Original article

DETECTION OF *CLOSTRIDIUM PERFRINGENS* TYPES THROUGH GENETIC PROFILING AND MOUSE NEUTRALISATION TEST

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Summary

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The aim of this study was to detect the types of *C. perfringens* using PCR and mouse neutralisation test (MNT) and find the genetic profile of virulence factors tpeL and netB isolated from animal samples in Iran. For this purpose, 46 *C. perfringens* isolates were tested using MNT. Detection was also performed using 16S rRNA-PCR and multiplex PCR for alpha, beta, epsilon and iota toxins. For *tpeL* and *netB* gene profiling, separate PCR experiments were carried out. MNT detected that all isolates were *C. perfringens* type A. The same results were obtained in 16SrRNA-PCR and multiplex-PCR. Based on the obtained results, the molecular test was 100% consistent with MNT. Samples were positive for *tpeL* and *netB* genes in 52.1% and 60.8% isolates, respectively. Overall, four genetic virulence factor profiles were found in *C. perfringens* type A isolates.

Key words: alpha toxin, *Clostridium perfringens*, netB, PCR, tpeL

INTRODUCTION

Due to the growing population and the increasing demand for protein sources, animal and poultry diseases, especially gastrointestinal diseases, have become important. *Clostridium* plays an important role in pathogenesis causing heavy economic losses in livestock industry. *C. perfringens* is the causative agent of food poisoning and gas gangrene, enteritis in animals and necrotic enteritis in poultry. *C. perfringens* are Gram-positive, anaerobic and spore-forming rod shaped bacteria producing major and minor toxins. These bacteria are divided into several types based on the possession of main toxins including alpha, beta, epsilon and iota (Rood *et al*., 2018). The role of other toxins such as netB and tpeL is not completely clear (Popoff & Bouvet, 2009). Previous research has shown the prevalence of *C. perfringens* especially type A from several sources including cattle (Hosseinzadeh *et al*., 2018), sheep (Gharibi *et al*., 2017; Ahmadi Rahnemoon *et*

al., 2020), ostrich (Zandi *et al*., 2014; Ezatkhah *et al*., 2016) and dogs (Sedigh *et al*., 2015) over the years in Iran.

Multiple tests have been used for the diagnosis of *C. perfringens*, such as culture and biochemical tests and ELISA (Naylor *et al*., 1997). Culture usually has low sensitivity and is time consuming. Although ELISA has been extensively used, it may not detect some infection cases (Mohiuddin *et al*., 2020). The mouse neutralisation test (MNT) is known as a gold standard for the toxin typing (Uzal *et al*., 2003), and has been commonly used for detection of toxins (Sterne & Batty, 1973; Nagahama *et al*., 1991). MNT has some ethical disadvantages, is costly and time consuming (Guerra *et al*., 2016). So, attempts to introduce new approaches as suitable alternative methods were made. Polymerase chain reaction (PCR) is widely used for detection of *C. perfringens* (Settanni & Corsetti, 2007; Coursodon *et al*., 2012), with high sensitivity and specificity (Meer & Songer, 1997). Because of the importance of human protein foodstuffs for clostridial growth and the necessity to prevent foodborne microbial infections, the study of diseases caused by *C. perfringens* is always important. Also, the gene profiles of *tpeL* and *netB* virulence factors in *C. perfringens* isolated from animals are very important to clarify and better understand their role in pathogenicity.

The aim of this study was to detect *C. perfringens* in animals isolates from Iran, to perform genetic profiling of virulence factors by PCR and compare them with MNT as a gold standard.

MATERIALS AND METHODS

Bacterial isolates and reference strains

In this study, 46 isolates of *C. perfringens* from livestock, poultry, and fish samples, previously detected by microbiological and biochemical methods, were used for analysis. Also, the reference strains *C. perfringens* type B (CN228), *C. perfringens* type C (CN301), *C. perfringens* type D (CN409), *C. perfringens* type E (CN1241) and *C. perfringens* type A were used as positive controls; *C. septicum* (CN913) and distilled water were used as negative controls. Isolates were inoculated in liver medium and incubated for 24 h under anaerobic conditions.

Mouse neutralisation test

Strains and isolates were cultured overnight in fresh toxigenic media under anaerobic conditions and the media were centrifuged at 4500 rpm for 15 minutes. Then, the supernatants were divided into two series. One series was treated with trypsin (Sigma, Germany) at 37 °C for 45 min, and the other was not trypsinised. Each of the two series was mixed with *C. perfringens* antitoxin types α, β and ε and normal saline. Seven groups were considered: treatment #1: with 1 mL toxin + 0.6 mL normal saline, treatment #2: with 1 mL toxin + 0.4 mL normal saline + 0.2 mL antitoxin α, treatment #3: with 1 mL toxin + 0.2 mL normal saline + 0.2 mL antitoxin α + 0.2 mL antitoxin β, treatment #4: with 1 mL toxin + 0.2 mL antitoxin α + 0.2 mL antitoxin β + 0.2 mL antitoxin ε, treatment #5: with 1 mL tripsinized toxin + 0.6 mL normal saline, treatment #6: with 1 mL tripsinised toxin + 0.4 mL normal saline + 0.2 mL antitoxin α and treatment #7: with 1 mL tripsinised toxin + 0.2 mL normal saline + 0.2 mL antitoxin α + 0.2 mL antitoxin ε. Samples were incubated at room temperature for 30 minutes. Two NMRI (Naval Medical Research Institute) mice (17–22 g body weight) were injected intravenously with 0.5 mL of each sample and

were monitored for 72 h. Death cases were recorded (Uzal *et al.*, 2003).

Ethical approval

Research regarding mouse injections was performed in compliance with the Animal Welfare Act and Regulations following the principles in the Guide for the Care and Use of Laboratory Animals.

PCR profiling

For DNA purification, cultures of isolates and reference strains of *C. perfringens* were centrifuged at 4000 rpm for 15 min and extraction was performed using the phenol/chloroform method (Pilehchian Langroudi *et al.*, 2011). Quality and quantity of DNA were analysed by Nanodrop Spectrophotometer and electrophoresis on 1% agarose/TAE gel.

Strains were confirmed as *C. perfringens* by amplification of a specific 231 bp fragment of *16s rRNA* gene. Then, in order to detect types of *C. perfringens* and the genetic profile of virulence factors, PCR was performed using specific primers for major toxins of alpha, beta, epsilon and iota and minor toxins of netB and

Tpel (Table 1). The primers for major toxins were used simultaneously in a multiplex PCR at a final volume of 50 μL containing 5 μL DNA template, 5 μL PCR buffer (10×), 1 μL dNTP (10 mM), 2 μL MgCl$_2$ (50 mM), 1 μL *Taq* DNA polymerase (5 units/μL), 1 μL of each primer (10 pmol/μL) and distilled water. The thermocycler (Qantarus) regimen was as followed: initial denaturation at 95 °C for 10 min, 35 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 10 min.

Each of the minor toxins (*NetB* and *TpeL*) and the pre-mentioned *16s rRNA* gene was amplified in a separate PCR at a final volume of 25 μL containing 1 μL DNA template, 2.5 μL PCR buffer (10×), 0.5 μL dNTP (10 mM), 0.75 μL MgCl$_2$ (50 mM), 0.4 μL *Taq* DNA polymerase (5 units/μL), 1 μL of each primer (10 pmol/μL) and distilled water. The reaction condition for *16s rRNA* was as followed: 95 °C for 2 min, 45 cycles of 95 °C for 20 s, 63 °C for 30 s and 72 °C for 45 s, and a final step at 72 °C for 15 min. The following regimen was considered for *NetB* and *TpeL*: denaturation at 94 °C

Table 1. Sequences of oligonucleotide primers, gene and PCR product length

Primer	Sequence	Product size (bp)	Reference
16S rRNA	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	231	Song *et al.*, 2004
cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	Van Asten *et al.*, 2009
cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	Van Asten *et al.*, 2009
etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	Van Asten *et al.*, 2009
iap	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	Van Asten *et al.*, 2009
netB	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	384	Keyburn *et al.*, 2008
tpeL	ATATAGAGTCAAGCAGTGGAG GGAATACCACTTGATATACCTG	466	Coursodon *et al.*, 2012

for 2 min, 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, and a final extension at 72 °C for 12 min. All amplified fragments were electrophoresed in 1% agarose gels and visualised under UV light.

RESULTS

MNT results confirmed that all isolated strains belonged to *C. perfringens* type A.

PCR results indicated that all isolates were positive for the 231 bp fragment of PCR *Clostridium* cluster I gene primers (*16SrRNA* gene), again confirming them as *C. perfringens* strains (Fig. 1).

Multiplex PCR resulted in amplification of only one 324 bp fragment belonging to *cpa* (alpha toxin) in all Iranian isolates (Fig. 2B). The reference strain *of C. septicum* was negative for the 324 bp fragment pointing to the specificity of *cpa* primers (Fig. 2A).
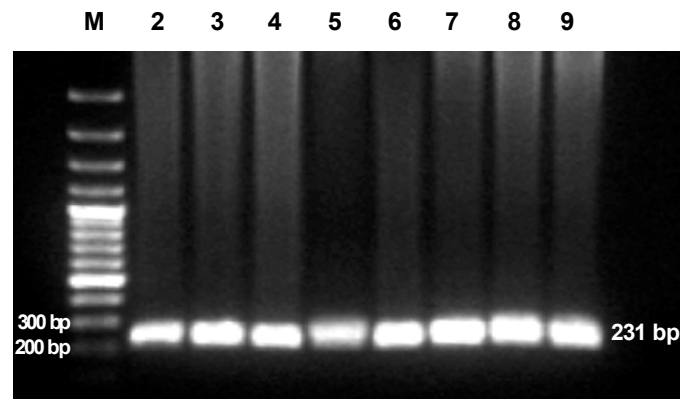

Fig. 1. 16S rRNA PCR for detection of *C. perfringens.* Lane M: 100 bp marker; lanes 2–9: field isolates.
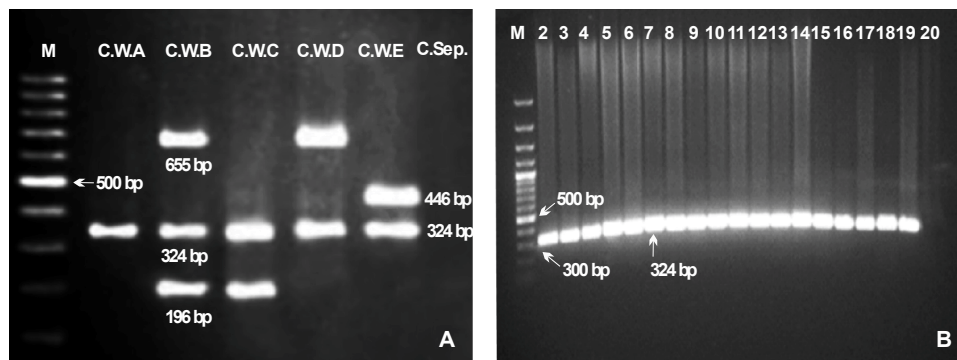

Fig. 2. Multiplex PCR for detection of *C. perfringens* types. **A.** Lane M: 100 bp marker; lane C.W.A: *C. perfringens* type A; lane C.W.B: *C. perfringens* type B; lane C.W.C: *C. perfringens* type C; lane C.W.D: *C. perfringens* type D; lane C.W.E: *C. perfringens* type E; lane *C. sep:* negative control; **B.** Lane M: 100 bp marker; lanes 2–18: field isolates; lane 19: *C. perfringens* type A (positive control); lane 20: *C. septicum* (negative control).
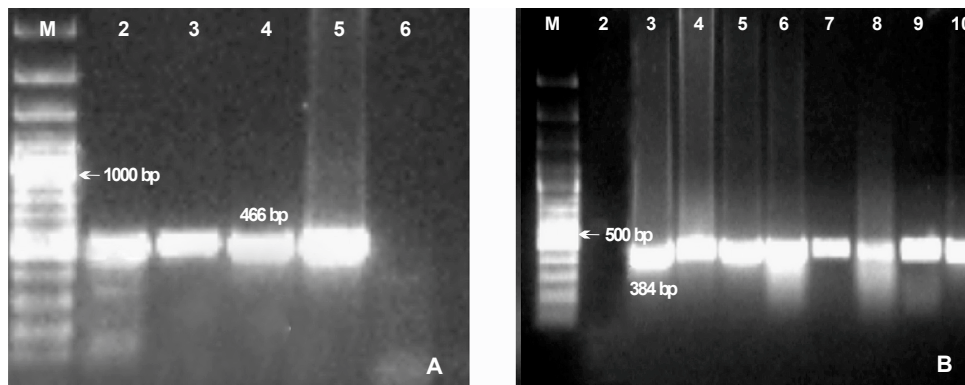
Fig. 3. PCR for detection of *tpeL* and *netB* genes. **A.** Lane M: 100 bp marker; lanes 2–4: field isolates; lane 5: *C. perfringens* type A field isolate (positive for *tpeL* gene); lane 6: distilled H₂O (negative control); **B.** Lane M: 100 bp marker; lane 2: distilled H₂O (negative control); lanes 3–9: field isolates; lane 10: *C. perfringens* type A field isolate (positive for *netB* gene).

PCR results were positive in 24 (52.1%) and 28 isolates (60.8%) for *tpeL* and *netB* gene, respectively (Fig. 3A,B). Overall, four genetic profile of virulence factors were found among Iranian isolates, namely: *tpeL⁺ netB⁺* (n=19), *netB⁺ tpeL⁻* (n=8), *netB⁻ tpeL⁺* (n=5) and *netB⁻ tpeL⁻* (n=14).

DISCUSSION

Clostridium spp. are recognised as the causative agents of many important diseases in humans and animals (Carter *et al*., 2014). *C. perfringens* type A produces several virulence factors, including alpha toxin, CPE, PFO, TpeL and NetB (Amimoto *et al*., 2007; Keyburn *et al*., 2010; Uzal *et al*., 2010). In spite of great economic losses, limited studies have been conducted to identify *C. perfringens* types in samples isolated from disease cases in Iran so far. Nowadays, several molecular tests are used for diagnosis. Al-Khaldi *et al*. (2004) carried out a multiple-oligonucleotide microarray hybridisation test for specific detection of *C. perfringens* types on isolates. Gurjar *et al.* (2008) also used real-time multiplex PCR on dairy cow samples for fast typing. In the present study, molecular tests were performed to identify and characterise *C. perfringens* types in 46 Iranian isolates. This will provide information for the prevalence of clostridial diseases and future control programmes in Iran. In several studies, *C. perfringens* have been identified using PCR approach. So, in this study, we used *Clostridium* cluster I gene primers (*16S rRNA*) for confirmation of *C. perfringens,* which was previously detected by microbiological and biochemical methods. PCR-based techniques are commonly being used for toxinotyping (Yoo *et al*., 1997; Van Asten *et al*., 2009; Hadimli *et al*., 2012). The results of multiplex PCR showed that all isolates and strains were *C. perfringens* type A, which was in accordance with other previous studies in different parts of Iran (Poursoltani *et al*., 2014; Sedigh *et al*., 2015; Ezatkhah *et al*., 2016). *Clostridium perfringens* type A is regarded as the most

frequent type isolated from animal intestinal tract worldwide (Yoo *et al*., 1997; Petit *et al*., 1999; Greco *et al*., 2005).

MNT has long been used and is known as a gold standard for the typing of toxin (Sterne & Batty, 1973; Nagahama *et al*., 1991). The test was used in this study for *C. perfringens* typing. Based on the obtained results, the designed multiplex PCR was 100% consistent with MNT. Songer & Meer (1996) and Yoo *et al*. (1997) also reported 100% correlation between genotype and toxinogenic phenotype in line with our study.

MNT has some disadvantages in terms of ethics and it is costly and time consuming (Guerra *et al*., 2016). So, attempts to introduce new approaches as suitable alternative methods are fully justified. The specificity of the multiplex-PCR was checked by samples of *C. septicum* strain and distilled water. The results confirmed the specificity of the primer. Due to high sensitivity and specificity of the multiplex-PCR, it is suggested as a proper alternative method for typing *C. perfringens*.

Our results showed that *tpeL* and *netB* genes were present in more than half of the cases. Keyburn *et al*. (2008) reported the *netB* gene in 77% of cases. Martin & Smyth (2009) also showed the presence of *netB* gene in 58.3% and 8.6% of poultry with necrotic enteritis and healthy poultry respectively. Abildgaard *et al*. (2010) showed that this gene existed in 60% of healthy poultries. Poursoltani *et al*. (2014) also reported *netB* and *tpeL* genes in 83.33% and 50% of cases, respectively. Nagahama *et al*. (2011) developed a PCR based method using *tpeL* gene primers in *C. perfringens* type A samples. Coursodon *et al*. (2012) showed that the *tpeL* gene played an important role in necrotic enteritis in poultry. Another study revealed that *netB* gene was present in

17.78% of necrotic enteritis-suspected cases (Ezatkhah *et al*., 2016). However, Sedigh *et al*. (2015) reported no positive cases for *netB* and *tpeL* genes in dogs with diarrhoea and healthy dogs.

Different studies have indicated the important role of *C. perfringens* types especially type A in contamination of meat and meat products and its transmission to humans (Afshari *et al*., 2015). Hence, reliable, specific, and quick methods such as PCR based approaches are the goal in food safety (Rodríguez-Lázaro *et al*., 2007) in order to reduce the bacterial transmission to humans. Also, identification of genetic profiles of virulence factors in *C. perfringens* isolates would be very helpful to clarify the role of *tpeL* and *netB* genes in contributing to pathogenic capacity.

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