

Clostridium perfringens isolate typing by multiplex PCR

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Abstract: *Clostridium perfringens* is an important pathogen that provokes numerous different diseases. This bacterium is classified into five different types, each of which capable of causing a different disease. There are various methods for the bacterial identification, many are labor-intensive, time-consuming, expensive and also present low sensitivity and specificity. The aim of this research was to identify the different types of *C. perfringens* using PCR molecular method. In this study, 130 sheep-dung samples were randomly collected from areas around the city of Kerman, southeastern Iran. After processing and culturing of samples, the produced colonies were morphologically studied, gram stain test was also carried out and the genera of these bacteria were identified through biochemical tests. DNA extracted from isolated bacteria for genotyping was tested by multiplex PCR with specific primers. Based on length of synthesized fragments by PCR, toxin types and bacterial strains were detected. *C. perfringens* isolated types were divided as follows: 17.39% type A, 21.74% type B, 34.78% type C and 26.09% type D. It should be emphasized that, up to the present moment, *C. perfringens* type A has not been reported in Iran.

Key words: *Clostridium perfringens*, multiplex PCR, biochemical tests, genetic typing.

INTRODUCTION

C. perfringens has a notorious reputation for possessing an extremely wide toxin arsenal, with over 15 toxins and may be the most widely occurring pathogenic in the globe (1, 2). The organism is therefore capable of producing different pathological pictures and of causing numerous different histotoxic and enteric diseases in both humans and other animals (1). Alpha (α), beta (β), epsilon (ϵ) and iota (ι) comprise the four main *C. perfringens* toxins which form the basis of categorizing this bacterium into five different types, from A to E (3- 5). These different bacterial types provoke diverse diseases and even one strain may cause the infection in numerous hosts (6, 7).

In sheep, enterotoxemia is caused by different toxin types of *C. perfringens* (8). This infection causes considerable economic loss to the sheep industry due to high fatality rates, increased treatment costs, and decreased productivity (9). On the other hand, domestic animals are known to be sources of human food poisoning. In order to reduce or eliminate this risk, strategies must be developed to diagnose and prevent infected animals from entering the food chain (10).

In some laboratories, a serum neutralization test on mice or guinea pigs is employed to determine and diagnose bacterial toxins (11). This method is tedious, time-consuming, expensive and monovalent. Furthermore, it is improper and unethical to apply it at the expense of laboratory animals (10-12).

According to Timoney *et al.* (13), the antitoxin ϵ was measured in sheep blood sera by ELISA, which proven to be a specific, quick and economical method that may replace the serum neutralization test. ELISA utilizes polyclonal antibodies to identify *C. perfringens* toxins (14). However, its disadvantage is that the interaction reaction among the produced antibodies works against the toxins, which may difficult the identification of toxin types. Moreover, ELISA falls short of identifying β_2 toxins and in order to identify toxins from spore-forming bacteria, it must be activated by means of special culture methods (15).

Biochemical tests are also incapable of distinguishing different types of *C. perfringens* (16). PCR is the most modern practical technology in diagnosing infectious diseases and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable (12, 14). PCR allows a faster bacterial identification directly from clinical samples (17). It should be noted that up to this moment, no research on this subject has been carried out in Iran.

MATERIALS AND METHODS

In the present study, sheep dung samples were obtained from 130 Kermani sheep, from nine locations in southeastern Iran. The sheep were from both genders and their ages ranged from 1 to 2.5 years. The samples were collected aseptically in sterile plastic bags and transferred to the laboratory within 1 to 2 hours, after which they were accordingly processed. Samples were diluted in PBS (1:10), the bath temperature was maintained at 80°C for ten minutes in order to eliminate the non-spore-forming bacteria, subsequently they were cultivated on 5% sheep blood agar and anaerobically incubated using Anoxomat® (Mart Microbiology, Netherlands) at 37°C for 48 hours.

The suspected colonies were identified by characteristic colony morphology, gram staining and biochemical tests as described by MacFaddin (16). Biochemical identification consisted of gelatin and lecithin hydrolyses; and catalase, lipase and motility tests. Moreover, indole production, litmus milk reaction and carbohydrate fermentation (sucrose, glucose, lactose and maltose) were carried out in order to identify the bacterial genus according to MacFaddin (16) (Table 1).

Table 1. Biochemical tests for identification some clostridia species

Biochemical tests	Catalase test	Egg yolk agar		Gelatin hydrolyzed	Indole produced	Carbohydrate fermentation				Motility	Milk reaction
		Lecithinase produced	Lipase produced			Glucose	Lactose	Sucrose	Maltose		
<i>C. perfringens</i>	-	+	-	+	-	+	+	+	+	-	dc
<i>C. baratii</i>	-	+	-	-	-	+	+w	+	+w	-	c
<i>C. absonum</i>	-	+	-	+	-	+	+	+	+	±	c
<i>C. bifermentans</i>	-	+	-	+	+	+	-	-	-w	+	d
<i>C. sporogenes</i>	-	-	+	+	-	+	-	-	-w	±	d
<i>C. leptum</i>	-	-	-	-	-	-	±	±	+	-	-
<i>C. aurantibutyricum</i>	-	-	+	+	-	+	+	+	+	+	c
<i>C. sporosphaeroides</i>	-	-	-	-	-	-	-	-	-	-	-
<i>C. symbiosum</i>	-	-	-	-	-	+	±	-	-	±	-c
<i>C. scatologenes</i>	-	-	-	-	±	+	-	-	-	+	-
<i>C. ramosum</i>	-	-	-	-	-	+	+	+	+	-	c
<i>C. sordellii</i>	-	+	-	+	+	+	-	-	+w	±	d

*c: curd; d: digestion; dc: first digestion then curd; w: weak.

DNA Extraction and PCR

Strains of *C. perfringens* types A, B, C, D and E were considered positive controls while *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. leptum* and *C. ramosum* were the negative controls. Isolated strains were cultured on blood agar and total DNA was extracted as described by Sambrook and Russell (18). Two or three colonies that grown on blood agar were suspended in 350 µL of STET buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 5% Triton® X-100) in 1.5-mL microtubes. Then, 25 µL of lysozyme (10 mg/mL) was added to each solution and the content was mixed. Subsequently, the microtubes were placed in a boiling water bath for 40 seconds.

The bacterial lysate was centrifuged at 13,000 rpm for 15 minutes at room temperature in a Microfuge® refrigerated microcentrifuge (Beckman Coulter, USA). After that, the supernatant was poured into a fresh microcentrifuge tube and the nucleic acids were precipitated from it by adding 40 µL of 2.5 M sodium acetate (pH 5.2) and 420 µL of isopropanol. Precipitated nucleic acids were recovered by centrifugation at 13,000 rpm for ten minutes at 4°C. Next, the supernatant was removed and the pellet of nucleic acid was rinsed

with 1 mL of 70% ethanol at 4°C. Finally, the pellet was dried and resuspended in 50 µL of TE buffer containing RNase.

Four specific primer sets corresponding to each toxin of *C. perfringens* were evaluated for their ability to detect *C. perfringens* and to discriminate between *C. perfringens* and other clostridia species (Table 2), according to Komoriya *et al.* (19) and van Asten *et al.* (20). The PCR was performed in a Thermal Cycler® (Bio-Rad, USA) in a total reaction volume of 50 µL containing: 5 µL of 10x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), 2 µL 50 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate, 5 U of Taq DNA polymerase, 100 pmol of primers and 5 µL of template DNA.

Amplification was obtained with 35 cycles following an initial denaturing step at 95°C for ten minutes. Each cycle comprised denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and synthesis at 72°C for 90 seconds. The final extension step occurred at 72°C for ten minutes. Then, 10 µL of the amplified product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

Table 2. Primers employed in multiplex PCR

Toxin	Gene	Sequence 5'-3'	Primer position (bp)	Amplicon (bp)
α	plc (cpa)	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGAAG	663-968	324
β	cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	871-1045	196
ε	etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCTACTAAC	267-862	655
ι	iap	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	1739-2161	446

RESULTS

The isolated *C. perfringens* comprised 30% of the samples, including *C. perfringens*, *C. baratii*, *C. absonum*, *C. bifermentans*, *C. sporogenes*, *C. leptum*, *C. aurantibutyricum*, *C. sporosphaeroides*, *C. symbiosum*, *C. scatologenes*, *C. ramosum* and

C. sordellii. The DNA extracted from all bacterial strains was identified by biochemical tests and standard strains. The quality of the extracted DNA was examined through 1% agarose gel and confirmed.

PCR was carried out with four sets of specific primers that were complementary to a fragment

of α , β , ϵ and ι encoding genes of *C. perfringens*. Primer annealing of complementary genes and synthesization of the fragment of interest revealed a specified toxin gene. Thus, different types were identified in accordance with their toxins. The toxin encoding gene was amplified by means of single PCR, the fragment of 324 bp belonged to the α toxin gene, shared by all the types. The 196 bp fragment, on the other hand, was from the β toxin encoding gene, existing in types B and C. The 655 bp fragment was from the ϵ toxin encoding gene and is observable in type D. The ι toxin encoding gene found in type E generates the fragment of 446 bp (Figure 1). PCR results corresponding to positive and negative controls are displayed in Figure 2. Out of 23 *C. perfringens* types, isolated by biochemical tests, four (17.39%) were type A, five (21.74%) were type B, eight (34.78%) were type C and six (26.09%) were type D, with type C as the dominant one (Figure 3).

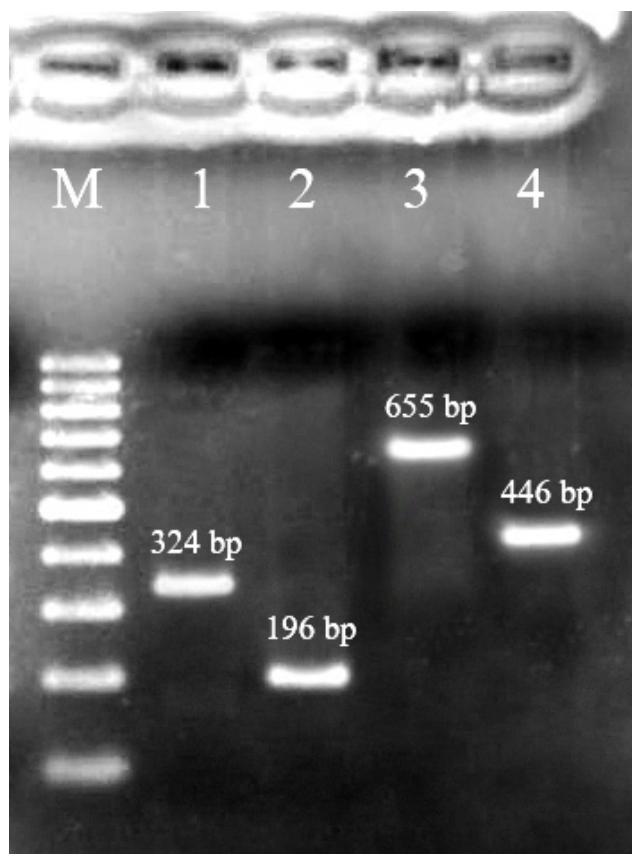


Figure 1. Detection of *C. perfringens* toxin genes amplified by single PCR. M: marker (DNA ladder, 100 bp); lane 1: a toxin encoding gene; lane 2: β toxin encoding gene; lane 3: ϵ toxin encoding gene; lane 4: ι toxin encoding gene.

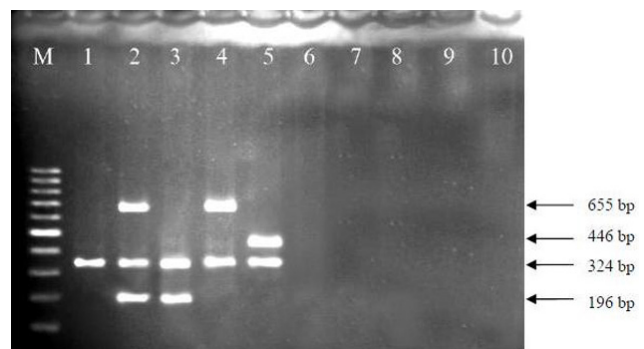


Figure 2. Standard strains, positive and negative controls. M: marker (DNA ladder, 100 bp); lanes 1 to 4: positive controls, standard strain of *C. perfringens* types A, B, C and D; lanes 5 to 9: negative controls, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. leptum* and *C. ramosum*.

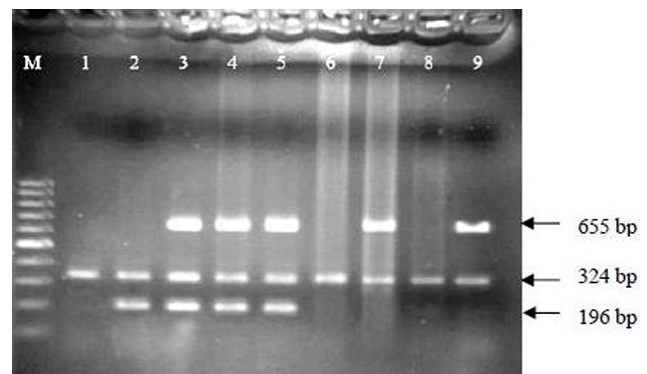


Figure 3. Agarose gel electrophoresis of PCR products obtained from *C. perfringens* isolated from Kermani sheep dong sample. M: marker (DNA ladder, 100 bp); lanes 1, 6 and 8: *C. perfringens* type A isolates; lane 2: *C. perfringens* type C isolates; lanes 3 to 5: *C. perfringens* type B isolates; lanes 7 and 9: *C. perfringens* type D isolates.

DISCUSSION

Toxins of *C. perfringens* are responsible for enterotoxaemia in sheep (21). Furthermore, it has been reported that the infection, which causes considerable economic losses to the sheep industry, may be provoked by all five types of *C. perfringens* (2, 11). It is well known that vaccination against *C. perfringens* comprises the main prophylactic measure to reduce losses or minimize the severity of enterotoxaemia (22). The vaccines should provide adequate protective

immunity against all *C. perfringens* types of a certain area, since other diseases that affect humans and domestic animals originate in this bacterium (11, 23).

Biological gauge comprises one of the important methods of identifying *C. perfringens*; however, it presents several disadvantages including the long time the test takes (24 to 72 hours) (13). Another limitation consists of non-specific deaths that may lead to false diagnosis. Moreover, some *C. perfringens* strains are incapable of producing toxins in measurable amounts under laboratory conditions, which creates an obstacle for typing by classical methods (21).

Sensitivity and specificity are the two main characteristics of an efficient and practical technique, two qualities that PCR presents. Rapidity constitutes one of the major advantages of this method, so that bacterium identification and type determination lasts no more than four hours. Hence, the toxicogenic strain in the sample can be identified by means of a rapid evaluation by the PCR technique before it produces toxin.

In the current study, *C. perfringens* types A, B, C, and D were isolated from sheep-dung samples, with type C being the most frequent in Kerman. Therefore, it is recommended that vaccination against enterotoxemia in this province should provide adequate immunity, especially against *C. perfringens* types A and C. Furthermore, enterotoxemia in sheep is asymptomatic; thus, molecular detection of *C. perfringens* in farms may be useful for prophylaxis.

Up to our knowledge, no similar research has been conducted in Iran and the findings of the present study disagree with those from different countries (3, 7, 21, 24). The difference comprises the fact that the existence and emergence of some bacterium types is closely related to geographic features, consequently several regions may totally lack one or more types of a certain bacterium. For instance, *C. perfringens* type A has been reported as most prevalent in North America, while types C, D and E are uncommon. Moreover, type A constitutes 97% of isolates in Belgium (8). Although previous studies suggested that types A and E did not exist in Iran, the present study detected and identified type A for the first time in Kerman, southeastern Iran (13).

Primers used in this study were perfectly

specific, only complementary to *C. perfringens* encoding toxin gene. As Figure 2 demonstrates, these primers do not identify other strains. In addition, PCR showed to be highly sensitive, so that the reaction was accomplished successfully with a small amount of DNA (less than 1 µL).

In general, molecular methods provide a new insight into bacterial classification. They offer essential genetic information about the organism of interest, which comprises one of the most practical and helpful aspects of PCR. As PCR employs specific primers, an individual species or strain may be traced among different species or types of organisms (17). Compared to other identification techniques that are based on large amounts of samples, PCR is safer for researchers; since sample preparation for PCR starts with cell lysis and DNA extraction. It is unnecessary to say that the cell cannot survive this stage and loses its pathogenic property (17). In conclusion, PCR has become an essential research and diagnostic tool, being a powerful technique with a vast and increasing range of applications.

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CONFLICTS OF INTEREST

There is no conflict.

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