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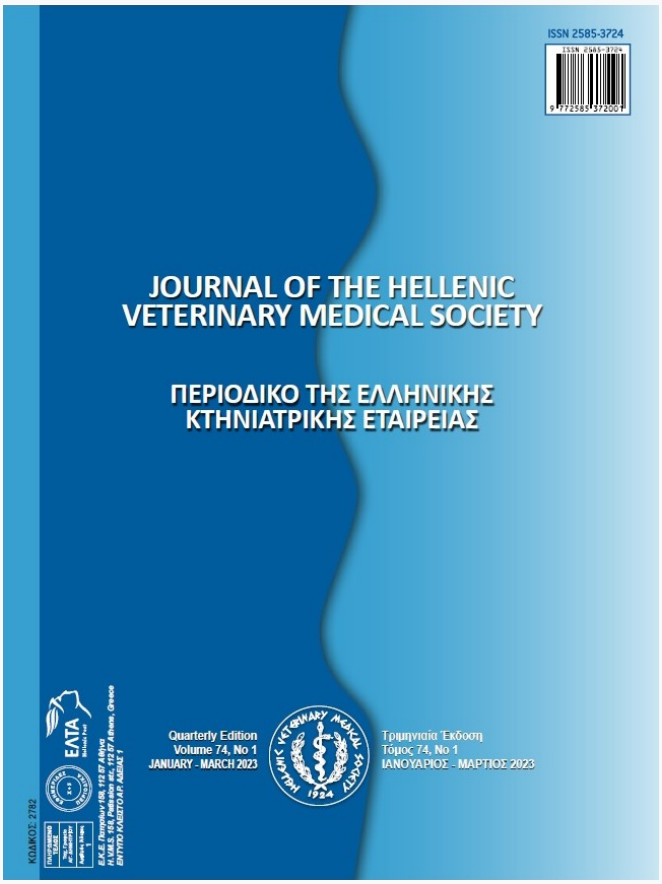

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Research article
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Molecular identification and sequence analysis of *Clostridium perfringens* virulence genes isolated from sheep and goats

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ABSTRACT: *Clostridium perfringens* (*C. perfringens*) belongs to the family of *Clostridiaceae* and produces a wide range of toxins (four major and a variety of minor toxins). Some toxins associated with virulence have been shown to participate in the pathogenesis of enteric diseases in sheep, goats, and other animals. This study aimed to determine the presence of minor virulence genes and their genetic diversity in the various toxin types of *C. perfringens* isolates. About 84 isolates collected from sheep and goat flocks were examined and sequenced for the presence of minor virulence genes. Results showed that *PFO* and *cpb2* genes were found in 79 out of 84 (94%), *cpe* gene was identified in 29 out of 84 (35%) and the presence of *tpeL* gene was confirmed in 28 out of 84 (33%) isolates, while none of the isolates were identified as carrying the *netB* gene. This study shows that the prevalence of genes varied among various types of *C. perfringens* isolates and also sheep and goat samples, furthermore these findings change the toxin types of isolates based on a modified scheme of toxin type, which incorporated CPE and NetB toxins. The results of this study showed that the dominant minor virulence genes were *PFO* and *cpb2* and the occurrence of *cpe* and *tpeL* genes was also diverse. DNA sequencing of toxinx genes revealed approximately a sequence similarity of 97−100% with the GenBank database and sequence analysis showed 3 mutations in *pfo* and *cpe* genes.

Keywords: *Clostridium perfringens*; Genetic diversity; Sequence; Toxin type; Virulence.

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INTRODUCTION

Clostridium perfringens (*C. perfringens)* consists of a well-defined group of Gram-positive, rod-shaped spore-forming anaerobic bacterium with a ubiquitous environmental distribution such as soil, food, feces, and the normal intestinal flora of humans and animals (Michel and Stiles 2005). It causes abundant gastrointestinal infections in animals and has different characteristics in sheep and goats (Revitt-Mills et al. 2015, Uzal and Songer 2008). More than 20 genes, encoding toxins, and extracellular enzymes have been identified, which are important in the pathogenesis of *C. perfringens* besides the predisposing environmental factors (Allaart et al. 2013, Mehdizadeh Gohari et al. 2021).

C. perfringens produces an array of extracellular toxins called major and minor toxins encoded by virulence associated genes, which have a vital contribution to disease; some of them act only locally, while others act both locally and systemically (Songer and Uzal 2005, Uzal et al. 2014). *C. perfringens* is divided into five toxin types (A-E), according to the presence and combination of the four major toxins: alpha (*CPA*), beta (*CPB*), epsilon (*ETX*), and iota (*ITXA* and *ITXB*).

This traditional classification has been changed based the presence of minor toxins including enterotoxin *(*CPE*)* and necrotic enteritis B-like *(*NetB) toxin to seven toxin types (A-G) (Michel and Stiles 2005, Rood et al. 2018). Each toxin type can produce one or more non-typing minor toxins, which are important in the pathogenicity of *C. perfringens* (Kiu and Hall 2018, Uzal et al. 2010)

Toxin perfringens large (TPEL), NetB, CPE, perfringolysin O (PFO), and Beta 2 toxin (CPB2) are some of the proven virulence factors of *C. perfringens* (Mehdizadeh Gohari et al. 2021). These minor toxins have been illustrated in a wide variety of wild and domestic animals; some of them are involved in the induction of enteric diseases like necrohemorrhagic lesions by synergy with other major toxins of *C. perfringens* (Freedman et al. 2016, Verherstraeten et al. 2015). There are few studies on minor virulence genes of *C. perfringens* in Iran (Yadegar et al. 2018, Jabbari et al. 2012), so detection of these genes across the different *C. perfringens* types would provide more information regarding the importance of these toxins and lead to a greater understanding of diseases caused by *C. perfringens*. Furthermore, it is important to assess minor virulence genes to provide a more complete genomic picture of *C. perfringens* and elucidate the contributions of these genes to pathogenicity.

Nucleic acid-based detection methods have the advantages of being fast, relatively inexpensive, and highly specific for the presence of certain toxin genes of *C. perfringens* (Duracova et al. 2019, Heikinheimo and Korkeala 2005) so these are the best methods for gene identification.

The aim of this study was molecular detection of *C. perfringens* minor virulence genes isolated from clinical samples of sheep and goats, as well as sequencing of toxin genes and phylogenetic analysis. All isolates were analyzed concerning toxin type, presence of genes coding for minor toxins, and source of isolation (sheep and goat).

MATERIALS AND METHODS

Samples

A total of 84 clinical isolates of *C. perfringens* types A, B, C, and D that originated from sheep (n = 51) and goats (n = 33) were provided by the microbial archive of Razi Institute (south-east branch) that located in Kerman (Ahsani et al. 2010, Ezatkhah et al. 2016). These isolates were identified by the diagnostic lab, as positive for *C. perfringens* by microbiological, biochemical, and molecular evaluations (Ahsani et al. 2011, Ezatkhah et al. 2016).

According to the molecular methods out of total 84 isolates, 22, 19, 22, and 21 were designated as toxin type A, B, C, and D, respectively, using standard *C. perfringens* reference strains (Ahsani et al. 2011, Ezatkhah et al. 2016)

Isolates were removed from the cryotube stored at -70°C and then, it smeared on blood agar medium containing 5% defibrinated sheep blood, which was incubated in an anaerobic jar, using Anoxomat (Mart Microbiology B.V Drachtenthe, The Netherlands) with an atmosphere of 10% CO_2, 10% H_2, and 80% N_2, for 48 hours at 37 °C.

DNA extraction

A single colony exhibiting double-zone hemolysis was selected and suspended in 1.5 ml microcentrifuge tubes, containing 100 µl of distilled water by gentle vortexing. Total DNA was extracted from the bacterial cells, using the Genomic DNA Purification kit (Sinacolon, Iran), according to the manufacturer's instructions. Chromosomal DNAs obtained were used

as templates for all PCR experiments. Quantification and purity of extracted DNA samples were carried out using a spectrophotometric reading (Pg Instruments Limited, Leicestershire UK) using 1μL of extracted DNA based on the manufacturer's instruction. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. The bacterial DNA was stored at -20 °C until further use for PCR reactions.

Molecular detection of virulence genes

PCR assay was used to identify the targeted virulence genes *(cpb2*, *cpe*, *tpeL*, *PFO,* and *netB*) by using five pairs of specific primers, Primer sequences and corresponding lengths of amplicons and also the amplification programs are shown in Table 1.

PCR reaction mixtures were performed in a final 25 μl reaction volumes containing 12.5 μl of mas-

Table 1. Oligonucleotide sequences and amplification programs of target genes

Target gene	Primers	Amplification programs	Product size (bp)	Reference
netB	*netB*R *netB*F	95 °C (5 min), 94 °C (1 min), 52.5 °C (1 min),72 °C (1.5 min), 72 °C (10 min)	384	(Keyburn et al., 2008)
cpb2	*cpb2*R *cpb2*F	95 °C (7 min), 94 °C (1 min), 52.5 °C (1 min), 72 °C (1.5 min), 72 °C (10 min)	548	(van Asten et al., 2010)
tpeL	*tpeL*R *tpeL*F	95 °C (4 min), 94 °C (45 S), 54 °C (45 S) ,72 °C (1min), 72 °C(7 min)	466	(Bailey et al., 2013)
cpe	*cpe*F *cpe*R	94 °C (5 min), 94 °C (1 min) 53 °C (1 min), 72 °C (30 sec), 72 °C (5 min)	485	(Hayati and Tahamtan, 2020)
PFO	*pfo*F *pfoa*R	95 °C (5 min), 95 °C (1 min), 55°C (1 min), 72 °C (1.5 min), 72 °C (5 min)	532	(Fisher et al., 2006)

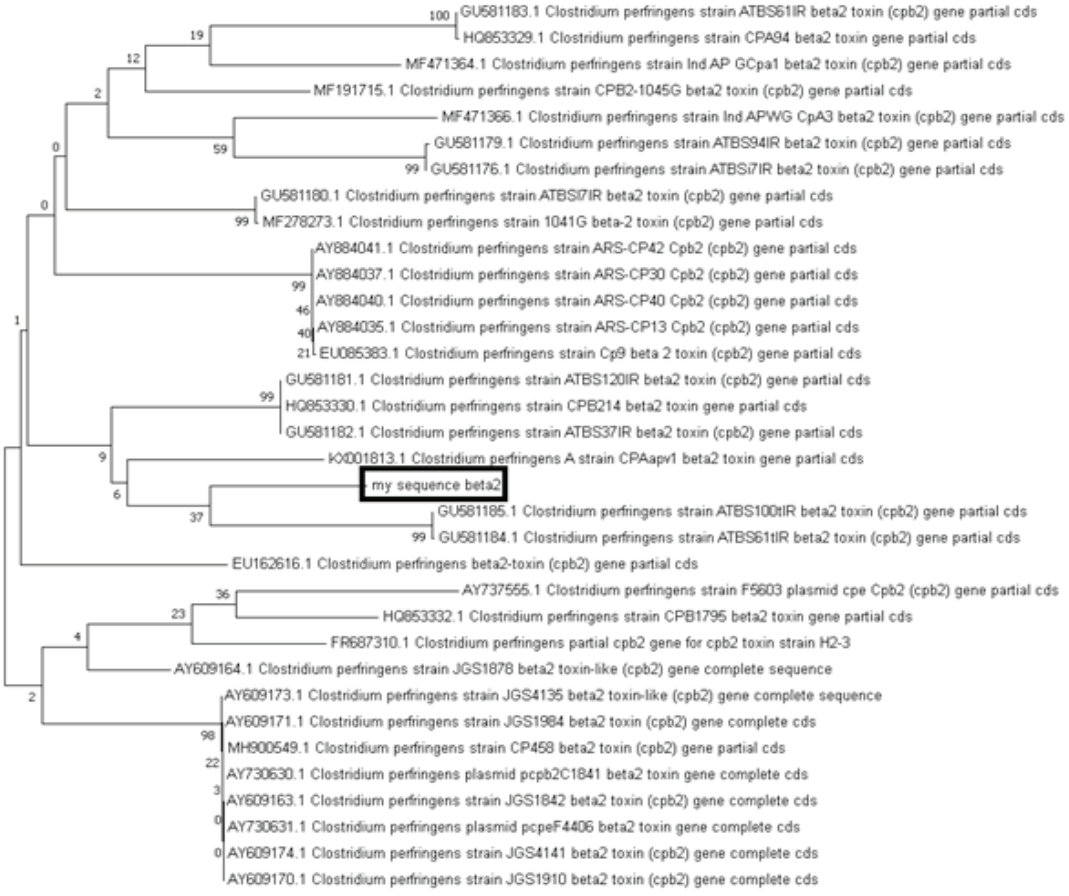

Figure 1. Phylogenetic tree of cpb2 gene in one of C. perfringens isolates based on nucleotide sequences.

ter mix, 1.5 µl of each primer (20 pmol/µl), 7 µl of DNAse free deionized water, and 2.5 µl of template DNA, in a thermocycler (Bio-Rad, California, USA). Subsequently, a fraction of each PCR product was analyzed by gel electrophoresis at 90 V in a 1.5% agarose gel for 2 hours. About 10 µl of safe stain color solution was added to the agarose for DNA visualization. Amplified bands were inspected under a UV transilluminator and then, photographed using the gel imaging system (Uvitec, Cambridge, UK). The PCR products were purified using the QIA quick kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction.

Sequencing and phylogenetic analysis

Purified PCR products were sequenced with forward and reverse primer by the Sinacolon facility (Tehran, Iran). After the conversion of the sequences to a FASTA format the genetic changes and multiple sequence alignment analysis were performed by using the computer program MEGAX (Kumar et al. 2020).

Sequence similarity of our genes with genes sequences available in the GenBank database was estimated by using NCBI-Blast (Basic Local Alignment Search Tool). The Phylogenetic tree was constructed with the neighbor-joining algorithm (Figure 1) Sequences used for comparison or phylogenetic analysis in this study were obtained from the GenBank databases.

Statistical analysis

To compare the frequencies obtained for virulence genes the Statistical analysis was performed using SPSS, 21 statistical software (SPSS Inc., Chicago, IL). The chi-square test (X^2) was applied to compare distribution among sheep and goat samples and the statistically significant difference was defined as $P< 0.05$ (Sharpe 2015).

RESULTS

Out of 84 samples, 79 (94%) were *cpb2* and *PFO* positive, 28 (33%) were *tpeL* positive, and *cpe* was detected in 29 (35%) isolates, as shown in Figure 2; meanwhile, none of the *C. perfringens* isolates carried the *netB* gene.

Prevalence of virulence genes among toxin types

Prevalence of virulence genes among toxin types indicated that *cpb2* (100%), *PFO* (100%), *tpeL* (84%),

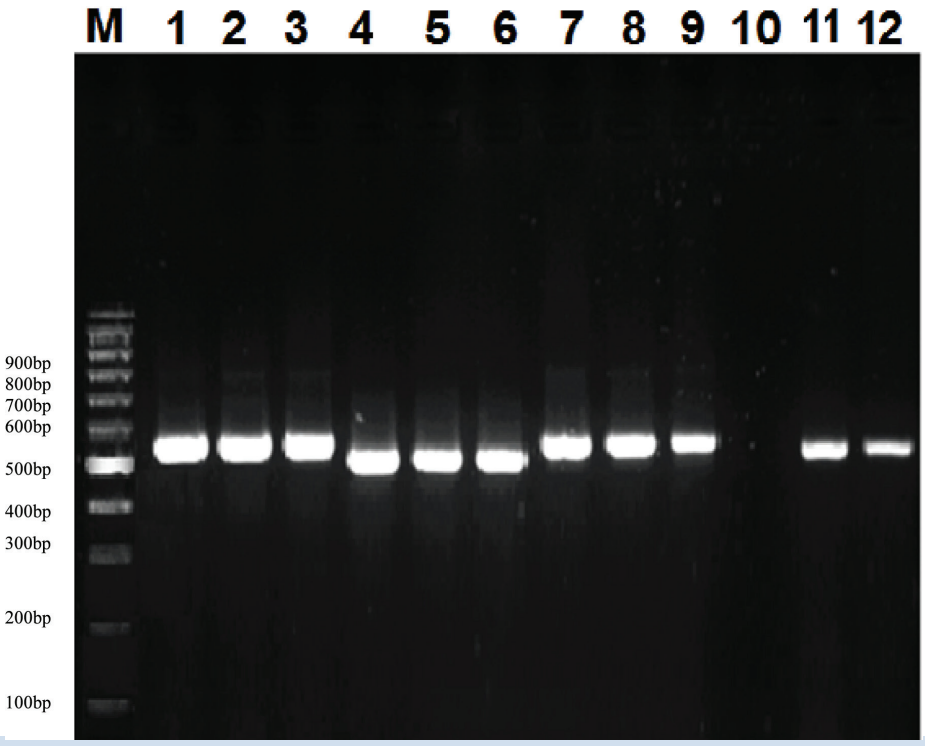

Figure 2. Gel electrophoresis obtained from PCR products of *C. perfringens* isolates

M : Marker (100 bp ladder); lane (1) : *cpb2* positive control; lane (2-3) : *cpb2* samples (548 bp); lane (4) : *tpeL* positive control; lane(5-6) : *tpeL* samples (466 bp); lane (7) : *PFO* positive control; lane (8-9) : *PFO* samples (532 bp); lane (10) : negative control; lane (11) : *cpe* positive control; lane (12) : *cpe* sample (485 bp).

and *cpe* (68%) were the most frequent in type D, B, B, and A, respectively. No *cpe*-positive type B and *tpeL* positive type D isolates have been identified in this study. The percentage of each toxin gene in the samples and also in various toxin types of *C. perfringens* isolates is represented in Figure 3.

Distribution of virulence genes in sheep and goat isolates

A comparison of the distribution of the studied virulence genes in *C. perfringens* isolates that originated from sheep and goats showed that out of 51 sheep isolates 48 (94%), 47 (92%),16 (31%), and 14 (27%) were positive for the *PFO, cpb2, cpe*, and *tpeL* genes, respectively; while *PFO* 31 (94%), *cpb2* 32 (97%), *tpeL* 14 (42%), and *cpe* 13 (39%), were the most common virulence genes in 33 goat isolates. The difference in the distribution and frequency of virulence genes among sheep and goat isolates concerning toxin types is shown in Table 2.

Sequence analysis

Sequence analysis of the successful PCR products confirmed closed relationships with others world strains that were previously deposited in the GenBank database (97-100%).Sequence homology of *PFO* gene in one isolate with the previously identified isolates is indicated in Table 3.

Sequence analysis showed 2 mutations in two sequenced sample of *cpe* genes that led to changes in amino acids. The first mutation in one sample converted TGC to TTC, which led to a protein change by placing the amino acid Phenylalanine, instead of Cysteine and the second one in another codon converted AAA to AGA and placed the amino acid Arginine, instead of Lysine.

The results of *pfo* gene sequence analysis in one sample showed a mutation that led to a change in one amino acid and subsequently convert CAG to CGG and Glutamine placed instead of Arginine. No muta-

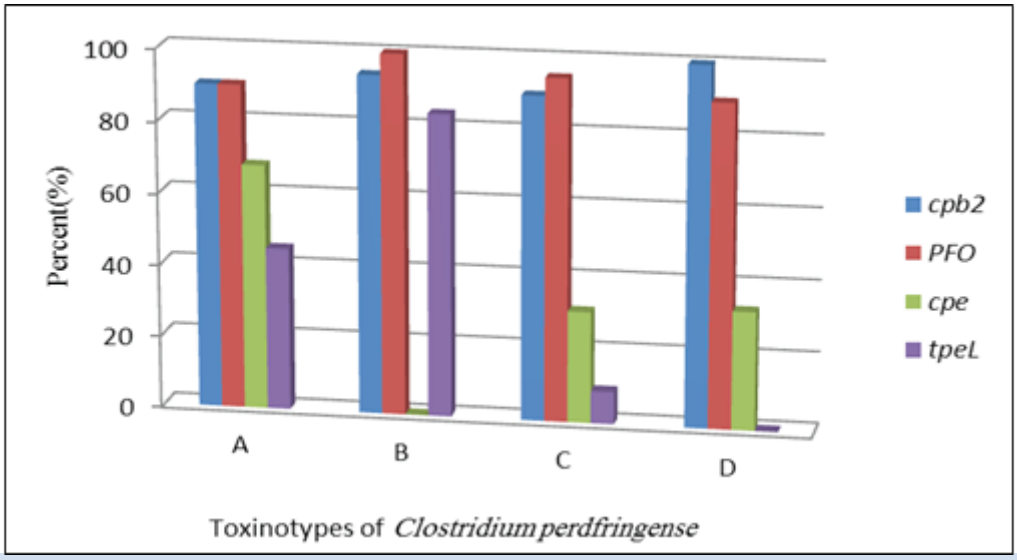

Figure 3. Percentage distribution of minor toxin genes in different toxinotypes of C. perfringens isolates.

Table 2. The distribution of minor toxin genes in different toxinotypes of C. perfringens in gaot and sheep isolates

Animal	Toxinotype	Toxin genes				
		cpb2	*PFO*	cpe	*tpeL*	*netB*
Sheep	A (n = 14)	13	13	8	4	0
	B (n = 10)	9	10	0	9	0
	C (n = 15)	13	14	4	1	0
	D (n = 12)	12	11	4	0	0
Goat	A (n = 8)	7	7	7	6	0
	B (n = 9)	9	9	0	7	0
	C (n = 7)	7	7	3	1	0
	D (n = 9)	9	8	3	0	0
Total		79	79	29	28	0

Table 3. Homology of pfo gene in one of C. perfringens isolate with the previously identified isolates

Row	Definition	Query Cover (%)	Identity (%)	Accession
1	MF-CA 1 (pfoA) gene	98	98.78	MK599270.1
2	CN5388 (pfoA) gene	98	98.78	DQ673100.1
3	BAR3 (pfoA) gene	98	98.78	DQ673097.1
4	NCTC 8533 (pfoA) gene	98	98.58	EF165972.1
5	(pfoR) gene, complete cds	98	98.37	M81080.1
6	(pfoA) gene, complete cds.	98	98.37	M36704.1
7	CN3947 (pfoA) gene	98	98.17	DQ673099.1
8	(pfoA) gene, partial cds.	98	98.08	AY304477.1
9	NCTC10719 (pfoA) gene,	98	97.76	DQ673098.1

tion was identified in *tpel* and *cpb2* genes and they were identical 100%.

DISCUSSION

Minor toxins cause the symptoms correlated with several gastrointestinal diseases by induction of necrohemorrhagic lesions with clinical signs, and they have a synergistic role with major toxins of *C. perfringens* in domestic and wild animals (Amimoto et al. 2007, Rood et al. 2016, van Asten et al. 2010).

In the present study, the abundance of 5 genes encoding minor toxins (*cpe*, *netB*, *cpb2*, *tpeL,* and *PFO*) of *C. perfringens* was revealed for isolates that originated from sheep and goat populations. Many of them are plasmid-encoded, including, *cpb2*, *tpeL,* and *netB*, while the *PFO* is located on the chromosome, and *cpe* can be either chromosomally or plasmid-encoded (Li et al. 2013, Navarro et al. 2018)

Our results showed that the frequency of toxin genes varied between the toxin types. In the current study we found that the predominant virulence genes among all isolates from different toxin types were *cpb2* and *PFO* (94%) and the occurrence of *cpe* and *tpeL* toxin genes in various toxin types was also diverse. Meanwhile based on *netB* gene, all *C. perfringens* isolates were negative. The present study is the first report of the characterization of the *PFO* gene in sheep and goats in Iran.

The detection rate for *cpb2* in this study was higher than those reported for similar, previously published studies. A high prevalence of *cpb2* has been reported in sheep (72.17%) and goats (61.11%) (Singh et al. 2018). The *cpb2* gene was reported in 51.8% of *C. perfringens* sheep isolates that suffered from enterotoxaemia in Iran (Jabbari et al. 2012). Another study in Iran reported that 52% of sheep isolates were *cpb2* positive in PCR assay, in which 9 of 13 (69%) and 4 of 13 (31%) isolates were taken from diseased and healthy sheep, respectively (Yadegar et al. 2018).

Mohiuddin et al. (2020), showed the percentage of positive isolates for *cpb2* was remarkably higher in the diseased population (64%) of sheep and goats, compared to the healthy population (37%), also Fayez et al (2020) indicate that all *C. perfringens* isolates were found to be associated with *cpb2*+ genes, were detected only in diarrheic cases.

Based on our results, the presence of *tpeL* was confirmed in 33% of isolates, and it was more dominant in type B isolates while none of *C. perfringens* type D isolates carried the *tpeL* gene. The study of Chen and McClane (2015) surveyed carriage of the *tpeL* gene among different *C. perfringens* strains presented similar results in the detection of this toxin gene in some type A, B, and C strains but not in any of type D or E. This pattern could be due to incompatibility issues between plasmids carrying *tpeL*gene and the plasmids carrying *etx* genes in type D strains (Chen and McClane 2015). Evaluation of the *tpeL* gene presence in wild-type strains of *C. perfringens* in some type A, B, and C, indicated the presence of the *tpeL* gene, from only two of 22 surveyed type A strains (Sayeed et al. 2010). The *tpeL* gene had been discovered from all sources of isolates but the frequency of that was higher in strains isolated from animals and soil (Park and Rafii 2019).

Gurjar et al.(2010) reported that the prevalence of *cpe* gene was 33%, which was higher in type A than the others, while it was not detected in any of *C. perfringens* type B isolates. It could be possibly because 65 or 95 kb *cpb* plasmids are incompatible with some or all *cpe* plasmids. The prevalence and genetic diversity of the *cpe* gene in clinical isolates of *C. perfringens* were reported as 34.2% which was similar to our detection rate for this gene (Matsuda et al. 2019). Another study was carried out in the south

of Italy to assess the role of *Clostridia* in neonatal diseases of lambs and kids and showed that only 2 out of 25 isolates of *C. perfringens* were positive for the presence of the *cpe* gene (Greco et al. 2005). *C. perfringens* types C and D (*cpe*+) was detected in Argentina (Mignaqui et al. 2017). The presence of the *cpe*-positive type A isolates decisively showed in the small intestines of a goat kid, suffering from necrotic enteritis (Deguchi et al. 2009). Out of the 259 neonatal calves *cpe* gene was determined in 38 (14.6%) isolates (Athira et al. 2018), and in another study *cpe* was reported in (9/184) of ovine specimens, as well as typified in caprine specimens (4/54) (Forti et al. 2020).

Similar to previous studies in ENT or in non-ENT sheep, the *netB* gene was not found in *C. perfringens* isolates of our study (Yadegar et al. 2018). The *netB* gene is strongly associated with necrotic enteritis-derived strains from poultry except an isolate recovered from a 3-year-old cow with liver abscesses (Keyburn et al. 2008, Martin and Smyth 2009).

The analysis of the *C. perfringens* isolates for comparison of minor toxin gene distribution in sheep and goat species showed that although no statistically significant difference exists among the c*pb2* and *PFO* genes in both species but *cpe* and *tpeL* gene percentage in goat isolates was higher than sheep (Figure 4).

The DNA sequencing examined the nucleotide sequences and relationships with other world strains. The NCBI- BLAST algorithm determined the identity percentages with other world strains and they ranged from 97-100%, while 3 mutations were detected.

Overall impressive distribution of these minor toxins genes across the different *C. perfringens* types were observed, showing the importance of these toxins. Although little information is available on the pathogenesis of minor virulence genes in animals, the importance of *PFO* in the pathogenesis of gas gangrene, myonecrosis, histotoxic and *cpe* and *netB* in the intestinal disease-associated toxins infections has been largely controversial (Uzal et al. 2014). PFO toxin might also have a synergistic effect with other toxins and can be produced by different toxin types during sporulation (Brito et al. 2019, Fernandez-Miyakawa et al. 2008, Woudstra et al. 2018), also some studies emphasized the importance of *cpb2* gene in the outbreak of clostridial GI diseases like enterotoxemia in domestic animals, due to the isolation of *cpb2* gene in cases with severe clinical signs (Greco et al. 2005, Jabbari et al. 2012).

However, there are no commercial vaccines currently available that confer protection against minor toxins in animals because *C. perfringens* diseases are multifactorial, the most appropriate vaccines are recombinant multivalent vaccines that have a mixture of major and minor toxoids that evoke full immunity and increase protective response. A recombinant NetB vaccine had proven to create significant protection when used in combination with traditional toxoid

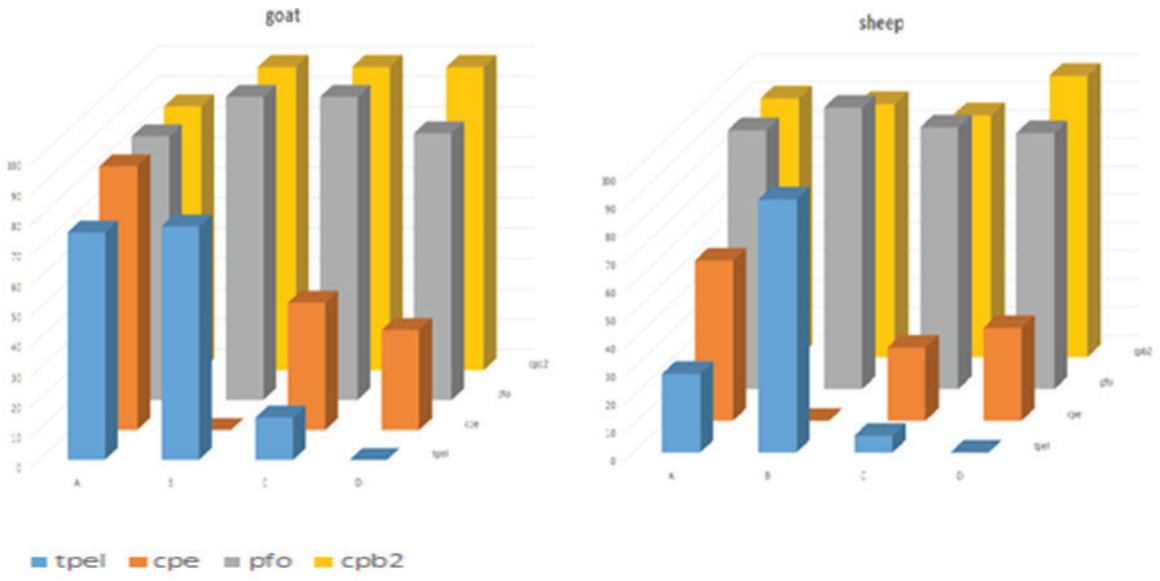

Figure 4. Comparison of minor toxin gene distribution among C. perfringens isolates in sheep and goat species.

vaccines against necrotic enteritis in birds (Keyburn et al. 2013, Zaragoza et al. 2019). Also, characterization of the exact pathogenic toxin types circulating in a region is necessary for assigning specific vaccines against *C. perferingens* to control this disease.

Considering the results of the present study, the first toxin typing of isolates was changed because the updated toxin typing scheme suggested that strains, producing CPA-toxin, as well as CPE is determined as type F, whereas those producing CPA and NetB toxins are categorized as type G (Rood et al. 2018). Accordingly, 14 *cpe* positive isolates that were primarily classified into type A, will categorize as type F based on the results of this research. Because certain toxins are associated with specific hosts and diseases the accurate typing of *C. perfringens* strains and classification into 7 toxin types is important for epidemiology and diagnosis and to differentiate the strains involved in enteric infection.

CONCLUSION

The present study revealed a relatively high minor virulence gene presentation, in various types of *C. perfringens* isolates in sheep and goats. In addition, this study changed the toxin types of isolates.

Determining the presence of these toxin genes in *C. perfringen*s isolates provides more information about genetic analysis of *C. perfringen*s and emphasizes the importance of these novel genes in sheep and goats in future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the study and publication of the article.

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