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Full Article

Molecular typing of toxigenic *Clostridum perfringens* isolated from sheep in Iran

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ABSTRACT

In this research a molecular method based on polymerase chain reaction for typing of *Clostridium perfringens* was developed and toxin genotypes of 64 isolates from sheep and goats in Iran were determined**.** The PCR assays were developed for detection of alpha (cpa), beta (cpb) and epsilon (etx) toxin genes, allowing classification of the isolates into genotypes A B, C and D. The field isolates were assigned to genotypes A (n=9, 14.07 %), B (n=20, 31.25%), C (n=17, 26.56%) and D (n=18, 28.12%). In this PCR system the fragments of 900, 611 and 402 bp were amplified using specific primers for alpha, beta and epsilon toxins, respectively. The fragments were confirmed by sequencing and blasting in GenBank. The sequence alignment of the fragments showed more than 98% similarity with other related published sequences from other sources. Our results suggest that PCR genotyping is an acceptable tool for in vitro typing of *C. perfringens*.

Keywords: *Clostridium perfringens*, typing, toxin, PCR

INTRODUCTION*

Clostridium perfringens, the spore-forming anaerobic bacterium is widespread in the environment and is frequently found in the intestinal tract of man and animals. It is responsible for gastrointestinal and enterotoxaemic diseases in animals and food-poisoning, gangrene and enteritis necroticans in man (Petit *et al* 1999).

Usually, *C. perfringens* has been classified into five toxigenic types (A through E) on the basis of its ability to produce major toxins (Yoo *et al* 1997). Type A produces only alpha toxin, type B produces alpha, beta and epsilon toxins, type C produces alpha and beta toxins, type D produces alpha and epsilon toxins and type E produces alpha and iota toxins. Each of these five types has been associated with enterotoxaemia in sheep (Nilo 1980, Warren *et al* 1999, Kalender *et al* 2005). Each type of *C. perfringens* can cause different

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diseases. The most commonly encountered type A strain causes gas gangrene (myonecrosis), diarrhea, and food-borne illness in humans (Hatheway 1990). Type B and D strains are causative agents of fatal enterotoxaemia in domestic animals and occasionally humans. The type C strains cause severe necrotic enteritis (pig- bel) in humans. The pathogenecity of type E strains is unclear (Yamagishi *et al* 1997).

Routinely the typing of *C.perfringens* is performed by the mouse neutralization test (MNT) after culture and isolation of the micro-organism (Stern & Batty 1975). This procedure is time consuming, expensive, using live animals and requires antisera for each toxin type which are not currently commercially available. Also, some strains may not produce toxins under laboratory conditions which make typing by MNT impossible (Warren *et al* 1999).

Many researchers have been used PCR method to detect sequences of interest in *C. perfringens* in purpose of identification and classification (Meer & Songer 1997, Baums *et al* 2004, Effat *et al* 2007, Goncuoglu *et al* 2008).

The aim of this study was to develop a PCR method for identification and toxinotyping of *C. perfringens* and to determine the types of field isolates of sheep and goats by this method in Iran.

MATERIALS AND METHODS

Bacterial strains. *C. perfringens* reference strains CPA094 (type A), CPB228 (type B), CPC303 (type C) and CPD409 (type D) were used to set up unique conditions for the PCR. Reference strains of the bacteria were obtained from Anaerobic Animal Bacterial Vaccines Department, Razi vaccine and Serum Research Institute (RVSRI), Karaj, Iran. In order to confirm the specificity of the PCR conditions, *C. chauvoei, C. septicum* and *C. tetani,* (all from our collection) were used, too. The toxin types of 64 field isolates of *C. perfringens* majority from diseased sheep and goats were investigated. The origins and characteristics of the strains and isolates are summarized in Table 1.

Biochemical Characterization. Catalase test, lecithinase activity on egg yolk salt agar, hemolytic activity on sheep blood agar and sugar fermentation (glucose, maltose, lactose, inulin, dulcitol, mannitol, inositol and salicin). The incubated sugar media were incubated anaerobically at 37 °C for 24 h and examined for acid and gas production (Effat *et al* 2007).

The confirmed isolates were smeared onto blood agar plates containing 5% defibrinated sheep blood, and the plates were incubated anaerobically at 37 °C for 24 h. A typical hemolytic colony was picked up and cultured in cooked meat medium. The pure bacterial suspensions were used for biochemical identification tests and DNA extraction.

DNA Extraction. The reference strains and field bacterial strains were cultured in a thioglycolate broth at 37 °C for 48 to 72 h in an anaerobic jar. One ml aliquot of each culture was centrifuged (13000 x g 15 min) and the then pellets were washed twice and resuspended in 200 ul of HPLC-grade water. After boiling for 20 min and centrifugation approximately 5 µl of lysate was used as template for PCR assay. A single colony suspended in 200 µl of HPLC-grade water could be substituted for washed cells obtained from liquid culture and microwaving (10 min at 700 W) could be used instead of boiling.

Primers. The primers which used in this research are presented in table 2. The sequences of the oligonuclotide primers for the toxin genes of *C.perfringens* were as previously described (Baums *et al* 2004, Songer & Meer 1996).

Because cpa (alpha toxin) is presented and amplified in all isolates of *C.perfringens*, it was considered as a confirmation, beyond biochemical properties, colony morphology, and hemolytic pattern of the identity of the isolates.

The expected sizes were 900 bp for the alpha toxin gene, 402 bp and 611 bp for the epsilon and beta toxin genes respectively.

PCR . The PCR reaction mixture (50 µl) contained 5 µl of bacterial lysate as template DNA (100 ng), 0.4 mM dNTPs, 5 µl 10 X PCR buffer, 0.25 µl of 5 U/µl Taq DNA polymerase, 0.4 uM of each primers (10 pmol/µl) and 2 mM MgCl2. The PCR reaction mixture were placed in a Eppendorf PCR thermal cycler.

PCR Condition. The PCR program was run with initial denaturation at 95 °C for 5 min, 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, followed by the final extension for 10 min. Five microlitres of the PCR products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide (0.25 µg/ml) and documented with a gel documentation system.

Sequencing. PCR products for sequencing was purified using the PCR product purification kit (Roche, Germany). All purified PCR products were sequenced by Applied Bioscience, France.

Analysis of the sequence. Searches for sequences in GenBank databases were performed by Blast. The comparison of the sequence alignments was done by Megalign software. The alignments of Iranian isolates were compared to each other and the toxin gene sequences of reference strains in the GenBank.

RESULTS

We examined 64 isolates of *C. perfringens* by PCR and they were readily placed into genotype A (n =9, 14.07%), genotype B (n=20, 31.25%), genotype C (n=17, 26.56%) and genotype D (n=18, 28.12%). Toxin genes were amplified with a single pair of primers based on the toxin types of the reference strains. The PCR consistently produced the predicted band patterns and no unexpected amplicon was observed. All of the *C. perfringens* isolates were re-identified by biochemical tests as described in Bergey's manual. The characteristics of the isolates were positive in fermentation of glucose, lactose, sucrose, and maltose, hydrolysis of gelatin; production of lecithinase and a positive reverse CAMP test results.

The fragment of cpa (alpha toxin) was amplified as a band of 900 bp from all isolates of *C. perfringens* with phenotypes previously determined by mice inoculate method. The bands corresponding to the beta and epsilon toxin genes were approximately 611 and 402 bp long respectively. Results were consistent throughout with repeated examination of isolates yielding the identical pattern. Genotyping results were (100%) in agreement with in vivo typing results, where this information was available.

The amplified PCR products from reference strains were confirmed by analysis of nucleotide sequences and comparison of the sequences with previously reported sequences in GenBank. It was shown that they had more than 98% similarity with the published nuclotide sequences from related genes. The specificity of the PCR was confirmed by specific amplification of the toxin genes from only *C. perfringens*. There was no nonspecific binding of the PCR primers to template derived from other clostridial species such as *C. chauvoei* and *C. septicum*.

As shown in Figure 1, the alpha toxin gene from all types, the beta toxin gene from types B and C, the epsilon toxin gene from types B and D were amplified with the primer pairs listed in Table 2 under unique conditions. It was demonstrated that the PCR could detect up to 10 pg of template DNA in each toxin gene. In comparison of DNA extraction methods, there was no difference in the PCR results, regardless of the culture method.

DISCUSSION

The pathogenecity of the *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon and iota) and other toxins including enterotoxin. The pattern of production of the toxins are different, depending on the *C. perfringens* type. Therefore, the patterns have been used to type the bacterium into types A, B, C, D and E (Songer & Meer 1996).

Table 1. Properties of *C.perfringens* isolates and results of PCR amplification of alpha (cpa), beta (cpb) and epsilon (etx) toxin gens.

Code of study	Animal	Organ	Alpha toxin	Beta toxin	Epsilon toxin	Toxin genotype
073	Sheep	Intestine	+	-	-	A
022	Sheep	Intestine	+	-	-	A
094	Lamb	Intestine	+	-	-	A
093	Cattle	Muscle	+	-	-	A
095	Calf	Muscle	+	-	-	A
105	Cattle	Muscle	+	-	-	A
122	Sheep	Intestine	+	-	-	A
123	Sheep	Intestine	+	-	-	A
020	Dog	Intestine	+	-	-	A
220	Sheep	Intestine	+	+	+	B
213	Lamb	Intestine	+	+	+	B
333	Sheep	Intestine	+	+	+	B
227	Lamb	Intestine	+	+	+	B
214	Sheep	Intestine	+	+	+	B
221	Sheep	Intestine	+	+	+	B
1795	Sheep	Intestine	+	+	+	B
236	Sheep	Intestine	+	+	+	B
222	Sheep	Intestine	+	+	+	B
8071	Sheep	Intestine	+	+	+	B
234	Sheep	Intestine	+	+	+	B
237	Sheep	Intestine	+	+	+	B
215	Sheep	Intestine	+	+	+	B
210	Sheep	Intestine	+	+	+	B
228	Sheep	Intestine	+	+	+	B
238	Sheep	Intestine	+	+	+	B
233	Sheep	Intestine	+	+	+	B
231	Sheep	Intestine	+	+	+	B
207	Sheep	Intestine	+	+	+	B
240	Sheep	Intestine	+	+	+	B
316	Lamb	Intestine	+	+	-	C
314	Sheep	Intestine	+	+	-	C
312	Pig	Intestine	+	+	-	C
305	Sheep	Intestine	+	+	-	C
302	Sheep	Intestine	+	+	-	C
301	Sheep	Intestine	+	+	-	C
356	Sheep	Intestine	+	+	-	C
337	Sheep	Intestine	+	+	-	C
327	Sheep	Intestine	+	+	-	C
326	Goat	Intestine	+	+	-	C
325	Sheep	Intestine	+	+	-	C
324	Sheep	Intestine	+	+	-	C
321	Sheep	Intestine	+	+	-	C
320	Sheep	Intestine	+	+	-	C
335	Sheep	Intestine	+	+	-	C
336	Sheep	Intestine	+	+	-	C
337	Sheep	Intestine	+	+	-	C
413	Sheep	Intestine	+	-	+	D
469	Sheep	Intestine	+	-	+	D
450	Sheep	Intestine	+	-	+	D
456	Sheep	Intestine	+	-	+	D
471	Sheep	Intestine	+	-	+	D
447	Sheep	Intestine	+	-	+	D
440	Sheep	Intestine	+	-	+	D
401	Sheep	Intestine	+	-	+	D
431	Sheep	Intestine	+	-	+	D
432	Sheep	Intestine	+	-	+	D
433	Sheep	Intestine	+	-	+	D
453	Sheep	Intestine	+	-	+	D
444	Sheep	Intestine	+	-	+	D
468	Sheep	Intestine	+	-	+	D
460	Sheep	Intestine	+	-	+	D
401	Sheep	Intestine	+	-	+	D
409	Sheep	Intestine	+	-	+	D
458	Sheep	Intestine	+	-	+	D

Table 2. Target toxin gene, oligonucleotide primer sequences and length of amplification products of the *C.perfringens* PCR.

Toxin Gene	Primers	Sequence(5'-3')	Amplicone	References
Cpa	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	900	Bauma et al (2004)
Cpb	CPBL CPBR	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	611	Bauma et al (2004)
Etx	ETXII-F ETXII-R	5'-TAC TCA TAC TGT GGG AAC TTC GAT ACA AGC-3' 5'-CTCATCTCCCATAACTGCACTATAATTTCC-3'	402	Songer and Meer (1996)

Enterotoxaemia caused by *C. perfringens* depends not only on the presence of the bacteria, but also on other factors that alter the balance in the digestive tract.(Aschfalk *et al* 2002). In sheep, enterotoxaemia has been reported to be produced by all five types *C.perfringens* (Songer 1996, Kalender *et al* 2005), although the role of type A in disease production is considered doubtful by some researchers (Nilo 1980). Detection of *C. perfringens* types in an area is important for the development of the most appropriate vaccines (Kalender *et al* 2005).

The presence of *C. perfringens* infections among sheep and goats in Iran was reported first in 1954 during an outbreak of enterotoxemia among the imported Merino sheep near Tehran. Further studies indicated that the diseases caused by *C. perfringens* types B, C and D were widespread all over the country and had been the cause of a very heavy losses for years. So far the existence of lamb dysentery, pulpy kidney, struck and haemorrhagic enteritis are diagnosed in different parts of the country (Ardehali *et al* 1988).

Thus, detection of *C. perfringens* toxin types is critical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the

development of effective preventive measures (*Baums et al* 2005). The typing of *C. perfringens* strains was originally established based on neutralization of the pathological effect of each major toxin, both trypsin treated and untreated, with appropriate antisera in laboratory animal models (Stern & Batty 1975). This toxin-typing technique requires continuous supply of laboratory animals and use of monovalent diagnostic sera, which are increasingly difficult to find and extremely expensive. Moreover, toxin-typing results are obtained in more than 24 or even 48 h observation. Some strains of *C. perfringens* may not be able to produce in measurable amounts under laboratory conditions and this causes an obstacle for typing by classical methods (Kalender *et al* 2005). PCR has been highlighted as a rapid and accurate method for the detection of low copy numbers of genes. This method is more accurate and faster than seroneutralization with mice and guinea pigs.

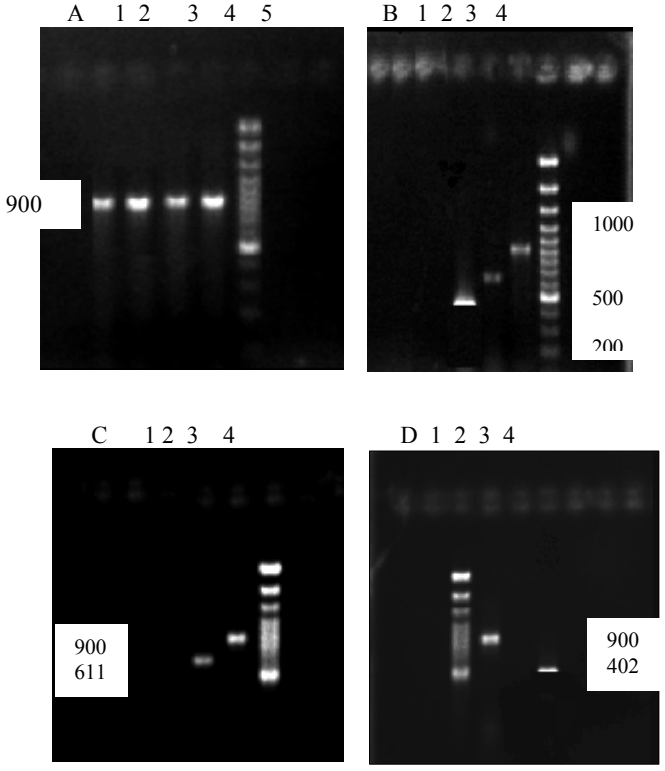

Figure 1. Amplification of *C.perfringens* toxin gens by PCR. Lanes in gel A(genotype A) are: 1-4 (cpa 900bp), lane 5 (Marker) , in gel B (genotype B) are: 1 (etx 402bp), 2 (cpb 611bp) 3(cpa), in gel C (genotype C) are: 1 (etx negative), 2 (cpb) 3 (cpa), 4 (Marker), in gel D: (genotype D) are: 1 (Marker), 2 (cpa), 3 (cpb negative), 4 (etx). The Marker is 100 bp ladder.

In the present study, all of the strains which had been examined by mouse inoculation for the presence of alpha, beta, and epsilon toxins were positive for these toxin genes by PCR. Songer and Mayer (1996) and Yoo et al. (1997) also developed a PCR assay for individual major toxin genes and they found 100% correlation between genotype and toxinogenic phenotype. The identity of the amplified PCR products from reference strains was confirmed by sequencing and by comparison of the sequence data obtained from GenBank.

These results confirmed that the PCR instead of seroneutralization could be used to type the bacterium. In conclusion the PCR that was developed in this study can be used to type *C. perfringens* isolates in epidemiological studies as an alternative to conventional procedures.

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