

**Short Communication**

**PREVALENCE AND TOXINOTYPING OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXINS IN SMALL RUMINANTS OF SAMSUN PROVINCE, NORTHERN TURKEY**

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**ABSTRACT**

Enterotoxemia, caused by *Clostridium perfringens*, is one of the most common problems of small ruminants' industry worldwide. *C. perfringens* is considered to be a normal resident of the intestines and when the intestinal environment changed, proliferates in large numbers and produces enterotoxins. Vaccination against *C. perfringens* enterotoxins is of paramount importance for preventing enterotoxemia in small ruminants. Therefore, detection of local enterotoxin types produced by *C. perfringens* could help deciding the suitable vaccine program. The present study was aimed to determine the types of *C. perfringens* and their toxins prevalent in small ruminant of Samsun province, Northern Turkey. For this purpose, 221 intestinal samples from sheep and goats with sudden death and/or suspected from enterotoxemia were examined by ELISA for detection of *C. perfringens* enterotoxins. According to ELISA results, 45.2% of the samples (100/221) were positive for enterotoxemia caused by *C. perfringens*. Overall, 133 enterotoxins were detected in this 100 positive samples. When evaluating the individual enterotoxins; alfa ( $\alpha$ ), beta ( $\beta$ ), and epsilon ( $\epsilon$ ) toxin were detected in 72.9%, 4.5%, and 22.6%, respectively. According to toxin types, 65 for *C. perfringens* type A (65.0%), 1 for type C (1.0%), 5 for type D (5.0%), and 29 for type E (29.0%) were determined. In conclusion, it was determined that enterotoxemia was high (45.2%), *C. perfringens* A and E type dominant strain in sheep and goats in Samsun province. A suitable vaccination program against *C. perfringens* type A and type E may provide adequate protection against the enterotoxemia of small ruminants in this area.

**Keywords:** *Clostridium perfringens*, enterotoxemia, enterotoxin types, sheep, goat.

**INTRODUCTION**

*Clostridium perfringens* is a Gram-positive, spore-forming, ubiquitous, anaerobic bacterium found widely in the environment and can be a normal inhabitant of the intestines of human and animals (Vinod *et al.*, 2014). When the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates in large numbers and produces several potent enterotoxins. The pathogenicity of this organism is associated with these enterotoxins (Islam *et al.*, 2010). *C. perfringens* infections are generally called as enterotoxemia in sheep and goats because toxins are produced in the intestine and then absorbed into the general circulation (Uzal and Songer 2008). Enterotoxemia is one of the most often occurring diseases of sheep and goats worldwide with prevalence rates ranging between 24.13% and 100% (El Idrissi and Ward 1992; Greco *et al.*, 2005).

*Clostridium perfringens* is classified into 5 types (A, B, C, D, and E) that produce 4 major toxins, namely alpha ( $\alpha$ ), beta ( $\beta$ ), epsilon ( $\epsilon$ ), and iota ( $\iota$ ) (Alves *et al.*, 2014). All five types of *C. perfringens* produce  $\alpha$ -toxin, whereas type B produces  $\beta$  and  $\epsilon$ -toxins in addition to  $\alpha$ -

toxin. Similarly, types C, D, and E produce  $\beta$ ,  $\epsilon$ , and  $\iota$ -toxins, respectively in addition to  $\alpha$ -toxin. Each of these types causes a specific disease in humans and animals (Songer, 1996). *C. perfringens* type A is commonly found as part of normal intestinal microflora and lacks production of powerful toxin as by other types. Types B and C both produce potent necrotizing and lethal  $\beta$  toxin responsible for severe intestinal damage, enteritis, dysentery, toxemia, and high mortality in young lambs, calves, pigs, and foals. Epsilon toxin produced by *C. perfringens* type D causes pulpy kidney disease in sheep and goats and, on rare occasions, in cattle (Uzal *et al.*, 2010).

The presumptive diagnosis of enterotoxemia is usually based on history, clinical signs, and gross postmortem pathological findings. Because *C. perfringens* is a natural commensal of intestines flora, it is not sufficient for the diagnosis of bacteria found in the intestine. Therefore, it is essential to determine the toxin typing and the strains of *C. perfringens*. The most commonly used diagnostic techniques for *C. perfringens* enterotoxins are neutralization test, PCR, latex agglutination, counter immunoelectrophoresis and

enzyme-linked immunosorbent assay (ELISA) (Fayez, *et al.*, 2013).

Samsun is one of the important provinces of small ruminant breeding in the Black Sea region. Vaccination against enterotoxins of *C. perfringens* is of paramount importance for preventing enterotoxemia; therefore, identification of types of *C. perfringens* and toxinotyping in an area could help for the development of the appropriate vaccination program (Bernath *et al.*, 2004, De la Rosa *et al.*, 1997). The types of *C. perfringens* and toxin typing should be determined in the small ruminants with enterotoxemia in an area because there are some differences in toxin types between regions. To the authors' knowledge, no reports on the prevalence and toxin typing of *C. perfringens* toxins in small ruminants in Samsun province. This research describes the prevalence of types of *C. perfringens* and its toxin typing in sheep and goats suspected for enterotoxemia in Samsun province, Northern Turkey.

## MATERIALS AND METHODS

**Animals and sampling:** A total of 221 intestinal samples from 153 sheep and 68 goats at different ages (2 wks to 3y) with the history of sudden death or suspected for enterotoxemia were examined for the detection of *C. perfringens* enterotoxins. Samples were collected from 12 sheep and 5 goat flocks (ranged with 70 to 320 animals in each farm) during the period from 2014 to 2015 in Samsun province. All the flocks were unvaccinated to *C. perfringens* enterotoxins, and the mortality rates were 2.9%-25.4% in the flock. Samsun province is located in the north of Turkey, on the coast of the Black Sea within 41° 8' 60" north, 36° 8' 60" east longitude, and 600 m average altitude. Samsun province has a temperate climate. Its average temperature is 14.2°C and the average rainfall is 664.9 mm annually.

**Intestinal samples:** Intestinal contents were collected aseptically from the ileum at necropsy and transferred to the laboratory under cold chain conditions. In the laboratory, the contents were diluted (1:5) with phosphate buffer saline and centrifuged at 2000 x g for 20 min at 4°C. After centrifugation, supernatants were removed and passed through 0.45 µm membrane filters (Millipore, Bedford, MA, USA), and then kept at -80°C until used. Samples were used to determine alpha, beta, and epsilon toxins of *C. perfringens* by ELISA test. Iota toxin could not be detected because it was not in this test content.

**ELISA procedure:** A commercial antigenic ELISA kit (Bio-X, Jemelle, Belgium) was used for the detection of *C. perfringens* enterotoxins according to the manufacturer's instructions. Briefly, 100 µl of the test sample, negative and positive controls were added to the

wells and incubated at room temperature for 1h. The plates were washed 3 times and 100 µl of the conjugate (1:50) was added to each well, and then incubated at room temperature for 1h again. After second incubation and washing of the plates, 100 µl of indicator solution was added to each well. The plates were incubated at room temperature for 20 min. After the last incubation, the reaction was stopped by 50 µl of stop solution (1M phosphoric acid) and the optical densities (OD) were recorded at 450 nm using a micro-plate reader (Tecan-spectra, Austria). The net OD for each sample was calculated by subtracting the OD of the corresponding negative control from the reading of each sample well and the samples in OD≥0.150 were considered as positive. According to toxin types *C. perfringens* strains were classified as in Table 1 (Uzal and Songer 2008).

**Statistical analysis:** A chi-square test was used to compare the differences between the numbers of positivity for *C. perfringens* types and P≤0.05 were considered as significant.

**Table 1. Toxin types of *C. perfringens*.**

Toxinotypes	Alpha	Beta	Epsilon	Iota
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	++

## RESULTS

ELISA results demonstrated that 45.2% (100/221) of the intestinal samples from sheep and goats were positive for enterotoxins of *C. perfringens*. Among these, 67% sheep and 33% goats were positive for *C. perfringens* enterotoxin. Of these 100 positive intestinal samples, 133 enterotoxins were detected. On toxin type, it was found that α-, β-, and ε-toxins were present in 72.9% (97/133), 4.5% (6/133), and 22.6% (30/133) in the intestinal samples, respectively. Based on toxinotype, the prevalence of *C. perfringens* types A, B, C and D was 65.0%, 1.0%, 5.0% and 29.0% in small ruminants, respectively. Statistical analysis revealed that prevalence of *C. perfringens* type A and *C. perfringens* type D was significantly higher (P<0.05) than other types. When evaluated according to animal species, in sheep the prevalence of *C. perfringens* type A 67.16%, type C 4.47% and type D 28.35% was determined while type B was not detected. On the other hand, in goats, 60.6% was positive for *C. perfringens* type A, 3.0% for type B, 6.06% for type C and 36.4% for type D (Table 2).

Table 2. *C. perfringens* types in found to be positive in sheep and goats.

<i>C. perfringens</i> types	Sheep, n (%)	Goats, n (%)	Total, n (%)
Type A ( $\alpha$ )	45(67.2) <sup>a</sup>	20 (60.6) <sup>a</sup>	65 (65.0) <sup>a</sup>
Type B ( $\alpha$ , $\beta$ , $\epsilon$ )	0(0.0) <sup>b</sup>	1 (3.0) <sup>b</sup>	1 (1.0) <sup>b</sup>
Type C ( $\alpha$ , $\beta$ )	3(4.4) <sup>b</sup>	2 (6.1) <sup>b</sup>	5 (5.0) <sup>b</sup>
Type D ( $\alpha$ , $\epsilon$ )	19(28.4) <sup>c</sup>	10 (30.3) <sup>c</sup>	29 (29.0) <sup>c</sup>
Total	67 (43.8)	33 (48.5)	100

<sup>a-c</sup> The significance of deviations of *C. perfringens* types within columns are indicated by different superscript letters (P<0.05).

## DISCUSSION

When large amounts of undigested carbohydrates, usually due to sudden changes in the diet, reach the intestine, *C. perfringens* multiply and produces large amounts of exotoxins that act locally or are absorbed into the systemic circulation (Uzal *et al.*, 2010). As aforementioned above *C. perfringens* is a natural commensal of intestines, meant isolation and identification of the bacterium are not enough for a tentative diagnosis. Through diagnostic algorithm determination of the enterotoxins from intestinal content is the most important part of the diagnosis of enterotoxemia (Fayez *et al.*, 2013). For the vast majority of techniques most commonly used to determine of the enterotoxins are neutralization test, PCR, latex agglutination, counter-immunoelectrophoresis, and ELISA (Jabbari *et al.*, 2011; Seyitoglu *et al.*, 2012). Indeed, ELISA test has been accepted as more efficient in enterotoxin typing of *C. perfringens* through the stability of the reagents, ease of manipulation, high sensitivity and specificity by researchers (Hadimli *et al.*, 2012; Islam *et al.*, 2010; Jabbari *et al.*, 2011; Fayez *et al.*, 2013; Vinod *et al.*, 2014). Moreover, ELISA test might be used as well to determine the presence of toxins in suspected samples, pure cultures, and culture supernatant fluids. Hence, in the present study, the toxigenic strains of *C. perfringens* were identified by ELISA via detection of  $\alpha$ ,  $\beta$ , and  $\epsilon$ -toxins. However, i-toxin was not analyzed, as it was not involved in the test content. Taking into account results of the present study, it may be safely demonstrated that ELISA might be used to determine the presence of toxins and the types of the *C. perfringens* within a few hours that is much quicker than conventional assays.

*C. perfringens* is classified into five types (A, B, C, D, and E) according to the production of 4 major toxins (Uzal and Songer 2008). Each type is associated with specific infections of various animal species. *C. perfringens* type A is the most common strain in the environment and it's responsible for hemorrhagic and necrotizing enterotoxaemia in ruminants, abomasitis in calves, and diffuse enteritis in all mammals. Types B and C both produce highly necrotizing and lethal  $\beta$ -toxin responsible for severe intestinal damage, enteritis, dysentery, toxemia, and high mortality in young lambs, calves, pigs, and foals. Epsilon toxin caused by C

*perfringens* type D causes pulpy kidney disease in sheep and goats and, on rare occasions, in cattle (Uzal *et al.*, 2010). Reports from countries around the world have reported prevalence rates of enterotoxemia ranging between 24.2% and 100% (El Idrissi and Ward 1992; Grecoet *et al.*, 2005). In Turkey, the prevalence of enterotoxemia in sheep and goats suspected with has been reported to vary 21.3% and 84.6% by PCR, ELISA and latex agglutination test techniques (Ozcan and Gurcay 2000; Gokce *et al.*, 2007; Hadimli *et al.*, 2008). According to ELISA results obtained in this study, 45.2% of the samples (100/221) were positive in sheep and goats with suspected enterotoxemia (Table 2). This result was lower than some research; Kalender *et al.* (2005) (50%) and Gokce *et al.* (2007) (84.6%), and higher than some research; Ozcan and Gurcay (2000) (38.6%) and Hadimli *et al.* (2012) (21.3%).

It has been reported that different toxin types have been identified in different regions. Although *C. perfringens* type A, B, C, and D have also been reported as the causal agents of enterotoxaemia in sheep and goats in Turkey (Ozcan and Gurcay 2000; Kalender *et al.*, 2005; Gokce *et al.*, 2007; Hadimli *et al.*, 2012; Seyitoglu *et al.*, 2012), recent studies indicated that *C. perfringens* type A is the dominant type identified in sheep and/or goat with suspected enterotoxemia in Turkey (Kalender *et al.*, 2005; Gokce *et al.*, 2007). This finding is consistent with the result of this study and enterotoxin types were determined as 65% for *C. perfringens* type A, 1% for type B, 5% for type C, and 29% for type D (29.0%). Similarly, Kalender *et al.* (2005) reported that *C. perfringens* type as 64% type A, 21% type D, and (15%) type C in sheep, however, types B and E were not identified. However, Hadimli *et al.* (2012) reported that 40.6% *C. perfringens* type A, 3.2% type B, 28.2% type C, and 28.2% type D. In another study, 32.4% of the samples were reported as A, 18.9% of type B, 24.3% of type C, and 24.3% of type D (Seyitoglu *et al.*, 2012). This variation could be proves geographical differences.

In conclusion, enterotoxemia was found to be high (45.2%) in small ruminant with suspected enterotoxemia in Samsun province and *C. perfringens* type A (65.0%) and type D (29.0%) were detected as the dominant strains in sheep and goats. In conclusion, the recommendation of a routine proper vaccination schedule especially against *C. perfringens* types A and D might

provide adequate protection against enterotoxemia in small ruminants in this province.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

**Animal Rights Statement:** The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

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