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

Masoumeh Hayati[1*] , Mehrdad Shamseddini[2], Yahya Tahamtan[1], Safar Sadeghzadeh[1], Mohsen Manavian[1], Davood Nikoo[3]

[1]Razi Vaccine and Serum Research Institute, Shiraz Branch, Agricultural Research, Education and Extension Organization, Shiraz, Iran
[2]Razi Vaccine and Serum Research Institute, Kerman Branch, Agricultural Research, Education and Extension Organization, Kerman, Iran
[3]Veterinary Private Sector Clinician, Shiraz, Iran

*Corresponding Author:
Masoumeh Hayati,
Razi Vaccine and Serum
Research Institute, Shiraz
Branch, Mirzaye Shirazi Street,
Sanaye Square, Shiraz, Iran
Tel: +989177148712;
Email: hayatimax@yahoo.co.uk

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 pathogenic microorganism 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.

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.[1] The bacteria can produce histotoxic and fatal diseases such as enterotoxaemia in livestock.[2,3] There are many reports of annual mortality and economic losses to the farming industry worldwide, including our country.[4,6] Today, nearly 16 different toxins in various combinations are known to be encoded by *C. perfringens* contributing to its pathogenicity.[7,8] 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.[7-10] Each toxin type is associated with different areas of pathology and specific enteric infection.[8]

Toxin type A is of importance in food poisoning, antibiotic-associated diarrhea, and gas gangrene in humans and enteritis in some animal species.[11] 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.[3,7,12]

Toxin typing is essential for diagnosing diseases and investigating the epidemiology of these bacteria.[13,14] 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.[15] 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 16srRNA 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.

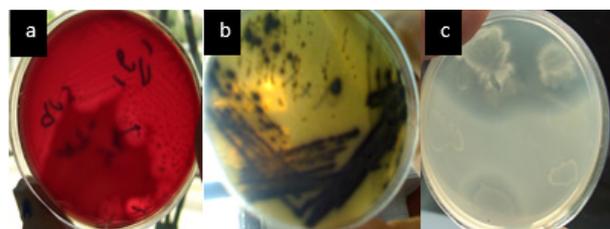Using specific primers for the detection of *C. perfringens* species, the PCR results showed that all isolates yielded

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.

Table 1. List of the Applied Primers

Primer Name	Specification	Sequence	Product Size (bp)	Reference
16SrRNA	*C. perfringens* species	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279	16
CPA	Alpha toxin	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	17
CPB	Beta toxin	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	17
ETX	Epsilon toxin	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	17
Ia	Iota toxin	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	17

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 Ia 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, χ^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.[18] 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.[4,5]

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.[5] Various primers have been designed and introduced in different studies. The primers, as described by Wang et al[16] and Meer and Songer[17] were used in the current study.

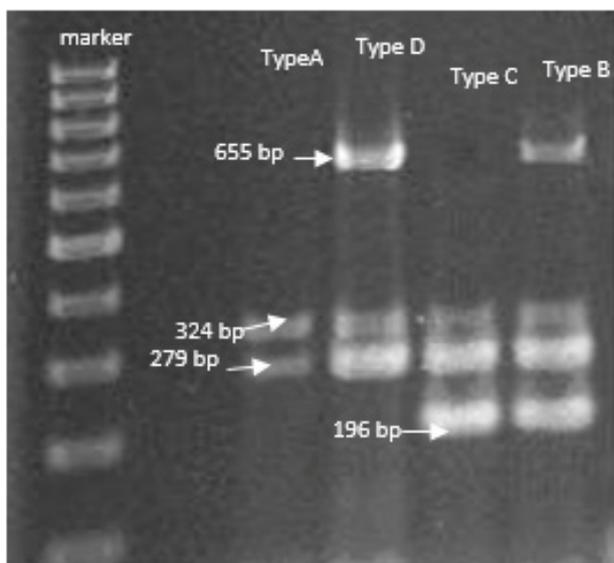Based on our results, all four types of *C. perfringens*

Figure 2. Gel Electrophoresis of the Amplified Fragments of Different Toxin Types of *Clostridium perfringens*, Along With the Genus Specific Band in a Multiplex PCR Assay. *Note*. PCR: Polymerase chain reaction. The left lane shows the 100 bp marker (CinnaGen, Iran).
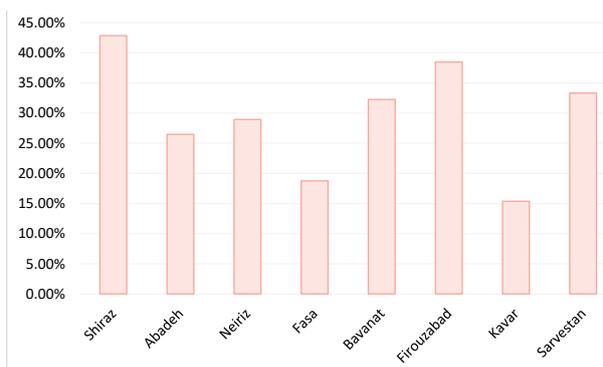

Figure 3. The Percentage of the Isolated *Clostridium perfringens* Strains From the Samples of Each Place in Fars Province.

(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.[19-22]

Afshari and Poursoltani reported the type C of *C. perfringens* as a predominant type in poultry in Mashhad

Table 2. The Number and Percentage of Different Toxin Types of *Clostridium perfringens* Isolates in Cattle, Sheep, and Goats

Toxin Types				Group
Type D	Type C	Type B	Type A	
6 (17.14%)	9 (25.71%)	1 (2.85%)	19 (54.28%)	Cattle
11 (15.94%)	14 (20.29%)	10 (14.49%)	34 (49.27%)	Sheep
6 (15.79%)	7 (18.42%)	2 (5.26%)	23 (60.52%)	Goats

although they pointed out that type A was the predominant type by several studies in other countries.[23]

According to the results of Hadimli et al on the genotyping of the isolated *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.[24]

Gökce et al reported a prevalence of 47.27%, 9.09%, 4.54%, and 39.07% of *C. perfringens* types A, B, C, and D, respectively, by ELISA tests in the Kars province of Tukey.[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.[26]

These results demonstrate that type A is a dominant genotype of *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 *C. perfringens* isolated from enterotoxemic sheep, goats, and cattle during 2014-2015. They reported that *C. perfringens* type A was the dominant type (67.2%), followed by types D (16.4%), B (13.4%), and C (3%).[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.[6]

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

These results regarding the higher frequency of type A *C. perfringens* are consistent with our results, representing the role of type A *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.[4]

This discrepancy can be due to the geographical difference.

Based on our results, type E of *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.[8,27]

However, Songer[27] and Miyashiro et al[28] reported the isolation of two strains of type E from the cattle.[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.[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%) *C. perfringens* strains were isolated from 119 samples in this region. The rate of *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 *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 *C. perfringens* into seven types in 2018. Based on this classification, the enterotoxin producing type A of *C. perfringens* was reclassified as type F, and those harboring *netB* genes (specific to poultry) were grouped as type G.[10] However, the current study did not investigate the presence of the *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 *C. perfringens* are conjugative, indicating the possibility of the toxin gene transfer to intestinal flora strains and the virulence plasticity of this microorganism.[8,29]

The detection of toxin types increases our understanding of the epidemiology of related diseases. On the other hand, vaccination against *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.

References
1. Kiu R, Hall LJ. An update on the human and animal enteric pathogen *Clostridium perfringens*. Emerg Microbes Infect. 2018;7(1):141. doi:10.1038/s41426-018-0144-8
2. Rood JI. *Clostridium perfringens* and histotoxic disease. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, eds. The Prokaryotes. New York, NY: Springer; 2006:753-770. doi:10.1007/0-387-30744-3_23
3. Shrestha A, Uzal FA, McClane BA. Enterotoxic clostridia: *Clostridium perfringens* enteric diseases. Microbiol Spectr. 2018;6(5). doi:10.1128/microbiolspec.GPP3-0003-2017
4. Ahsani MR, Mohammadabadi MR, Shamsaddini MB. *Clostridium perfringens* isolate typing by multiplex PCR. J Venom Anim Toxins incl Trop Dis. 2010;16(4):573-578. doi:10.1590/s1678-91992010000400006
5. Moosawi Shooshtari M, Pilehchian Langroudi R, Esmaelizad M, Afshari Far S, Jabbari AR, Abdolmohammadi Khiav L. Molecular typing of toxigenic *Clostridum perfringens* isolated from sheep in Iran. Arch Razi Inst. 2011;66(2):81-86. doi:10.22092/ari.2016.103869
6. Nazki S, Wani SA, Parveen R, et al. Isolation, molecular characterization and prevalence of *Clostridium perfringens* in sheep and goats of Kashmir Himalayas, India. Vet World. 2017;10(12):1501-1507. doi:10.14202/vetworld.2017.1501-1507
7. Uzal FA, Vidal JE, McClane BA, Gurjar AA. *Clostridium perfringens* toxins involved in mammalian veterinary diseases. Open Toxinology J. 2010;2:24-42.
8. Li J, Adams V, Bannam TL, et al. Toxin plasmids of *Clostridium perfringens*. Microbiol Mol Biol Rev. 2013;77(2):208-233. doi:10.1128/mmbr.00062-12
9. Sayeed S, Li J, McClane BA. Characterization of virulence plasmid diversity among *Clostridium perfringens* type B isolates. Infect Immun. 2010;78(1):495-504. doi:10.1128/iai.00838-09
10. Rood JI, Adams V, Lacey J, et al. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe. 2018;53:5-10. doi:10.1016/j.anaerobe.2018.04.011
11. Azimirad M, Gholami F, Yadegar A, et al. Prevalence and characterization of *Clostridium perfringens* toxinotypes among patients with antibiotic-associated diarrhea in Iran. Sci Rep. 2019;9(1):7792. doi:10.1038/s41598-019-44281-5
12. Ali Nasir A, Younus M, Rashid A, et al. Clinico-pathological findings of *Clostridium perfringens* type D enterotoxaemia in goats and its hemolytic activity in different erythrocytes. Iran J Vet Res. 2015;16(1):94-99.
13. Omer SA, Babiker SEH, Aljulaifi MZN, Al-Olayan EM, Alagaili AN, Mohammed OB. Epidemiology of enterotoxaemia in livestock in the Kingdom of Saudi Arabia. J King Saud Univ Sci. 2020;32(5):2662-2668. doi:10.1016/j.jksus.2020.06.001
14. Uzal FA, Songer JG. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. J Vet Diagn Invest. 2008;20(3):253-265. doi:10.1177/104063870802000301
15. Jouseimies-Somer H, Summanen P, Citron D, Baron E, Wexler H, Finegold S. Wadsworth-KTL Anaerobic Bacteriology Manual. 6th ed. Star Publishing; 2002.
16. Wang RF, Cao WW, Franklin W, Campbell W, Cerniglia CE. A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. Mol Cell Probes. 1994;8(2):131-137. doi:10.1006/mcpr.1994.1018
17. Meer RR, Songer JG. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am J Vet Res. 1997;58(7):702-705.
18. Bokori-Brown M, Savva CG, Fernandes da Costa SP, Naylor CE, Basak AK, Titball RW. Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. FEBS J. 2011;278(23):4589-4601. doi:10.1111/j.1742-4658.2011.08140.x
19. Gkiourtzidis K, Frey J, Bourtzi-Hatzopoulou E, Iliadis N, Sarris K. PCR detection and prevalence of alpha-, beta-, beta 2-, epsilon-, iota- and enterotoxin genes in *Clostridium perfringens* isolated from lambs with clostridial dysentery. Vet Microbiol. 2001;82(1):39-43. doi:10.1016/s0378-1135(01)00327-3
20. Albini S, Brodard I, Jaussi A, et al. Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. Vet Microbiol. 2008;127(1-2):179-185. doi:10.1016/j.vetmic.2007.07.024
21. Kalender H, Ertas HB, Cetinkaya B, Muz A, Arslan N, Kilic A. Typing of isolates of *Clostridium perfringens* from healthy and diseased sheep by multiplex PCR. Vet Med Czech. 2005;50(10):439-442.
22. Koç R, Gökce HI. Determination of the toxins and biotypes of *Clostridium perfringens* in diarrhoeic calves in the Kars district of Turkey. Turk J Vet Anim Sci. 2007;31(3):207-211.
23. Afshari A, Jamshidi A, Razmyar J, Rad M. Genotyping of *Clostridium perfringens* isolated from broiler meat in northeastern of Iran. Vet Res Forum. 2015;6(4):279-284.
24. Hadimli HH, Erganiş O, Sayin Z, Aras Z. Toxinotyping of *Clostridium perfringens* isolates by ELISA and PCR from lambs suspected of enterotoxemia. Turk J Vet Anim Sci. 2012;36(4):409-415.
25. Gökce HI, Genç O, Sözmen M, Gökçe G. Determination of *Clostridium perfringens* toxin-types in sheep with suspected enterotoxemia in Kars province, Turkey. Turk J Vet Anim Sci. 2007;31(5):355-360.
26. Tutuncu M, Kilicoglu Y, Guzel M, Pekmezci D, Gulhan T. Prevalence and toxinotyping of *Clostridium perfringens* enterotoxins in small ruminants of Samsun province, Northern Turkey. J Anim Plant Sci. 2018;28(4):1204-1207.
27. Songer JG, Miskimmins DW. *Clostridium perfringens* type E enteritis in calves: two cases and a brief review of the literature. Anaerobe. 2004;10(4):239-242. doi:10.1016/j.anaerobe.2004.05.00
28. Miyashiro S, Baldassi L, Nassar AFC. Genotyping of *Clostridium perfringens* associated with sudden death in cattle. J Venom Anim Toxins Incl Trop Dis. 2009;15(3):491-497. doi:10.1590/s1678-91992009000300010
29. Hughes ML, Poon R, Adams V, et al. Epsilon-toxin plasmids of *Clostridium perfringens* type D are conjugative. J Bacteriol. 2007;189(21):7531-7538. doi:10.1128/jb.00767-07