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# Toxinotyping of Clostridium perfringens isolates by ELISA and PCR from lambs suspected of enterotoxemia

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**Abstract:** *Clostridium perfringens* is a gram-positive, anaerobic bacterium that causes a wide range of diseases in humans and animals. The purposes of this study were to determine, using the enzyme-linked immunosorbent assay (ELISA), the clostridial toxins in intestinal samples of lambs with suspected enterotoxemia; to conduct molecular typing of *C. perfringens* isolates; and to investigate the presence of the genes of 4 major toxins (α, β, ε, and ι) in the isolates by polymerase chain reaction (PCR). According to the ELISA results, clostridial toxins were determined in 32 intestinal samples. Of the samples, 13 (40.62%) were *C. perfringens* type A, 1 (3.125%) was *C. perfringens* type B, 9 (28.125%) were *C. perfringens* type C, and 9 (28.125%) were *C. perfringens* type D. *C. perfringens* was isolated from 13 (8.66%) out of 150 intestinal samples. A total of 13 *C. perfringens* isolates from lambs were genotyped by PCR. Of the isolates, 10 (76.92%) were type A, 2 (%15.38) were type D, and 1 (7.69%) was type C. Type B and type E were not identified. The results showed that *C. perfringens* type A was the most common type by both ELISA and PCR in lambs with enterotoxemia.

**Key words:** *Clostridium perfringens*, lamb, enterotoxemia, ELISA, PCR

## Introduction

*Clostridium perfringens* is a gram-positive, anaerobic bacterium that causes a wide range of diseases in humans and animals (1). It is widely spread in the environment (e.g., in soil and sewage) and is commonly found in the gastrointestinal tract of animals (2-4).

This species is assigned to 5 toxinotypes (A, B, C, D, and E) on the basis of the production of 4 major toxins, namely *alpha* (α), *beta* (β), *epsilon* (ε), and *iota* (ι) (5,6). Each toxin type is associated with specific enteric infections of various animal species (6-10). The α-toxin is produced by all toxinotypes, while the β-toxin is produced by type B and C strains, the ε-toxin is produced by type B and D strains, and the ι-toxin is produced by type E strains (11-13). The α-, β-, ε-, and ι-toxins are encoded by the *cpa*, *cpb*, *etx*, and *iap* genes, respectively (7,9). The enterotoxin and β2-toxin are further important toxins produced by *C. perfringens*, and both have been reported in the last decade (10,14).

Enterotoxemia is caused by all 5 types of *C. perfringens* (1,2,7). Different biotypes of *C. perfringens* cause different diseases in humans and animals (4,15). Type A strains are commonly found as part of the normal intestinal microflora of lambs and have been associated with gas gangrene and food poisoning in humans (16). Type C strains cause

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enterotoxemia and necrotic enteritis in sheep, lambs, calves, piglets, and fowl (1,7,10,17). Type D strains are thought to cause dysentery and pulpy kidney disease in sheep and lambs (12,18).

Since 1931, the identification and toxinotyping of *C. perfringens* biotypes have been carried out using an in vivo toxin neutralization test in mice (3). However, this conventional method has certain disadvantages, in that it requires the use of antitoxin antibodies and is ethically unacceptable and time-consuming (14,19). In addition, enzyme-linked immunosorbent assay (ELISA) kits have been used for the detection of clostridial toxins (18,20,21). DNA-based techniques, such as polymerase chain reaction (PCR), have been developed for *C. perfringens* genotyping and are a reliable alternative method to testing in laboratory animals. Various PCR protocols have been established to genotype *C. perfringens* isolates with respect to the genes *cpa*, *cpb*, *etx*, *iap*, *cpe*, and *cpb*2, which encode the α-, β-, ε-, ι-, entero- and β2-toxins, respectively (3,6,9-11,14,17,19,22,23).

The present study was aimed at determining, by ELISA, the clostridial toxins in intestinal samples of lambs with suspected enterotoxemia; conducting molecular typing of *C. perfringens* isolates by PCR; and investigating the presence of the genes of 4 major toxins (α, β, ε, and ι) in the isolates.

## Materials and methods

### Intestinal samples

The intestinal samples were collected at necropsy from 149 lambs and 1 kid with suspected enterotoxemia or mortality due to sudden death syndrome in 2005 and 2006. The lambs had belonged to different herds raised in Konya Province and its vicinity. All of the samples were taken aseptically from the ileum, transferred to the laboratory (Department of Microbiology, Faculty of Veterinary Medicine, Selçuk University) under cold chain conditions, and immediately processed. The processed samples were kept at –20 °C until used (13).

### The detection of C. perfringens toxins (α, β, and ε) from intestinal samples by ELISA

A total of 150 intestinal samples (from 149 lambs and 1 kid) were used for the detection of *C. perfringens* toxins (α, β, and ε) using a commercial ELISA Kit (Bio-X, Jemelle, Belgium) according to the manufacturer's instructions.

### Bacteriological examination of the samples

A total of 150 intestinal samples from 149 lambs and 1 kid were cultured onto blood agar base (CM271, Oxoid, Basingstoke, UK) containing 5% defibrinated sheep blood and perfringens agar base (TSC agar; CM0587, Oxoid) containing egg yolk emulsion (SR47, Oxoid) and a selective supplement (SR88, Oxoid), and they were incubated for 24 h at 37 °C under anaerobic conditions. The suspected colonies were identified by Gram staining, characteristic colony morphology, and biochemical tests (1,4).

### Reference strains

*C. perfringens* type C (NCTC 10719), type D (NCTC 8346), and type E (NCTC 8084) reference strains were provided by the Konya Veterinary Control and Research Institute, Ministry of Agriculture and Rural Affairs, Konya, Turkey.

### DNA extraction from C. perfringens isolates

*C. perfringens* isolates and the reference strains were grown on blood agar base containing 5% sheep blood, and 4 or 5 colonies of strains were suspended in 500 µL of distilled water. Bacteria were killed by the addition of 0.5% formaldehyde. Subsequently, DNA was extracted using the protocol provided in the Fermentas genomic purification kit (Cat No: K0512, Fermentas, Vilnius, Lithuania). The DNA concentration was determined spectrophotometrically (biophotometer model 6131, Eppendorf, Hamburg, Germany) by absorbance readings performed at 260 and 280 nm. The samples were stored at –20 °C until used as templates for amplification.

### The detection of C. perfringens toxin genes by PCR

Molecular typing of *C. perfringens* was performed by the PCR amplification of the genes of 4 major toxins (α, β, ε, and ι), as described by Gkiourtzidis et al. (10). Briefly, the PCR assay was performed using a thermal cycler (Mastercycler gradient, Eppendorf) with a total reaction volume of 50 µL containing 5 µL of 10X PCR buffer, 1.5 mM of $MgCl_2$, 170 µM of each of the 4 dNTPs (R0182, Fermentas), 1.25 U of Taq DNA polymerase (EP0401, Fermentas), 0.25 mM of each of the primers (Integrated DNA Technologies, Coralville,

IA, USA), and 5 µL of template DNA. The reactions were subjected to 35 cycles of amplification consisting of 30 s of denaturation at 94 °C, 30 s of annealing at different temperatures (46 °C for the *cpa* gene, 39 °C for the *cpb* gene, 46 °C for the *etx* gene, and 46 °C for the *iap* gene), and 30 s of extension at 72 °C. The primers used for the different PCR protocols are listed in Table 1. The positive and negative controls used were DNA isolated from *C. perfringens* reference strains (strain NCTC 10719 for the α-toxin gene *cpa*, the β-toxin gene *cpb*, and the ε-toxin gene *etx*; strain NCTC 8346 for *cpa* and *etx*; strain NCTC 8084 for *cpa* and the ι-toxin gene *iap*) and water, respectively. Afterwards, the PCR products (10 µL) were analyzed by electrophoresis on 1.6% agarose gel, and the gel was stained with ethidium bromide (1.5 µg/mL) and photographed.

## Results

### The detection of C. perfringens toxins (α, β, and ε) by ELISA

Based on the ELISA results, at least 1 of the clostridial toxins was determined in 40 samples. When evaluating toxin types, α-, β-, and ε-toxins were found to be positive in 19, 10, and 11 samples, respectively. Samples were examined for all 3 toxins; accordingly, 32 of the 150 samples were positive for *C. perfringens* toxins.

The number of samples determined to be positive for different *C. perfringens* types by ELISA was 13 for *C. perfringens* type A (40.625%), 1 for *C. perfringens* type B (3.125%), 9 for *C. perfringens* type C (28.125%), and 9 for *C. perfringens* type D (28.125%) (Table 2).

Table 1. Oligonucleotide sequence of the primers used (10).

| Toxin/gene | Primer | Oligonucleotide sequence | Fragment length (bp) |
|---|---|---|---|
| α/*cpa* | CPALPHATOX-L<br>CPALPHATOX-R | 5’-AAGATTTGTAAGGCGCTT-3’<br>5’-ATTTCCTGAAATCCACTC-3’ | 1167 |
| β/*cpb* | CPBETATOX-L<br>CPBETATOX-R | 5’-AGGAGGTTTTTTTATGAAG-3’<br>5’-TCTAAATAGCTGTTACTTTGTG-3’ | 1025 |
| ε/*etx* | CPETOXIN-L<br>CPETOXIN-R | 5’-AAGTTTAGCAATCGCATC-3’<br>5’-TATTCCTGGTGCCTTAATT-3’ | 961 |
| ι/*iap* | CPIOTA-L<br>CPIOTA-R | 5’-AATGCCATATCAAAAAATAA-3’<br>5’-TTAGCAAATGCACTCATATT-3’ | 821 |

Table 2. *C. perfringens* types according to ELISA results.

| *C. perfringens* types | Positive samples | % |
|---|---|---|
| A | 13 | 40.625 |
| B | 1 | 3.125 |
| C | 9 | 28.125 |
| D | 9 | 28.125 |
| Total | 32 | 100 |

**Bacteriological examination of samples**

While *C. perfringens* was isolated from 13 (8.66%) of the 150 intestinal samples, 137 (91.34%) of the samples were bacteriologically negative. The *cpa* gene was detected in all 13 *C. perfringens* isolates by PCR; therefore, all of the isolates were confirmed as *C. perfringens*.

A total of 13 *C. perfringens* isolates from lambs were genotyped by PCR. Of these isolates, 10 (76.92%) were type A (possessing the *cpa* gene only), 2 (15.38%) were type D (possessing the *cpa* and *etx* genes), and 1 (7.69%) was type C (possessing the *cpa* and *cpb* genes) (Table 2). Types B and E were not identified (Table 3).

## Discussion

*C. perfringens* infections in lambs, sheep, goats, and other species are generically called enterotoxemia (4). In animals, *C. perfringens* is a normal member of the gastrointestinal tract, but when the intestines are altered by sudden changes in various conditions such as diet or other factors, *C. perfringens* proliferates in large numbers and produces several toxins (1). It has been reported that animals having high levels of enterotoxins could either lack the signs of illness until found dead or exhibit the acute form of the disease, enterotoxemia (21).

It is a common view that *Clostridium perfringens* types B, C, and D are mostly responsible for enterotoxemia, while *C. perfringens* type A alone is not involved or has very limited influence. Recently, several researchers have reported on the importance of C. *perfringens* type A (6,24). Greco et al. (6) reported that while *C. perfringens* types A and D were detected at rates of 84% and 16% by PCR, respectively, in 87 lambs and 15 kids and/or goats suspected of having enterotoxemia, C. *perfringens* types B and C were not encountered.

The use of ELISA for the detection of α-, ε-, and β-toxins allows the differential diagnosis of enterotoxemia cases caused by *C. perfringens* types A, B, C, and D from samples of intestinal content as well as the typing of *C perfringens* cultures (20). Although measurable levels of toxins did not fully reveal the cause of death, the ELISA kits used in the present study could be evaluated semiquantitatively. As enterotoxemia is a complex intestinal illness, the disease may not only be related to the presence of toxins. The occurrence of illness depends on toxin synthesis and on the amount of bacteria ($>10^4$-$10^5$ cfu/mL), the presence of toxigenic strains, and other conditions (environmental and microbiological) (1,24).

El Idrissi and Ward (8) reported that the comparison of ELISA with conventional assays (mouse assays and culturing of microorganisms) gave sensitivity and specificity rates of 90.5% and 89.2%, respectively, for β-toxin assays and 97.4% and 94.6% for ε-toxin assays, respectively. Vaikosen and Ikhatua (21) reported that *C. perfringens* types C and D, and particularly the type D strains, were responsible for enterotoxemia in Nigeria. They reported that 91 isolates isolated from 342 fecal samples were lecithinase-positive and that these

Table 3. Distributions of the toxin genes and types of *C. perfringens* from lambs suspected of enterotoxemia.

| *C. perfringens* toxin types | *C. perfringens* toxin genes by PCR | Number of isolates (%) | Percent of isolates |
|---|---|---|---|
| A | *cpa* | 10 | 76.92% |
| B | *cpa*, *cpb*, *etx* | 0 | 0% |
| C | *cpa*, *cpb* | 1 | 7.69% |
| D | *cpa*, *etx* | 2 | 15.38% |
| E | *cpa*, *iap* | 0 | 0% |
| Total isolates | | 13 | 100% |

strains were characterized by ELISA as types C and D. Koç and Gökçe (25) reported that *C. perfringens* was identified as types A, B, C, and D in 40.66%, 2.66%, 18.66%, and 9.33% of toxin-positive samples from diarrheic calves, respectively. Furthermore, 30.76% and 69.23% of intestinal samples taken from dead calves were positive for *C. perfringens* types B and C, respectively.

The measurement of toxin is the most important criterion in the diagnosis of enterotoxemia from intestinal content (1). Therefore, the determination of the rates shown in parentheses for type A (40.625%), type B, (3.125%), type C (28.125%), and type D (28.125%) demonstrates the increasing importance of type A in enterotoxemia of lambs.

The present study was aimed at direct detection of clostridial toxins in the intestines of lambs. At least 1 of 3 toxins was detected by ELISA in intestinal samples belonging to 32 animals. The particular *C. perfringens* type existing was identified as type A in 13 (40.625%), type B in 1 (3.125%), type C in 9 (28.125%), and type D in 9 (28.125%) of the positive samples by ELISA. Gökçe et al. (26) reported that *C. perfringens* types A and D were the dominant types detected by ELISA in sheep with suspected enterotoxemia, while type E was not encountered. The results obtained in the present study were in compliance with those reported in previous studies. Accordingly, it was determined that *C. perfringens* type E was not involved in enterotoxemia cases of lambs and sheep in Turkey.

In the present study, 13 *C. perfringens* isolates from lambs were genotyped by PCR and, of these isolates, 10 (76.92%) were type A, 2 (15.38%) were type D, and 1 (7.69%) was type C. Types B and E were not identified. The results of the present study indicate that *C. perfringens* type A is the most predominant cause of enterotoxemia in lambs in central Anatolia. In contrast, *C. perfringens* type B is the main cause of dysentery in lambs in the UK, South Africa, and Greece, where type B infection is widespread (10,27). On the other hand, it has been reported in various studies that type A is the dominant type of *C. perfringens* in lambs and sheep in worldwide (6,19). *C. perfringens* type E not being identified in our study was in agreement with previous data and confirms that *C. perfringens* type E is rare in lambs (2). Enterotoxemia in sheep has been reported to be caused by all 5 types of *C. perfringens* (2). However, the role of type A in disease production is considered doubtful by some researchers (15).

In Turkey, *C. perfringens* types A, B, C, and D have been isolated from sheep with enterotoxemia (26,28). Kalender et al. (23) reported that of 52 *C. perfringens* isolates from diseased sheep, 33 (64%) were type A, 11 (21%) were type D, and 8 (15%) were type C, while types B and E were not identified. PCR genotyping constitutes a useful alternative to in vivo toxin neutralization tests for the typing of *C. perfringens* isolates (4). Gkiourtzidis et al. (10) reported that 46% of *C. perfringens* isolates from 117 lambs with enterotoxemia were identified as type B by PCR in Greece, and the toxin genes of other strains were not detected. The data obtained in previous studies conducted by Gkiourtzidis et al. (10) and Kalender et al. (23) seem to contradict each other, yet the presence of the toxin gene can be explained by the toxin not being expressed.

Compared to conventional techniques, the PCR method has been shown to be much more rapid, giving results in a few hours, and it is much more reliable (17). In addition, it is stated that PCR should be chosen in the first place for the identification and typing of *C. perfringens* isolates from different sources (17). Gkiourtzidis et al. (10) and Yamagashi et al. (5) have developed PCR protocols with individual reactions for each toxin gene. Furthermore, multiplex PCR has been developed by some researchers (6,11). In this study, the molecular typing of all *C. perfringens* isolates was performed successfully by PCR.

Bacterial genes and the presence of toxins have been investigated in previously conducted PCR studies. In cases where bacteria possessing toxin genes do not produce toxins (no expression), the exact cause of death may not be identified in the field. In the present study, clostridial toxins were detected by ELISA in 40 out of 150 intestinal samples, whereas clostridial toxin genes were detected in 13 out of 150 intestinal samples.

Miserez et al. (11) reported that the presence of the α- and ε-toxin genes, which is typical for type D, was demonstrated by PCR in *C. perfringens* isolated from 52 animals displaying pathological signs of enterotoxemia. Gkiourtzidis et al. (10) indicated that

*C. perfringens* type B (containing α-, β-, and ε- toxin genes) was most prevalent in Greece and was isolated in 46% of clostridial dysentery cases. On the other hand, *C. perfringens* type C (α- and β- toxin genes) and type D (α- and ε- toxin genes) were isolated at rates of 20% and 28%, respectively.

In conclusion, the results obtained in the present study, using ELISA and PCR, demonstrated that *C. perfringens* type A predominates in clostridial enterotoxemia cases of lambs in Konya Province and its vicinity. PCR is a simple, effective, and rapid method for the typing of *C. perfringens*. Thus, PCR can be recommended for use in epidemiological studies on *C. perfringens*.

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