

Determination of the Toxins and Biotypes of *Clostridium perfringens* in Diarrhoeic Calves in the Kars District of Turkey

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Abstract: This study was designed to determine the types of *Clostridium perfringens* and their toxins in diarrhoeic calves in Kars. For this purpose, 150 calves with diarrhoea, 13 dead calves, and 25 healthy calves of less than 1-month old were used. Faecal samples from the diarrhoeic and control calves and intestinal contents from the dead calves were collected and used to determine alpha, beta, and epsilon toxins produced by *C. perfringens* types using a commercially available ELISA test kit.

A total of 122 (81.33%) faecal samples collected from the diarrhoeic calves were found to be positive for *C. perfringens* toxins. According to the toxins produced by *C. perfringens*, 61 (40.66%), 19 (12.66%), 28 (18.66%), and 14 (9.33%) toxin-positive samples were identified as types A, B, C, and D, respectively. Furthermore, 4 (30.76%) and 9 (69.23%) intestinal samples collected from the dead calves were also positive for *C. perfringens* types B and C, respectively. All the faecal samples collected from healthy calves were negative for the *C. perfringens* toxins tested. It is recommended that an appropriate vaccination schedule against *C. perfringens* should be applied to pregnant cattle and new-born calves in Kars. This vaccine should provide an adequate protective immunity against *C. perfringens* types A, B, and C.

Key Words: Calves, *Clostridium perfringens*, alpha toxin, beta toxin, epsilon toxin, diarrhoea, enterotoxaemia, enzyme-linked immunosorbent assay (ELISA)

Kars ve Yöresinde İshalli Buzağılarda *Clostridium perfringens*'in Toksinlerinin ve Biotiplerinin Belirlenmesi

Özet: Çalışmada ishallerli buzağılarda *Clostridium perfringens*'in biyotiplerinin ve bu bakterinin ürettiği toksinlerin belirlenmesi amaçlandı. Bu amaçla çalışmada, Kars Yöresinde bir aya kadar olan 150 ishallerli, 13 aniden ölmüş ve 25 adet de sağlıklı buzağı kullanıldı. İshallerli ve sağlıklı buzağılardan dışkı örnekleri, ölen buzağılardan ise ince bağırsak içerikleri toplandı. Bu örneklerde Enzyme-Linked Immunosorbent Assay (ELISA) test kiti kullanılarak *C. perfringens* tiplerinin ürettiği alfa, beta ve epsilon toksinlerinin varlığı araştırıldı.

Çalışmada kullanılan 150 ishallerli buzağıdan toplanan dışkı örneklerinin 122'sinde (% 81,33) *C. perfringens* tipleri tarafından üretilen toksinler belirlendi. Bakterinin ürettiği toksinler dikkate alınarak yapılan biyotiplendirmede pozitif olarak belirlenen örneklerin 61'i (% 40,66) tip A, 19'u (% 12,66) tip B, 28'i (% 18,66) tip C ve 14'ü (% 9,33) tip D olarak tanımlandı. Ayrıca, ölmüş olan buzağılardan alınan bağırsak içeriklerinde sırasıyla 4 (% 30,76) ve 9'unda (% 69,23) *C. perfringens* tip B ve C belirlendi. Sağlıklı buzağılardan toplanan dışkı örneklerinde ise *C. perfringens* toksinlerine rastlanmadı. Bu nedenle Kars ve yöresinde gebe inek ve yeni doğmuş buzağılar mutlaka aşılmalı ve bu aşılar *C. perfringens* tip A, B, ve C'ye karşı bağışıklık oluşturacak şekilde düzenlenmelidir.

Anahtar Sözcükler: Buzağı, *Clostridium perfringens*, alfa toksin, beta toksin, epsilon toksin, ishal, enterotoksemi, enzyme-linked immunosorbent assay (ELISA)

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Diarrhoea is a common complaint in calves, particularly in the first few months of life. It causes major economic losses directly through mortality and the need for treatment, and indirectly from poor growth after clinical disease. It has been estimated that neonatal calf diarrhoea accounts for approximately 75% of the mortality of dairy calves under 3 weeks of age. Moreover, the possible long-term effects of neonatal diarrhoea on the health and performance of calves that survive clinical episodes might constitute an even greater loss (1). The diarrhoeal syndrome has a complex etiopathogenesis, because various infectious agents, either alone or in combination, may be associated with field outbreaks. In addition, environmental, management, and nutritional factors influence the severity and outcome of the disease. Rotavirus, coronavirus, enterotoxigenic *E. coli*, *Clostridium perfringens*, *Salmonella* spp., and *Cryptosporidium parvum* are the major pathogens associated with neonatal calf diarrhoea worldwide (1-6). *C. perfringens* is one of these pathogens of diarrhoea in calves (2,6-8), and it is widespread in terrestrial and aquatic environments. This bacterium is also frequently found in the intestines of humans and animals, where it can be pathogenic in certain circumstances. The organism can cause gastrointestinal and enterotoxaemic diseases in animals, and food poisoning, gangrene and necrotic enteritis in humans (7,9). *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 expression of the toxins is closely related to the virulence and pathogenicity of the organism (7,9,10).

Large amounts of toxin as well as large numbers of *C. perfringens* cells can usually be demonstrated in the intestinal fluid of the diseased or dead animals (7,9). As *C. perfringens* is naturally found in human and animal intestines, identification of the bacterium is not sufficient. The diagnosis of enterotoxaemia is usually based on clinical signs and pathological findings but demonstration of toxins in the gut is necessary for confirmatory diagnosis. The most widely used method for toxin detection is the mouse protection test, which is cumbersome, expensive, and time consuming, and the treatment of animals involved in the test is inhumane. A number of serological and molecular techniques have also been used to type toxins, including counterimmuno-electrophoresis (11), ELISA (12), latex agglutination test

(13), and PCR (14). For instance, ELISA has been used for laboratory diagnosis of enterotoxaemia, especially in cases of sudden death in sheep and cattle. It is simple, reliable, of limited cost, and gives quantitative results. These tests can be used for toxin typing and differential diagnosis of *C. perfringens* types A, B, C, and D enterotoxaemias (12,13).

There are published reports on enterotoxaemia in sheep in Turkey (15,16). However, to the best of our knowledge, no report has been published to date on the bovine enterotoxaemia and toxin typing of *C. perfringens* in diarrhoeic calves in Turkey. Therefore, the purpose of the present study was to determine the toxins and biotypes of *C. perfringens* in diarrhoeic calves in Kars district.

In our study, 150 calves with diarrhoea, 13 calves that died suddenly, and 25 healthy calves of less than 1-month old were used. Faecal samples from the diarrhoeic and control calves and intestinal samples from the dead calves were collected and submitted to the laboratory on the same day. Samples were immediately processed or refrigerated (4-6 °C) for no longer than 24 h until processing. Processed samples were kept at -20 °C until used. Faecal samples of the control calves were used to determine the background level of activity present in normal faecal samples.

All the 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 mm membrane filters (Millipore, Bedford, MA, USA) and kept at -70 °C until used for ELISA.

C. perfringens toxins were investigated in the supernatants using an indirect ELISA commercial kit (Bio-X Diagnostics, Belgium) according to the manufacturer's instructions. Briefly, 100 µl of test samples, and negative and positive controls were added to 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 to each well, followed by incubation at room temperature for 1 h. After the second incubation, the plates were washed again and 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, the reaction was stopped by

adding 50 µl of stop solution (1 M phosphoric acid). Finally, the optical densities (ODs) were recorded at 450 nm using a micro plate reader (Tecan Spectra, Austria).

The net OD for each sample was calculated by subtracting from the reading for each sample's well the OD of the corresponding negative control. According to the manufacturer's QC data sheet, the limit of positivity of OD for the alpha, beta, and epsilon toxins is 0.150. Therefore, any sample that yielded a difference in OD that is greater than or equal to 0.150 was considered positive for the toxins tested. Conversely, any sample that yielded a difference in the OD less than 0.150 was considered negative.

The results were expressed as the percentage of positivity for *C. perfringens* types. A chi-squared test was used to compare the differences between the percentages of biotypes.

In the present study, faecal samples from 25 healthy neonatal calves were found to be negative for alpha, beta, and epsilon toxins by ELISA. The means of OD of these control animals were 0.071 ± 0.015 for alpha toxin, 0.070 ± 0.012 for beta toxin, and 0.075 ± 0.006 for epsilon toxin. The mean of the OD of control animals was less than the limit of positivity of OD suggested by the manufacturer. In our study, a total of 122 (81.33%) samples were positive for the toxins produced by *C. perfringens*, while 28 samples (18.66%) were negative. According to the toxins produced by the bacterium, 61 (40.66%), 19 (12.66%), 28 (18.66%), and 14 (9.33%)

of these positive samples were identified as *C. perfringens* types A, B, C, and D, respectively. *C. perfringens* type A was higher than the other types detected in the samples ($P < 0.001$; Table).

In the present study, congestion and enlargement of the spleen and liver, tympanites, and haemorrhages and ulcers on the abomasum and intestines were obtained upon post-mortem examination of the dead calves. Furthermore, epicardial haemorrhages and pericardial gelatinous effusion were also detected in these calves. All of the samples collected from the dead calves were also positive for *C. perfringens* toxins. Four (30.76%) and 9 (69.23%) of these toxin-positive samples were identified as types B and C, respectively (Table).

In the present study, a total of 122 (81.33%) samples collected from diarrhoeic calves were positive for the toxins produced by *C. perfringens*. *C. perfringens* types A, B, C, and D were identified in the samples but type A was the dominant type as detected by ELISA. Furthermore, all of the samples collected from dead calves suspected of having enterotoxaemia were also positive for *C. perfringens* toxins. Four (30.76%) and 9 (69.23%) of these toxin-positive samples were identified as types B and C, respectively. *C. perfringens* may have played a role in the development of diarrhoea either alone or in combination with other bacteria, viruses, or parasites in the neonatal calves studied. Furthermore, it may also cause bovine enterotoxaemia as its toxins were detected in the dead calves in this study.

Table. Number and percentages of *C. perfringens* biotypes in the diarrhoeic, dead, and healthy calves.

<i>C. perfringens</i> types	Number (%) of positive samples		
	Diarrhoeic calves (n = 150)	Dead calves (n = 13)	Healthy calves (n = 25)
Type A (α)	61 (40.66%) ^a	0	0
Type B (α - β - ϵ)	19 (12.66%) ^{bd}	4 (30.76%)	0
Type C (α - β)	28 (18.66%) ^c	9 (69.23%)	0
Type D (α - ϵ)	14 (9.33%) ^d	0	0
Total positive samples	122 (81.33%)	13 (100%)	0
Negative samples	28 (18.66%)	0	25 (100%)

^{a-d}: The significance of differences of *C. perfringens* types within column is indicated by different superscript letters ($P < 0.001$).

Several bacteria, viruses, and parasites can play a role in the aetiology of neonatal diarrhoea in calves (1-3,5). However, there are few studies on the aetiology of neonatal diarrhoea in calves in Kars district. In these studies, the incidence of neonatal diarrhoea was reported to be 28.8%, and *E. coli*, *Salmonella* spp., *Campylobacter* spp., Rotavirus, Coronavirus, *Eimeria* spp., and *C. parvum* were detected as the causative agents of diarrhoea in calves (15,16). *C. perfringens* is one of the causative agents of neonatal diarrhoea and bovine enterotoxaemia (2,6-8). Clostridial infections usually occur in young animals, particularly in calves less than 2 weeks of age, although it has been reported in calves up to 2 months of age (7-9). The incidence of *C. perfringens* in bovine diarrhoea and enterotoxaemia has been reported to range between 19% and 80% worldwide. In these studies, *C. perfringens* types A, B, C, and D were detected in calves and the majority of isolates were identified as type A, in agreement with the results obtained in this study (6,8,9,17,18).

In Kars, most farmers believe that the colostrum induces neonatal diarrhoea in calves and therefore newborn calves are not allowed to consume adequate amounts of colostrum in first days of life. However, it is well known that the cow produces antibodies that are then passed into the colostrum, which should be consumed in sufficient amounts after birth. These maternal antibodies may protect newborn calves against clostridial infections in first weeks of their life (1,2).

It is very important that the vaccine should be applied in time and it should stimulate strong immunological reaction for all the *C. perfringens* types causing diarrhoea or enterotoxaemia. However, in this district, most farmers do not vaccinate their animals against *C. perfringens* and more than half of the owners who apply

the vaccine give one dose. It has been indicated that the response to clostridial antigens is greater when the second immunising dose is delayed 2 to 6 weeks after the initial dose (19). It has also been reported that antibody titres for clostridial diseases in colostrum can be enhanced if dams are vaccinated approximately 4 months before calving (20). Therefore, insufficient consumption of colostrum in the first days of life and lack of vaccination in calves against *C. perfringens* may be responsible for the high incidence of neonatal diarrhoeal and clostridial infections in Kars. It is well known that enterotoxaemia causes considerable economic losses to the dairy industry because of high fatality, decreased productive efficiency, and increased treatment cost (7,9). On the other hand, calf losses caused by clostridial diseases can be prevented with appropriate management and vaccination timing (20).

In conclusion, the results of the present study indicated that *C. perfringens* types A, B, and C should be considered the common cause of clostridial diarrhoea and/or enterotoxaemia either alone or in combination with other pathogens in neonatal calves in Kars. It is strongly recommended that a vaccination schedule be applied to reduce the incidence of neonatal diarrhoea and enterotoxaemia caused by *C. perfringens* types in the district. These vaccines should be applied to dairy cattle and newborn calves to provide an adequate protective immunity, especially against *C. perfringens* types A, B, and C.

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