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

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**Summary**


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 (Gharib et al., 2017; Ahmadi Rahnemoon et
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Multipe 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 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 tcpL 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 trypsinized toxin + 0.6 mL normal saline, treatment #6: with 1 mL trypsinised toxin + 0.4 mL normal saline + 0.2 mL antitoxin α and treatment #7: with 1 mL trypsinised 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 MgCl₂ (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 MgCl₂ (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>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>16S rRNA</td>
<td>TACCHRAGGAGGAAGCCAC GTTCTTCCTAAATCTCTACCGCAT</td>
<td>231</td>
<td>Song et al., 2004</td>
</tr>
<tr>
<td>cpa</td>
<td>GCTAATGGTACTGCGGTGTA CCCTGATGCTACGTTGAAG</td>
<td>324</td>
<td>Van Asten et al., 2009</td>
</tr>
<tr>
<td>cpb</td>
<td>GCAGAAATGCTGAATCATCTAG CGAGAACATTAGTATATCTTC</td>
<td>196</td>
<td>Van Asten et al., 2009</td>
</tr>
<tr>
<td>ext</td>
<td>GCAGTTGATATCCATCTATTC CACTTACTTGTCCCTCTAAC</td>
<td>655</td>
<td>Van Asten et al., 2009</td>
</tr>
<tr>
<td>iap</td>
<td>ACTACTCTCAAGACAAGAGAC CGTTCCTTCATTACTATACG</td>
<td>446</td>
<td>Van Asten et al., 2009</td>
</tr>
<tr>
<td>netB</td>
<td>GCTGGTGCTGGAATAAATATGG CTGCCATGAGGTAGTGTTC</td>
<td>384</td>
<td>Keyburn et al., 2008</td>
</tr>
<tr>
<td>tpeL</td>
<td>ATATAGAGTCAAGCAGTGGA GGAATACCCTTGATATACCTG</td>
<td>466</td>
<td>Coursodon et al., 2012</td>
</tr>
</tbody>
</table>
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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 (16S rRNA 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).
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 toxino-typing (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*
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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 & Batt, 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. Courson 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 (Rodriguez-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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REFERENCES


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