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Detection of the ADP-ribosyltransferase toxin gene (*cdt*A) and its activity in *Clostridium difficile* isolates from Equidae

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Abstract

Clostridium difficile is an antibiotic-associated emerging pathogen of humans and animals. Thus far three toxins of *C. difficile* have been described: an enterotoxin (ToxA), a cytotoxin (ToxB) and an ADP-ribosyltransferase (CDT). In the present work we describe the first isolation of CDT producing *C. difficile* from Equidae with gastro-intestinal disease. Out of 17 *C. difficile* strains isolated from Equidae, 11 were positive for the genes *tcd*A and *tcd*B encoding ToxA and ToxB. In addition four of these 11 isolates were positive for the *cdt*A gene encoding the catalytic subunit of the ADP-ribosyltransferase CDT. Interestingly none of the isolates derived from canines (41 isolates) and felines (4 isolates) harboured the *cdt*A gene. In *C. difficile* field isolates which contained the *cdt*A gene, ADP-ribosyltransferase activity could also be detected in culture supernatants indicating expression and secretion of CDT. All strains were associated with intestinal disorders, but no association was found for the occurrence of toxins with a specific clinical diagnosis.

Keywords: *Clostridium difficile*; ADP-ribosyltransferase toxin; Diseased Equidae

1. Introduction

Acute colitis and diarrhoea in mature horses is a severe sporadic disease with a high mortality rate and has been associated with *Clostridium difficile* [1,2]. Due to the recent antibiotic-associated occurrence of *C. difficile* in humans and animals, *C. difficile* is considered an emerging pathogen [3]. Three toxins produced by *C. difficile* have been described so far: an enterotoxin (ToxA), a cytotoxin (ToxB) and an ADP-ribosyltransferase (CDT). ToxA and ToxB both belong to the large clostridial cytotoxin family of glucosyltransferases which UDP-glucosylate proteins of the Rho family [4,5]. Members of the Rho family are small GTP binding proteins which regulate the organisation of the actin cytoskeleton [6]. The third known toxin of *C. difficile*, the binary ADP-ribosyltransferase (CDT), consists of two subunits, homologous to the iota toxin of *Clostridium perfringens* type E and *Clostridium spiroforme* toxin. The catalytic subunit CDTa and the binding subunit CDTb are encoded by separate genes *cdt*A and *cdt*B [7,8], homologous to the closely related iota toxin genes *iap* and *ibp* which encode the two subunits of iota toxin from *C. perfringens* type E. As reported for other binary ADP-ribosylating toxins monomeric actin is the target substrate of CDT. Ribosylated actin induces a disorganisation of the cytoskeleton. Several binary-toxin-producing *Clostridia* have been implicated in digestive diseases: *C. perfringens* type E in animal enterotoxaemia [9] and toxigenic *C. spiroforme* in rabbit enteritis [9]. The *C. difficile* strain CD196, which produces CDT, was isolated from a human patient with severe pseudomembranous colitis [8].

Studies on the occurrence of CDT-producing *C. difficile* in animals have not been done as far. We therefore analysed *C. difficile* isolates from various animals with gastro-intestinal disease for the presence of the *cdt*A gene and for expression of ADP-ribosyltransferase activity.

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2. Materials and methods

2.1. Clinical observations, bacterial strains and growth conditions

C. difficile strains were isolated from Equidae suffering of various gastro-intestinal disorders (Table 1). Strains were routinely isolated on a selective medium (*C. difficile* agar; Biomérieux Suisse, Geneva, Switzerland) and subcultured on blood agar plates (Trypticase soy agar; BBL, Becton Dickson, Meylan, France, supplemented with 5% sheep blood) at 37°C under anaerobic conditions.

2.2. PCR, DNA sequencing and sequence data analysis

Sequences of primers used to amplify segments of the *tcd*A, *tcd*B, *cdt*A and *iap* toxin genes are given in Table 2. Amplification was done on a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, CT, USA), using 0.5 μl *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), 0.25 mM of each dNTP (Boehringer Mannheim), 400 nM of each primer, an annealing temperature of 52°C, 45 s extension time, and 5 μl lysate as the template in a total volume of 50 μl 1×PCR buffer (Boehringer Mannheim). Lysates were prepared by incubation of 3–10 colonies, depending on colony size, in lysis buffer (0.1 mM Tris–HCl pH 8.5/0.05% Tween 20; Merck, Darmstadt, Germany/240 μg ml^{-1} proteinase K; Boehringer Mannheim) for 1 h at 60°C followed by 15 min at 95°C. Lysates of *C. perfringens* NCTC8084 and *C. difficile* ATCC43255 were used as positive controls for amplification of the *iap* (*cdt*A), *tcd*A and *tcd*B fragments, respectively. Five μl of each PCR product was analysed on ethidium bromide-stained 1.5% agarose gels (Fig. 1).

Sequencing reactions were performed with the *Taq* Dye Deoxy Terminator cycle sequencing kit (PE Biosystems, Norwalk, CT, USA) using one of the PCR oligonucleotides as sequencing primer and the PCR product as template. Reaction products were analysed on an ABI Prism 310 genetic analyser (PE Biosystems). Sequence alignment and editing were done with the software Sequencher™ (Gene Codes Corporation, Ann Arbor, MI, USA). Sequence comparisons were done as described by Altschul et al. [10].

2.3. DNA extraction and Southern blot analyses

Cells from 10–50 bacterial colonies from blood agar plates were dissolved in 500 μl of 0.9% NaCl and pelleted by centrifugation at 7000 rpm for 10 min. The pellet was resuspended in 200 μl 0.05 M Tris pH 8 containing 25% sucrose. 1 mg lysozyme, 1 mg proteinase K and 5 μg

Table 1
C. difficile strains isolated from Equidae, their clinical diagnosis and type strains used

C. difficile	*tcd*A PCR	*tcd*B PCR	*cdt*A PCR	Patient, sex, age[a]	Hosp. days	Antibiotics[b]	Clinical diagnosis	Euthanasia	cpm[c]
Clinical strains									
E422/97	−	−	−	horse, G, 2y	7	−	afebrile diarrhoea	−	
D2798/97	−	−	−	horse, G, 8y	0.2	−	duodeno-jejunitis	+	
D877/98	−	−	−	horse, G, 13y	8	P/GM	duodeno-jejunitis	+	
D1456/98	−	−	−	horse, G, 6y	7	P/GM	typhlocolitis	+	
E114/99	−	−	−	horse, M, 12y	0.2	P	colon infarction	+	
D734/99	−	−	−	horse, C, 2d	2	P	febrile diarrhoea, jejunitis, *E. coli* sepsis	−	
D1292/97	+	+	−	horse, M, 26y	0.2	−	jejunal infarction	+	
D2423/97	+	+	−	horse, M, 10y	0.2	−	hepato-cerebral syndrome, afebrile diarrhoea	+	
E283/98	+	+	−	horse, M, 3y	3	−	hepatopathy, afebrile diarrhoea	+	
D1268/98	+	+	−	horse, G, 1y	0.2	−	febrile diarrhoea	+	
E3/99	+	+	−	pony, M, 15y	1	−	jejunal infarction	+	
D306/99	+	+	−	horse, M, 1y	0.2	−	adynamic ileus	+	
E334/99	+	+	−	horse, G, 1y	3	P	afebrile diarrhoea	−	
E315/98	+	+	+	horse, C, 10d	16	P/GM	afebrile diarrhoea	−	
E327/98	+	+	+	horse, M, 14y	9	P/GM	duodeno-jejunitis	+	108 ± 13
E1060/98	+	+	+	horse, G, 4y	3	P	adynamic ileus	+	100 ± 20
E133/99	+	+	+	pony, M, 18y	0.2	−	jejunal infarction	+	
Total 17	11	11	4						
Type strains				Origin[d]					
C. difficile	+	+	−	ATCC43255					2 ± 3
C. perfringens (E)	−	−	+[e]	NCTC8084					395 ± 41

[a]G: gelding, C: colt, M: mare, y: years, d: days.
[b]P: penicillin, GM: gentamicin.
[c]Detection of ADP-ribosyltransferase activity in cpm ± standard deviation.
[d]ATCC: American Type Culture Collection, Rockville, MD, USA; NCTC: National Collection of Type Cultures, London, UK.
[e]*iap* gene.

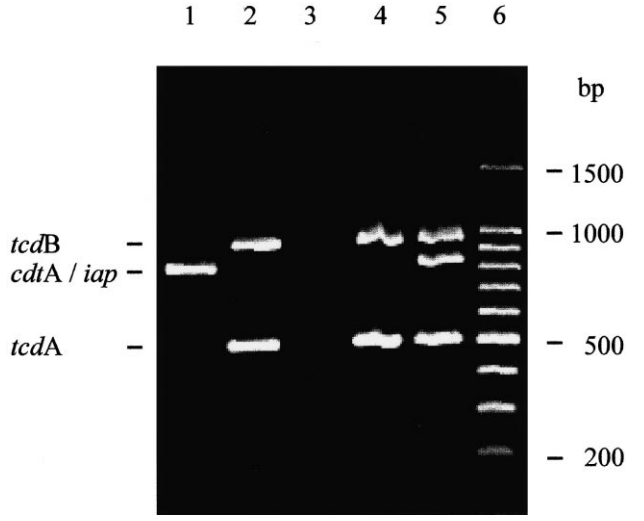

Fig. 1. PCR fragment analysis on an ethidium bromide-stained 1.5% agarose gel. PCR fragments were amplified as described in Section 2. For each analysed strain 5 μl of each PCR reaction (Table 2) were mixed and separated by gel electrophoresis. The corresponding genes of the specific amplification products are indicated at the left. Lane 1: *C. perfringens* type E NCTC8084^T, lane 2: *C. difficile* ATCC43255^T, lane 3: *C. difficile* E114/99, lane 4: *C. difficile* E3/99, lane 5: *C. difficile* E1060/98, and lane 6: 100-bp ladder marker (Promega, Madison, WI, USA).

RNase were added and the solution then was incubated for 1 h at 37°C. 5 μl (125 U) achromopeptidase and 200 μl 2% lauroylsarcosine in 0.05 M Tris and 0.05 M EDTA pH 8 were added and incubated again at 37°C until the solution was clear and viscous. DNA was purified with each 600 μl of PCIA/CIA and then precipitated with 600 μl 2-propanol and subsequent centrifugation for 15 min at 15 000 rpm. The pellet was redissolved in 50 μl water and 3 μl was digested with *Eco*RI or *Hin*dIII and checked on a 0.7% agarose gel for quality and quantification. 100–500 ng of DNA was digested with *Hin*dIII and *Pst*I and analysed on Southern blots. A probe for the specific detection of the *tcd*B gene was synthesised by PCR using the primer pair CDTOXB-L and CDTOXB-R (Table 2), genomic DNA of *C. difficile* ATCC43255 as template and supplementing the reaction with 40 μM digoxigenin-11-dUTP (Boehringer Mannheim).

2.4. ADP-ribosyltransferase assay

Clostridia were cultivated either in TGY (30 g l^{-1} trypticase soy broth without dextrose; BBL/20 g l^{-1} Bacto yeast extract; Difco Laboratories, Detroit, MI, USA/5 g l^{-1} α-D-glucose and 0.5 g l^{-1} L-cysteine hydrochloride pH 7.5; Fluka, Buchs, Switzerland) or in BHI (37 g l^{-1} Bacto brain heart infusion medium; Difco) for 48 h at 37°C. Supernatants were harvested by centrifugation for 10 min at 2500 rpm. The PCR using primer pair CPIOTA-L and CPIOTA-R (Table 2) was used for confirmation of the presence of *cdt*A. ADP-ribosyltransferase assays contained in a total of 20 μl: 50 μM [^{14}C]NAD (specific activity: 12 Ci mol^{-1}); Amersham Life Science, Buckinghamshire, UK/100 mM HEPES pH 7.5; Gibco BRL Life Technologies, Basel, Switzerland and 5 μg G-actin from bovine muscle; Sigma-Aldrich, St. Louis, MO, USA. The reaction was started by adding 10 μl supernatant. The reaction was performed at room temperature for 1.5 h and stopped by addition of 500 μl 10% trichloroacetic acid (TCA). The mixtures were blotted onto filters (GS 0.22 μm, Millipore, Bedford, MA, USA) using a vacuum pump and washed 5 times with 0.75 ml 10% TCA. The filters were air dried and scintillation liquid (Emulsifier scintillator plus, Packard Instrument Company, Meriden, CT, USA) was added. Scintillation was detected on a liquid scintillation counter (Wallac 1410, Pharmacia, Dübendorf, Switzerland). Activity derived from supernatants of *C. perfringens* NCTC8084 was taken as 100%. Activity was measured twice from each of the cultures done in two different growth media resulting in quadruplicates for each strain.

2.5. Detection of C. difficile toxins ToxA and ToxB by ELISA

Assessment of biosynthesis of the protein toxins ToxA and ToxB in *C. difficile* cultures was performed by a commercially available ELISA Kit, TOX A/B TEST (TechLab Inc., Blacksburg, VA, USA). For this purpose *C. difficile* isolates were grown in alkaline trypticase yeast extract medium (pH 8.5) supplemented with 1% mannitol [11] at 37°C for 48 h under anaerobic conditions. Content of

Table 2
Oligonucleotide primers for the toxin gene PCRs

Gene	Name	Sequence	Position	Product length (bp)
*cdt*A, *iap*	CPIOTA-L	5′-AATGCCATATCAAAAAATAA-3′	477–496[a]	821
	CPIOTA-R	5′-TTAGCAAATGCACTCATATT-3′	1 297–1 278[a]	
*tcd*A	AM2 [12]	5′-GGATAGGTGGAGAAGTCAGTG-3′	12 607–12 627[b]	484
	AMB [12]	5′-CCACGATTTAACAACTCCTGAC-3′	13 090–13 069[b]	
*tcd*B	CDTOXB-L	5′-TGGGAAATGACAAAGTTAGA-3′	4 789–4 808[b]	947
	CDTOXB-R	5′-TCACCATCTCCAGGATTATA-3′	5 735–5 716[b]	

[a]On sequences EMBL/GenBank accession number L76081 for *cdt*A and X73562 for *iap*.
[b]On sequence accession number X92982.

Table 3
Detection of *tcd*A, *tcd*B and ADP-ribosyltransferase toxin (*cdt*A) gene by PCR in *C. difficile* derived from various animal patients

		Total	*tcd*A$^-$, *tcd*B$^-$, *cdt*A$^-$	*tcd*A$^+$, *tcd*B$^+$, *cdt*A$^-$	*tcd*A$^+$, *tcd*B$^+$, *cdt*A$^+$
C. difficile ATCC43255		1	0	1	0
C. difficile CD196		1	0	0	1
Cat		4	2	2	0
Dog		41	22	19	0
Equidae:	Horse	15	6	6	**3**
	Pony	2	0	1	**1**
Equidae total		17	6	7	**4**

ToxA and ToxB was then determined from the supernatants using the TOX A/B TEST following the supplier's instructions.

3. Results

3.1. Detection of tcdA and tcdB genes by PCR

For the detection of the *tcd*A gene primers AM2 and AMB (Table 2) were used as described by Chachaty et al. [12] and Saulnier et al. [13]. For a PCR assay that specifically detects the *C. difficile tcd*B gene we designed the primers CDTOXB-L and CDTOXB-R (Table 2). Specificity and sensitivity of the *tcd*B PCR were tested by parallel Southern blot analyses. Genomic DNA of 10 *tcd*B-negative and two *tcd*B-positive strains, as determined by PCR, were analysed using a DIG-labelled *tcd*B probe. The results obtained by Southern blot were in agreement with the results obtained by PCR. In another study PCR results were also compared with results obtained by ELISA using the TOX A/B TEST. A total of 51 *C. difficile* isolates were analysed by PCR and ELISA. Sixteen strains were positive by PCR for the *tcd*A and *tcd*B genes and 14 thereof were shown to produce the toxins A and B as determined by the ELISA assay. None of the PCR-negative strains secreted either ToxA or ToxB.

3.2. Detection of the cdtA gene in C. difficile

For the detection of the *cdt*A gene in *C. difficile* we developed a PCR assay based on primers CPIOTA-L and CPIOTA-R (Table 2) which match segments that are conserved between the *cdt*A gene of *C. difficile* [7] and the *iap* toxin gene of *C. perfringens* type E. This PCR detected the toxin genes in both the *cdt*A-positive *C. difficile* strain CD196 [7] and the iota toxin-positive *C. perfringens* type E strain.

3.3. Screening of clinical C. difficile strains for tcdA/tcdB and cdtA toxin genes

In a second study *C. difficile* isolated from cats, dogs and the Equidae family were analysed by PCR for the occurrence of the *tcd*A/*tcd*B and *cdt*A toxin genes. While two of four isolates from cats and 19 of 41 isolates (46%) from dogs were positive for both *tcd*A and *tcd*B, none was positive for the *cdt*A toxin gene (Table 3). For the Equidae family 11 of 17 isolates (65%) were positive for *tcd*A and *tcd*B. Of interest is that four of the *tcd*A/B-positive strains were also positive for the *cdt*A toxin gene (Table 1). The PCR product of the *cdt*A gene of *C. difficile* strain E1060/98, isolated from a horse, was sequenced and compared with the known sequences on the EMBL/GenBank database. It showed 98.3% identical nucleotide (nt) positions within the corresponding region of the *cdt*A gene of *C. difficile* strain CD196 (nt 518–1240 on EMBL/GenBank accession number L76081). The corresponding amino acid sequences showed 98.3% identity (99.5% similarity).

C. difficile strains isolated from Equidae were associated with a very broad range of gastro-intestinal disease (Table 1). We found no association of the toxin-harbouring strains with sex, age, time of hospitalisation, antibiotic therapy or outcome.

3.4. Expression of ADP-ribosyltransferase activity by cdtA-positive C. difficile strains

Culture supernatants derived from *C. perfringens* NCTC8084 expressing ADP-ribosyltransferase were used as positive control and supernatants derived from *cdt*A-negative *C. difficile* ATCC43255^T as negative control. Non-inoculated medium was used for background determination. The horse isolates *C. difficile* E327/98 and E1060/98 both produced an ADP-ribosyltransferase activity which corresponded to 26% of that measured in *C. perfringens* type E strain NCTC8084 (Table 1). No activity was found in the type strain of *C. difficile* which is devoid of the *cdt*A gene.

4. Discussion

Various PCR assays for the detection of *tcd*A and *tcd*B genes have been proposed [13–15]. In this study we use the method described by Chachaty et al. [12] for the detection of *tcd*A and a newly developed PCR assay using the primers CDTOXB-L and CDTOXA-R which is specific for *tcd*B. Since no assay has been described for the detection of the *cdt*A gene, we developed a PCR assay using con-

served primers CPIOTA-L and CPIOTA-R, which detects both the *cdt*A of *C. difficile* and the *iap* toxin gene of *C. perfringens* type E. An advantage of these three PCR assays is their common annealing temperature of 52°C and extension time of 45 s. In addition, due to their divergence in size, analysis of the products can be done in one lane of an agarose gel. The consistency of the PCR results for *tcd*A/*tcd*B with the results obtained by ELISA for the detection of ToxA/B shows the PCR to be highly reliable.

The three PCR assays were used for screening of clinical *C. difficile* isolates for the presence of *tcd*A, *tcd*B and *cdt*A. In combination the three genes were detected only in Equidae isolates but not in examined canine or feline strains. Interestingly, in all cases the *cdt*A was found to be present in combination with *tcd*A and *tcd*B as was also observed in human isolates [7]. Whereas only one of four human *C. difficile* isolates expressed ADP-ribosyltransferase activity [7] both isolates of horse analysed expressed activity in their supernatants. The activity of the two *C. difficile* supernatants was approximately fourfold less than that of supernatants derived from *C. perfringens* NCTC8084.

All strains were associated with various intestinal disorders. However, the small number of clinical isolates analysed does not allow us to correlate occurrence of toxins with specific clinical diagnosis or with antibiotic therapy (Table 1). As suggested by Perelle et al. [7], CDT is probably not responsible alone for the virulence of *C. difficile*. Whether or not CDT-producing strains correspond to a particular pathogenic *C. difficile* group remains a matter of debate, and will need more samples to be analysed for statistical evaluation.

This is the first report on the occurrence of the ADP-ribosyltransferase toxin gene (*cdt*A) and its corresponding ADP-ribosyltransferase activity in *C. difficile* isolates of animal origin.

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