Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Detection of Different Genotypes of *Clostridium perfringens* in Feces of Healthy Dairy Cattle from China using Real-Time Duplex PCR Assay

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ARTICLE HISTORY

Received: September 11, 2010
Revised: October 23, 2010
Accepted: November 02, 2010

Key words:
Beta2-toxin
Clostridium perfringens
Duplex qPCR
Toxin typing

ABSTRACT

Dual-labeled fluorescence hybridization probe-based multiplex quantitative real-time polymerase chain reaction (qPCR) assay was used for the detection of *Clostridium perfringens* toxin genes alpha (*cpa*), beta (*cpb*), iota (*ia*), epsilon (*etx*), beta2 (*cpb2*) and enterotoxin (*cpe*) directly from the feces of cattle. Fecal samples from 261 lactating cattle, belonging to three dairy herds in Ningxia (China), were examined using the developed assays. The duplex qPCR assay revealed that *cpa*, *etx*, *cpb2* and *cpe* toxin genes were detected in 176 (100%), 15 (8.5%), 142 (80.7%) and 4 (2.3%) of 176 PCR positive samples, respectively. The findings of this study revealed that *C. perfringens* beta2-toxin-producing strains were widely prevalent in lactating cows in Ningxia, possibly playing an important role in *C. perfringens*-associated diarrheal disease.

To Cite This Article: Wang G, J Zhou, F Zheng, G Lin, X Cao, X Gong and C Qiu, 2011. Detection of different genotypes of *Clostridium perfringens* in feces of healthy dairy cattle from china using real-time duplex PCR assay. Pak Vet J, 31(2): 120-124.

INTRODUCTION

Depending on the production of the four major toxins, alpha (α), beta (β), epsilon (ε) and iota (ι), *Clostridium perfringens* are divided into five toxigenic types, A, B, C, D and E. The α toxin is commonly produced by all five types and is a predominant product of *C. perfringens* type A. When properly equipped genetically and placed in an opportune situation, the organism can cause gas gangrene, food poisoning, and gastrointestinal illness in humans, necrotic enteritis in chickens, necrotizing enteritis in piglets, and abomasitis, tympany and hemorrhagic enteritis in calves (Awad *et al*., 1995; Songer, 1996; Sawires and Songer, 2006). The β toxin is a major lethal toxin produced by types B and C and is known to play a major role in the pathogenesis of necrotic enteritis in humans and sheep. The ε toxin is produced by types B and D and is responsible for rapid fatal enterotoxemia in livestock. Finally, the ι toxin is produced only by type E and has been implicated in calf and lamb enterotoxemia. Some *C. perfringens* strains produce an enterotoxin encoded by the gene *cpe*, which cause diarrhea in humans, sheep, goats and pigs (McClane, 1996; Songer, 1996; Sarker *et al*., 1999). Furthermore, in 1997, a formerly unknown toxin (β2) of *C. perfringens* was identified. Strains producing β2 toxin seem to be involved in some cases of intestinal disorders in horses, piglets, dogs and calves (Herholz *et al*., 1999; Manteca *et al*., 2002). Detection of *C. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the development of effective preventative measures.

The classical typing method is based on neutralization of the pathological effect of each major toxin. This is done by using appropriate antisera to neutralize the toxin, which was untreated or incubated with trypsin (Sterne and Batty,1975). An alternative approach is toxin detection by immunologic or molecular methods. However, there is a major problem in using immunological methods, due to the variability of toxin production by *in vitro* cultures of *C. perfringens*. Molecular genotyping methods, which are predominantly polymerase chain reaction (PCR)-based (Hunter *et al*., 1992; Augustynowicz *et al*., 2000), have become the standard for toxin typing of *C. perfringens*. Multiplex quantitative real-time PCR (qPCR) technology allows for simultaneous detection and amplification of parts of specific gene targets, such as *C. perfringens* toxin genes, and provides higher sensitivity over conventional PCR. It

is also less time consuming, as post-PCR processing is not necessary. In this study, the duplex qPCR protocol of Gurjar and Hegde (2007) was chosen in order to determine the presence of α (*cpa*), β (*cpb*), ε (*etx*), ι (*ia*), enterotoxin (*cpe*) and β2 (*cpb2*) toxin genes from *C. perfringens* in the feces of dairy cattle for the purpose of providing direction regarding the correct use of vaccines.

MATERIALS AND METHODS

Fecal samples

Fecal samples (n = 261) were from lactating cattle of three dairy herds in Ningxia (China). Specimens were collected using sterile disposable rectal sleeves. Then the samples were transferred to a centrifuge tube and transported to the Lab in TGY broth (30 g/l casein tryptic peptone, 20 g/l yeast extract, 1 g/l glucose, 1 g/l L-cysteine and 30% glycerol), until required.

Preparation of bacterial DNA and plasmids

Four reference strains of *C. perfringens* (α, ATCC 13124; α, ε, ATCC 3626; α, β, ATCC 51880; and ι, entero, ATCC 27324) were purchased from the American Type Culture Collection. A *C. perfringens* strain that produced the β2 toxin was a field isolate that had been preserved by the Veterinary Etiological Biology State Key Laboratory of China. DNA from the five *C. perfringens* strains was extracted from pure cultures, grown in Reinforced *Clostridium* Medium (RCM; Oxoid, USA) for 20 h at 37°C under anaerobic conditions, using a routine phenol-chloroform extraction method (Pospiech and Neumann, 1995). To obtain standard positive controls and for the determination of analytical sensitivity, the sequences of control strains were amplified with the primers listed in Table 1 and cloned into a pMD18-T Simple Vector (Takara Corporation, Kyoto, Japan) by conventional methods. The nucleotide sequences were verified by sequencing on an ABI PRISM™ 3730XL DNA Analyzer. The plasmids were designated pMDalpha, pMDbeta, pMDbeta2, pMDepsilon, pMDentero and pMDiota. For use in the duplex qPCR assays, the plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen, Basel, Switzerland) and the DNA content measured by spectroscopic analysis and serially diluted 10-fold to determine analytic sensitivity. Aliquots of the diluted plasmid solutions were used as positive controls in the experiment.

Isolation of the bacterial DNA from feces

The second day after specimens were collected, approximately 2–5 g of feces was taken from the TGY broth to a sterile 10 ml centrifuge tube. All fecal samples were examined for the presence of *C. perfringens* toxin genes (α, β, ε, ι, β2, and enterotoxin) using duplex qPCR. All collected fecal samples were subjected to a 4 h pre-enrichment in RCM by inoculating approximately 1 g of feces into 9 ml medium and incubating at 37°C for 4 h. Following incubation, 200 µl aliquots of the 4 h pre-enrichment broth culture were used for DNA purification using a QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen, CA, USA). The eluent from the spin column containing purified DNA was used immediately in PCR assays.

Primer and acid hydrolysis (TaqMan) probes for qPCR

Based on published DNA sequences of *cpb2* (Genbank accession number L77965), a primer pair and acid hydrolysis probe for amplification of *cpb2* were designed with Beacon Designer 5. All primers and probes were synthesized commercially by Invitrogen (Carlsbad, CA, USA). A sequence homology search was conducted using BLAST (http://www.ncbi.nlm.nih.gov/ blast) to confirm the uniqueness of the sequence. The probe and primer sequences and their respective product sizes are shown in Table 1.

Duplex qPCR conditions

The duplex qPCR assays were performed in three reaction tubes, with reaction 1 able to detect genes encoding *C. perfringens* α and β2 toxins, reaction 2 could detect the β and ε toxins, and reaction 3 was used to detect the enterotoxin and the ι toxin. Briefly, each 25 µl duplex PCR mix consisted of 6 µl ddH_2O, 12.5 µl 2× Premix *Ex Taq* (Takara), 0.5 µl each forward and reverse primers (50 pM final concentration) for the appropriate toxin genes, 1 µl (25 pM) of the appropriate TaqMan hybridization probe for each toxin gene and 0.5 µl 50× ROX Reference Dye II. A 2 µl volume of sample lysate (diluted 1:10 in sterile water) was added to each reaction and subjected to qPCR using a Stratagene MX3005P instrument (Stratagene, CA, USA). Thermal cycling conditions comprised an initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, then annealing and extension at 55°C for 1 min. Fluorescence was measured at 515 and 556 nm during the annealing step.

Sensitivity and specificity of duplex assay

The sensitivity and specificity of the duplex qPCR assays were determined according to a previously described method (Gurjar *et al*., 2007), and standard curves were established.

RESULTS AND DISCUSSION

Sensitivity and specificity of duplex qPCR assay

The standard positive plasmid prepared from the reference *C. perfringens* strain, was 10-fold serially diluted covering the range from 10^5 to 10^1 plasmid molecules and used as template to determine the sensitivity of real-time PCR and to construct standard curves by plotting fluorescence values (dR) versus threshold cycle (C_q) produced for *cpa* and *cpb2* genes. Standard curves showed a linear relationship between the fluorescence values and the C_q. The six primer and probe sets produced similar amplification yields and detection of the target genes. The conventional simplex PCRs for *C. perfringens* toxin genes were able to detect 10^4 copies per reaction, the real-time duplex PCRs in this study were always able to detect about 10 copies of toxin genes per reaction (Fig. 1).

Table 1: Primer and probe sequences used in the duplex qPCR assay

Primers and probes	Product Sequence (5′--3′)	size (bp)	Gene	Reference
CPA F	TGCACTATTTTGGAGATATAGATAC			
CPA R	CTGCTGTGTTTATTTTATACTGTTC	128	*cpa*	Gurjar and Hegde (2007)
CPA Pr	FAM-TCCTGCTAATGTTACTGCCGTTGA-TAMRA			
CPB2 F	CGGATTTTCACCATATACCCATTGA			
CPB2 R	CAAGATCCTTAAAGGTAAAATACAG	91	*cpb2*	Present study
CPB2 Pr	HEX-AAGCTCTAATGTCATCCCCCCAC-TAMRA			
CPB F	ATTTCATTAGTTATAGTTAGTTCAC			
CPB R	TTATAGTAGTAGTTTTGCCTATATC	93	*cpb*	Gurjar and Hegde (2007)
CPB Pr	HEX-AACGGATGCCTATTATCACCAACT-TAMRA			
ETX F	TTAACTAATGATACTCAACAAGAAC			
ETX R	GTTTCATTAAAAGGAACAGTAAAC	145	*etx*	Gurjar and Hegde (2007)
ETX Pr	FAM-TGCTTGTATCGAAGTTCCCACAGT-TAMRA			
IA F	CAAGATGGATTTAAGGATGTTTC			
IA R	TTTTGGTAATTTCAAATGTATAAGTAG	89	*ia*	Gurjar and Hegde (2007)
IA Pr	FAM-TTTCATCGCCATTACCTGGTTCAT-TAMRA			
CPE F	AACTATAGGAGAACAAAATACAATAG			
CPE R	TGCATAAACCTTATAATATACATATTC	84	*cpe*	Gurjar and Hegde (2007)
CPE Pr	HEX-FTCTGTATCTACAACTGCTGGTCCA- TAMRA			

F, forward primer; R, reverse primer, Pr, probe; TAMRA, tetramethyl-6-Carboxyrhodamine

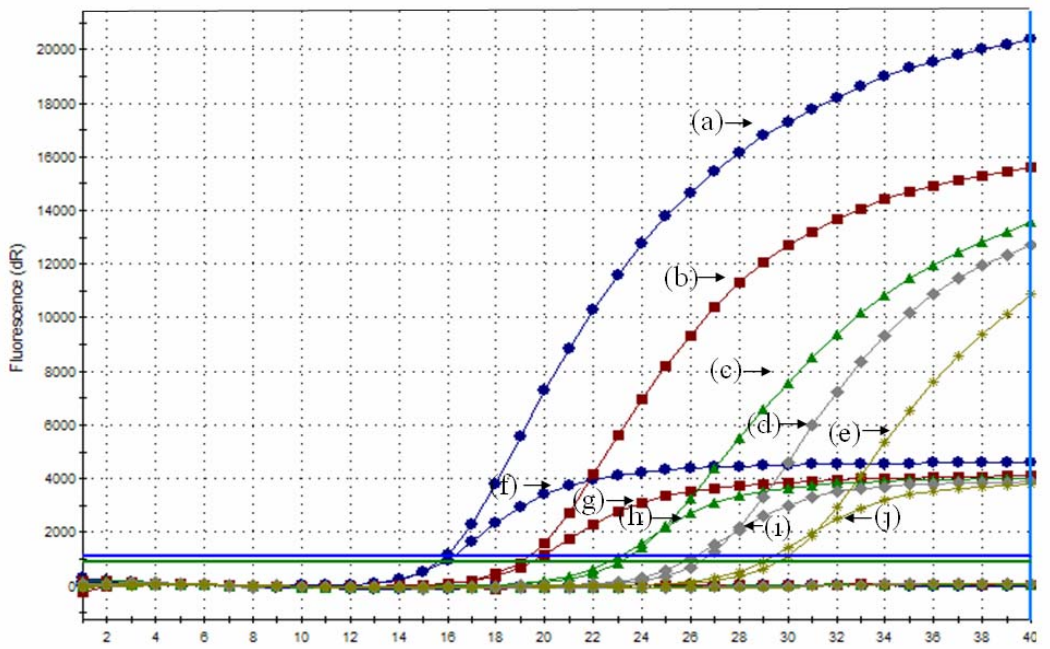

Fig. 1: Amplification curves of duplex real-time PCR for detection *cpa* and *cpb2* gene of *Clostrdium perfringens.* (a)4×10^5 copies of *cpa* DNA,(b) 4×10^4 copies of *cpa* DNA, (c) 4×10^3 copies of of *cpa* DNA ,(d) 4×10^2 copies of *cpa* DNA ,(e) 4×10^1 copies of *cpa* DNA,(f)4.5×10^5 copies of *cpb* DNA,(g)4.5×10^4 copies of *cpb* DNA,(h)4.5×10^3 copies of *cpb* DNA,(i) 4.5×10^2 copies of *cpb* DNA,(j)4.5×10^1 copies of *cpb* DNA.

The assay's specificity was demonstrated by its ability to exclude non-*C. perfringens* and non-clostridial species. The reference *C. perfringens* strain, used as positive controls in establishing the duplex qPCR assay yielded the expected amplification products, whereas, the non-*C. perfringens* and non-clostridial species, such as *C. difficile*, *C.novyi*, *C. septicum*, *Escherichia coli*, *Salmonella typhimurium*, were found negative (Table 2).

Detection of *C. perfringens* toxin-producing strains in feces of dairy cattle

The number of fecal samples where *C. perfringens* was detected and their respective toxin types are shown in Table 3. The duplex qPCR assay revealed that *cpa*, *etx*, *cpb2* and *cpe* toxin genes were detected in 176 (100%), 15 (8.5%), 142 (80.7%) and 4 (2.3%) samples, respectively. Toxin gene typing of *C. perfringens* isolates from 176 PCR-positive fecal samples revealed the presence of toxin type A to be widely prevalent (91.5%), followed by type D (8.5%). The quantification cycle for the duplex qPCR positive samples ranged from 22–36, indicating a wide variation in the bacterial load of toxigenic *C. perfringens* within the feces of lactating cattle.

As reported previously, the majority of enteric pathogens (Belanger *et al*., 2003), including *Clostridia*, are present in very low numbers in environmental samples

such as feces. To improve the detection of *Clostridium* in feces, the samples were cultured in an appropriate pre-enrichment broth. In this study, a pre-enrichment broth comprising RCM supplemented with neomycin (Tansuphasiri *et al*., 2005) was used to enrich fecal samples. The broth inhibited the growth of non-clostridial organisms and allowed for concentration and purification of the target bacteria from crude fecal samples. The multiplex qPCR assay protocol published by Gurjar and Hegde (2007) was used in this study to determine the presence of major toxin genes from *C. perfringens* isolates in the feces of lactating cattle.

Table 2: Specificity of the real-time duplex PCR assay

Reference strains	Toxins					
	cpa	*cpb*	*cpb2*	*etx*	*ia*	*cpe*
C. perfringens ATCC 13124	+	-	-	-	-	-
C. perfringens ATCC 3626	+	-	-	+	-	-
C. perfringens ATCC 51880	+	+	-	-	-	-
C. perfringens ATCC 27324	+		-	-	+	+
C. perfringens beta2	+	-	+	-	-	-
C. difficile	-	-	-	-	-	-
C. septicum	-		-	-	-	-
C. novyi	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-
Salmonella typhimurium	-	-	-	-	-	-

The qPCR assay for detection of major toxin-encoding genes of *C. perfringens* was highly specific. It can detect about 10 copies toxin genes, as described previously, more rapid and considerably more convenient as compared to mouse neutralization tests or other *in vivo* methods. In this study the *cpb2* primers of Gurjar and Hegde (2007) did not amplify any products from isolated and purified DNA despite several optimization attempts and testing with a wild strain. A BLAST search revealed that the most likely reason for this failure was that the *cpb2* gene in the reference strain utilized in this study only demonstrated approximately 70% nucleotide identity with the isolates described by Gurjar and Hegde (2007). Therefore a new *cpb2* primer pair and probe were designed, and the qPCR was then able to detect atypical β2 toxin genes from field isolates.

Table 3: Prevalence and distribution of *C. perfringens* toxin types in feces

Toxin type	Toxin Gene	Cows	
		Positive/total	%
A	α	32/176	18.2
	α+β2	125/176	71
	α+β2+*cpe*	4/176	2.3
D	α+ε	2/176	1.1
	α+ε+β2	13/176	7.4

In this study, only types A and D *C. perfringens* were isolated from the feces of healthy dairy cattle. None of the identified strains carried the *cpb* and *ia* genes. However, the high prevalence of the β2 toxin gene in our isolates was surprising. Of the 176 PCR-positive fecal samples tested by the assay, 80.7% (142) were positive for the presence of the *cpb2* gene. This gene is thought to be associated with increased bacterial virulence in some animals (Gibert *et al*.,1997; Herholz *et al*., 1999; Garmory *et al*., 2000; Schotte *et al*., 2004). Previous studies have reported the isolation of *C. perfringens* harbouring the β2-toxin gene from healthy animals. Bueschel *et al*. (2003) screened 3270 field isolates of *C. perfringens* from various animal species and observed that 37.2% of PCR-positive *C. perfringens* isolates harbouring the *cpb2* toxin gene. They also reported 35.1% prevalence of the type A genotype, harboring the *cpa* and *cpb2* genes. These findings clearly suggest that *C. perfringens* genotypes containing α and β2 toxin genes are widely distributed among animal species. The present study was not designed to assess the prevalence of the β2 toxin gene in a *C. perfringens* population, but its high prevalence in our dairy cattle isolates is noticeable when considering that all cattle analyzed were healthy. The presence of *C. perfringens* strains harbouring *cpb2* in healthy animals may not be considered a risk by itself, but may possibly be considered an emerging threat to animal health if the physiological equilibrium of the intestine and resident microflora is disturbed due to changes in feed or antibiotic therapy (Herholz *et al*., 1999).

In conclusion, our results indicate that the duplex qPCR assay is a rapid and sensitive method for detection of toxigenic *C. perfringens* in fecal samples. Additionally we demonstrated for the first time that *C. perfringens* genotypes corresponding to α, β2, ε and enterotoxin toxin-producing strains were present in the feces of healthy calves raised in China. These initial findings could be further used for epidemiological studies, prophylaxis programs, and to formulate strategies for correct use of vaccines.

Acknowledgments

The authors would like to thank Jizhang Zhou and Guozhen Lin for their valuable technical assistance. Financial support was provided by the Foundation of National Science and Technology Ministry of China (2006BAD04A05; 2008FY210200).

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