholni šok pred obogatitvijo in po njej. V obeh primerih so po obogatitvi uporabili fruktozni agar s cikloserinom in cefoksitinom ter gojišče TSA. Ugotovili so, da je uporaba dvojnega alkoholnega šoka boljša za prašičje vzorce, medtem ko je za goveje vzorce primernejši samo en alkoholni šok. Izbira medija je imela manjši vpliv, vendar vseeno priporočajo uporabo selektivnega gojišča za *C. difficile*, saj lažje pridemo do čiste kulture *C. difficile*.

Na začetku je tudi v veterini prevladovala uporaba testov EIA, ki pa so validirani le na humanih vzorcih. Chouicha in Marks (2006) sta primerjala pet različnih testov EIA s testom

citotoksičnosti na pasjih vzorcih blata in ugotovila, da občutljivost in specifičnost nista zadostni za dokazovanje toksinov v pasjih vzorcih. Neujemanje med rezultati kulture oziroma PCR in testi EIA so ugotovili tudi pri vzorcih konjev z okužbo s *C. difficile* (Magdesian in sod., 2002; Magdesian in sod., 2006; Arroyo in sod., 2007; Ruby in sod., 2009). Medtem ko so Medina-Torres in sod. (2010) ugotovili ujemanje rezultatov med EIA in testom citotoksičnosti v 93 % vzorcev konjev z drisko. Neujemanje med rezultati kulture in testom EIA so opisali tudi pri vzorcih blata telet (Rodriguez-Palacious in sod., 2006; Pirš in sod., 2008). Dve študiji sta primerjali teste EIA in test citotoksičnosti za dokazovanje toksinov v blatu prašičev (Post in sod., 2002; Anderson in Songer, 2008). Prvi test je imel občutljivost 91 % v primerjavi s testom citotoksičnosti, vendar je imel drugi test EIA občutljivost le 39 % v primerjavi s prvim testom EIA. Keessen in sod. (2011b) so primerjali tri teste EIA in komercialni rtPCR BD GeneOhm Cdiff na prašičjih vzorcih in ugotovili, da noben test ni primeren kot samostojna metoda za dokazovanje *C. difficile* pri prašičih. Ujemanje rezultatov testov in referenčne metode je bilo samo pri 16,9 % vzorcev. Najboljšo občutljivost (93 %) in NNV (87,5 %) je imel rtPCR, zato avtorji predlagajo dvostopenjski algoritem za dokazovanje *C. difficile* pri prašičih (rtPCR kot presejalni test in toksigena kultura kot potrditveni test rtPCR-pozitivnih vzorcev). Na splošno imajo testi EIA slabšo občutljivost za dokazovanje toksinov pri živalih kot pri ljudeh in uporaba EIA, namenjenih za diagnostiko pri ljudeh, ni priporočljiva brez predhodne validacije na živalskih vzorcih.

Hopman in sod. (2011b) so testirali komercialni rtPCR BD GeneOhm Cdiff za dokazovanje *C. difficile* v blatu prašičev in rezultate primerjali s toksigeno obogatitveno kulturo: 28 % vzorcev je bilo pozitivnih s toksigeno kulturo, vsi pa so bili negativni z rtPCR. Do sedaj je opisan le en TaqMan rtPCR (*tcdA, tcdB, cdtA, cdtB*) za uporabo v veterini (blato telet) in za dokazovanje *C. difficile* v hrani (Houser in sod., 2010). Neposredno z rtPCR so pregledali 71 vzorcev blata in ugotovili, da je smiselno rtPCR-negativne vzorce saditi v selektivni bujon in jih inkubirati 5–7 dni, nato izolirati DNA iz bujona ter ponovno narediti rtPCR. S predobogatitvijo so dobili 7 % več rtPCR-pozitivnih vzorcev.

2.6 GENOTIPIZACIJSKE METODE BAKTERIJE *CLOSTRIDIUM DIFFICILE*

Za tipizacijo *C. difficile* je opisanih več genotipizacijskih metod, ki temeljijo na:

- restrikciji z restrikcijskimi encimi in nadaljnji analizi na agaroznem gelu – polimorfizem restrikcijskih fragmentov celotne DNA (REA; angl. restriction endonuclease analysis), gelska elektroforeza v pulzirajočem električnem polju (PFGE; angl. pulse-field gel electrophoresis) in PCR-polimorfizem dolžin restrikcijskih fragmentov (PCR-RFLP; angl. PCR-restriction fragment length polymorphism, npr. toksinotipizacija *C. difficile*);

- pomnoževanju specifičnih odsekov genoma z metodo PCR – PCR-ribotipizacija, multilokusno spremenljivo število tandemskih ponovitev (MLVA; angl. multilocus variable number tandem repeat analysis) in polimorfizem dolžin pomnoženih fragmentov (AFLP; angl. amplified fragment lenght polymorphism);

- določitvi in analizi nukleotidnega zaporedja izbranih odsekov DNA – tipizacija na osnovi zaporedij več lokusov (MLST; angl. multilocus sequence typing), alternativna metoda serotipizacije *slpA* (*slpA*ST; angl. surface layer protein A gene sequnce typing) in tipizacija sekvenc tandemskih ponovitev (angl. tandem repeat sequence typing) (Kuijper in sod., 2009; Janežič in Rupnik, 2010).

Killgore in sod. (2008) so primerjali sedem različnih metod. Za vsako so določili sposobnost razlikovanja in odstotek sevov, ki so jih z določeno metodo lahko tipizirali. Z vsemi metodami so lahko tipizirali vse izbrane izolate *C. difficile*, medtem ko se je sposobnost razlikovanja gibala med 0,964 in 0,631 v naslednjem vrstnem redu: MLVA, REA, PFGE, *slpA*ST, PCR-ribotipizacija, MLST, AFLP. Izkazalo se je, da so vse metode sposobne dokazati seve, ki povzročijo izbruh, vendar sta samo metodi REA in MLVA pokazali zadostno sposobnost razlikovanja, da ločimo seve iz različnih izbruhov. Metoda REA ima zelo visoko sposobnost razlikovanja, vendar je ovrednotenje rezultatov zaradi velikega števila dobljenih fragmentov težko in subjektivno. Poleg tega je treba primerjati profil izolata z referenčnim izolatom na istem gelu in rezultati niso primerljivi med laboratoriji. Medlaboratorijska primerljivost rezultatov pa je vedno bolj pomembna lastnost tipizacijskih metod (Clabots in sod., 1993; Killgore in sod., 2008). MLVA je novejša in perspektivna metoda, ki kljub svoji odlični sposobnosti razlikovanja in enostavnosti še ni široko sprejeta v laboratorijih (van den Berg in sod., 2007b). Največkrat uporabljena metoda za primerjavo tipov z drugimi laboratoriji je PCR-ribotipizacija, vendar je treba imeti knjižnico znanih PCR-

ribotipov, da lahko preiskovani izolat poimenujemo po standardni nomenklaturi referenčnega laboratorija za anaerobe v Cardiffu (Kuijper in sod., 2009).

3 IZVIRNI ZNANSTVENI ČLANKI

3.1 DIVERSITY OF *CLOSTRIDIUM DIFFICILE* IN PIGS AND OTHER ANIMALS IN SLOVENIA

Raznolikost bakterije *Clostridium difficile* pri prašičih in drugih živalih v Sloveniji

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Izvleček:

Opravljena je bila študija raznolikosti *Clostridium difficile* pri prašičih, teletih in konjih v Sloveniji. Skupno je bilo pregledanih 547 vzorcev. *C. difficile* smo izolirali iz 247/485 (50,9 %) sesnih pujskov, 4/42 (9,5 %) telet in 1/20 (5 %) konjev. Izolate smo genotipizirali s toksinotipizacijo, PCR-ribotipizacijo in gelsko elektroforezo v pulzirajočem električnem polju (PFGE) z uporabo restrikcijskega encima *Sma*I. Prašičji izolati so pripadali dvema toksinotipoma (V in 0), štirim PCR-ribotipom (066, 029, SI 011, SI 010) in šestim pulzotipom. Govejim izolatom smo določili dva toksinotipa (XIa in 0), tri PCR-ribotipe (077, 002, 033) in tri pulzotipe. Toksinotip, PCR-ribotip in pulzotip edinega konjskega izolata je bil enak kot pri enem izolatu *C. difficile* iz teleta (XIa/033). Nobeden od dokazanih genotipov ni bil prisoten pri vseh treh živalskih vrstah.

Opomba: Po objavi tega članka so na Zavodu za zdravstveno varstvo Maribor (laboratorij dr. Maje Rupnik) pridobili nove referenčne seve iz Cardiffa in preizkusili PCR-ribotipizacijo na sekvenatorju ter ugotovili, da so nekateri PCR-ribotipi naših izolatov narobe določeni. Tako so vsi prašičji izolati PCR-ribotip 066 postali 045, PCR-ribotip SI 011 pa 150; goveji izolati PCR-ribotip 077 so po novem 014/020.

3.2 DETECTION OF *CLOSTRIDIUM DIFFICILE* IN ANIMALS: COMPARISON OF REAL-TIME PCR ASSAYS WITH THE CULTURE METHOD

Dokazovanje bakterije *Clostridium difficile* pri živalih: primerjava testov PCR v realnem času z metodo izolacije bakterije

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Izvleček:

Clostridium difficile postaja pomemben patogen in komenzal različnih živali. Živali bi lahko bile rezervoar *C. difficile*, saj se humani, živalski in izolati iz mesa ne razlikujejo. Razvili smo nov TaqMan PCR v realnem času (TMrtPCR) za neposredno dokazovanje variantnih sevov *C. difficile* (geni *tcdA*, *tcdB*, *cdtB*) v blatu živali z drisko in zdravih živali. Metodo smo primerjali z obogatitveno kulturo in z dvema že obstoječima testoma PCR v realnem času, BrtPCR in PCRFast, s katerima dokazujemo gen *tcdB* oziroma *tcdA*/*tcdB*. S TMrtPCR smo dokazali vseh deset testiranih toksinotipov, medtem ko s PCRFast nismo zaznali toksinotipa XIa, z BrtPCR pa toksinotipov X in XIa. Z metodo izolacije bakterije in TMrtPCR smo skupno pregledali 340 (100 %) vzorcev. Rezultati so se ujemali pri 75,3 % vzorcev. Štirideset (11,8 %) vzorcev je bilo kulturapozitivnih/TMrtPCR-negativnih. Najverjetnejša razloga sta nizko število bakterij v vzorcu ali neuspešna izolacija DNA. Štirideset (11,8 %) vzorcev pa je bilo kulturanegativnih/TMrtPCR-pozitivnih. V primerjavo treh testov rtPCR je bilo vključenih 79 vzorcev in rezultati so se popolnoma ujemali v 50,6 %. Rezultati kažejo, da je TMrtPCR boljši od BrtPCR in PCRFast, saj je bilo 67 % kulturapozitivnih vzorcev pozitivnih tudi s TMrtPCR, medtem ko je bilo le 40 % oziroma 7 % teh vzorcev pozitivnih z BrtPCR oziroma s PCRFast. Dodatna prednost TMrtPCR je tudi možnost dokazovanja gena za binarni toksin, saj s tem lahko dobimo prvo informacijo o prisotnem toksinskem tipu. Rezultati študije kažejo, da bi lahko TMrtPCR uporabljali kot presejalno metodo za hitro dokazovanje *C. difficile* v živalskih vzorcih blata, vendar je treba vzorce z negativnim ali dvomljivim rezultatom nadaljnje testirati z izolacijo bakterije v obogatitvenem gojišču.

3.3 IMPROVED DETECTION OF *CLOSTRIDIUM DIFFICILE* IN ANIMALS BY USING ENRICHMENT CULTURE FOLLOWED BY LIGHTCYCLER REAL-TIME PCR

Izboljšano dokazovanje prisotnosti bakterije *Clostridium difficile* pri živalih z uporabo kombinacije obogatitvene kulture in LightCycler PCR v realnem času

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Izvleček:

Uporaba našega predhodno objavljenega TaqMan PCR v realnem času (TMrtPCR) za dokazovanje *Clostridium difficile* neposredno v blatu živali ni zadovoljiva zaradi lažno negativnih rezultatov z metodo TMrtPCR. Zato smo razvili nov LightCycler PCR v realnem času (LC rtPCR) z interno kontrolo za dokazovanje variantnih sevov pri živalih z drisko in zdravih živalih. LC rtPCR uporablja dve hibridizacijski sondi za razliko od ene hidrolizirajoče sonde, uporabljene v TMrtPCR. Ker z LC rtPCR nismo dobili boljših rezultatov kot s TMrtPCR, smo želeli izboljšati postopek dokazovanja *C. difficile* v vzorcih z nizkim številom bakterij z vpeljavo obogatitve v bujonu pred pomnoževanjem z rtPCR. V ta namen smo vzorčili 40 (100 %) sesnih pujskov (rektalni brisi) in jih neposredno testirali z LC rtPCR in z izolacijo bakterij brez obogatitve in z obogatitvijo. Po enem, dveh, treh in sedmih dneh inkubacije smo iz bujona izolirali DNA in jo pomnoževali z LC rtPCR. Samo en vzorec (2,5 %) je bil kultura- in LC rtPCR-pozitiven (neposredno dokazovanje), medtem ko je bilo po sedemdnevni obogatitvi 33 (82,5 %) vzorcev kulturapozitivnih. Samo en dan predobogatitve je izrazito povečal število kultura- (15; 37,5 %) in LC rtPCR-pozitivnih vzorcev (28; 70 %). Rezultati te študije kažejo, da je enodnevna predobogatitev, ki ji sledi dokazovanje vseh treh toksinskih genov *C. difficile* z LC rtPCR, zanesljiv in hiter presejalni test, predvsem za vzorce z nizkim številom *C. difficile,* saj ni bilo kulturapozitivnih/LC rtPCR-negativnih vzorcev.

4 RAZPRAVA

Bakterija *C. difficile* povzroča neonatalne driske pri sesnih pujskih, vendar je njena prevalenca visoka tudi pri subkliničnih sesnih pujskih, ki so zgolj kolonizirani in so prenašalci bakterije. V Sloveniji smo *C. difficile* izolirali iz različnih živali z drisko ali brez nje. V naši raziskavi smo pregledali devet različnih prašičjih farm in izolirali *C. difficile* v 37,3–80 %. Raznolikost sevov na prašičjih farmah ni velika – dokazali smo le dva toksinotipa oziroma tri PCR-ribotipe (V/045, 0/150, 0/029), ki so specifični za določeno geografsko področje v Sloveniji. Toksinotip V ima vse tri toksine (A+B+CDT+), medtem ko ima toksinotip 0 samo dva (A+B+CDT–). Tip V/045 je prisoten v osrednji in južni Sloveniji (okolica Ljubljane, Dolenjska), medtem ko sta druga dva genotipa specifična za vzhodno Slovenijo (Prlekija, Prekmurje). Vsi dokazani PCR-ribotipi so drugačni kot drugje po svetu, kjer je pri prašičih najpogostejši PCR-ribotip 078 (Keel in sod., 2007; Goorhuis in sod., 2008b; Gould in Limbago, 2010; Weese in sod., 2010a). Pri preostalih živalskih vrstah je v Sloveniji prevalenca *C. difficile* veliko manjša, razen pri perutnini, kjer je tudi raznolikost sevov velika, vendar večina sevov nima zapisa za binarni toksin (CDT–).

Ugotavljanje prisotnosti *C. difficile* pri različnih živalskih vrstah je pomembno, saj so lahko tudi subklinične živali, ki so lahko rezervoar oziroma so izločevalci te bakterije, vir potencialno pomembnih genotipov *C. difficile*, ki so sposobni povzročiti hude okužbe pri živalih in ljudeh. Ker so koncentracije *C. difficile* v vzorcih zdravih živali majhne, je nujna obogatitvena kultura, ki pa je dolgotrajna (devet dni) in ne loči netoksigenih sevov od toksigenih. Da bi izboljšali postopek dokazovanja prisotnosti *C. difficile* pri živalih, smo razvili in validirali dve novi metodi rtPCR za hitrejše dokazovanje *C. difficile* neposredno v blatu (TMrtPCR in LC rtPCR), ju primerjali z dvema že obstoječima testoma rtPCR (PCRFast *Clostridium difficile* A/B test – PCRFast in rtPCR za dokazovanje *tcdB* – BrtPCR) (van den Berg in sod., 2006), z obogatitveno kulturo in med seboj.

Uporaba komercialnih testov razvitih in validiranih na humanih vzorcih je omejena pri testiranju živalskih vzorcev (Hopman in sod., 2011b; Keessen in sod., 2011b). Tudi rezultati naše primerjave dveh novih metod rtPCR, validiranih na živalskih vzorcih, in dveh že obstoječih testov rtPCR kažejo enake ugotovitve. TMrtPCR in LC rtPCR sta primernejša za dokazovanje *C. difficile* v blatu živali kot BrtPCR in PCRFast, saj se je pri prvih dveh testih

rtPCR najvišji odstotek rezultatov ujemal z rezultati kulture. BrtPCR je bil validiran na humanih vzorcih, medtem ko za PCRFast ni podatka, na kakšnih vzorcih je bil preizkušen oziroma za kakšne vzorce je namenjen.

Rezultati primerjave TMrtPCR in LC rtPCR z obogatitveno kulturo so bili podobni (ujemanje v 75,0 % pri LC rtPCR in 75,3 % pri TMrtPCR). Podoben odstotek (71,4 %) ujemanja rezultatov metode rtPCR (*tcdB*) s kulturo so dobili v študiji van den Bergove in sod. (2007a). Kljub temu da metodi TMrtPCR in LC rtPCR temeljita na različnem načinu dokazovanja pridelkov PCR je medsebojna primerjava pokazala, da so se rezultati ujemali v 97,7 % pregledanih vzorcev. Teoretično naj bi imel LC rtPCR boljšo specifičnost, saj temelji na zaznavanju z dvema hibridizacijskima sondama, vendar tega v naši raziskavi nismo potrdili. Se je pa razlikovala meja zaznave za dokazovanje gena *tcdA* in *tcdB*, ki je bila nižja pri uporabi TMrtPCR. Poleg tega je vpeljava interne kontrole v dupleks-PCR zvišala mejo zaznave samo v primeru LC rtPCR. Validacija obeh metod rtPCR na vzorcih blata živali je pokazala, da kljub ugotovljenim razlikam v meji zaznave ni večjih odstopanj, kar kaže, da je uporaba obeh metod enakovredna. Izbira tako temelji na razpoložljivosti in zmogljivosti aparatur v določenem laboratoriju.

Z obema metodama rtPCR smo dobili 11–12 % kulturapozitivnih/rtPCR-negativnih vzorcev. To bi lahko bilo povezano s številom celic *C. difficile* v vzorcu blata. Ker blato ni homogen material, se lahko koncentracija bakterij razlikuje v vzorcih za različne preiskave. Poleg tega smo za vzorčenje sesnih pujskov uporabili rektalne brise, ki vsebujejo majhno količino blata, ki smo jo naknadno s spiranjem brisa še razredčili. Razlog bi lahko bila tudi neuspešna izolacija DNA, saj subklinične živali izločajo majhno količino bakterij, predvsem v sporogeni obliki. Po drugi strani pa je bilo 11–12 % kulturanegativnih/rtPCR-pozitivnih vzorcev. V tem primeru bi težko trditi, da gre za lažno pozitivne vzorce z metodo rtPCR, saj smo vsak vzorec testirali na tri gene z dvema metodama rtPCR. Poleg tega so se rezultati rtPCR ujemali z dokazanim toksinotipom na vzorčeni farmi. 45 % kulturanegativnih/rtPCR-pozitivnih vzorcev je bilo testiranih tudi s testoma PCRFast in BrtPCR in 89 % od teh vzorcev je bilo pozitivnih tudi z vsaj enim od teh dveh testov. Neskladnosti bi lahko razložili s tem, da sta TMrtPCR in LC rtPCR zmožna zaznati nizke koncentracije tarčne DNA v vzorcih z malo bakterij, ki pa jih je premalo, da bi se lahko dovolj namnožile v obogatitvenem gojišču in bi bila kultura pozitivna, ali pa da smo izolirali DNA iz propadlih bakterij, ki niso preživele vzorčenja in

transporta v laboratorij. Treba je poudariti, da metoda izolacije *C. difficile* na gojiščih ni standardizirana metoda in lahko kljub predhodnemu alkoholnemu šoku druge prisotne anaerobne sporogene bakterije prerastejo *C. difficile* na gojišču. Rezultati naše študije se ujemajo z ugotovitvami študije, ki opisuje metodo rtPCR za neposredno dokazovanje *C. difficile* v blatu telet, kjer poročajo o 7 % kulturapozitivnih/rtPCR-negativnih in 11 % kulturanegativnih/rtPCR-pozitivnih vzorcih (Houser in sod., 2010). Prav zaradi kulturanegativnih/rtPCR-pozitivnih vzorcev, ki jih ne moremo upoštevati kot lažne rtPCR-pozitivne vzorce, izračun občutljivosti, specifičnosti, PNV in NNV v našem primeru ni smiseln.

Pri dokazovanju *C. difficile* v vzorcih živali je pomembno zaznavanje variantnih tipov. Obstoječi protokoli rtPCR ne zaznajo vseh variantnih tipov *C. difficile* oziroma niso validirani z različnimi toksinotipi, ki so pogosti pri živalih in jih vedno pogosteje najdemo tudi pri ljudeh. Z obema novima metodama smo zaznali vse testirane toksinotipe, medtem ko komercialni test PCRFast in BrtPCR nista zaznala enega oziroma dveh testiranih toksinotipov. Toksinotip XIa (A–B–CDT+) je bil z obema testoma negativen, ker ne zaznata gena za binarni toksin. Rezultat TMrtPCR in LC rtPCR nam lahko poda že prvo informacijo o tipu *C. difficile* v vzorcu, vendar ni tako zanesljiv kot toksinotipizacija, saj smo z obema novima metodama rtPCR zaznali tudi nefunkcionalen gen *tcdA* pri toksinotipu VII in XIa. Prednost TMrtPCR in LC rtPCR je tudi, da lahko ločeno dokazujemo vse tri toksinske gene, medtem ko so začetni oligonukleotidi in sonde za *tcdA* in *tcdB* pri testu PCRFast zmešane skupaj, tako da ne moremo ločiti sevov A+B+ in A–B+.

Glede na naše rezultate in podatke iz literature neposredno dokazovanje *C. difficile* v blatu živali izključno z uporabo rtPCR ni primerno, zaradi lažno negativnih rezultatov z metodo rtPCR. V nadaljevanju študije smo zato izboljšali postopek tako, da smo pred pomnoževanjem z rtPCR vključili obogatitev v selektivnem bujonu in s tem povečali količino *C. difficile* v vzorcu. Prvi del raziskav nam je dal rezultate o razširjenosti *C. difficile* pri različnih živalih v Sloveniji in vpogled o prisotnosti različnih genotipov. Ker smo želeli testirati metodo rtPCR, s katero lahko zaznamo variantne seve z binarnim toksinom, smo za nadaljnje raziskave o izboljšavi postopkov vzorčili sesne pujske, ki so v velikem številu pozitivni na *C. difficile*, na farmi s prisotnim genotipom V/045 (A+B+CDT+).

V literaturi sta opisani dve študiji, ki sta vpeljali obogatitev (5–7 oziroma 3 dni) pred izolacijo DNA in pomnoževanjem z rtPCR. Houser in sod. (2010) so testirali 71 vzorcev blata telet in 7 % vzorcev je bilo rtPCR-pozitivnih samo po obogatitvi, medtem ko Curry in sod. (2011) niso primerjali obogatitve pred rtPCR z neposrednim dokazovanjem z rtPCR. V naši raziskavi smo čas predobogatitve optimizirali, tako da smo zmanjšali celoten čas obogatitvene kulture (devet dni) na minimum. Vzorce smo inkubirali v obogatitvenem gojišču en, dva, tri in sedem dni, nato izolirali DNA in jo pomnoževali z LC rtPCR. Vzorce smo obdelali na dva načina pred inokulacijo obogatitvenega gojišča – s predhodnim alkoholnim šokom in brez. V vzorcih brez alkoholnega šoka so ostale žive vegetativne celice in spore, zato smo predvidevali, da bo začetno število bakterij *C. difficile* višje in se bo še bolj povišalo med obogatitvijo, kar se bo odražalo v več kulturapozitivnih vzorcih po enem in dveh dnevih inkubacije. Po drugi strani pa smo v vzorcih z alkoholnim šokom zmanjšali ostalo mikrobno floro in s tem omogočili lažje dokazovanje kolonij *C. difficile* na gojišču. Slednje se je v naši raziskavi pokazalo kot pomembnejše od začetne koncentracije *C. difficile* v vzorcu. Vseeno pa prisotnost živih vegetativnih celic in spor v vzorcu brez alkoholnega šoka vpliva na višje število kulturanegativnih/LC rtPCR-pozitivnih vzorcev v primerjavi z vzorci z alkoholnim šokom, saj se med obogatitvijo namnožijo vegetativne celice in spore ter s tem povečajo koncentracijo *C. difficile*/DNA v vzorcu.

Primerjava rezultatov obogatitvene kulture in LC rtPCR za vzorce z alkoholnim šokom in brez njega po različnih dneh inkubacije je pokazala, da v splošnem vsak dodatni dan inkubacije poveča število tako kultura- kot rtPCR-pozitivnih vzorcev. Med različnimi dnevi inkubacije je število pozitivnih in negativnih vzorcev rahlo padalo in naraščalo, kar bi lahko pojasnili z naslednjimi razlogi: i) rektalni brisi vsebujejo malo blata, poleg tega se koncentracija bakterij zmanjša v pripravljeni suspenziji in imamo tako v vzorcu malo *C. difficile*, ki je pod mejo zaznave metode rtPCR; ii) različna koncentracija bakterij in razmerje med vegetativnimi celicami in sporami v inokulumu (pripravljeni suspenziji) posamezne epruvete; iii) slabo razmnoževanje *C. difficile* med obogatitvijo zaradi morebitne prisotnosti metabolitov, ki jih proizvajajo druge bakterije v vzorcu; iiii) sporulacija *C. difficile* v bujonu in s tem neprimerna izolacija DNA iz spor. Kljub tem razlikam po različnih dnevih inkubacije lahko opazimo, da že en dan predobogatitve občutno poveča število kultura- in LC rtPCR-pozitivnih vzorcev. Število rtPCR-pozitivnih vzorcev pa se po dvo- in tridnevni inkubaciji ne poveča, medtem ko je za kulturo pomemben vsak dodatni dan. Rezultati kažejo,

da je enodnevna predobogatitev v bujonu, ki ji sledi dokazovanje vseh treh toksinskih genov *C. diffiicle* z metodo rtPCR, lahko samostojna metoda, saj ni bilo kulturapozitivnih/LC rtPCR-negativnih vzorcev – t. i. lažno negativnih vzorcev z metodo rtPCR.

DNA iz obogatitvene kulture smo izolirali na dva načina: s komercialnim kompletom foodproof Sample Preparation Kit I (BIOTECON Diagnostics) in s hitro metodo izolacije s spiranjem, segrevanjem in centrifugiranjem. Rezultati so pokazali, da je primernejša uporaba komercialnega kompleta, vendar je potrebna previdnost pri ovrednotenju rezultatov pri vzorcih, kjer je pozitiven samo en gen. Velika pomanjkljivost uporabljenega komercialnega kompleta je tudi visoka cena, zato bi bilo smiselno testirati še cenejše komercialne kite drugih proizvajalcev.

Kljub uporabnosti predlaganega postopka predobogatitve v kombinaciji z metodo rtPCR je za testiranje odpornosti proti antibiotikom in genotipizacijo še vedno treba pridobiti izolat *C. difficile* na gojiščih. Rezultati naše raziskave kažejo, da je za ta namen najbolj optimalna obogatitvena kultura po sedmih dneh inkubacije s predhodnim alkoholnim šokom, ki ji sledi *in vitro* dokazovanje toksina z EIA. Proizvajanje toksinov smo dokazovali z EIA v dveh različnih gojiščih in rezultati so se razlikovali. Ker je uporabljeni pozitivni kontrolni sev proizvajal toksine v obeh gojiščih, sklepamo da morebitni pomanjkljivi anaerobni pogoji in samo gojišče niso bili razlog za razlike. Le-te pa bi lahko bile povezane z različno rastjo in/ali proizvajanjem toksinov specifičnega genotipa *C. difficile* v določenem gojišču. Naši izolati so pripadali toksinotipu V, medtem ko je bila pozitivna kontrola referenčni sev toksinotipa 0.

Uporaba opisanega postopka rtPCR s predobogatitvijo je priporočljiva predvsem za vzorce z majhnim številom *C. difficile* (npr. živali brez kliničnih znakov). S tem postopkom bi lahko dobili tudi nove pomembne informacije o prisotnosti *C. difficile* v hrani, saj za te vzorce ni standardnega postopka in je tako primerjava rezultatov različnih študij omejena (Rupnik in Songer, 2010). Tudi okolje (zemlja, voda, krma, površine idr.) je lahko pomemben vir klostridijskih spor. Posebno vlogo pri tem ima bolnišnično okolje in domovi za starejše. Clabots in sod. (1992) so ugotovili, da so asimptomatski prenašalci *C. difficile* vir te bakterije pri 84 % v bolnišnici pridobljenih okužb. Prenašalci pa pogosto ostanejo neodkriti oziroma so rezultati negativni s konvencionalnimi metodami za dokazovanje *C. difficile*. Bolniki brez simptomov (driske) v bolnišnicah niso izolirani niti zdravljeni in tako predstavljajo vir okužb (neomejeno širijo spore po bolnišnici). Izboljšan postopek, opisan v tem doktorskem delu, bi

bil tako lahko uporaben za odkrivanje človeških rezervoarjev *C. difficile* v bolnišničnem okolju. Kljub temu bi bilo treba narediti dodatne validacije na človeških vzorcih, saj ni podatkov, ali so testi, validirani na živalskih vzorcih, primerni tudi za človeške vzorce. Običajno je v okolju odvzetih več vzorcev naenkrat in že en pozitiven vzorec po enodnevni predobogatitvi bi lahko pomenil, da je treba uvesti ukrepe za preprečevanje širjenja spor v bolnišnici.

V naši raziskavi smo razvili metodi, s katerima dokazujemo vse tri toksinske gene, saj menimo, da je to nujno, ker so pred kratkim poročali o prvem primeru seva s toksinom A in brez toksina B (A+B–), kar je do sedaj veljalo le kot hipotetična možnost, poleg tega pa postajajo sevi z binarnim toksinom klinično pomembni in se jih povezuje z doma pridobljeno okužbo (McEllistrem in sod., 2005; Limbago in sod., 2009; Lemire in sod., 2012). Metodi TMrtPCR in LC rtPCR bi se lahko v kombinaciji z enodnevno predobogatitvijo v bujonu uporabljali kot samostojna metoda, ker je postopek hiter, zanesljiv in nam poda prvo informacijo o toksinskem tipu.

Čeprav smo uspeli izboljšati postopek dokazovanja *C. difficile*, bi bilo v bodoče smiselno raziskave usmeriti v iskanje optimalne izolacije DNA iz spor in s tem izboljšati neposredno dokazovanje *C. difficile* v blatu. Večina komercialnih kompletov in sistemov za izolacijo DNA temelji na encimski in kemični lizi bakterij, s katerima težko liziramo odporne spore in je tako izolacija DNA iz spor omejena. Mehansko razbitje s kroglicami je najučinkovitejša tehnika za izolacijo DNA v vzorcih zemlje in iz spor *Bacillus* sp. (Kuske in sod., 1998; Miller in sod., 1999; Dauphin in sod., 2009). Freifeld in sod. (2012) so razvili mikroreaktor za izolacijo DNA, ki ji sledi pomnoževanje DNA *C. difficile* s PCR. Liza v mikroreaktorju združuje segrevanje, kemično in fizično razbitje celic, vendar aparatura še ni na tržišču. Menimo, da bi kombinacija mehanskega razbitja s steklenimi ali cirkonij/silicijevimi kroglicami, encimske in kemične lize izboljšala izplen izolirane DNA iz spor in bakterij z odporno celično steno in s tem izboljšala dokazovanje *C. difficile* z molekularnimi metodami (Miller in sod., 1999; Kim in sod., 2012).

5 ZAKLJUČKI

- Razvili smo dve novi metodi rtPCR: TMrtPCR in LC rtPCR za dokazovanje variantnih tipov *C. difficile* neposredno v blatu živali in ju validirali na živalskih vzorcih. LC rtPCR je prvi rtPCR za dokazovanje vseh treh toksinskih genov *C. difficile*, ki uporablja dve hibridizacijski sondi. Rezultati obeh novih metod rtPCR so se ujemali v 97,7 %, kar kaže na podobno občutljivost in specifičnost obeh metod in hkrati enakovredno uporabnost obeh za neposredno dokazovanje *C. difficile*. Poleg tega imata oba testa rtPCR vključeno interno kontrolo za ugotavljanje prisotnosti inhibitorjev DNA-polimeraze v dupleks-PCR, vendar se je pri LC rtPCR meja zaznave na ta račun znižala.

- Izključna uporaba rtPCR za neposredno dokazovanje *C. difficile* v blatu ni dovolj dobra zaradi lažno negativnih rezultatov v primerjavi z obogatitveno kulturo (11–12 %), zato smo opisali najbolj optimalno kombinacijo klasične gojiščne in molekularne preiskave glede na zanesljivost in hitrost. Enodnevna inkubacija vzorca v obogatitvenem bujonu, ki ji sledi dokazovanje *C. difficile* s pomnoževanjem DNA z metodo rtPCR, se lahko uporablja kot samostojna metoda, saj ni lažno negativnih rezultatov z metodo rtPCR. Vseeno pa ne smemo zanemariti pomena izolacije bakterije, saj z njo pridobimo izolat *C. difficile* za nadaljnje tipizacije. Najbolj optimalna je sedemdnevna inkubacija vzorca, predhodno obdelanega z alkoholnim šokom, v obogatitvenem gojišču, ki ji sledi dvodnevna inkubacija na selektivnem gojišču.

- Pri razvoju novih testov za dokazovanje *C. difficile* pri živalih je nujna validacija na živalskih vzorcih, saj so testi rtPCR, validirani na humanih vzorcih, oziroma komercialno dostopni kompleti rtPCR za dokazovanje *C. difficile* le pogojno primerni za uporabo na živalskih vzorcih.

- Za dokazovanje *C. difficile* tako pri ljudeh kot pri živalih je priporočljiva uporaba testov rtPCR, s katerimi lahko zaznamo vse tri toksinske gene, saj se pojavljajo sevi z različnimi kombinacijami genskih zapisov za toksine oziroma toksinotipi.

6 POVZETEK

Bakterija *Clostridium difficile* (*C. difficile*) je pomemben povzročitelj v bolnišnici pridobljenih okužb pri ljudeh, vendar poročajo tudi o povečanem številu doma pridobljenih okužb pri mladih ljudeh, ki niso prejemali antibiotične terapije ali bili v bolnišnici. Pri različnih živalskih vrstah je lahko *C. difficile* prisoten kot komenzal ali pa povzroča drisko in enterokolitis predvsem v neonatalnem obdobju. Kolonizacija s *C. difficile* s starostjo živali pada. Enake seve *C. difficile* so dokazali pri ljudeh, živalih, v hrani in okolju, kar nakazuje na možen zoonotski potencial bakterije. Predvsem zdrave živali in okolje pa bi lahko bili rezervoar *C. difficile* v primerih doma pridobljenih okužb.

Diagnostika pri ljudeh temelji na kombinaciji različnih metod (toksigena kultura, test citotoksičnosti, encimskoimunski testi). Dostopne metode so hitre in manj občutljive oziroma specifične ali pa občutljive in specifične, vendar dolgotrajne, zato se uveljavljajo molekularne metode. Uporabnost komercialnih testov za dokazovanje toksinov ali DNA, namenjenih za diagnostiko pri ljudeh, je v veterini omejena, saj se rezultati slabo ujemajo z metodo izolacije. Do sedaj je bil razvit le en PCR v realnem času (rtPCR) za neposredno dokazovanje *C. difficile* pri živalih.

Namen našega dela je bil izboljšati postopek dokazovanja *C. difficile* pri živalih. Želeli smo najti najučinkovitejšo kombinacijo klasične gojiščne in molekularne preiskave, tako da bi dosegli zadovoljivo občutljivost in specifičnost metode in dokazali *C. difficile* pri živalih hitreje, kot to omogočajo dostopne metode. V prvem delu študije smo z metodo izolacije bakterije v obogatitvenem gojišču pregledali več živali z drisko in zdravih živali na različnih farmah ter ugotovili, da je prevalenca največja pri sesnih pujskih, ki so poleg tega edini, katerih sevi imajo zapis za binarni toksin. Za hitrejše dokazovanje *C. difficile* smo v nadaljevanju razvili dva nova testa rtPCR za dokazovanje vseh treh toksinskih genov (*tcdA*, *tcdB*, *cdtB*), enega s hidrolizirajočo sondo TaqMan (TMrtPCR) in drugega s hibridizacijskima sondama na aparaturi LightCycler (LC rtPCR). Rezultate obeh smo primerjali med seboj in z obogatitveno kulturo. Istočasno pa smo testirali še dva že obstoječa testa rtPCR (BrtPCR, PCRFast) ter rezultate primerjali z rezultati TMrtPCR, LC rtPCR in kulture.

Rezultati TMrtPCR in LC rtPCR so se ujemali v 97,7 % vzorcev, medtem ko je bilo ujemanje s kulturo 75 %. Najboljše ujemanje z rezultati kulture je bilo v primeru TMrtPCR oziroma

LC rtPCR, slabše z BrtPCR in najslabše s PCRFast, kar potrjuje nujnost validacije testov na živalskih vzorcih. Poleg tega smo samo s TMrtPCR in LC rtPCR zaznali vse testirane variantne seve *C. difficile*. Meja zaznave je bila nižja za TMrtPCR kot za LC rtPCR, vendar preverjanje na realnih vzorcih tega ni pokazala. Ocenjujemo, da je uporaba TMrtPCR in LC rtPCR enakovredna. RtPCR za neposredno dokazovanje *C. difficile* v blatu živali ni uporaben kot samostojna metoda, saj je bilo 11–12 % vzorcev kulturapozitivnih/rtPCR-negativnih, zato smo v nadaljevanju študije izboljšali postopek za dokazovanje *C. difficile* v vzorcih z nizkim številom *C. difficile* (subklinične živali). Vpeljali smo obogatitev v selektivnem gojišču pred pomnoževanjem DNA z metodo rtPCR z namenom povečati število *C. difficile*. DNA smo izolirali iz bujona po enem, dveh, treh in sedmih dneh inkubacije in jo pomnoževali z LC rtPCR. Čas obogatitve smo optimizirali, tako da smo skrajšali čas, potreben za izvedbo celotne obogatitvene kulture, na minimum. Brez predhodne obogatitve je bil samo en (2,5 %) vzorec kultura- in rtPCR-pozitiven, medtem ko je bilo na koncu (po sedmih dneh obogatitve v bujonu) 33 (82,5 %) vzorcev kulturapozitivnih. Že enodnevna inkubacija v bujonu pred rtPCR je izrazito povečala število kultura- (37,5 %) in rtPCR-pozitivnih vzorcev (70 %).

Opisani postopek z enodnevno obogatitvijo, ki ji sledi rtPCR za dokazovanje vseh treh toksinskih genov, je zanesljiv in hiter presejalni test, tudi za vzorce z nizkim številom *C. difficile*, saj ni bilo kulturapozitivnih/LC rtPCR-negativnih vzorcev. Vseeno ne smemo pozabiti na pomen izolacije bakterije, saj samo na tak način pridobimo izolat za nadaljnje tipizacije. Ugotovili smo, da je najbolj optimalna sedemdnevna inkubacija vzorca, predhodno obdelanega z alkoholnim šokom, v obogatitvenem gojišču, ki ji sledi izolacija *C. difficile* na selektivnem gojišču.

7 SUMMARY

Clostridium difficile (*C. difficile*) is known as an important cause of hospital-acquired infections in humans. Recent reports have indicated an increased frequency of community-acquired infections in young people without antibiotic therapy and previous hospitalisation. *C. difficile* infections (diarrhoea and enterocolitis, especially in neonatal animals) or subclinical carriage of *C. difficile* has been described for numerous animals. Colonisation rates decreased with animal age. The overlap of human, animal, food, and environment *C. difficile* strains suggests the zoonotic potential of *C. difficile*. Furthermore, especially subclinical animals and the environment could be the reservoir of *C. difficile* in case of community-acquired infections.

The diagnosis of a disease in humans is usually based on a combination of different methods (toxigenic culture, cytotoxicity assay, enzyme immunoassays), all of which are either labour intensive and time-consuming or lack sensitivity and specificity, therefore molecular methods are becoming more and more important. The use of the commercial test for toxin and DNA detection intended for human diagnostic is limited for the testing of animal samples, as the test results often poorly correlate with culture results. Up to date, only one study dealing with the development of real-time PCR (rtPCR) for direct detection of *C. difficile* in animal samples has been published.

The aim of our study was to improve the procedure for the detection of *C. difficile* in animals. We want to find a reliable combination of bacteriological cultivation and a molecular method with satisfying sensitivity and specificity, capable of faster detection of *C. difficile* than the existing methods. In the first part of our study we screened different diarrhoeic and subclinical animals on different farms using the enrichment culture method. Results showed that the highest prevalence is among piglets and only strains from piglets possess binary toxin genes. Furthermore, for faster detection of *C. difficile* we developed two rtPCR assays targeting the *tcdA*, *tcdB*, and *cdtB* toxin genes, the first using TaqMan hydrolysis probe (TMrtPCR) and the second using hybridisation probes on LightCycler (LC rtPCR). We compared both methods to each other, to culture and to two previously existing rtPCR assays (BrtPCR, PCRFast).

The results of the comparison of LC rtPCR with TMrtPCR showed complete concordance in 97.7 % of the samples, while the correlation with the culture was in 75 % of the samples. The

best correlation with culture results was observed with TMrtPCR and LC rtPCR in comparison with BrtPCR and PCRFast, which confirms the importance of test validation on animal samples. Furthermore, only TMrtPCR and LC rtPCR detected all tested variant *C. difficile* strains. The limit of detection was lower for TMrtPCR than for LC rtPCR, but it was not proven on field animal samples, hence both rtPCR assays can be used as equivalent methods. Real-time PCR assays for direct detection of *C. difficile* in animal faecal samples are not adequate as a stand-alone test due to 11-12 % of culture positive/rtPCR negative samples. Therefore, we improved the procedure for the detection of *C. difficile* in the samples with a low number of bacteria (e.g. sublinical animals). A pre-enrichment step was added prior to rtPCR with the purpose to increase the number of *C. difficile*. DNA was extracted from broth culture after one, two, three, and seven days of incubation, followed by LC rtPCR amplification. The enrichment time was optimised to reduce the necessary time for the complete enrichment culture to a minimum. Only one (2.5 %) sample was culture and rtPCR positive without enrichment, while 33 (82.5 %) samples were culture positive after seven days of enrichment. One day of enrichment already evidently increased the number of culture (37.5 %) and rtPCR (70 %) positive samples.

The described procedure with one day enrichment followed by the rtPCR targeting of all three toxin genes is accurate and a rapid screening test also for samples with a low number of *C. difficile*, as no culture positive/rtPCR negative samples were observed. However, the importance of microorganism cultivation should not be underestimated, it being the only way to obtain the isolate for further typing. The optimal cultivation method was broth enrichment for seven days with a previous alcohol shock, followed by the isolation of *C. difficile* on selective agar.

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10 PRILOGE

Priloga 1: Objavljeni izvirni znanstveni članki

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Veterinary anaerobes and diseases

Diversity of *Clostridium difficile* in pigs and other animals in Slovenia

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ABSTRACT

A study of *Clostridium difficile* diversity in pigs, calves and horses in Slovenia was conducted. A total of 547 samples were collected and *C. difficile* was isolated from 247/485 (50.9%) piglet samples, from 4/42 (9.5%) calf samples, and 1/20 (5%) horse samples. The isolates were characterized by toxinotyping, PCR-ribotyping, and pulsed-field gel electrophoresis (PFGE) using restriction endonuclease *Sma*I. Piglet isolates belonged to two toxinotypes (V and 0), four PCR-ribotypes (066, 029, SI 011, SI 010), and six pulsotypes. Bovine isolates were grouped into two toxinotypes (XIa and 0), three PCR-ribotypes (077, 002, 033), and three pulsotypes. The only equine isolate was indistinguishable from one calf isolate (XIa/033) in toxinotype, PCR-ribotype, and pulsotype. None of detected genotypes was present in all three animal hosts.

1. Introduction

Clostridium difficile is an anaerobic Gram-positive, spore-forming environmental bacillus that has been known as an important bacterial pathogen of humans. Toxigenic strains of *C. difficile* are well-recognized nosocomial pathogens responsible for antibiotic-associated diarrhea and pseudomembraneous colitis [1]. In the past years, it has also emerged as an animal pathogen or commensal, perhaps most prominently in pigs and cattle [2–6], and horses [7–10]. Recently reported overlap between isolates from animals, retail meat and human isolates suggests that animals may be a reservoir and *C. difficile* infection a zoonosis [4,6,11–16]. Typing of the microorganisms can be useful for strain characterization and epidemiological investigations. Different molecular approaches have been used for typing *C. difficile*, although PCR-ribotyping, pulsed-field gel electrophoresis (PFGE), and toxinotyping are most widely used for animal isolates [4–6,10,11,15–19].

The objective of our work was to evaluate the diversity of *C. difficile* isolated mostly from pigs and some other farm animals *i.e.* calves and horses in Slovenia, using three different typing methods – toxinotyping, PCR-ribotyping, and PFGE. Pirs et al., 2008 [18] have reported the isolation of previously unreported animal *C. difficile* toxinotype V/ribotype 066 from piglets from two Slovenian farms. In this study we document the presence of *C. difficile* in pigs from additional Slovenian piggeries, and in other farm animals.

2. Materials and methods

2.1. Animals and sampling

A total of 547 samples from different farm animals were collected. From the piglets (< ten days old) 485 rectal swabs were obtained. The samples were taken from five piglets per litter. Of 94 litters sampled 77.7% were with at least one diarrheic animal, while 22.3% litters were with animals without diarrhea. Fifteen samples were from individual animals. Piglets were from five different large farms (A to E, >1000 total animal number) and from four different small farms (F to I, <500 total animal number) (Table 1). Seven piglet samples were of unknown origin.

Fecal samples from 42 calves (<12 weeks old) from 12 different farms and 20 samples from horses (14 stool samples from adult horses and 6 rectal swabs from foals up to one month of age) were obtained. Most of the investigated calves (76.1%) had diarrhea, while 92.9% adult horses and 50% foals were non-diarrheic.

2.2. Bacteriological cultivation and identification

The pig samples were inoculated directly onto pre-reduced plates of standard selective medium with cycloserine and cefoxitin

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Table 1
Recovery and strain characterization of *Clostridium difficile* isolates from farm animals in Slovenia.

Animal	Farm	No. of tested samples	Culture positive	Toxinotype/PCR-ribotype	Toxin genes	PFGE types
Pig ($n = 485$)	A	150	56 (37.3%)	V/066	A + B + CDT+	VF1a, VF1b, VF4, VF7
	B	53	36 (67.9%)	V/066	A + B + CDT+	VF1, VF1a, VF1b
	C	94	71 (75.6%)	V/066	A + B + CDT+	VF1, VF1a, VF1b, VF1c
	D	106	46 (43.4%)	0/SI 011	A + B + CDT−	VF2a, VF2b
	E	35	12 (34.3%)	0/SI 011	A + B + CDT−	VF2c, VF2d
	F	10	7 (70%)	0/029	A + B + CDT−	VF6a, VF6b
	G	10	1 (10%)	0/SI 011	A + B + CDT−	VF2a
	H	10	8 (80%)	0/SI 011	A + B + CDT−	VF2a
	I	10	7 (70%)	0/SI 011	A + B + CDT−	VF2a
	Unknown	7	3 (42.9%)	V/066 (2 isolates)	A + B + CDT+	VF1a
				tox-[a] /SI 010 (1 isolate)	A − B − CDT−	VF8
Calf		42	4 (9.5%)	0/002	A + B + CDT−	VF9
				0/077 (2 isolates)	A + B + CDT−	VF3, VF3a
				XIa/033	A − B − CDT+	VF5
Horse		20	1 (5%)	XIa/ 033	A − B − CDT+	VF5

[a] tox–: non-toxigenic strain

within 4 h after sampling. The samples were mixed with approximately equal amount of ethanol and left at room temperature for half an hour and then inoculated onto standard selective medium with cycloserine and cefoxitin (*C. difficile* agar base and *C. difficile* selective supplement; Oxoid, UK). Inoculated selective agar plates were incubated in anaerobic jars at 37 °C for two days. The calf and horse samples were inoculated in cyloserine-cefoxitin fructose enrichment broth (Oxoid, UK) supplemented with 0.1% sodium taurocholate (Sigma-Aldrich, USA) and processed as described before [20].

The isolates were identified on the basis of morphological criteria, typical odor, and confirmed by amplification of *C. difficile*-specific gene *cdd3* using the primer pair Tim6/Struppi6 [21] or by multiplex PCR targeting *tpi*, *tcdA*, and *tcdB* [22].

2.3. Toxinotyping, binary toxin gene detection, and PCR-ribotyping

All isolates were characterized by toxinotyping which involved amplification and enzymatic restriction of PCR fragment A3 of *tcdA* and PCR fragment B1 of *tcdB* [23]. Binary toxin gene (*cdtB*) was detected as previously described [24]. Thirty six randomly selected piglet isolates from farms A, B, and C, 15 from farms D and E, and 16 from farms F, G, H, and I were PCR-ribotyped. Additionally, three isolates from piglets of unknown origin and all isolates from calves and foal were PCR-ribotyped. PCR-ribotyping was preformed as described previously [25]. PCR-ribotypes for which the reference strains were available were designated by standard Cardiff nomenclature, while others were designated by internal nomenclature (SI and number).

2.4. Pulsed-field gel electrophoresis

A total of 131 isolates were subjected to PFGE analysis using restriction endonuclease *Sma*I: 125 randomly selected isolates from piglets (farm A: 25, farm B: 20, farm C: 28, farm D: 25, farm E: 9, farm F: 2, farm G: 1, farm H: 8, farm I: 5, and three isolates from piglets of unknown origin) and six isolates from calves and foal. Bacterial cultures grown on sheep blood agar were inoculated into brain heart infusion (BHI) broth (Oxoid, UK) and incubated anaerobically at 37 °C for 24 h. Cultures were grown to an optical density (OD_{600}) of 1.0–1.2. Cells were suspended in PIV (1 M Tris with NaCl) buffer with 25 mg/ml lysozyme (Sigma-Aldrich, USA) and incorporated into 1% SeaKem Gold agarose plugs (Cambrex, USA). Plugs were first incubated in cell lysis buffer with lysozyme for 1 h and then with 5 mg/ml proteinase K (Sigma-Aldrich, USA) overnight at 53 °C. Plugs were washed four times with Tris-EDTA buffer at 37 °C for 15 minutes with gentle agitation and stored in TE buffer at 4 °C until used. DNA in plugs was digested with *Sma*I (40 U/μl) (Roche Diagnostics, Germany) for 5 h at 25 °C. Electrophoresis was performed on a CHEF-DRII system (BioRad, USA) with the following conditions: switch time from 5 s to 55 s running for 21 h at 6 V/cm and 14 °C.

After electrophoresis agarose gels were stained with ethidium bromide (Invitrogen, USA) and DNA fragments were visualized with UV light. PFGE results were analyzed using BioNumerics software (version 5.0; Applied Maths, Belgium). Chromosomal DNA of *Salmonella* Braenderup H9812, digested with *Xba*I (Sigma, USA), was used for band normalization. Dendrograms with 0.9% position tolerance were created by the unweighted-pair group method with arithmetic mean using Dice coefficients. We considered clusters with ≥80% similarity to represent distinct PFGE types and clusters with ≥95% similarity within a type to be distinct subtypes. PFGE types were assigned based on the internal nomenclature system (VF for Veterinary Faculty and number).

3. Results

3.1. Bacteriology

A total of 547 samples were studied: 485 from piglets, 42 from calves, and 20 from horses. *C. difficile* was isolated from 247 (50.9%) piglets, 4 (9.5%) calves, and one (5%) foal. The proportion of positive samples on different pig farms ranged from 10% to 80% (Table 1). Among four positive calf samples one *C. difficile* strain was isolated from a non-diarrheic animal. None of the non-diarrheic adult horses or foals (more than 3 weeks old) was positive for *C. difficile*. Among three diarrheic foals (two days to one month of age) one was positive for *C. difficile*. It was isolated from a severely diseased foal in absence of any other searched bacterial pathogen (*Escherichia coli, Salmonella sp., Campylobacter sp.*).

3.2. Toxinotyping, binary toxin gene detection, and PCR-ribotyping

Typed strains belonged to three different toxinotypes, type 0 which is characterized by toxin A and toxin B production, but no binary toxin [A + B + CDT−], type V which also produces toxins A & B, but has binary toxin genes [A + B + CDT+], and type XIa which has a remnant of the toxin A gene, but does not produce either toxins A or B, and has binary toxin genes [A − B − CDT+]. Seven different PCR-ribotypes were identified of which five corresponded to the reference type strains of ribotypes 066, 029, 077, 002, 033. Two types could not be identified with the collection of available

PCR-ribotype strains and are designated with internal nomenclature SI 011 and SI 010.

Piglet isolates belonged to two toxinotypes (type V and type 0) and four PCR-ribotypes. One isolate was non-toxigenic, therefore, did not have a toxinotype. Calf isolates belonged to two toxinotypes (type 0 and type XIa), and three PCR-ribotypes (Table 1). The only isolate from a foal was of toxinotype XIa and PCR-ribotype 033. Piglet isolates belonging to toxinotype 0 showed different PCR-ribotypes than calf isolates of the same toxinotype.

3.3. *Pulsed-field gel electrophoresis*

Among 131 isolates a total of nine PFGE types were found, designated VF 1–9 (Table 1). Four of these types were further divided into subtypes (1a–c, 2a–d, 3a, 6a–b) (Fig. 1).

All studied pig isolates from farms A, B and C belonged to toxinotype V and PCR-ribotype 066. However, with PFGE they could be further subdivided into three types (VF 1, 4 and 7) and three subtypes (1a–c). Similarly, PFGE provided subtyping of PCR-ribotypes 0/SI 011, 0/077 and 0/029. Type 0/SI 011, found in pig isolates from farms D and E was subdivided into four PFGE subtypes (2a–d). Type 0/077 (calf isolates of unknown origin) comprised one subtype of PFGE type VF 3 while type 0/029 was subdivided into two PFGE subtypes (6a and 6b).

4. Discussion

C. difficile has been isolated from a variety of diarrheic or non-diarrheic farm animals, *e.g.* pigs and cattle [2–6], horses [7–10], and poultry [19,26]. In our work *C. difficile* has been isolated from neonatal pigs, calves, and a foal. Although *C. difficile* gained importance as a causing agent of neonatal diarrhea in pigs [2,3], the recent study showed that *C. difficile* prevalence in non-diarrheic piglets wasn't significantly different than in diarrheic piglets [27], which indicates the high proportion of carriers without clinical signs. In our study, the sampling plan was optimized to isolate high number of strains, therefore no farms without diarrheic pigs were included. However, large proportion of piglets was non-diarrheic at the time of sampling although they belonged to litters with present diarrhea. Our data suggest that piglets are colonized in different proportions regardless of the presence of disease.

As shown in other studies [15,28], PFGE revealed greater diversity among isolates than toxinotyping and PCR-ribotyping. To the best of our knowledge, the only previously published PFGE typing of animal strains was reported by Jhung et al., 2008 [15]; this study revealed a variety of PFGE types among toxinotype V/PCR-ribotype 078 isolates from pigs and cattle.

Some of five internationally comparable ribotypes found during this survey (066, 029, 077, 002, 033) were previously reported in animals; 002 in bovine, equine and swine isolates [6], 033 in bovine and swine isolates [6,4], 077 in dog [6] and bovine isolates [4]. PCR-ribotype 077 was also found in ground beef [16]. Both prevalent *C. difficile* ribotypes (066 and SI 011) are different from the PCR-ribotype 078 reported in piglets elsewhere [6,14].

All piglet isolates from large farms (A–E) belonged to two toxinotypes and two PCR-ribotypes (toxinotype V/PCR-ribotype 066 and toxinotype 0 and PCR-ribotype SI 011, respectively), which indicates low variability of strains on the farms. The same distribution of *C. difficile* type V/066 among pigs in two connected Slovenian farms was reported previously [18]. Farms A, B, and C are of the same owner and there is occasional exchange of animals between these farms going on, which could be the reason for the presence of the same type of *C. difficile* isolates. However, PFGE revealed three different types of C. difficile type V/066 on farm A, B and C. One of them (VF1) was further divided into subtypes, which were distributed on all three farms (1a–1b) or were farm-specific (1c). It appears that the same type can persist on a given farm for a longer time and differentiate to different subtypes, similarly as reported for human strains within hospitals [18,29]. However, the occurrence of isolates with different PFGE types on the same farm was also noticed, *e.g.* in case of three isolates of types VF 4 and VF 7 which expressed low similarity with more commonly identified subtypes on farm A. This suggests different origin (*e.g.* environment) of *C. difficile* on this farm.

The farms D–I are all located at least 100 km away from farms A–C and isolates from these farms belonged to different types (toxinotype 0/PCR-ribotype SI 011 or 029). However, no connection was established for these farms in terms of animal, food, and

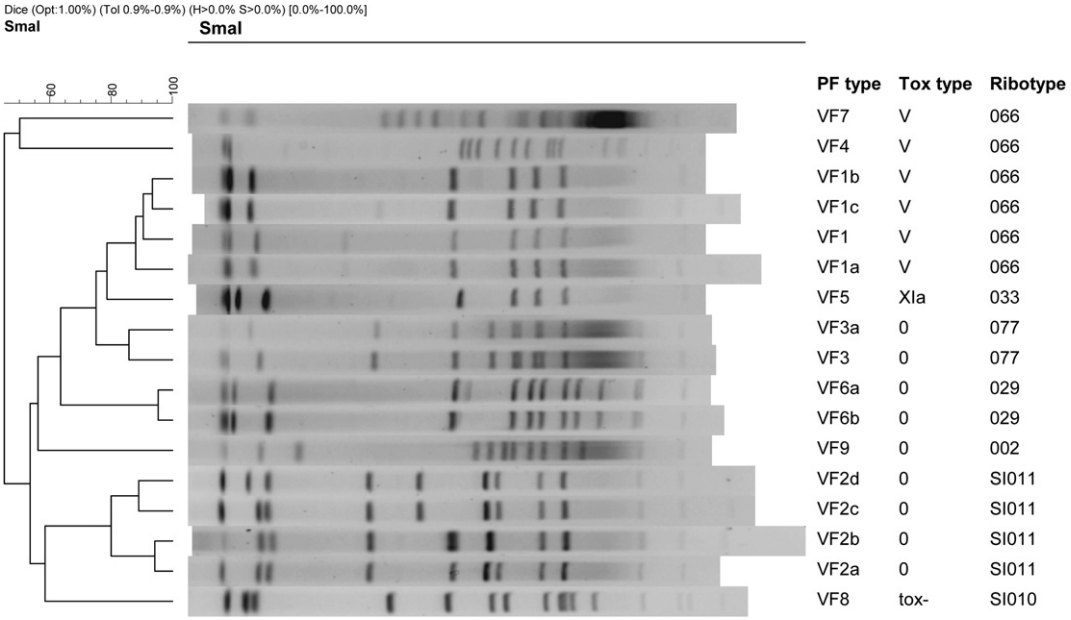

Fig 1. PFGE types of *C. difficile* isolates from pigs, calves and horse obtained after *Sma*I macrorestriction. PF type – pulsed-field gel electrophoresis type, Toxtype – toxinotype.

personnel exchange, which suggests the presence of certain genotypes in different geographical regions.

C. difficile was less frequently present in tested cattle farms, but the results suggest greater diversity (three different PCR-ribotypes among four isolates). The only equine *C. difficile* isolate was A − B − CDT+ and was isolated from a diarrheic foal of one week of age. In case of two isolates belonging to toxinotype XIa and PCR-ribotype 033, PFGE did not provide further subtyping as the isolates shared a common PFGE type (VF 5), although the isolates were obtained from two different animals (calf and foal) of different origin.

In conclusion, this study confirms the presence of *C. difficile* on large and small farms in Slovenia. The diversity among the isolates with the same PCR-ribotype was observed. One of the first extensive PFGE typing of animal strains has been performed and the results showed similarity of strains inside one farm. These findings suggest that strains could spread among animals on farm (horizontal and vertical transmission). On the other hand, similar strains were found also on different farm without known contacts, which indicates prevalence of certain genotypes in different geographic regions.

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Detection of *Clostridium difficile* in animals: comparison of real-time PCR assays with the culture method

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Clostridium difficile has emerged as a pathogen or commensal in food animals. There is overlap between isolates from animals, retail meats and humans, suggesting that animals may be a *C. difficile* reservoir. For direct detection of variant *C. difficile* strains in faecal samples of symptomatic and asymptomatic animals, we developed and validated a new TaqMan real-time PCR (TMrtPCR) assay targeting the *tcdA*, *tcdB* and *cdtB* genes. We compared it with the enrichment culture method and with two real-time PCR (rtPCR) assays, BrtPCR and PCRFast, targeting *tcdB* and *tcdA*/*tcdB*, respectively. All ten tested *C. difficile* toxinotypes, except one (XIa) with PCRFast and two (X, XIa) with BrtPCR, were detected with the test assays. A total of 340 (100 %) samples were cultured and amplified with TMrtPCR. Results correlated in 75.3 % samples. Forty (11.8 %) samples were culture positive/TMrtPCR negative, possibly because of the low numbers of bacteria in the samples or because of DNA extraction failure. Forty (11.8 %) samples were TMrtPCR positive/culture negative. Among 79 samples included in the rtPCR assays/culture comparison, 50.6 % were in complete concordance. The results showed that TMrtPCR performed better than BrtPCR and PCRFast, and 67 % of the culture-positive samples were TMrtPCR positive in comparison to 40 % of the samples positive in BrtPCR and 7 % of the samples positive in PCRFast, respectively. Another advantage of TMrtPCR over BrtPCR and PCRFast is its ability to detect a binary toxin gene. Therefore, the TMrtPCR results can provide the first information about the toxin type present in the sample. According to the results of our study, TMrtPCR could be a preferred screening method for the rapid detection of *C. difficile* in animal faecal samples, although an enrichment culture has to be performed for the specimens with negative or inconclusive rtPCR results.

INTRODUCTION

Clostridium difficile-associated disease (CDAD) or asymptomatic carriage of toxigenic *C. difficile* has been described for numerous animals, including piglets, calves and broiler chickens (Rodriguez-Palacios *et al.*, 2006; Songer & Anderson, 2006; Hammitt *et al.*, 2008; Pirs *et al.*, 2008; Simango & Mwakurudza, 2008; Zidaric *et al.*, 2008; Alvarez-Perez *et al.*, 2009). Variant *C. difficile* strains with binary toxin CDT are often isolated from animals and these strains seem to be associated with community-acquired *C. difficile* infections (CA-CDIs) in humans (Rupnik, 2007; Jhung *et al.*, 2008; Limbago *et al.*, 2009). The increased frequency and severity of CA-CDIs has resulted in an increasing interest in understanding the relationship between animal and human strains of *C. difficile*, and consequently in diagnostic methods (Barbut *et al.*, 2005; Pituch, 2009).

C. difficile or its toxins, are usually diagnosed by a combination of cell cytotoxicity assay, specific culture followed by *in vitro* toxin detection of the isolated strains (toxigenic culture) and enzyme immunoassays (Crobach *et al.*, 2009). These methods are either labour intensive and time-consuming or lacking in sensitivity and specificity. In contrast to the situation in humans, the results of commercial toxin tests in animal faecal samples often correlate poorly with culture results (Rodriguez-Palacios *et al.*, 2006; Pirs *et al.*, 2008). Rapid real-time PCR (rtPCR) methods have been described for the detection of *C. difficile* directly from human faeces. These rtPCR protocols target either 16S rRNA (Rinttilä *et al.*, 2004; Penders *et al.*, 2005; Tonooka *et al.*, 2005) or only one gene (*tcdB*, *tcdC*) (Van den Berg *et al.*, 2006; Peterson *et al.*, 2007; Sloan *et al.*, 2008; Larson *et al.*, 2010). SYBR Green is also commonly used, even though it has its drawbacks in terms of specificity (Rinttilä *et al.*, 2004; Tonooka *et al.*, 2005;

Abbreviations: CA-CDI, community-acquired *Clostridium difficile* infection; CDAD, *Clostridium difficile*-associated disease; FAM, 6-carboxyfluorescein; LOD, limit of detection; rtPCR, real-time PCR; TMrtPCR, TaqMan real-time PCR.

Peterson *et al.*, 2007). Wroblewski *et al.* (2009) developed a TaqMan-based multiplex rtPCR targeting the genes encoding toxins A and B, and binary toxin, but the method has been validated only for human samples. Three commercially available rtPCR assays have been recently approved by the US Food and Drug Administration – BD GeneOhm Cdiff (BD Diagnostics) (Stamper *et al.*, 2009); ProGastro Cd (Prodesse) (Doing *et al.*, 2010); and Cepheid Xpert *C. difficile* (Cepheid) (Huang *et al.*, 2009). These systems require special instruments and kits, which represent additional costs for diagnostic laboratories. Furthermore, all commercial tests (enzyme immunoassays and rtPCR) have been validated only for humans with CDAD. A rapid, simple and sensitive method, capable of detecting variant strains, is required for laboratory detection of *C. difficile* in symptomatic and asymptomatic animals. To the best of our knowledge, only one study dealing with the comparison of a rtPCR with the culture method for the detection of *C. difficile* in animal samples has so far been published (Houser *et al.*, 2010).

The objectives of this study were (i) to develop and validate a new TaqMan real-time PCR (TMrtPCR) targeting genes for toxins A and B, and binary toxin, for direct detection of *C. difficile* in animal faecal samples; (ii) to compare it with a commercial PCRFast *Clostridium difficile* A/B test (PCRFast) (ifp Institut für Produktqualitet) and with rtPCR targeting *tcdB* (BrtPCR), using published primers and probe (Van den Berg *et al.*, 2006); and (iii) to compare rtPCR assays with the culture method.

METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The specificity of the TMrtPCR was demonstrated by testing 28 non-*C. difficile* strains, among them 18 *Clostridium* sp. strains (Table 1).

Animals and sampling. A total of 340 samples from different farm animals were collected. From piglets (<10 days old) 285 rectal swabs in duplicate (one for cultivation and one for DNA extraction) were obtained. The samples were taken from five piglets per litter on four different large farms (total animal number >1000) and four different small farms (total animal number <500). Of the 52 litters sampled, 53.8 % were with at least one diarrhoeic animal, while 46.2 % litters were with animals without diarrhoea. Faecal samples from 51 diarrhoeic calves (<10 days old) from two different farms and four samples from diarrhoeic foals (up to 1 month of age) were also investigated.

Bacteriological cultivation and identification. Samples were inoculated into a 9 ml cycloserine cefoxitin fructose enrichment broth (Oxoid) supplemented with 0.1 % sodium taurocholate (Sigma-Aldrich). After 7 days of incubation at 37 °C in anaerobic jars, 1 ml was transferred into a sterile test tube and mixed with an equal amount of absolute ethanol and left at room temperature for 30 min. Samples were centrifuged and the pellet was plated onto a standard selective medium with cycloserine and cefoxitin (*C. difficile* agar base and *C. difficile* selective supplement; Oxoid). The inoculated plates were incubated anaerobically at 37 °C for 2 days (Arroyo *et al.*, 2005). The isolates were identified on the basis of morphological criteria, typical odour, and confirmed with multiplex PCR targeting *tpi*, *tcdA* and *tcdB* (Lemee *et al.*, 2004).

Table 1. Bacterial strains used for specificity testing of rtPCR for *C. difficile* toxin genes

Species	Source
Clostridium botulinum A	ICVF*
Clostridium botulinum B	NCTC 7273
Clostridium botulinum C	ICVF*
Clostridium botulinum E	NCTC 8266
Clostridium butyricum	ICVF*
Clostridium chauvoei	ATCC 10092
Clostridium novyi	ATCC 19402
Clostridium perfringens A	ATCC 25768
Clostridium perfringens A (*cpe*$^+$)†	ICVF*
Clostridium perfringens B	CCUG 2035
Clostridium perfringens C	ATCC 51880
Clostridium perfringens D (*cpb2*$^+$)‡	ICVF*
Clostridium perfringens E	CCUG 44727
Clostridium septicum	ATCC 12464
Clostridium sordellii	ATCC 9714
Clostridium spiroforme	ATCC 29900
Clostridium sporogenes	ATCC 3584
Clostridium tetani	ATCC 19406
Bacteroides fragilis	ATCC 25285
Campylobacter coli	ICVF*
Campylobacter jejuni	ATCC 33560
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 25922
Escherichia coli O157	ICVF*
Proteus mirabilis	DSM 788
Staphylococcus aureus	ATCC 25923
Salmonella Enteritidis	CAPM 5439
Salmonella Typhimurium	ATCC 14028
Clostridium difficile	ATCC 9689
Clostridium difficile VPI 10463(toxinotype 0)	IPH MB§
Clostridium difficile (toxinotype I)	IPH MB§
Clostridium difficile (toxinotype IIIb)	IPH MB§
Clostridium difficile (toxinotype IV)	IPH MB§
Clostridium difficile (toxinotype V)	ICVF*
Clostridium difficile 51377 (toxinotype VI)	IPH MB§
Clostridium difficile (toxinotype VIII)	IPH MB§
Clostridium difficile 8864 (toxinotype X)	IPH MB§
Clostridium difficile (toxinotype XIa)	ICVF*
Clostridium difficile (toxinotype XII)	IPH MB§

*Internal collection of the Veterinary Faculty, University of Ljubljana.
†Strain with gene encoding enterotoxin.
‡Strain with gene encoding β2 toxin.
§Strains provided by Maja Rupnik (Institute of Public Health Maribor, Maribor, Slovenia).

DNA extraction. Rectal swabs were washed in 700 µl sterile distilled water and 200 µl of the suspension obtained was used for DNA extraction. DNA from rectal swabs and faecal samples was extracted using the QIAamp DNA stool mini kit (Qiagen).

DNA from bacterial isolates was purified from colonies grown on blood agar (BBL Columbia agar base supplemented with 5 % ovine

blood; Becton Dickinson) by heating the bacterial suspensions at 95 °C for 15 min and then centrifuging for 2 min at 14 000 ***g***. Supernatants were used as a source of DNA for rtPCR.

TMrtPCR

Primers and probes. The *tcd*A, *tcd*B and *cdt*B *C. difficile* toxin gene sequences available from public databases and sequences kindly provided by Maja Rupnik (Institute for Public Health, Maribor, Slovenia) were analysed. Sequence alignments revealed conserved and specific regions for all genes. Using sequences within these regions, primers and probes were designed and synthesized by Applied Biosystems. The sequences of the amplification primers and probes are listed in Table 2.

PCR conditions. For the detection of *tcd*A, *tcd*B and *cdt*B genes, three reactions were prepared in a total volume of 10 µl, containing 1 × TaqMan universal PCR master mix (Applied Biosystems), 1 × assay mix [mix of 900 nM both PCR primers and 200 nM TaqMan MGB (minor groove binder) probe – FAM (6-carboxyfluorescein) dye-labelled], and 1 µl DNA template. PCR amplification (2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C) and detection were carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems).

Internal inhibition control. The TaqMan exogenous internal positive control reagents (Applied Biosystems) were used for the detection of DNA polymerase inhibitors, possibly present in DNA preparations from faecal samples. The kit provides an internal positive control DNA template (Exo IPC DNA) and a mix of primers and VIC dye-labelled probes for amplification and specific detection of internal positive control DNA (Exo IPC mix). To analyse the presence of potential PCR inhibitors in each of the faecal samples tested, an amplification control reaction was carried out. Each 25 µl reaction mixture comprised 12.5 µl TaqMan universal PCR master mix (Applied Biosystems), 1.5 µl assay mix for the detection of *tcd*A, *tcd*B or *cdt*B genes, 2.5 µl Exo IPC mix, 5.5 µl sterile water, 0.5 µl Exo IPC DNA (diluted 1 : 500) and 2.5 µl DNA template. PCR amplification and detection conditions were the same as described above.

Specificity and analytical sensitivity. The specificity of the assay was determined by testing the DNA from 39 bacterial strains, as shown in Table 1. The limit of detection (LOD) of the TMrtPCR for each target gene, with and without internal control, was determined by spiking *C. difficile*-negative faecal specimens with 10-fold serial dilutions (10^{-1} to 10^{-6}) of *C. difficile* cells (strain 51377, $A^+B^+CDT^+$), grown in a brain heart infusion broth (Oxoid). Each *C. difficile* dilution was spiked in triplicate. The number of *C. difficile* cells in the broth was counted with a microscope. DNA extraction and amplification were performed as described above. Each DNA extract was tested three times with TMrtPCR.

PCRFast *Clostridium difficile* A/B test. A total of 79 samples (42 culture positive and 37 culture negative) were subjected to PCRFast, performed according to the manufacturer's instructions on an ABI Prism 7000 sequence detection system with the TaqMan universal PCR master mix (Applied Biosystems). An initial step (2 min at 50 °C) for AmpErase UNG (uracil *N*-glycosylase) activity of the master mix was added to the amplification programme according to the manufacturer's instructions.

rtPCR targeting *tcdB*. The same 79 samples that were used for PCRFast were also amplified with BrtPCR. Primers 398CLDs/399CLDas and probe 551CLD-tq-FAM were used (Van den Berg *et al.*, 2006). The reaction mixture and cycling conditions were the same as for TMrtPCR (described above).

RESULTS

Specificity and analytical sensitivity of TMrtPCR

The specificity of the primers and probes used for TMrtPCR was determined to be 100 %, as all non-*C. difficile* bacterial strains were negative for all three genes and all *C. difficile* strains were positive for the respective genes (Table 3). In case of toxinotypes VIII ($A^-B^+CDT^-$) and XIa ($A^-B^-CDT^+$), the assay targeting *tcdA* was positive, since the gene for toxin A is present but not expressed in these toxinotypes.

The LOD of the TMrtPCR for *tcdB* and *cdtB* genes was found to be 440 copies *C. difficile* DNA (g faeces)$^{-1}$ (100 % probability of detection). The LOD for *tcdA* gene was below 440 copies DNA (g faeces)$^{-1}$, while two triplicates with 44 copies DNA (g faeces)$^{-1}$, were positive in TMrtPCR. When using the internal control in a duplex assay with rtPCR for *tcdA* or *tcdB* gene, the LOD was the same as for rtPCR without the internal control. However, in the case of *cdtB* gene detection, not only was the LOD higher [at least 4400 copies DNA (g faeces^{-1})], but also two triplicates with 440 copies DNA (g faeces)$^{-1}$ were positive in TMrtPCR.

Table 2. Primers and probes used for TMrtPCR

Gene target	Primer or probe	Oligonucleotide sequence (5′→3′)
tcdA	tcdA-F	TGCCAACTATAACTACTAACGAAATTAGAAACAA
	tcdA-R	TATTGGATATGAAGATAATAATAAAGAGTAAGTTCCT
	tcdA-P	FAM-CCTGCTCCATCAAATG-NFQ
tcdB	tcdB-F	GTGTAAGTTTAGGTGCAGCAATCAA
	tcdB-R	CCATTATACCTATCTTAGCTTCTATTTCTTGTCT
	tcdB-P	FAM-ATGGGTCACTCGTTTCAC-NFQ
cdtB	BinTox-F	ACTTCCCCTGAATATGATTTTAACAAACTAGAAA
	BinTox-R	TCACACTACCAATACTAGTACTAATAAAGTTTGGA
	BinTox-P	FAM-ACAGTGCTTGTCCTTCC-NFQ

NFQ, Nonfluorescent quencher.

Table 3. Results of the amplification of toxin genes of different *C. difficile* toxinotypes used for the validation of rtPCR assays

Assay	Toxinotype									
	0	I	IIIb	IV	V	VI	VIII	X	XIa	XII
Toxin production	$A^+B^+CDT^-$	$A^+B^+CDT^-$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^-B^+CDT^-$	$A^-B^+CDT^+$	$A^-B^-CDT^+$	$A^+B^+CDT^-$
TMrtPCR*	$A^+B^+CDT^-$	$A^+B^+CDT^-$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^+B^+CDT^+$	$A^+B^+CDT^-$	$A^-B^+CDT^+$	$A^+B^-CDT^+$	$A^+B^+CDT^-$
PCRFast†	+	+	+	+	+	+	+	+	–	+
BrtPCR‡	+	+	+	+	+	+	+	–	–	+

*Detection of genes encoding toxin A, toxin B and binary toxin.
†PCRFast *Clostridium difficile* A/B test.
‡*tcdB* rtPCR.

Detection of variant *C. difficile* toxinotypes with rtPCR assays

Eight tested toxinotypes (0, I, IIIb, IV, V, VI, VIII, XII) of *C. difficile* were detected with all three rtPCR assays (Table 3). Toxinotype XIa ($A^-B^-CDT^+$) was negative in PCRFast and BrtPCR, while toxinotype X ($A^-B^+CDT^+$) was negative in BrtPCR (Table 3).

Detection of toxigenic *C. difficile* with TMrtPCR and culture

Among the 340 samples, 113 (33.2 %) were positive and 223 (65.6 %) were negative in the TMrtPCR assay. Inhibition was not observed. Samples were defined as positive with TMrtPCR if the test was positive for all genes belonging to a defined toxinotype present on the sampled farm (data not shown; Avbersek *et al.*, 2009). Three different types of toxigenic *C. difficile* ($A^+B^+CDT^+$, $A^+B^+CDT^-$ and $A^+B^-CDT^+$) were detected with TMrtPCR. The proportion of these types among all the positive samples is shown in Table 4.

The correlation of TMrtPCR with the cultivation results is shown in Table 5. Results of TMrtPCR for four culture-positive samples were not in complete concordance with the $A^+B^+CDT^+$ type defined for *C. difficile* isolates, while TMrtPCR for *tcdA* or *cdtB* was negative.

Comparison of all three rtPCR assays and the culture method

The highest number of culture-positive samples gave positive results also in TMrtPCR (57 %), while only 40 and 7 % of the samples were positive in BrtPCR and PCRFast, respectively (Table 6). Four culture-positive samples (10 %) that were unresolved by TMrtPCR, as described above, were negative in both BrtPCR and PCRFast assays.

Inhibitors were not detected for TMrtPCR, while inhibition was observed in four samples for PCRFast. One inhibited sample was culture/TMrtPCR/BrtPCR positive, two were culture positive/rtPCR negative and one was culture/rtPCR negative.

Among 79 samples, 40 (50.6 %) were in complete concordance (all three rtPCR assays showed the same result) – 19 were culture negative/rtPCR negative, 4 were culture negative/rtPCR positive, 14 were culture positive/rtPCR negative and 3 were culture positive/rtPCR positive. However, two culture-positive samples (toxinotype XIa; data not shown) yielded an $A^+B^-CDT^+$ result with TMrtPCR, while BrtPCR and PCRFast, targeting only *tcdA* and/or *tcdB*, were negative. These two samples were designated rtPCR positive, as the negative result with BrtPCR and PCRFast was correct. Additionally, among the 39 (49.4 %) samples that were not in complete concordance, BrtPCR results of 23 (59.0 %) samples were in accordance with TMrtPCR results, but not with PCRFast results. However, five culture-negative samples (12.8 %)

Table 4. Proportion of *C. difficile* types among TMrtPCR-positive samples

Toxin genes detected with TMrtPCR			No. of samples	Percentage of toxin gene-positive samples	Percentage of total samples (n=340, 100 %)
tcdA	*tcdB*	*cdtB*			
+	+	+	48	42.48	14.12
+	+	−	63	55.75	18.53
+	−	+	2	1.77	0.59
Total samples positive for toxin genes			113	100	33.24

were positive in TMrtPCR and three of them were also positive in PCRFast, while all five samples were negative in BrtPCR. Of the 39 samples, only these 3 (7.7 %) gave the same result in PCRFast and TMrtPCR.

DISCUSSION

Low carriage rates of toxigenic *C. difficile* strains with no detectable level of toxin might indicate carriage rather than an infection of the animal. These animals could serve as reservoirs for other animals (e.g. during hospitalization) and also for humans (Indra *et al.*, 2009; Gould & Limbago, 2010). Detection of *C. difficile* in asymptomatic animals with potentially severe strains is therefore very important. In such cases enrichment culture is necessary but it is time-consuming and not specific for identification of toxigenic strains (Båverud, 2002; Arroyo *et al.*, 2005). Therefore, we developed and validated a new TMrtPCR for faster detection of toxigenic strains directly in the faeces of symptomatic and asymptomatic animals, and performed a comparison with the enrichment culture method and with two existing rtPCR assays.

The results obtained with TMrtPCR correlate with culture in 75.3 % samples. Forty (11.8 %) culture-positive samples were TMrtPCR negative. This could be connected with the number of *C. difficile* cells contained in the faecal sample. As the latter is never a homogeneous medium, the concentration of bacteria may vary. Rectal swabs used for sampling young animals contain a smaller amount of faeces, the swab washing prior to the DNA extraction employed in our study may have reduced the concentration of bacteria. It could also be a DNA extraction failure, since in case of samples from asymptomatic animals, a small amount of bacteria, especially clostridial spores, is expected. Forty samples (11.8 %) were TMrtPCR positive but were not detected by the enrichment culture. TMrtPCR results were not regarded as false positive as every sample was tested three times (detection of three toxin genes) and as the toxin type results correlated with the type presented on the sampled farm (Avbersek *et al.*, 2009). However, 18/40 culture-negative/TMrtPCR-positive samples were also tested with BrtPCR and PCRFast – 16 samples were also positive with BrtPCR and/or PCRFast. Only two samples were negative with BrtPCR and PCRFast. Due to its high sensitivity, the TMrtPCR assay was able to detect low concentrations of target DNA in samples in which the amount of *C. difficile* was insufficient to grow in enrichment media or in which the DNA was extracted from bacteria that did not survive in the sample before cultivation. It should be noted that the culture method is not designated a 'gold standard' and it is not a standardized procedure. In spite of alcohol shock after enrichment, other anaerobic sporogenic bacterial flora could be present, which could overgrow *C. difficile* colonies. The results of our study agree with the findings published by Houser *et al.* (2010). This study reported 7.0 % culture-positive/rtPCR-negative and 11.3 % culture-negative/rtPCR-positive faecal samples from calves. The sensitivity of the rtPCR assay was increased if faecal samples were enriched before DNA extraction (Houser *et al.*, 2010).

As variant toxinotypes have emerged in humans and animals, it is important for diagnostic methods to detect as many different toxinotypes as possible (Rupnik, 2007). In this study, ten toxinotypes were tested in all three rtPCR assays. With the exception of one (XIa) in PCRFast and two (X, XIa) in BrtPCR, all toxinotypes were detected with the test assays. Toxinotype XIa (only binary toxin positive) was negative, because PCRFast and BrtPCR do not detect the

Table 5. Comparison of TMrtPCR with the culture method for the detection of toxigenic *C. difficile* in rectal swabs and faecal samples

Culture result	TMrtPCR positive	TMrtPCR negative	TMrtPCR unresolved	No. (%) of total samples
Culture positive	73 (21.5 %)	40 (11.8 %)	4 (1.2 %)	340 (100 %)
Culture negative	40 (11.8 %)	183 (53.8 %)	0	

Table 6. Comparison of different rtPCR assays with the culture method

Culture result		No. (%) of samples with the following assay result:					
		TMrtPCR		*tcdB* rtPCR		PCRFast *Clostridium difficile* A/B test	
Positive	42 (53 %)	Positive	24 (57 %)	Positive	15 + 2* (40 %)	Positive	1 + 2* (7 %)
		Negative	14 (33 %)	Negative	25 (60 %)	Negative	39 (93 %)
		Unresolved	4 (10 %)	Unresolved	0	Unresolved	0
Negative	37 (47 %)	Positive	18 (49 %)	Positive	13 (35 %)	Positive	7 (19 %)
		Negative	19 (51 %)	Negative	24 (65 %)	Negative	30 (81 %)
		Unresolved	0	Unresolved	0	Unresolved	0
Total samples	79 (100 %)	–	–	–	–	–	–

*Two samples had the $A^-B^-CDT^+$ type with negative rtPCR result, but were designated correctly positive.

gene for binary toxin. The results of a TMrtPCR assay could give us the first information about the toxin type present in a sample, although it is not as reliable as toxinotyping. However, TMrtPCR detected a nonfunctional *tcdA* gene in case of toxinotypes VII and XIa. The TMrtPCR assay enabled the detection of all three toxin genes separately for one sample. In contrast, the PCRFast test, in which the primers and probes targeting the toxin A and B genes are mixed in one amplification reaction, does not allow the distinction between A^+B^+ and A^-B^+ strains.

The results of the comparison of rtPCR assays indicate that TMrtPCR is more suitable than BrtPCR and PCRFast for the detection of *C. difficile* directly in animal faecal samples. The highest percentage of culture-positive samples was positive in TMrtPCR and the lowest percentage of culture-positive samples was TMrtPCR negative compared to the other rtPCR assays tested. The four samples with unresolved TMrtPCR results (culture positive) and negative BrtPCR and PCRFast results support our preference of TMrtPCR. Furthermore, the added value of TMrtPCR is also the ability to detect the binary toxin gene, which is often present in animals and contributes to the severity of CDAD in humans (McEllistrem *et al.*, 2005), although the detection of three genes raises the price of the assay. Results for the samples that were not in complete concordance (49.4 %) show that BrtPCR performed better than PCRFast, as the results correlated better with the culture and TMrtPCR results. However, BrtPCR was not able to detect toxinotype X, which could lead to a false-negative rtPCR result. The manufacturer's instructions for PCRFast, however, do not specify whether the kit has been validated on animal samples and, to the best of our knowledge, BrtPCR was used here on animal samples for the first time. It should be noted that a different master mix than the one suggested in the manufacturer's instructions was used for PCRFast, as the results obtained by using the AmpliTaq Gold PCR master mix (Applied Biosystems) were invalid. Therefore, further testing of PCRFast with other PCR reagents is required.

In conclusion, this study describes a novel TMrtPCR for the direct detection of *C. difficile* in animal faecal samples. TMrtPCR targets the genes for toxins A and B, and binary toxin; detection of the latter is becoming more important due to its potential clinical significance and association with CA-CDI. TMrtPCR could be the preferred screening method, because of its advantages: it is faster and it provides the first information about the toxin type. Because of possible false negative TMrtPCR results, enrichment culture should be performed for the specimens with negative or inconclusive rtPCR results as a second step for *C. difficile* detection in animal samples. However, as the isolate is often necessary for genotyping studies, the importance of the culture should not be underestimated. This study also demonstrated that the validation of rtPCR with animal samples should be carried out, as rtPCR assays designed for CDAD diagnosis in humans may be inadequate. The new TMrtPCR could also be used for the detection of *C. difficile* in environmental samples (e.g. water, soil, surface swabs taken in hospitals) or in food samples, in which low numbers of *C. difficile* are expected. To prevent false-negative rtPCR results DNA extraction from enriched samples should be performed. Further studies should also evaluate different DNA extraction methods from spores, which could result in more rtPCR-positive samples.

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Improved detection of *Clostridium difficile* in animals by using enrichment culture followed by LightCycler real-time PCR

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ABSTRACT

The performance of our previously published TaqMan real-time PCR (TMrtPCR) for the detection of *Clostridium difficile* directly from animal faeces was found to be inadequate due to TMrtPCR false negative results. Therefore, we developed a new internally controlled LightCycler real-time PCR (LC rtPCR) capable of detecting variant strains in diarrhoeic and subclinical animals by using two hybridisation probes instead of one hydrolysis probe used in TMrtPCR. While LC rtPCR did not provide better results than TMrtPCR, improved detection of *C. difficile* in samples with low number of bacteria was introduced, using a pre-enrichment step followed by LC rtPCR. Hence, 40 (100%) rectal swabs were sampled and subjected to direct LC rtPCR, culture without enrichment and enrichment culture; after 24, 48 and 72 h, and seven days of incubation, DNA was extracted and amplified with LC rtPCR. Only one (2.5%) sample was culture positive/LC rtPCR positive without the enrichment step, while 33 (82.5%) samples were culture positive after seven days of enrichment. Only one day of enrichment evidently increases the number of culture positive (15; 37.5%) and LC rtPCR positive (28; 70%) samples. Results of this study demonstrate that one day enrichment culture for *C. difficile* followed by LC rtPCR assay targeting multiple genes can be applied as accurate and rapid screening test, especially in samples with low number of *C. difficile*, as no culture positive/LC rtPCR negative samples were observed.

1. Introduction

Clostridium difficile is known as an important cause of hospital acquired diarrhoea in humans (Brazier, 1998). Recent reports have indicated an increased frequency and severity of community-acquired *C. difficile*-associated disease (CA-CDAD) (Kuntz et al., 2011; Khanna et al., 2012). Toxigenic strains of *C. difficile* have also been recognised as a cause of disease in different animal species. On the other hand, a high percentage of subclinical animal carriers are reported (Båverud et al., 2003; Rodriguez-Palacios et al., 2006; Simango and Mwakurudza, 2008; Alvarez-Perez et al., 2009). The overlap of animal strains with those from some human cases and isolation of the organism from food and the environment suggests that animals may be a reservoir and CDAD a zoonosis (Debast et al., 2009; Rupnik and Songer, 2010; Koene et al., 2012; Janezic et al., 2012).

The diagnosis of a disease caused by toxigenic *C. difficile* is usually based on a combination of cytotoxicity assay, bacteriological cultivation, and enzyme immunoassays (EIAs), all of which are either labour intensive and time-consuming or lack sensitivity and specificity (Crobach et al., 2009). Several in-house and commercially available real-time PCR (rtPCR) assays have also been described for direct detection of toxin genes mostly in human samples (Barbut et al., 2011; Deshpande et al., 2011; Knetsch et al., 2011; Hoegh et al., 2012). The use of commercial EIAs and rtPCR tests intended for diagnosing CDAD in humans is limited for testing animal faeces, as the test results often poorly correlate with culture results (Rodriguez-Palacios et al., 2006; Pirs et al., 2008; Hopman et al., 2011; Keessen

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et al., 2011). To the best of our knowledge and up to date, only two studies dealing with the development of rtPCR used for direct detection of *C. difficile* in animal samples have been published (Houser et al., 2010; Avbersek et al., 2011).

The aim of this study was to optimise the procedure for the detection of *C. difficile* in animal faecal samples, especially for samples with low number of *C. difficile* (e.g. subclinical animals). Since our previously published Taq-Man real-time PCR (TMrtPCR) used for direct detection of *C. difficile* cannot be used as a stand-alone test, we developed and validated a new LightCycler real-time PCR (LC rtPCR) by using two hybridisation probes instead of one hydrolysis probe used in TMrtPCR. Subsequently, enrichment step was added prior to LC rtPCR with the purpose to increase the number of *C. difficile* in samples.

2. Materials and methods

2.1. LightCycler real-time PCR design and verification

2.1.1. Primers and probes

The *tcdA*, *tcdB* and *cdtB C. difficile* toxin gene sequences available from public databases and sequences kindly provided by Dr. Maja Rupnik (Institute of Public Health, Maribor, Slovenia) were aligned and analysed. Within the conserved and specific regions for all three genes primers and probes were designed and synthesised by TIB Molbiol (Germany). The sequences of the primers and probes are listed in Table 1.

2.1.2. Specificity and analytical sensitivity

The specificity of the primers and probes used in LC rtPCR and analytical sensitivity (limit of detection) of the assay was determined as previously described (Avbersek et al., 2011). Briefly, the specificity of the assay was demonstrated by testing the DNA isolated from 11 *C. difficile* strains (toxinotypes 0, I, IIIb, IV, V, VI, VIII, X, XIa, XII) and 28 non-*C. difficile* strains, which comprise 18 *Clostridium* spp. strains. Limit of detection (LOD) for each target gene, with and without internal control, was determined by amplifying DNA extracted from spiked *C. difficile*-negative faeces with serial dilutions of a known number of *C. difficile* cells used in previously published study, which was stored at −70 °C for four months (Avbersek et al., 2011). LC rtPCR amplification was performed three times for each tested gene, for each dilution as described further in the text.

Table 1
Primers and probes used for LightCycler real-time PCR.

Gene target	Primer or probe	Oligonucleotide sequence (5′ → 3′)[a]
tcdA	tcdA-S	ATGACGGAACTAGATTACTTGAT
	tcdA-R	ATAAGATAATTTGTTTCTAATTTCGT
	tcdA-LC	LC640-AGTTATTGCATAATCGAAAAAA GCAT-PH
	tcdA-FL	ATTAGTGTCTTCATAAACTGGTTTTAA TG-FL
tcdB	tcdB-S	TTACTTCCTACATTATCTGAAGGAT
	tcdB-R	TGCAGTTGTAGCTGTTGTTA
	tcdB-LC	LC640-TTGATTGCTGCACCTAAACTT ACACCA-PH
	tcdB-FL	TGGGTCACTCGTTTCACTTAGCTC-FL
cdtB	cdtB-A	TCTCCAGCAGTATTTGACTCTAAA
	cdtB-F	CTAATCTTTATTGGGAATTAGATGGT
	cdtB-LC	LC640-AAGATAGTTTTGCAGAACAAGGC TATAAGA-PH
	cdtB-FL	ACTATTAAGGACTTAATTGCAGTTAAG TGG-FL

[a] LC640, LightCycler Red 640; PH, phosphate; FL, fluorescein.

2.1.3. PCR conditions

For the detection of *tcdA*, *tcdB* and *cdtB* genes, three reaction mixtures were prepared in a total volume of 20 μl, containing 1× LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Germany), 3 mM $MgCl_2$, 0.5 μM of each primer, 0.2 μM of each hybridisation probe, and 2 μl of DNA template. The PCR amplification and detection were carried out on a LightCycler 2.0 (Roche Diagnostics, Germany). Temperature transition rate/slope was 20 °C/s and acquisition mode none, except where indicated. An initial pre-incubation cycle of 95 °C for 10 min was followed by 45 cycles of 95 °C for 10 s, 54 °C (*tcdA*)/55 °C (*tcdB*)/58 °C (*cdtB*) for 10 s (temperature transition rate/slope – 18 °C/s, acquisition mode – single), and 72 °C for 10 s; and by cooling step of 40 °C for 30 s.

2.1.4. Internal inhibition control

LightCycler Control Kit DNA (Roche Diagnostics, Germany) was used for the detection of possibly present DNA polymerase inhibitors in DNA extracts from faeces. The kit contains primers and probes for human β-globin gene used as internal positive control. Amplification control reaction was carried out as a duplex LC rtPCR with *tcdA* or *tcdB* amplification. A final volume of 20 μl was used containing 1× LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Germany), 4 mM $MgCl_2$, 0.5 μM of each *tcdA* or *tcdB* primers, 0.2 μM of each *tcdA* or *tcdB* hybridisation probes, 0.5× β-globin primer mix, 0.5× HybProbe Mix (LightCycler Red 705-labelled), 2 μl of DNA template, and 2 μl of human genomic DNA (diluted 1:1000). LC rtPCR amplification and detection conditions were the same as described above.

2.2. LightCycler real-time PCR validation

LC rtPCR was performed on the same 340 animal samples (285 rectal swabs from piglets, faecal samples from 51 calves and 4 foals) that were tested in our previous study and compared with previously published results of bacteriological cultivation and TMrtPCR amplification (Avbersek et al., 2011). Extracted DNA obtained in our previously study was aliquoted to avoid repeat freezing and thawing, stored at −70 °C for two to four months and then amplified with LightCycler. All genes belonging to a defined toxinotype present on the sampled farm were required for designating LC rtPCR result as positive (data not shown, Avbersek et al., 2009); otherwise the samples with at least one positive gene were designated as unresolved.

2.3. Improvement of C. difficile detection by using pre-enrichment step followed by LightCycler real-time PCR

2.3.1. Animal samples and rectal swab processing

Additionally, rectal swabs from 20 diarrhoeic and 20 non-diarrhoeic piglets (8–10 days old) were taken from a large farm, where *C. difficile* toxinotype V/ribotype 066 had previously been detected (Avbersek et al., 2009). Animals on this farm are constantly surveyed for *C. difficile* and only above mentioned PCR-ribotype was detected up to date (Avbersek and Ocepek, unpublished data). From one piglet four rectal swabs were obtained. Rectal swabs from one animal were washed in 1.6 ml sterile distilled water (SDW) and used for further testing (culture and DNA extraction).

2.3.2. DNA extraction and real-time PCR amplification

DNA from rectal swabs (200 μl of the obtained suspension) were extracted by using QIAamp DNA Stool Mini Kit (Qiagen, Germany) and amplified by LC rtPCR (detection of *tcdA*, *tcdB* and *cdtB* genes).

2.3.3. Culture without alcohol shock

The obtained suspension was inoculated into four test tubes with 9 ml of cycloserine–cefoxitin fructose enrichment broth (Oxoid, UK) supplemented with 0.1% sodium tauroholate (Sigma-Aldrich, USA) (CCFB) (four tubes were used, because incubation times differed), 100 μl in each tube. After 24, 48 and 72 h of anaerobic incubation, 100 μl of the sample was inoculated onto a standard selective medium with cycloserine and cefoxitin (*C. difficile* agar base and *C. difficile* selective supplement, Oxoid, UK) (CCA) and incubated for two days in anaerobic jars. Samples incubated for 7 days were subjected to alcohol shock and then inoculated onto CCA. After two days of anaerobic incubation, the isolates were identified on the basis of morphological criteria and typical odour, and confirmed with multiplex PCR targeting *tpi*, *tcdA* and *tcdB* (Lemee et al., 2004).

2.3.4. Culture with alcohol shock

The remaining suspension (approx. 500 μl) was subjected to alcohol shock by adding an equal amount of absolute ethanol and left at room temperature for 30 min. The resulting pellet was first inoculated onto CCA and then suspended in 500 μl of the CCFB and inoculated into four test tubes with 9 ml of CCFB (four tubes were used, as incubation times differed), 100 μl in each tube. After 24, 48 and 72 h, and 7 days of anaerobic incubation, 100 μl of the sample was inoculated onto CCA and incubated for two days in anaerobic jars. Identification and confirmation of isolates were the same as described above.

2.3.5. DNA extraction from enrichment culture and real-time PCR amplification

After 24, 48 and 72 h, and 7 days of anaerobic incubation, DNA was extracted from enriched culture (from tubes with and without alcohol shock) using two different methods: 1) a commercial kit foodproof Sample Preparation Kit I (BIOTECON Diagnostics, Germany) performed according to the manufacturer's instructions; and 2) a washing/heating/centrifuging method. Briefly, 1 ml of enriched culture was centrifuged for 5 min at 14,000 × *g*, the supernatant was discarded, a pellet was suspended in 1 ml of SDW, the suspension was centrifuged for 3 min at 14,000 × *g*, the pellet was suspended in 200 μl of SDW, heated at 95 °C for 15 min and then centrifuged for 2 min at 14,000 × *g*. Supernatants were used as a source of DNA. All extracted DNA samples were amplified by LC rtPCR (detection of *tcdA*, *tcdB* and *cdtB* genes) and additionally, for detection of potential PCR inhibitors in samples, amplification control reaction was carried out as a duplex rtPCR with *tcdA* amplification as described above.

2.3.6. In vitro toxin detection of isolated strains (EIA)

All *C. difficile* isolates were tested for toxin A and B production by Premier Toxins A&B *Clostridium difficile* (Meridian Bioscience, USA) EIA kit according to the manufacturer's instructions. Positive control (reference strain VPI 10463, toxinotype 0) was used. Before EIA, isolates and positive control were prepared in three ways. Briefly, *C. difficile* was cultured in brain heart infusion (BHI) broth (Oxoid, UK) and in cooked meat medium (Oxoid, UK) and incubated anaerobically at 37 °C for 48 h. The broth was centrifuged and 100 μl of the supernatant was used for EIA. Furthermore, five colonies were suspended in 200 μl EIA kit sample diluent, mixed, centrifuged and 100 μl of supernatant was tested directly by EIA (Wren, 2010).

3. Results

3.1. Specificity and analytical sensitivity of LightCycler real-time PCR

LC rtPCR efficiently detected all tested *C. difficile* toxinotypes. Eight toxinotypes were positive for respective genes, while toxinotypes VIII ($A^-B^+CDT^-$) and XIa ($A^-B^-CDT^+$) were positive for *tcdA*, which is present but not expressed in these strains. All non-*C. difficile* bacterial strains showed no amplification signal for any of the tested genes and thereby yielded 100% specificity of LC rtPCR.

The analytical sensitivity at 100% probability of detection for the singleplex LC rtPCR assay targeting *tcdA*, *cdtB* and *tcdB* was 4400, 440 and 4400 copies of *C. difficile* DNA/g of faeces. In singleplex *tcdB* assay, two triplicates with 440 copies of DNA/g of faeces were also positive, indicating that LOD is between 440 and 4400 copies of DNA/g of faeces. In duplex rtPCR assay with the internal control, the LOD for *tcdB* and *tcdA* gene detection were 4400 and 44,000 copies of *C. difficile* DNA/g of faeces (100% probability of detection). LC rtPCR for *tcdA* gene was positive also in two triplicates with 4400 copies of DNA/g of faeces, showing that LOD is between 4400 and 44,000 copies of *C. difficile* DNA/g of faeces.

3.2. LightCycler real-time PCR validation

A total of 340 samples were amplified for each gene, of which 108 (31.8%) were positive and 224 (65.9%) were negative. PCR inhibitors were detected in 4 (1.2%) of culture negative samples. There were no differences in LC rtPCR performance, irrespective of tested animal species.

Table 2
Proportion of *Clostridium difficile* types among LightCycler real-time PCR (LC rtPCR) positive samples.

Toxin genes detected with LC rtPCR					
tcdA	*tcdB*	*cdtB*	Number of samples	% of Toxin gene positive samples	% of Total samples (n = 340, 100%)
+	+	+	48	44.4	14.1
+	+	–	58	53.7	17.1
+	–	+	2	1.9	0.6
Total of samples positive for toxin genes			108	100.0	31.8

The only observed difference, namely presence of PCR inhibitors, was regarded to the type of specimen. If DNA was extracted from faecal samples, inhibitors were detected in 3.6% of samples, while only in 0.7% of rectal swab samples. Table 2 shows the types of toxigenic *C. difficile* and the proportion among all positive samples that were detected with LC rtPCR. Isolates from two samples belonging to toxinotype XIa were *tcdA* + /*tcdB*−/*cdt* + in LC rtPCR assay, which is in concordance with the LC rtPCR specificity testing. LC rtPCR results were further compared with enrichment culture and correlate in 75% samples (Table 3). Furthermore, comparison of LC rtPCR with TMrtPCR results showed complete concordance in 97.7% of cases. Different result were observed in eight samples (five culture positive vs. rtPCR positive but unresolved samples either with LC rtPCR or TMrtPCR; one culture positive vs. TMrtPCR negative vs. LC rtPCR unresolved sample; two culture negative vs. TMrtPCR positive vs. LC rtPCR negative samples). In two cases with different rtPCR results in comparison with culture, contamination could be the reason for such a result; meanwhile the TMrtPCR false negative result in one culture positive sample could be due to the low number of *C. difficile* in the sample, since LC rtPCR was positive only for the *tcdB* gene.

3.3. Improvement of *C. difficile* detection by using pre-enrichment step followed by LightCycler real-time PCR

After seven days of enrichment culture, 33 (82.5%) samples were culture positive and 7 (17.5%) samples were culture negative (six samples from diarrhoeic and one from non-diarrhoeic piglet). However, one culture negative sample was already LC rtPCR positive after one day of enrichment. While *C. difficile* was isolated from diarrhoeic and non-diarrhoeic piglets, further classification of samples, based on the presence or absence of diarrhoea, was not established.

On the other side, *C. difficile* was isolated from only one (2.5%) sample without an enrichment step and was also *tcdB* and *cdtB* positive with LC rtPCR. Additionally, three samples were *tcdB* positive with LC rtPCR but were culture negative. From all three samples *C. difficile* isolate was obtained after the enrichment step. Inhibitors of LC rtPCR were not detected.

Comparison of two different DNA extraction methods from enrichment broth showed that commercial kit foodproof Sample Preparation Kit I gave better results. Among 320 samples, 57 (17.8%) samples were *tcdA* positive, 52 (16.3%) samples were *tcdB* positive, and 37 (11.6%) samples were *cdtB* positive only after DNA extraction with commercial kit. Therefore, LC rtPCR results for samples using commercial kit as the DNA extraction method were considered for further processing of results. In general, approximately 2–3 cycles lower Ct values (the number of cycles required for the fluorescent signal to cross the threshold) were observed for LC rtPCR positive samples, if DNA was extracted with commercial kit in comparison to a washing/heating/centrifuging method (data not shown).

Only one day of enrichment significantly increased the number of culture and LC rtPCR positive samples (Table 4). However, the highest number of culture and LC rtPCR positive samples was after 7 days of incubation with previous alcohol shock. Comparison of culture results with or without previous alcohol shock, before the end of enrichment, showed that more samples were positive after one and two days of enrichment, when samples were treated with alcohol shock. After day one, 70% of samples were LC rtPCR positive irrespective of alcohol shock but after two and three days, the number of LC rtPCR positive samples was lower if the samples were treated with alcohol shock. Furthermore, during the whole experiment the number of culture negative/LC rtPCR positive was higher if the samples were not subjected to alcohol shock but the number of culture positive/LC rtPCR positive samples was lower, except after day three. The correlation of LC rtPCR with the enrichment culture results after days 1, 2, 3, and 7 without alcohol shock is shown in Table 5 and with alcohol shock in Table 6. Inhibition was not observed.

Table 3
Comparison of LightCycler real-time PCR (LC rtPCR) with the culture method for the detection of toxigenic *Clostridium difficile* in rectal swabs and faecal samples.

	LC rtPCR positive	LC rtPCR negative	LC rtPCR unresolved[a]	number (%) of total samples
Culture positive	70 (20.6%)	39 (11.5%)	8 (2.3%)	340 (100%)
Culture negative	38 (11.2%)	185 (54.4%)	0	

[a] LC rtPCR result was not in complete concordance with toxinotype defined for the isolate.

Table 4
Comparison of results for culture and LC rtPCR results for preamplified broth cultures without and with alcohol shock after one, two, three or seven day's incubation.

Days of enrichment broth incubation	Culture positive – without alcohol shock ($n = 40$)	LC rtPCR positive[a] – without alcohol shock ($n = 40$)	Culture positive – with alcohol shock ($n = 40$)	LC rtPCR positive[a] – with alcohol shock ($n = 40$)
0	/	4 (10.0%)[b]	1 (2.5%)[c]	/
1	11 (27.5%)	28 (70.0%)	15 (37.5%)	28 (70.0%)
2	13 (32.5%)	30 (75.0%)	14 (35.0%)	25 (62.5%)
3	19 (47.5%)	30 (75.0%)	17 (42.5%)	24 (60.0%)
7	18 (45.0%)	27 (67.5%)	26 (65.0%)	32 (80.0%)

[a] LC rtPCR was considered positive if at least one gene was positive.
[b] Direct DNA extraction from rectal swabs (without enrichment and alcohol shock).
[c] Culture without enrichment.

Table 5
Correlation of LightCycler real-time PCR (LC rtPCR) with the enrichment culture method without alcohol shock for the detection of toxigenic *Clostridium difficile* in rectal swabs.

Days of incubation	Culture positive/LC rtPCR positive[a]	Culture negative/LC rtPCR negative	Culture positive/LC rtPCR negative	Culture negative/LC rtPCR positive[a]	Number (%) of total samples
1	11 (27.5%)	12 (30.0%)	0 (0%)	17 (42.5%)	40 (100%)
2	13 (32.5%)	10 (25.0%)	0 (0%)	17 (42.5%)	40 (100%)
3	19 (47.5%)	10 (25.0%)	0 (0%)	11 (27.5%)	40 (100%)
7	18 (45.0%)	13 (32.5%)	0 (0%)	9 (22.5%)	40 (100%)

[a] LC rtPCR was considered positive if at least one gene was positive.

Table 6
Correlation of LightCycler real-time PCR (LC rtPCR) with the enrichment culture method with alcohol shock for the detection of toxigenic *Clostridium difficile* in rectal swabs.

Days of incubation	Culture positive/LC rtPCR positive[a]	Culture negative/LC rtPCR negative	Culture positive/LC rtPCR negative	Culture negative/LC rtPCR positive[a]	Number (%) of total samples
1	15 (37.5%)	12 (30.0%)	0 (0%)	13 (32.5%)	40 (100%)
2	14 (35.0%)	15 (37.5%)	0 (0%)	11 (27.5%)	40 (100%)
3	17 (42.5%)	16 (40.0%)	0 (0%)	7 (17.5%)	40 (100%)
7	26 (65.0%)	8 (20.0%)	0 (0%)	6 (15.0%)	40 (100%)

[a] LC rtPCR was considered positive if at least one gene was positive.

A total of 115 (71.9%) DNA samples extracted from enrichment broth treated without alcohol shock were LC rtPCR positive. Among these positive samples, 19 (16.5%) tested positive for only two genes and 11 (9.6%) only for one. Furthermore, a total of 112 (70%) DNA samples extracted from broth with previous alcohol shock were LC rtPCR positive; 5 (4.5%) of them were positive for only two genes and 9 (8.0%) samples were positive for only one gene. Among these nine samples, in three (negative at the end of study, only once was one gene found positive with rtPCR) contamination during DNA extraction cannot be excluded and it seems very likely to be the reason for the false-positive rtPCR result. Therefore, the rtPCR result for these three samples was designated as negative. Among samples with two positive genes, 22 were *tcdA* negative and two samples were *cdtB* negative. All one-positive gene samples were *tcdB* positive, except one sample with a *tcdA* positive result. All *tcdA* negative samples (*tcdB*+, *cdtB*+) were tested with previously published rtPCR (Avbersek et al., 2011) and yielded *tcdA* positive results with TaqMan real-time PCR (data not shown).

All 33 *C. difficile* isolates tested positive for toxin A and B by EIA, when prepared with a cooked meat medium or by direct testing of *C. difficile* colonies, while cultivation of isolates in BHI before EIA resulted in only 20 positive samples for toxin A and B. Positive control was toxin positive by EIA independently of the procedure.

4. Discussion and conclusion

Detection of *C. difficile* in animals is important due to the pathogenic potential of the bacterium for the animals and impacts on the economics of food production (e.g. worse quality of the meat from weak animals and consequently lower buying-in price). A fundamental understanding of disease in both humans and domestic animals will be the basis for strategies to prevent the potential spread of *C. difficile* via food chains or via animal contact. Commonly, low numbers of *C. difficile* in animal samples are expected and therefore enrichment culture is required but it is time-consuming, lacks specificity for identification of toxigenic strains and is difficult to perform adequately. Application of rtPCR assays that are validated for human use only, to animal and environmental samples is inadequate. Hopman et al. (2011) tested pig faecal samples with BD GeneOhm Cdiff

assay (BD Diagnostics); 28% were enrichment culture positive, while all samples were rtPCR negative. Furthermore, Keessen et al. (2011) evaluated three different commercially available EIAs and BD GeneOhm Cdiff assay and concluded that all tests had an unacceptably low performance for pig faecal samples. It has been previously shown, that rtPCR developed and validated for animal samples did not give satisfactory results, when used as a single method (Avbersek et al., 2011). Notwithstanding, in the first part of this study, a new LC rtPCR using hybridisation probes was developed and validated for the detection of toxigenic strains directly in the faeces of animals. To the best of the authors' knowledge, this is the first rtPCR which uses hybridisation detection probes (fluorescence resonance energy transfer/FRET probes) for detection of all three toxin genes of *C. difficile*. LC rtPCR assay offers a rapid screening method and can shorten the time for detection of toxigenic *C. difficile* in faecal samples. However, 11.2% of culture negative/LC rtPCR positive and especially 11.5% of culture positive/LC rtPCR negative samples represent drawback of the method. All culture negative/LC rtPCR positive samples were tested three times (for three toxin genes), toxin type correlated with the type present on the sampled farm and the results were in complete concordance with TMrtPCR testing. Therefore, these samples could be regarded as true positive. Results of direct testing with LC rtPCR concur with previously published studies dealing with rtPCR for direct detection in faecal samples from animals and also from humans (Houser et al., 2010; Barbut et al., 2011; Curry et al., 2011; Hoegh et al., 2012). As LC rtPCR did not provide better results than TMrtPCR from our previously study (Avbersek et al., 2011), one of the most important aims of this study was to introduce the enrichment step prior to LC rtPCR to improve the detection of *C. difficile* strains directly from animal faeces. Houser et al. (2010) and Curry et al. (2011) introduced enrichment culture before DNA extraction. Samples were enriched for 5–7 days and 3 days, respectively. Houser et al. (2010) tested 71 calf faeces samples and 7% were rtPCR positive only after enrichment. Furthermore, Curry et al. (2011) performed only rtPCR for preamplified broth culture without comparison with direct rtPCR. In the present study samples were enriched for one, two, three, and seven days prior LC rtPCR amplification. Samples were processed in two ways – with and without alcohol shock before enrichment. It was presumed that in samples without alcohol shock vegetative cells remain undamaged and therefore initial concentration of *C. difficile* is higher, which will result in more culture positive samples after one and two days of enrichment. On the other side, alcohol shock reduces the bacterial flora and the detection of *C. difficile* colonies can be easier, which was shown in this study to be more important than initial concentration of *C. difficile* in the sample. Anyway, presence of live vegetative cells and spores in initial samples without alcohol shock could influence higher number of culture negative/LC rtPCR positive results in comparison with samples with alcohol shock, while also multiplying of vegetative cells during enrichment contribute to the increased number of *C. difficile*.

In 91.7% of samples with *tcdB*+ and *cdtB*+ LC rtPCR result, *tcdA* was negative, which is expected, as LOD of LC rtPCR for *tcdA* was the highest among tested genes. Seventeen samples had only one positive gene with LC rtPCR. Among these, 5 (29.4%) samples showed one gene positive result in the first testing tube and all further tubes (after additional days of incubation) were rtPCR positive. The reason could be that *C. difficile* did not multiply enough during enrichment, which lead to a culture negative result for these samples. Sampling of piglets using rectal swabs could be a limitation of this study, as swabs containing a smaller amount of faeces and concentration of bacteria in obtained suspension may be reduced. Furthermore, concentration of bacteria and proportion of vegetative cells and spores in suspension may vary between different tubes. This could explain the *tcdB* positive (*tcdA*, *cdtB* negative) result in the remaining 12 (70.6%) samples.

Comparison of enrichment culture results and LC rtPCR results for preamplified broth cultures, with and without alcohol shock after different days of incubation, showed that in general the number of culture positive and LC rtPCR positive samples increased after every additional day of incubation (Table 4). After 7 days of incubation without previous alcohol shock, 18 (45.0%) samples were culture positive and 27 (67.5%) were LC rtPCR positive, while after 3 days of incubation 19 (47.5%) and 30 (75.0%) samples were positive, respectively. The reason for this observation could be reduced anaerobic atmosphere in an anaerobic jar during incubation, as the indicator of atmosphere did not completely change colour. Otherwise, a higher number of positive samples after seven days of incubation should be observed, as was shown for samples with alcohol shock. The reasons for increasing or decreasing the number of positive or negative samples between different days could be i) limitation of this study, as discussed before and therefore, presence of *C. difficile* below the LOD of LC rtPCR assay; ii) poor *C. difficile* multiplying during enrichment due to the presence of potential metabolites produced by other bacteria in the sample; iii) sporulation of *C. difficile* in broth and consequently DNA extraction failure from spores. Despite these deviations results showed that only one day preamplification broth culture notably increased the number of culture and LC rtPCR positive samples. The results obtained after two and three days of incubation indicate that there had been no increase of LC rtPCR positive samples, while every additional day is essential for the culture. The authors believe that the LC rtPCR test, targeting multiple genes and carried out on samples enriched for *C. difficile* for one day, can be applied as a stand-alone screening test, as no culture positive/LC rtPCR negative samples were observed. The use of commercial kit foodproof Sample Preparation Kit I for DNA extraction is preferred. Special precaution is needed, when only one gene is positive, as it is difficult to distinguish whether it is due to contamination or the presence of a low number of bacteria in the sample. The use of LC rtPCR for detection of *C. difficile* will not necessarily eliminate the need for culture, as it provides isolates for antimicrobial susceptibility testing and strain typing. Results of this study showed that enrichment culture incubated for seven days with previous alcohol shock is recommended, followed by

in vitro toxin detection of the isolated strains. Toxin production of the isolates was detected by EIA in two different media and the results differed. Anaerobic conditions and *C. difficile* growth were controlled with positive control used in both media. As it produces toxins in both media, we assume that conditions were not a reason for discrepancy between used media, but it could be connected with toxin production and/or growth of specific *C. difficile* genotype in different media.

According to the results of this study, LC rtPCR assay for preamplified broth cultures for the detection of *C. difficile* could be recommended mostly for samples with low numbers of *C. difficile* (e.g. subclinical animals), while detected *C. difficile* in veterinary important animals with diarrhoea is not necessarily the cause of the disease. Improved procedure applied to environmental and food samples could broaden the knowledge about prevalence of contamination in retail meat in other food, since there is no standard approach for the detection of *C. difficile* in food (Rupnik and Songer, 2010). Furthermore, environment (soil, water, feed, etc.) could also play an important role as a source of clostridial spores, with special attention to hospitals, homes for the aged, nursing homes, etc. Clabots et al. (1992) demonstrated that asymptomatic carriers of *C. difficile* are the source of 84% of hospital-acquired infections, which are often undiagnosed or tested negative by conventional methods. Patients without symptoms are neither isolated nor treated and may still be at risk of spreading spores in the hospital setting, leading to unlimited transmission in hospitals. Therefore, improved procedure with LC rtPCR may serve as a reliable method for identifying the human reservoirs of *C. difficile* and/or spores in a hospital environment. However, additional validation on such samples is required, as there is no data whether the assays developed for animal samples are adequate for testing human samples. Moreover, usually more samples are taken from the environment and a single positive result with LC rtPCR from one day enriched samples could lead to the initiation of infection control measures to prevent hospital spread.

In conclusion, addition of enrichment culture prior LC rtPCR is necessary for the method to be a preferred screening test for the rapid detection of *C. difficile*, especially in samples with a low number of bacteria. Described method could be applied to monitoring a lot of samples, as samples with LC rtPCR negative result after one day of enrichment do not need to be cultivated to get the isolate for further studies. We also believe that the use of rtPCR targeting all three toxin genes is required, while the emergence of a previously hypothetical A+B− strain was recently reported (Lemire et al., 2012). Although we described an improved protocol for detection of *C. difficile* in animal samples, further studies should be focused on different DNA extraction methods from spores to improve detection of *C. difficile* with direct rtPCR.

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