Isolation and Toxin Typing of Clostridium Perfringens From Sheep, Goats, and Cattle in Fars Province, Iran

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Abstract

Background: Clostridium perfringens is an important anaerobic bacterium found in the intestine of some livestock. It is concerned with the etiology of some diseases including enterotoxaemia. Various diseases are caused by different types of C. perfringens. Nonetheless, there is no published research on molecular typing and distribution of this pathogen in Fars province. Objectives: Accordingly, our study focused on the isolation and toxin typing of C. perfringens from sheep, cattle, and goats in different parts of Fars province by the culture and the polymerase chain reaction (PCR) method. Materials and Methods: Approximately 459 fecal samples were collected and cultured on defined media for the isolation of C. perfringens. The confirmed isolates were genotyped by the PCR method using specific primers. Results: C. perfringens was isolated from 30.93% of the total samples. The results of toxin typing showed a total of 76 (54%), 13 (9%), 30 (21%), and 23 (16%) isolates as types A, B, C, and D, respectively. Conclusion: Our results indicated that C. perfringens type A was the most common type in sheep, cattle, and goats while the lowest number of isolates belonged to type B. Finally, the isolation of C. perfringens and toxin typing increase our knowledge of the epidemiology of these diseases and can help in the vaccine industry and better controlling related diseases.

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Background

Clostridium perfringens is a gram-positive anaerobic bacterium that is widely distributed in the environment and can be considered as an opportunistic intestinal microflora of many domestic animals. The bacteria can produce histotoxic and fatal diseases such as enterotoxaemia in livestock. There are many reports of annual mortality and economic losses to the farming industry worldwide, including our country. Today, nearly 16 different toxins in various combinations are known to be encoded by C. perfringens contributing to its pathogenicity. However, based on the ability of C. perfringens for producing four major toxins (including alpha, beta, epsilon, and iota), this microorganism is divided into five toxin types of A, B, C, D, and E. Alpha toxin is common in all types and the beta toxin is present in types B and C while epsilon toxin is produced by types B and D. Finally, iota toxin is specific for type E of C. perfringens. Each toxin type is associated with different areas of pathology and specific enteric infection.

Toxin type A is of importance in food poisoning, antibiotic-associated diarrhea, and gangrene in humans and enteritis in some animal species. Toxin type D is important in the etiology of enterotoxaemia and enterocolitis in sheep and goats and pulpy kidney disease in lambs. Types B and C are responsible for enterotoxaemia and dysentery in sheep, goats, lambs, and calves. Toxin typing is essential for diagnosing diseases and investigating the epidemiology of these bacteria. Therefore, improving knowledge about the prevalence of the toxin types of C. perfringens in a region is helpful for the improvement of the enterotoxaemia polyvalent vaccine industry. Considering that limited published data about the toxin typing of C. perfringens in the Fars province of Iran, this study was designed with the purpose...
of isolating and toxin typing of *C. perfringens* from sheep, goats, and cattle in this province.

Materials and Methods

**Sampling, Culture, and Isolation**

The study was conducted during 2014-2016. Nearly 459 fecal samples of sheep, goats, and cattle from different parts of Fars province, including Shiraz suburb, Abadeh, Sarvestan, Fasa, Bavanat, Firouzabad, Kavar, Neiriz were randomly collected in the thioglycolate medium and transferred to the microbiology laboratory at Razi Vaccine and Serum Research Institute. The samples were sub-cultured on blood agar containing 40 µg/mL neomycin and tryptose sulphite cycloserine agar (TSC, Oxoid, Germany), containing 400 mg/L cycloserine (SR88, Oxoid), incubated anaerobically at 37°C for the isolation of suspected *C. perfringens* from hemolytic and black colonies, respectively. After Gram-staining and purification, several biochemical tests were performed, including catalase test, lecithinase activity on egg yolk salt agar, sugar fermentation, gelatinase activity, nitrate reduction, indole and H2S production, motility, lipase, and urease tests. The isolates were confirmed and submitted to toxin typing by the polymerase chain reaction (PCR) method.

**Polymerase Chain Reaction**

The PCR tests were carried out in the Eppendorf gradient thermocycler (Germany). The applied primer pairs for the species-specific 16s rRNA and each toxin gene including alpha, beta, epsilon, and iota are listed in Table 1.

DNA was extracted by the boiling method. The quantitative and qualitative assessments of the extracted DNA were performed by a spectrophotometer and gel electrophoresis. The purity of DNA was suitable where the ratio of optical density A260/A280 and A260/A230 were 1.8-2 and 1.8-2.2, respectively. Then, the DNA template from each isolate was used in a PCR mixture of totally 25 µL reaction volumes containing 1x PCR buffer, 200 µM dNTPs mix, 2 mM MgCl2, 1.25-unit Taq DNA polymerase, and 0.5 µM of each primer. The reference isolates for toxin types were included as positive controls in the PCR tests. The PCR program was set up and conducted for 30 cycles of 95°C, 55°C, and 72°C for 1 minute each, after the initial denaturation of 95°C for 3 minutes, and followed by the final extension of 72°C for 5 minutes. The PCR products were run on 1.5% agarose gels (Cleaver electrophoresis apparatus, England) and visualized under a UV light in the gel documentation of the Kodak GL200 imaging system (USA).

**Statistical Analysis**

The obtained data were statistically analyzed through IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA) using descriptive statistics and the chi-square test for testing relationships between categorical variables.

**Results**

*Clostridium perfringens* was isolated from a total of 142 (30.93%) of 459 samples from sheep, goats, and cattle and confirmed by biochemical tests. The isolated colonies were gray, smooth, occasionally rhizoid with a double zone of hemolysis on blood agar (Figure 1a), and black colored on TSC agar (Figure 1b). They were characterized as non-motile, catalase-negative, sucrose and lactose fermentation positive, urease and indole negative, lecithinase and gelatinase positive (Figure 1c), and lipase-negative.

Among the 138, 149, and 172 samples collected from cattle, goats, and sheep, 35 (26.36%), 38 (25.50%), and 69 (40.11%) *C. perfringens* strains were isolated, respectively.

Using specific primers for the detection of *C. perfringens* species, the PCR results showed that all isolates yielded

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Table 1. List of the Applied Primers

| Primer Name | Specification | Sequence | Product Size (bp) | Reference |
|-------------|---------------|----------|-------------------|-----------|
| 16SrRNA C. perfringens species | | AAGATGCGCATCATCATCAAC TACGGTCAATATATTCUGCAAA | 279 | 16 |
| CPA | Alpha toxin | GTCTATGTTGACGCTTGA CACTCTGATACGCGTGAAG | 324 | 17 |
| CPB | Beta toxin | GCGAATATGCTGAATCATCTACGCAGGAACATTAGTATCTTC | 196 | 17 |
| ETX | Epsilon toxin | GCCGGTGATATCCATATTTCA CCACTTACGTGCTGCTTACAC | 655 | 17 |
| Ia | Iota toxin | ACTCTCAGAAGCAGCAG CTTCTTTTATACATACAG | 446 | 17 |

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Figure 1. Colony Characteristics of *Clostridium perfringens* Isolates on Blood Agar (a), on TSC Agar (b), and Gelatinase Activity on Gelatin Agar (c). Note: TSC: Tryptose sulphite cycloserine.
the PCR products of the 279 bp segment of the 16S rRNA gene, confirming the identity of these isolates as *C. perfringens* isolates (Figure 2).

The PCR products of toxin typing for alpha, beta, and epsilon toxins were 324 bp, 196 bp, 655 bp, respectively (Figure 2). The la primers revealed no PCR products with all the field isolates. The toxin typing of *C. perfringens* isolates by the PCR demonstrated that 76 (54%), 13 (9%), 30 (21%), and 23 (16%) strains of overall isolates were of types A, B, C, and D, respectively.

The results of toxin typing among different individual groups are shown in Table 2. *C. perfringens* type A was the most common genotype isolated in the three groups of cattle, sheep, and goats while type B was the least common genotype recovered in each group.

The percentage of the isolated *C. perfringens* strains from the samples of each place in Fars province is depicted in Figure 3. According to the frequency distribution of the positive samples of *C. perfringens* in different places, the most isolated strains belonged to Shiraz suburbs.

The result of the Pearson chi-square test represented a significant association between the place and the number of *C. perfringens* isolates, namely, $\chi^2 (1, N = 459) = 25.045$, $P = 0.001$.

**Discussion**

*Clostridium perfringens* is found in many environments, as well as the gastrointestinal tract of humans and animals, and under some specific circumstances, can cause some important diseases in humans and animals by producing a variety of toxins. Despite many annual reports of diseases caused by *C. perfringens*, including enterotoxaemia in all parts of our country, to the best of our knowledge, there are few published reports on the toxin typing of this microorganism from the three groups of sheep, cattle, and goats, especially in Fars province, located in the south of Iran.

In this study, a total of 459 samples from sheep, goats, and cattle were processed to assess the frequency of *C. perfringens* isolation and their genotypes in these three groups of livestock in Fars province by the PCR method.

The PCR is a useful method for the genotyping of *C. perfringens* isolates. Various primers have been designed and introduced in different studies. The primers, as described by Wang et al. and Meer and Songer were used in the current study.

Based on our results, all four types of *C. perfringens*

(i.e., A, B, C, and D) were detected in the guts of the screened groups of healthy sheep, cattle, and goats except for type E. It was obvious that the isolates of type A were dominant in all groups, followed by type C. Type B was the least dominant toxin type recovered in all groups. There are some reports on the prevalence and typing of *C. perfringens* isolates in different parts of the world.

Afshari and Poursoltani reported the type C of *C. perfringens* as a predominant type in poultry in Mashhad.
although they pointed out that type A was the predominant type by several studies in other countries.\textsuperscript{23}

According to the results of Hadimili et al on the genotyping of the isolated \textit{C. perfringens} from enterotoxemic lambs in Turkey by the PCR, 76.92%, 15.38%, and 7.69% of the isolates were of types A, D, and C, respectively. However, no types B and E were identified in this study.\textsuperscript{24}

Gökce et al reported a prevalence of 47.27%, 9.09%, 4.54%, and 39.07% of \textit{C. perfringens} types A, B, C, and D, respectively, by ELISA tests in the Kars province of Tukey.\textsuperscript{25}

Tutuncu et al also showed that 65% of the isolates from enterotoxemic sheep and goats in Turkey were of type A. Types B, C, and D included 1%, 5%, and 29%, respectively.\textsuperscript{26}

These results demonstrate that type A is a dominant genotype of \textit{C. perfringens} in sheep and goats in Turkey as our northern-west neighboring country.

In the other neighboring country, Saudi Arabia, Omer et al conducted a study on the toxin typing of \textit{C. perfringens} isolated from enterotoxemic sheep, goats, and cattle during 2014-2015. They reported that \textit{C. perfringens} type A was the dominant type (67.2%), followed by types D (16.4%), B (13.4%), and C (3%).\textsuperscript{13}

The results of a survey by Nazki et al in Kashmir Himalayas, India revealed that type A had the highest frequency in sheep and goats and type D was observed in lambs and kids. Eventually, types B, C, and E were absent among their isolates.\textsuperscript{6}

In another study, Kalender et al reported that 40.6%, 21%, and 15% of \textit{C. perfringens} isolates from sheep were types A, D, and C, respectively.\textsuperscript{21} Similarly, Meer and Songer found type A as a more prevalent type.\textsuperscript{17}

These results regarding the higher frequency of type A \textit{C. perfringens} are consistent with our results, representing the role of type A \textit{C. perfringens} as a dominant normal flora in the livestock intestine.

On the other hand, our result is in contrast with that of Ahsani et al, demonstrating type A as the least dominant isolated toxin type while type C as the dominant type. According to their results, the prevalence of types A, B, C, and D were 17.39%, 21.74%, 34.78%, and 26.09%, respectively.\textsuperscript{4}

This discrepancy can be due to the geographical difference.

Based on our results, type E of \textit{C. perfringens} was not recovered from any samples, indicating that it is an uncommon type in our region. Based on the reports of some previous studies, type E is an uncommon cause of enterotoxaemia in lambs, calves, and rabbits.\textsuperscript{8,27}

However, Songer\textsuperscript{22} and Miyashiro et al\textsuperscript{28} reported the isolation of two strains of type E from the cattle.\textsuperscript{27,28} Miyashiro et al found two cases of genotype E by the PCR analysis of the obtained intestinal samples in the post-mortem examination of 23 bovines in Brazil although most strains were identified as type A.\textsuperscript{28}

Based on the findings of the current study, the most isolated strains (35.91%) belonged to the Shiraz suburb when evaluating different places in the province. Fifty-one (42.8%) \textit{C. perfringens} strains were isolated from 119 samples in this region. The rate of \textit{C. perfringens} isolation in Firouzabad, Sarvestan, Bavanat, Neiriz, Abadeh, Fasa, and Kavar was 38.46%, 33.33%, 32.25%, 28.94%, 26.47%, 18.75%, and 15.38%, respectively. Various numbers of samples at different times were collected from each place although sampling from all groups was done in every region in the province. Type A of \textit{C. perfringens} was isolated in all places and type C was dominant in Bavanat. This type was isolated from 13 out of a total of 93 collected samples in this area in June, implying 43.33% out of 30 positive samples.

It is well-established that genotyping is based on the toxin typing of four major toxins including alpha, beta, epsilon, and iota. Rood et al have recently described a new classification of \textit{C. perfringens} into seven types in 2018. Based on this classification, the enterotoxin producing type A of \textit{C. perfringens} was reclassified as type E; and those harboring \textit{netB} genes (specific to poultry) were grouped as type G.\textsuperscript{20} However, the current study did not investigate the presence of the \textit{cpe} gene in our type A isolates thus the typing of our isolates was reported according to the ordinary toxin typing scheme.

Beta, epsilon, and iota toxins are plasmid-borne and alpha-toxin can be chromosomally or plasmid-encoded. The toxin plasmids of \textit{C. perfringens} are conjugative, indicating the possibility of the toxin gene transfer to intestinal flora strains and the virulence plasticity of this microorganism.\textsuperscript{8,29}

The detection of toxin types increases our understanding of the epidemiology of related diseases. On the other hand, vaccination against \textit{C. perfringens} toxins is important in preventing enterotoxaemia in livestock. The related vaccine is a polyvalent vaccine with a specific formulation of types B, C, and D. Therefore, the determination of the prevalence of different toxin types in a region can help in managing a suitable vaccination strategy and achieving a proper and more efficient polyvalent vaccine formulation.

\textbf{Authors’ Contributions}
This research was conducted in Fars province as part of the national project No. 0-85-18-90010/2. MH and M. Sh were the provincial project administrator and the national project administrator, respectively. All authors participated in conducting the project and approval of the final manuscript.

\textbf{Ethical Approval}
Not applicable in this study.

\textbf{Conflict of Interest Disclosures}
The authors declare that they have no conflict of interests.
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