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 (Xla) with PCRFast and two (X, Xla) 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.

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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 Produktqualität) 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

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

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

*Internal collection of the Veterinary Faculty, University of Ljubljana.

†Strain with gene encoding enterotoxin.

‡Strain with gene encoding β 2 toxin.

§Strains provided by Maja Rupnik (Institute of Public Health Maribor, Maribor, Slovenia).

criteria, typical odour, and confirmed with multiplex PCR targeting *tpi*, *tcdA* and *tcdB* (Lemee *et al.*, 2004).

DNA extraction. Rectal swabs were washed in 700 μ l sterile distilled water and 200 μ l 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 *tcdA*, *tcdB* and *cdtB* *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 *tcdA*, *tcdB* and *cdtB* 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 *tcdA*, *tcdB* or *cdtB* 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/399CLDs 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')
<i>tcdA</i>	tcdA-F	TGCCAACTATACTACTAACGAAATTAGAAACAA
	tcdA-R	TATTGGATATGAAGATAATAATAAGAGTAAGTTCCT
	tcdA-P	FAM-CCTGCTCCATCAAATG-NFQ
<i>tcdB</i>	tcdB-F	GTGTAAGTTTATAGGTGCAGCAATCAA
	tcdB-R	CCATTATACCTATCTTAGCTTCTATTCTTGTCT
	tcdB-P	FAM-ATGGGTCACTCGTTTCAC-NFQ
<i>cdtB</i>	BinTox-F	ACTTCCCCTGAATATGATTTTAAACAACTAGAAA
	BinTox-R	TCACACTACCAATACTAGTACTAATAAAAGTTTGGGA
	BinTox-P	FAM-ACAGTGCTTGTCTTCC-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/BrtpPCR 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 %)
<i>tcdA</i>	<i>tcdB</i>	<i>cdtB</i>			
+	+	+	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		<i>tcdB</i> rtPCR		PCRFast <i>Clostridium difficile</i> 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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