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Research Article

Determination of *Clostridium perfringens* Toxin-Types in Sheep with Suspected Enterotoxemia in Kars Province, Turkey

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Abstract: The study was designed to determine the types of *Clostridium perfringens* and their toxins in sheep with suspected enterotoxemia in Kars province. For this purpose, 260 dead sheep with suspected enterotoxemia and 35 clinically healthy sheep (controls) of different ages and breeds were used. Intestinal contents of each sheep were collected and used to determine alpha, beta, and epsilon toxins of *C. perfringens* using enzyme-linked immunosorbent assay (ELISA) and the latex agglutination test (LAT).

Based on ELISA and LAT, 220 (84.61%) and 152 (58.46%) sheep, respectively, were positive for *C. perfringens* toxins. In contrast, 40 (15.38%) sheep were toxin-negative based on ELISA and 108 (41.53%) were toxin-negative based on LAT. With ELISA, 104 (47.27%), 20 (9.09%), 10 (4.54%), and 86 (39.07%) toxin-positive samples were identified as *C. perfringens* type A, B, C, and D, respectively, whereas with LAT, 79 (51.97%), 15 (9.86%), 7 (4.60%), and 51 (33.55%) toxin-positive samples were identified as type A, B, C, and D, respectively. *C. perfringens* type A and type D were the dominant types detected by both tests in sheep with suspected enterotoxemia, in Kars province.

It is concluded that enterotoxemia causes considerable economic loss to the sheep industry in Kars province, where 300,000 sheep are present. Therefore, it is recommended that a proper vaccination schedule against enterotoxemia be implemented for flocks in Kars province. These vaccines should provide adequate protective immunity against both *C. perfringens* type A and type D.

Key Words: Sheep, *Clostridium perfringens*, alpha toxin, beta toxin, epsilon toxin, enterotoxaemia, enzyme-linked immunosorbent assay (ELISA), latex agglutination test (LAT)

Kars İlinde Enterotoksemi Şüphesi ile Ölen Koyunlarda *Clostridium perfringens*'in Toksinlerinin Belirlenmesi

Özet: Bu çalışmada Kars ilinde aniden ölen ve enterotoksemi şüphesi bulunan koyunlarda bu hastalığa neden olan *Clostridium perfringens* tiplerini ve bu bakterinin üretmiş olduğu toksinlerin belirlenmesi amaçlandı. Çalışmada Kars ilinde enterotoksemi şüphesi ile ölmüş olan çeşitli ırk ve yaştaki 260 adet ve mezbahada kesilen 35 adet sağlıklı koyun kullanıldı. Bu amaçla her bir koyunun ince bağırsak içeriği toplandı ve bu içerikde enzyme-linked immunosorbent assay (ELISA) ve lateks agglutinasyon testi (LAT) ile *C. perfringens* tipleri tarafından üretilen alfa, beta ve epsilon toksinlerin varlığı araştırıldı.

Çalışmada, toplanan 260 bağırsak içeriğinden ELISA yöntemiyle ile 220'sinde (% 84,61) ve LAT ile ise 152'sinde (% 58,46) *C. perfringens* toksinlerine rastlandı. Bu örneklerin 40'ı (% 15,38) ELISA ve 108'i (% 41,53) LAT ile negatif olarak belirlendi. Yapılan biyo-tiplendirmede ELISA yöntemi ile pozitif olarak belirlenen örneklerin sırasıyla 104'ü (% 47,27), 20'si (% 9,09), 10'u (% 4,54) ve 86'sı (% 39,07) *C. perfringens* tip A, B, C ve D olarak idenfiye edildi. LAT testi ile bakterinin yapılan biyo-tiplendirilmesinde ise örneklerin 79'u (% 51,97) tip A, 15'i (% 9,86) tip B, 7'si (% 4,60) tip C ve 51'i (% 33,55) tip D olarak idenfiye edildi. Her iki testte de en yüksek pozitiflik *C. perfringens* tip A ve tip D için bulundu.

Yapılan bu çalışmadan elde edilen sonuçlara göre ve Kars ilinde 300.000 civarında koyunun bulunması nedeniyle bu ilde enterotoksemiden kaynaklanan ekonomik kaybın epeyce büyük olduğu ortaya çıkmaktadır. Bu nedenle de Kars ilinde bulunan koyunların enterotoksemiye karşı düzenli bir şekilde aşılanması ve aşıların mutlaka *C. perfringens* tip A ve D'ye karşı bağışıklık sağlayacak şekilde düzenlenmesi önerilmektedir.

Anahtar Sözcükler: Koyun, *Clostridium perfringens*, alfa toxin, beta toxin, epsilon toxin, enterotoksemi, enzyme-linked immunosorbent assay (ELISA), lateks agglutinasyon testi (LAT)

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Introduction

Enterotoxemia caused by *Clostridium perfringens* is a fatal enteric disease that affects all species of domestic animals and humans. The organism can cause gastrointestinal and enterotoxemic diseases in animals, and food poisoning, gangrene, and necrotic enteritis in humans (1-4). Enterotoxemia is one of the most frequently occurring diseases of sheep and goats worldwide. Reports from countries around the world have reported prevalence rates of enterotoxemia ranging between 24.13% and 100% (5-7). In Turkey, there are a few published reports on enterotoxemia in sheep. In these studies, its prevalence has been shown to be between 38.63% and 50%. Furthermore, *C. perfringens* type A, B, C, and D have been detected in sheep, although the majority of these isolates were identified as type D (8,9).

C. perfringens has been classified into 5 toxigenic types (A, B, C, D, and E) on the basis of the expression of 4 major toxins: alpha, beta, epsilon, and iota. The bacterium is a normal inhabitant of the intestine, but is usually present in low numbers. These organisms produce little toxin and under normal conditions are removed by normal gut movements or are inactivated by circulating antibodies. Sudden changes in diet (grazing lush, rapidly growing pastures or young cereal crops) or heavy grain feeding (as in feedlots) enables the bacteria to multiply rapidly. Toxemia occurs when the movement of food in the intestine slows or the organisms multiply and produce toxin faster than can be removed or neutralized (1,3).

Large amounts of toxin as well as large numbers of *C. perfringens* can usually be observed in the intestinal contents of diseased or dead animals (1,3). As *C. perfringens* is a natural host of human and animal intestines, identification of the bacterium is not sufficient. The diagnosis of enterotoxemia is usually based on clinical signs and pathological findings, but identification of toxins in intestinal contents is necessary to confirm the diagnosis. The most widely used method for toxin detection is the mouse protection test, which is cumbersome, expensive, and time consuming. Moreover, as the treatment of animals involved in the test is inhumane, alternative in vitro tests are required. On the other hand, a number of serological and molecular techniques have been used to type toxins, including counterimmunoelectrophoresis (CIE) (10), ELISA (5,11), latex agglutination test (LAT) (12,13), and PCR (7,14,15). Among these tests, ELISA and LAT have been used for laboratory diagnosis of enterotoxemia, especially in cases of sudden death outbreaks in sheep. These tests are simple, of limited cost, and provide quantitative results. They can be used for toxin-typing and for the differential diagnosis of *C. perfringens* types A, B, C, and D enterotoxemias (5,11-13). For instance, ELISA has been found to be 95% reliable for the detection of *C. perfringens* toxins in the intestinal contents of sheep suspected of having enterotoxemia. The comparison of ELISA to LAT showed that it had 96.5% sensitivity and 95.2% specificity (13).

All the published studies on enterotoxemia in Turkey have been focused on the isolation and mouse protection test (8,9). To the best of our knowledge, there are no published reports of toxinotyping *C. perfringens* in the intestinal contents of sheep suspected of enterotoxemia in Turkey. It is well known that vaccinating sheep against *C. perfringens* is very important for preventing enterotoxemia (16,17); however, *C. perfringens* biotypes causing enterotoxemia in a particular region should be identified so as to formulate a proper vaccine. Therefore, the biotypes of *C. perfringens* must be determined in the sheep with enterotoxemia in Kars province.

The aim of the present study was to determine the biotypes of *C. perfringens* and their toxins in sheep with suspected enterotoxemia in Kars province, using ELISA and LAT.

Materials and Methods

Animals

The intestinal contents were collected from 260 sheep with suspected enterotoxemia and 35 healthy sheep (controls) of different ages. Sheep with suspected enterotoxemia belonged to 16 different herds from Kars province, Turkey, and its vicinity. The number of sheep in the flocks ranged between 70 and 370. Furthermore, the mortality rate of the flocks ranged between 3.2% and 23.3%, and post-mortem examination of some of the dead animals was performed at the Department of Veterinary Pathology, Kafkas University, Kars.

Intestinal samples

Intestinal samples were collected from sheep with suspected enterotoxemia during post-mortem examinations, while control samples were collected from the healthy animals slaughtered at the abattoir. All of the

samples were submitted to the laboratory on the same day and immediately processed as described below, or refrigerated (4-6 °C) for no longer than 24 h before processing. Processed samples were kept at –20 °C until used.

Specimens were diluted (1:5) with endotoxin-tested distilled water (Sigma-Aldrich Chemie Gmbh, Germany) and centrifuged at 2000 ×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 kept at –70 °C until used. Samples collected from sheep with suspected enterotoxemia were used to determine alpha, beta, and epsilon toxins of *C. perfringens*, using ELISA and LAT. Furthermore, control samples collected from healthy sheep were used to determine the background level activity of these toxins present in normal intestinal contents.

ELISA procedure

The presence of *C. perfringens* toxins in the supernatants was determined by an indirect ELISA commercial kit (Bio-X Diagnostics, Belgium), according to the manufacturer's instructions. In the test, rows A, C, E, and G were sensitized with the specific antibodies for alpha, beta, or epsilon toxin, while rows B, D, F, and H contained non-specific antibodies.

Briefly, 100 µl of test sample, and negative and positive controls were added to the appropriate wells and the plates were then incubated at room temperature for 1 h. After this first incubation the plates were washed 3 times and 100 µl of conjugate (1:50) was added into each well and then incubated at room temperature for 1 h. After this incubation and washing of the plates, 100 µl of indicator solution (a mixture of chromogen and substrate) was added to each well. All the plates were then incubated at room temperature for 20 min. After this incubation, reaction was stopped by adding 50 µl of stop solution (1 M phosphoric acid). Finally, 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. According to the manufacturer QC data sheet, the limit of OD positivity for the alpha, beta, and epsilon toxins is 0.150; therefore, any sample that yielded a difference in OD ≥ 0.150 was considered positive for the toxins tested. Conversely, any sample that yielded a difference in the OD < 0.150 was considered negative.

Sensitization of latex particles

Latex particles were sensitized with alpha, beta, or epsilon antitoxin as described by Martin and Naylor (13). Briefly, freeze-dried antitoxin of equine origin against *C. perfringens* alpha, epsilon, and beta toxins was obtained from Pendik Veterinary Research Institute, İstanbul, Turkey. These sera were reconstituted in 1 ml of endotoxin-tested distilled water, and the protein content of each of the sera was measured using a bicinchoninic acid protein assay kit (Sigma-Aldrich Chemie Gmbh, Germany) and adjusted to 10 mg/ml. Each antitoxin was then used to sensitize the latex particles as follows: 0.4 ml of a 2.5% suspension of 1.0-µm red carboxylate modified latex particles (Sigma-Aldrich Chemie Gmbh, Germany) in water was pipetted into a plastic centrifuge tube and 5 ml of MES buffer (0.05 M 2-[N-morpholino] ethane sulfonic acid in distilled water, pH 5.5) was added. The contents were agitated vigorously on a vortex and then centrifuged at 5000 × g for 6 min. After centrifugation the supernatant was removed carefully and discarded. The pellet was resuspended in 5 ml of MES buffer and centrifuged again at 5000 × g for 6 min. Washing was repeated 3 more times.

After the final wash the latex pellet was thoroughly resuspended in 0.4 ml of MES buffer in a glass tube. A fresh 1% solution of water soluble carbodiimide (Sigma-Aldrich Chemie Gmbh, Germany) was prepared (0.1 g of 1-cyclohexyl-3-[2-morpholinoethyl] carbodiimide metho-p-toluene sulfonate in 10 ml of distilled water) and 0.4 ml of the solution was mixed with the latex suspension. This mixture was kept in a water bath at 50 °C for 10 min and then transferred to the plastic centrifuge tube to which 5 ml of MES buffer was added. After centrifugation at 5000 × g for 6 min the supernatant was removed and discarded. The pellet was resuspended in 1 ml of MES buffer in a glass tube and 300 µg of alpha, beta, or epsilon antitoxin was added. After thorough mixing, the latex/antitoxin mixture was kept in a water bath at 50 °C for 10 min and then transferred to a plastic tube for centrifugation. The mixture was washed once in 20 ml of MES buffer before being resuspended in 10 ml of AMP buffer (0.2 M 2-amino-2-methyl [1-propanol] hydrochloride in distilled water [pH 7.8] with 0.5 % bovine serum albumin and 0.1% sodium azide). All the sensitized latex particles were kept at 4 °C until used.

Latex agglutination test (LAT) procedure

The LAT was performed as described by Martin and Naylor (13). In the LAT for the intestinal contents, known positive and negative controls for alpha, beta, or epsilon toxins (Bio-X Diagnostics, Belgium) were diluted 1:5 in AMP buffer before use. For each test 25 µl of AMP buffer was added to the test well and 25 µl of AMP buffer plus 2% alpha, beta, or epsilon antiserum were added to the control well. Sample supernatant (25 µl) was then added to each well of the micro well plates (Greiner bio-one, Germany, U bottom). Finally, 25 µl of sensitized latex particles was then added to each test and control well. Then, the plate was shaken mechanically for 30 s, sealed with plastic, and left at room temperature for 18-20 h before it was examined. After overnight incubation, specimens showing ≥ 50% more agglutination in the test well than in the neutralization well were considered positive, whereas those showing no agglutination in the test well or no neutralization were regarded as negative.

Statistical analysis

The results were expressed as the percentage and the number of positivity for *C. perfringens* types. A chi-square test was used to compare the differences between the numbers of biotypes (Table).

Results

Post-mortem findings

At post-mortem examination, a moderate amount of reddish gelatinous fluid was observed in the abdominal and pericardial cavities. Congestion, small ulcers, and edema were observed in the mucous membranes of the abomasum, and small and large intestines. The duodenum was markedly hemorrhagic. Congestion and enlargement were also observed in the liver, kidney, lung, and spleen. In addition to these, many petechiae were observed in the small intestine, kidney, epicardium, and bladder.

Determination of toxins

In the present study, the intestinal contents collected from 35 slaughtered healthy sheep were found to be negative for alpha, beta, and epsilon toxins, by both ELISA and LAT. With ELISA, the mean ODs of these control samples were 0.063 ± 0.009 for alpha toxin, 0.063 ± 0.008 for beta toxin, and 0.073 ± 0.004 for epsilon toxin. The mean OD of the control animals was less than the limit of OD positivity suggested by the manufacturer. In all, 220 (84.61%) samples collected from dead sheep were positive for *C. perfringens* toxins, while 40 samples (15.38%) were negative for the toxins we tested for. According to the toxins produced by the bacterium, 104 (47.27%), 20 (9.09%), 10 (4.54%), and 86 (39.09%) of the positive samples were identified as *C. perfringens* type A, B, C, and D, respectively. *C. perfringens* type A and D were the 2 most prevalent types detected in the samples ($P < 0.001$, Table). According to LAT, 152 (58.46%) samples were positive for *C. perfringens* toxins, while 108 (41.53%) samples were negative. Moreover, 51.97%, 9.86%, 4.60%, and 33.55% of the positive samples were identified as *C. perfringens* type A, B, C, and D, respectively. *C. perfringens* type A and D were also the dominant types identified by LAT ($P < 0.001$, Table). In the present study ELISA appeared to be more sensitive than LAT for the detection of the *C. perfringens* toxins we tested for.

Discussion

In the present study 220 (84.61 %) samples tested by ELISA and 152 (58.46 %) samples tested by LAT were positive for the toxins produced by *C. perfringens*. *C. perfringens* type A, B, C, and D were identified in the samples, but types A and D were the predominant types in the toxin-positive samples according to both test methods.

Table. Number and percentage of *C. perfringens* biotypes in the intestinal contents of sheep (n = 260) with suspected enterotoxemia.

C. perfringens types	The number of ELISA-positive samples	The number of LAT-positive samples
Type A (α)	104 (47.27%)[a]*	79 (51.97%)[a]
Type B (α-β-ε)	20 (9.09%)[b]	15 (9.86%)[b]
Type C (α-β)	10 (4.54%)[bc]	7 (4.60%)[bc]
Type D (α-ε)	86 (39.07%)[ad]*	51 (33.55%)[d]
Total positive samples	220 (84.61%)*	152 (58.46%)
Negative samples	40 (15.38%)	108 (41.53%)

[a-d]The significance of deviations of *C. perfringens* types within columns are indicated by different superscript letters ($P < 0.001$).

*The significance of deviations of *C. perfringens* types between ELISA and LAT are indicated by an asterisk ($P < 0.05$).

ELISA and LAT have been used for laboratory diagnosis of enterotoxemia, especially in cases of sudden death outbreaks in sheep (5,11-13). It has been reported that ELISA is 95% reliable for the detection of *C. perfringens* toxins in the intestinal contents of sheep suspected of having enterotoxemia (5,11). LAT is less expensive and quicker to perform than ELISA; however, the sensitivity and specificity of LAT is less than that of ELISA (13). LAT is open to operator interpretation, unlike ELISA, which is read by a spectrophotometer. Therefore, the ELISA remains the most specific test for *C. perfringens* toxins, although LAT, as a qualitative test, is an alternative that can be used as a screen or by laboratories that need a quick indication of the presence of the toxins when there is no facility for ELISA (13). In the present study ELISA was also found to be more sensitive than LAT; however, polyclonal antibodies for alpha, beta, and epsilon toxins were used to sensitize latex particles instead of monoclonal antibodies, most probably resulting in a decrease in the sensitivity of this test for detecting the toxins. Furthermore, the reason for more positive samples based on ELISA could also have been due to the presence of low amounts of toxins, which may not be detectable in LAT. Therefore, the results obtained by ELISA can be accepted as the true percentage of enterotoxemia in sheep that died suddenly in Kars province.

Reports from countries around the world have reported prevalence rates of enterotoxemia ranging between 24.13% and 100% (4-7). In these studies, *C. perfringens* type A has been reported to be the most dominant type identified in sheep with enterotoxemia (6,7). In Turkey, the prevalence of enterotoxemia has been reported to be range between 38.63% and 50%. In these studies various results were obtained and type D was reported to be the most dominant *C. perfringens* type causing enterotoxemia in sheep (8,9). However, in contrast to these studies, a recently published molecular study indicated that *C. perfringens* type A is the dominant type identified in sheep with suspected enterotoxemia in Elazığ, Turkey, which supports the results obtained in the present study (15). Moreover, the prevalence of enterotoxemia was higher in Kars province than reported in other studies published in Turkey.

The winter season is very long in Kars province and flocks are kept indoors for more than 6 months. During the spring, plants grow very fast in pastures and the flocks are put out to pasture and graze large amounts of green plants in a short period. In contrast, summer is very short and cereal crops are harvested in a short period. Grazing animals in the pastures are moved to these fields and allowed to consume large amounts of grain, mostly wheat and barley, in a short period of time, which are well-known predisposing factors for enterotoxemia in sheep (1,3). Therefore, consuming non-tolerable amounts of lush graze and grains in a short period of time may play a role in the high occurrence of enterotoxemia in Kars province.

Enterotoxemia causes more economic loss among feedlot- and pasture-reared lambs than all other diseases combined, if vaccination is not applied (1,3). Unfortunately, in Kars province, most sheep farmers do not vaccinate their ewes against enterotoxemia and more than half of the owners that do vaccinate give only a single dose of vaccine. Nonetheless, it is well known that initial vaccination should be followed by a booster 4-6 weeks later in order to promote protective immunity (16,17). Additionally, vaccination of pregnant ewes is also important for transferring the passive immunity through colostrums to the newborn lambs. Therefore, lack of vaccination programs against enterotoxemia in sheep is most probably another reason for the high incidence of the disease in Kars. Furthermore, *C. perfringens* type D has been reported to be the dominant type causing enterotoxemia in sheep in Turkey; however, the result of the present study indicated that both *C. perfringens* type A and D should be considered the common cause of enterotoxemia in sheep in Kars. On the other hand, to date, most vaccines prepared and administered to immunize sheep against type D and other types have been neglected in the province, which may also be another reason for the high incidence of the disease in Kars.

It is well known that enterotoxemia causes considerable economic loss to the sheep industry due to a high fatality rate, decreased productivity, and increased treatment costs (7,9). In the present study the mortality rate in the flocks ranged between 3.2% and 23.3%. In addition, 84.61% of the samples collected from sheep were positive for enterotoxemia by ELISA. Therefore, the result of the present study suggests that the disease causes major economic loss to the sheep industry in Kars, where 300,000 sheep are present. On the other hand, economic loss caused by clostridial diseases can be

prevented with proper management and vaccination timing (1-3). Thus, it can be suggested that a proper vaccine should be prepared and administered to provide strong immunity against both type A and D in the sheep of Kars province.

In conclusion, the intestinal contents collected from 220 (84.61%) and 152 (58.46%) sheep with suspected enterotoxemia were positive for *C. perfringens* toxins using ELISA and LAT, respectively. The majority of toxins were identified as *C. perfringens* type A and D, with both tests. The results of the study indicated that *C. perfringens* type A, B, C, and D may cause enterotoxemia, but type A and D were the predominant causative agents of enterotoxemia in the sheep in Kars. Therefore, it is strongly recommended that a vaccination schedule be implemented to reduce the incidence of enterotoxemia in Kars province. This vaccine should provide adequate protective immunity, especially against *C. perfringens* type A and D.

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