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Original Article

Isolation and Molecular Characterization of *Clostridium perfringens* Toxinotypes F & G in Diarrhoeic Sheep (*Ovis aries*) Flocks in Southeast of Iran

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Abstract

Clostridial enteric diseases, called enterotoxemia, are caused by *Clostridium perfringens* toxinotypes in sheep and other ruminants. This study aimed to describe the molecular characterization of *C. perfringens* isolates in diarrhoeic sheep (Ovis aries) flocks in the southeast of Iran. Fecal/intestinal samples were collected from diarrhoeic (n=116), dead (n= 13), and healthy (n=63) sheep over four years (2016-2020) and subjected to bacteriological and molecular examinations. The *C. perfringens* isolates were typed by polymerase chain reaction targeting genes, namely *16SrRNA*, *CPA*, *CPB*, *ETX*, *IAP*, *CPE*, and *NetB*. The overall prevalence of *C. perfringens* was 28.6% among the studied sheep, and there was a significant relationship between its isolation rate and diarrhea (P<0.001). The *C. perfringens* isolation rate also decreased with animal age (P=0.012) and was significantly higher in late winter and spring (P=0.000). The most prevalent toxinotypes were types A (52.4%), D (22.2%), and F (18.5%), in that order. Moreover, C, G, and B types were found in 4.2%, 1.6%, and 1.1% of the isolates, respectively, and no type E was detected. The *CPE* gene was detected in 32.3% of all isolates, and the diarrhoeic sheep were most likely to yield *CPE+* strains of *C. perfringens* (93.1%). These findings highlight the importance of *CPE+* strains of *C. perfringens* in sheep enteritis and suggest that the high presence of type F needs to be considered in new clostridial vaccines containing this toxinotype. It is noteworthy that the present study reported the isolation of *C. perfringens* type F, type G, and the *CPE+* strains of type B from diarrhoeic sheep for the first time.

Keywords: *Clostridium perfringens*, Diarrhoea, Iran, Sheep (Ovis aries), Toxinotype

1. Introduction

Enterotoxemia (overeating disease) is a fatal clostridial disease in sheep and other ruminants. It is one of the most prevalent infectious diseases of small ruminants worldwide, caused by *Clostridium perfringens*. This anaerobic spore-forming bacterium produces at least 20 different extracellular toxins/enzymes, six of which (alpha [α], beta [β], epsilon [ε], iota [ι], enterotoxin [*CPE*], and necrotic enteritis toxin B-like [*NetB*]) are currently used for typing it A-G (1).

Sheep enterotoxaemia prevalence rate is within the range of 24-100% in different countries (2), while in Iran, it is between 35.7% and 62.5% (3-10). In a study conducted by Ahsani, Bafti (9), *C. perfringens* was isolated from 27 out of 50 unvaccinated sheep (54.0%) and from 2 out of 90 vaccinated sheep (2.2%). They reported that the most prevalent toxinotype of *C.*

perfringens in sheep-dung samples was type C (34.8%) (10). In a study performed by Fahimeh, Peyman (5), the isolation rate of *C. perfringens* was 62.5% (25/40), and the most predominant toxinotype was type A with a 64.0% rate.

In another study, *C. perfringens* was isolated from 61.07% (102/167) of the suspected enterotoxaemia cases in Iranian sheep. In that study, the polymerase chain reaction (PCR) results showed that type A was the most prevalent toxinotype in both enterotoxaemia and healthy sheep (3). In another study conducted in Iran, the most dominant toxinotype was B (31.25%) (6). Sheep enterotoxemia is mainly induced by *C. perfringens* type D, which produces *α*- and *ε*-toxins, to a lesser extent by type B strain, which produces *α*-, *β*-, and *ε*-toxins and type C strain which produces *α*- and *β*-toxins (1). The role of the newly defined *C. perfringens* types (F and G) in the pathogenesis of sheep enterotoxaemia has not been addressed yet.

Except for the proven role of *β*- and *ε*-toxins in lamb dysentery and sheep enterotoxemia, the association of the other toxins with these diseases is questionable. The *β*-toxin of type B leads to lamb dysentery in young lambs (<14 days of age), however, *β*-toxin of type C leads to hemorrhagic enteritis in lambs and struck in adult sheep. The *ε*-toxin of type D is a common cause of enterotoxemia in older lambs and sheep. Moreover, particular *C. perfringens* strains produce an assortment of other pathogenic toxins (11).

The CPE is an influential toxin of *C. perfringens* specially produced by type F strains and can be produced with other types (1). It should be noted that type F is a vital cause of foodborne illness (12). CPE is a virulence factor for several human gastrointestinal disorders, such as food poisoning diarrhea, non-foodborne gastrointestinal diseases (antibiotic-associated diarrhea and sporadic non-food-borne diarrhea), and enteritis necroticans (1). This toxin may play a role in lamb dysentery or enterotoxemia (13). In contrast to the determining role of *NetB* in avian necrotic enteritis, its role in the pathogenesis of animal enterotoxemia has been less defined (11).

Therefore, the detection of *C. perfringens* toxinotypes associated with enterotoxemia is critical for a better understanding of the epidemiology of *C. perfringens* infections in sheep. There is no reliable information about the role of *C. perfringens* types F and G in sheep enteritis. This study aimed to describe the prevalence and importance of these types in diarrhoeic sheep (Ovis aries) flocks suspected of enterotoxemia in southeast Iran.

2. Materials and Methods

2.1. Samples

Over four years (from May 2016 to May 2020), a total of 192 fecal/intestinal samples were collected from 69 different sheep flocks, each comprising 40-200 animals (more than 9,700 animals) in the southeast of Iran. Most of these flocks (46 out of 69) were vaccinated against clostridial diseases with the available commercial vaccines. Samples were obtained from diarrhoeic sheep (n=116), dead sheep due to enterotoxemia (n=13), and healthy sheep with a history of current diarrhea (n=63) according to the inclusion and exclusion criteria.

The criteria for the diagnosis of enterotoxemia in cases of the sudden death of sheep were clinical signs, necropsy findings, and differential diagnosis of the other diarrhoeal diseases. Small intestinal samples were quickly obtained from the duodenum of the recently dead animals in sterile plastic containers and transported immediately to the research *Clostridia* laboratory of Razi Vaccine and Serum Research Institute (RVSRI), Kerman branch, Iran under refrigerated conditions for bacteriological investigations.

2.2. Isolation and Identification of *Clostridium perfringens*

Fecal samples were processed according to the standard operating procedures of RVSRI (14). For sample enrichment, a portion of specimens was inoculated in 10 mL of Robertson's cooked meat medium (HiMedia, M149). Robertson's cooked meat media was reheated at 80 ˚C for 10 min and incubated

at 37 ˚C for 24 h under anaerobic conditions (10% CO_2, 80% N_2, and 10% H_2).

Later, the turbid broth was cultured on blood agar plates (supplemented by 75 µg/ml neomycin sulfate) and incubated anaerobically at 37 ˚C for 48 h to isolate *C. perfringens*. Pure colonies were obtained by anaerobic plating of the subcultures on selective tryptose-sulfite cycloserine agar (Merck, Germany, 111972) for 24 h as black colonies were observed. The *C. perfringens* isolates were analyzed according to their colony morphology, type of hemolysis, gram staining smears, and the biochemical characteristics (catalase, lecithinase, lipase, gelatinase, motility, and skim milk coagulation) as described in Bergey's manual. Pure, confirmed *C. perfringens* isolates were preserved in cryopreservative beads, Microorganism Preservation System-Protect (Technical Service Consultants Ltd, Lancashire, UK), at -70 ˚C for further molecular characterization.

2.3. *Clostridium perfringens* Toxinotyping

The following strains were used as positive controls for the multiplex and duplex PCRs: *C. perfringens* ATCC®$_{13124}$™ for type A (*CPA* gene positive), *C. perfringens* CN_{228} for type B (*CPA*, *CPB*, and *ETX* genes positive), *C. perfringens* CN_{301} for type C (*CPA* and *CPB* genes positive), and *C. perfringens* CN_{409} for type D (*CPA* and *ETX* genes positive). The *C. perfringens* IHP-MA_{1183} and IBP-MA_{08} strains (RVSRI-Kerman branch laboratory collection) were used as the positive controls for the new type F (*CPA* and *CPE* genes positive) and G (*CPA* and *NetB* genes positive), respectively. Moreover, *C. septicum* CN_{913} was used as a negative control.

The DNA of the recovered *C. perfringens* isolates was extracted by the CinnaPure DNA kit for gram-positive bacteria (EX6021, CinnaGen, Iran) according to the instructions of the manufacturer. The *C. perfringens* isolates were toxinotyped by multiplex PCR targeting genes, namely α-toxin (*CPA*), β-toxin (*CPB*), ε-toxin (*ETX*), and ι-toxin (*IAP*). The species-specific primers of the 16SrRNA gene were used to confirm isolates as *C. perfringens* (15). All isolates were also screened for the presence of *CPA* and *CPE* genes or the *CPA* and *NetB* genes by duplex PCR assays. The *CPE+* strains of *C. perfringens* toxinotypes were confirmed by a multiplex PCR assay. It should be noted that the primer sequences are summarized in table 1.

Table 1. List of primers used to toxinotyping *C. perfringens* isolates

Gene (toxin)	PCR	Primers	Primers Sequence (5'–3')	Amplicon size (bp)	Final primer concentration (µM)	Reference
16s-rRNA cluster	M	CP1-F CP1-R	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279	0.4	(15)
cpa (Alpha)	M	Cpa-F Cpa-R	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	0.5	(16)
cpb (Beta)	M	Cpb-F Cpb-R	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	0.6	(16)
etx (Epsilon)	M	Etx-F Etx-R	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	0.45	(16)
iap (Iota)	M	Iap-F Iap-R	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	0.4	(16)
cpe (CPE)	D/M	Cpe-F Cpe-R	ACAGGTACCTTTAGCCAATC ACCAGCTGGATTTGAGTTTAATG	462	0.55	(17) (18)
netB (NetB)	D	NetB-F NetB-R	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	384	0.4	(19)

M: Multiplex PCR set up and optimized for this study; D: Duplex PCR

The PCR protocols were as follows: the multiplex PCR reaction was performed for toxinotyping in 50 µl volumes containing 25 µL of 2x PCR master mix (PR901638, Sinaclon, Iran), 1.5 µL of forward and reverse primers for 16s rRNA-cluster, *CPA*, *CPB*, *ETX*, and *IAP* genes, 5 µL of the sample DNA (at least 50 ng of DNA/50 µl), and 5 µL of DNA free distilled water. The PCR products were amplified under an initial pre-denaturation step at 95 ˚C for 5 min, followed by 35 cycles at 95 ˚C for 60 s, at 52.5 ˚C for 60 s, at 72 ˚C for 90 s, and the final extension step at 72 ˚C for 10 min.

Duplex PCRs to identify *C. perfringens* types F (*CPA* and *CPE* genes positive) and G (*CPA* and *NetB* genes positive) were performed in 25 µl reaction mixtures containing 12.5 µl of PCR master mix (PR901638, Sinaclon, Iran), 0.5 µM primers for *CPA* gene, 0.55 µM primers for *CPE* gene, or 0.4 µM primers for *NetB* gene, 2.5 µL of template DNA solution (at least 20 ng DNA/25 µl), and dH_2O till 25 µL volume. The following PCR protocol was used: initial denaturation step at 95 ˚C for 5 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at the respective temperature (at 55 ˚C for *CPE* gene and 52.5 ˚C for *NetB* gene) for 30 s, extension at 72 ˚C for 60 s, and a final extension step at 72 ˚C for 10 min. The multiplex PCR for the *CPE*+ strains of different *C. perfringens* toxinotypes were performed as the toxinotyping PCR assay (described above) with *CPA*, *CPB*, *ETX*, *IAP*, and *CPE* genes primers in similar conditions, except for the annealing temperature which was 55 ˚C.

Amplified products were subjected to 1.7% agarose gel electrophoresis, and the amplicons were visualized and photographed under UV illumination using the Uvitec system (Cambridge, UK). The 100 bp DNA ladder (PR911653, Sinaclon, Iran) and 50 bp DNA ladder (PR901633, Sinaclon, Iran) were used as markers to indicate the size of the amplicons.

2.4. Statistical Analysis

The *C. perfringens* strains isolated from diarrhoeic and healthy sheep were screened by PCR, and results were presented in a tabularized form. Pearson's chi-square (χ^2) and Fisher's exact tests were applied to compare differences among categorical variables and groups using IBM SPSS statistics software, version 19.0 (IBM, New York, USA). The statistical significance was defined as a *P*-value < 0.05.

3. Results

During the four-year study period, a total of 192 enterotoxemia-suspected sheep were tested. In terms of gender, 52 (27.1%) of them were male and 140 (72.9%) of them were female. About 28.6% (n=55) of the samples were positive for *C. perfringens* by the PCR assay (Table 2). The positive cases were located in 26 out of 69 (37.7%) flocks, while *C. perfringens* were not isolated from the other 43 flocks. The isolation rates in male and female sheep were 30.8% and 27.9%, respectively, which showed no significant difference (*P*>0.05).

The sheep were stratified into five groups based on their age: 1-12 months, 13–24 months, 25-36 months, 37-48 months, and ≥ 49 months with *C. perfringens* isolation rates of 40.0%, 30.2%, 27.5%, 8.7%, and 6.3%, respectively (Table 2). It should be noted that the isolation rates significantly decreased with age (*P*=0.012). Moreover, the isolation rates of *C. perfringens* from sheep in different seasons were 32.5%, 10.4%, 25.0%, and 58.3% for spring, summer, autumn, and winter, respectively, which was significantly high in late winter and spring (*P*=0.000).

There was a significant difference (*P*<0.001) between the presence rate of *C. perfringens* or the isolated toxinotypes and that of diarrhea. The *C. perfringens* types A, B, C, D, F, and G were isolated from 28.9% (20/69), 1.4% (1/69), 5.8% (4/69), 21.7% (15/69), 17.4% (12/69), and 4.3% (3/69) of the flocks, respectively. The multiplex PCR for the specific major toxins resulted in the predicted amplicons according to the type of tested strains (Figure 1). Furthermore, the results of duplex PCRs for types F and G predicted the correct size for each *CPE* and *NetB* gene (Figure 2). Moreover, the multiplex PCR for *CPE*+ strains confirmed the harboring of this gene by different *C. perfringens* toxinotypes, and highlighted the isolation

of *CPE+ C. perfringens* toxinotype B from diarrhoeic sheep for the first time (Figure 3).

There were 13 sudden deaths due to enterotoxemia, nine of which were positive for *C. perfringens*. It is noteworthy that seven of the dead sheep were not vaccinated for enterotoxemia. In addition, two of the dead sheep were positive for type C, four were positive for both types A and D, and two were positive for both types F and G.

The isolates of *C. perfringens* from dead/diarrhoeic and healthy sheep were subtyped into 10 genotypes based on their possession of the major toxin (*CPA*, *CPB*, *ETX*, and *IAP*), *CPE*, and *NetB* toxin genes (Table 4). There was a significant difference between the presence of different genotypes in isolates from dead/diarrhoeic and healthy sheep (P<0.05).

The *CPE* gene encoding *C. perfringens* enterotoxin was detected in 32.3% of all genotyped isolates (61 out of 189) in this study (Table 3), 35 of which were type F. The *CPE* gene was also present in 52.3% of type D isolates (22 out of 42) and 25.0% of type C isolates (two out of eight), while all type B strains were *CPE+*. However, it must be noted that only a few type B isolates (n=2) were screened. Twenty-one sheep with diarrhea (suspected of enterotoxemia), six dead sheep due to enterotoxemia, and two sheep without diarrhea had *CPE+* strains of *C. perfringens*. Therefore, the sheep with enterotoxemia were most likely to yield *CPE+* strains of *C. perfringens* (93.1%). These results showed a significant association between the possession of this gene and diarrhea in sheep (P<0.05).

Table 2. *C. perfringens* isolation from enterotoxemia-suspected sheep based on health status, sex, age, and season

Variable	Group	Positive		Negative		Total		95% CI for positive samples	*P*-value*
		N	%	N	%	N	%		
Health status	Diarrhoeic	38	32.8	78	67.2	116	100	24.3-41.3	0.000
	Dead	9	69.2	4	30.8	13	100	44.1-94.3	
	Healthy	8	12.7	55	87.3	63	100	4.5-20.9	
	Sum.	55	28.6	137	71.4	192	100	22.2-35.0	
Sex	Male	16	30.8	36	69.2	52	100	18.3-43.3	>0.05
	Female	39	27.9	101	72.1	140	100	20.5-35.3	
	Sum.	55	28.6	137	71.4	192	100	22.2-35.0	
Age groups	1-12 months	28	40.0	42	60.0	70	100	28.5-51.5	0.012
	13–24 months	13	30.2	30	69.8	43	100	16.5-43.9	
	25-36 months	11	27.5	29	72.5	40	100	13.7-41.3	
	37-48 months	2	8.7	21	91.3	23	100	-2.8-20.2	
	≥49 months	1	6.3	15	93.8	16	100	-5.6-18.2	
	Sum.	55	28.6	137	71.4	192	100	22.2-35.0	
Seasons	Spring	26	32.5	54	67.5	80	100	22.2-42.8	0.000
	Summer	5	10.4	43	89.6	48	100	1.8-19.0	
	Autumn	10	25.0	30	75.0	40	100	11.6-38.4	
	Late winter	14	58.3	10	41.7	24	100	38.6-78.0	
	Sum.	55	28.6	137	71.4	192	100	22.2-35.0	

CI, Confidence Interval; %, Percent; N, number; * Statistical analysis was performed by Chi-square and Fisher's exact tests. The *P*-value less than 0.05 indicates a significant association between *C. perfringens* isolation and variables

Table 3. Toxinotypes of *C. perfringens* isolates in diarrhoeic and healthy sheep

	Total no. of sheep	No (%) of sheep with *C. perfringens*	No. of *C. perfringens* isolates screened	Toxinotypes*						
				A	B	C	D	E	F	G
Diarrhoeic	116	38 (32.7)	135	73	2	3	29	0	27	1
Dead	13	9 (69.2)	34	14	0	3	11	0	4	2
Control	63	8 (12.7)	20	12	0	2	2	0	4	0
Total	192	55 (28.6)	189	99	2	8	42	0	35	3

* A, B, C, D, E, F, and G refers to *C. perfringens* toxinotypes. P< 0.001 using the Chi-square test

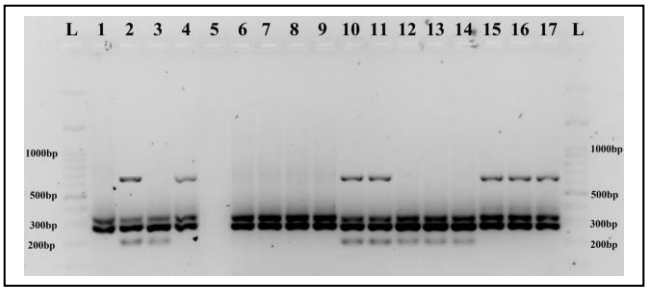

Figure 1. Multiplex PCR detection of *C. perfringens* toxinotypes in a 1.5% agarose gel electrophoresis with specific primers for *cpa* (324 bp), *cpb* (196 bp), *etx* (655 bp), *iap* (446 bp) and 16s rRNA cluster (279 bp) genes. Lane L: 100 bp DNA ladder (Sinaclon, Iran, Cat. No.: PR911653); Lane 1: *C. perfringens* type A reference strain (ATCC®$_{13124}$™; 16s rRNA and *cpa* genes positive); Lane 2: *C. perfringens* type B reference strain (CN_{228}; 16s rRNA, *cpa*, *cpb*, and *etx* genes positive); Lane 3: *C. perfringens* type C reference strain (CN_{301}; 16s rRNA, *cpa* and *cpb* genes positive); Lane 4: *C. perfringens* type D reference strain (CN_{409}; 16s rRNA, *cpa* and *etx* genes positive); Lanes 5: negative control (dH_2O); Lanes 6-9: *C. perfringens* type A field isolates; Lanes 10 and 11: *C. perfringens* type B field isolates; Lanes 12-14: *C. perfringens* type C field isolates; Lanes 15-17: *C. perfringens* type D field isolates. The reference strains were received from the bacterial isolate archive of RVSRI-Kerman branch of Iran

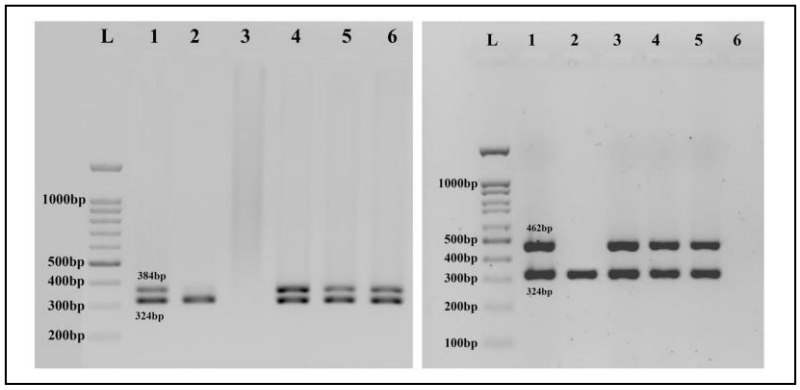

Figure 2. Agarose gel picture showing specific amplification of *cpa* (324bp) and *netB* (384bp) genes in *C. perfringens* type G (in the left side) and *cpa* (324bp) and *cpe* (462bp) genes in *C. perfringens* type F (in the right side) isolated from diarrhoeic sheep. Left: Lane L: 100 bp DNA ladder (Sinaclon, Iran, Cat. No.: PR911653); Lane 1: *C. perfringens* type G (IBP-MA_{08}; *cpa* and *netB* genes positive) as the positive control, provided by Dr. Alimolaei, RVSRI, Iran; Lane 2: *C. perfringens* type A reference strain (ATCC®$_{13124}$™; *cpa* gene positive) as the negative control; Lanes 3: negative control (dH_2O); Lanes 4-6: *C. perfringens* type G field isolates are positive for *cpa* and *netB* genes. Right: Lane L: 100 bp DNA ladder (Sinaclon, Iran, Cat. No.: PR911653); Lane 1: *C. perfringens* type F positive control strain (IHP-MA_{1183}; *cpa* and *cpe* genes positive), provided by Dr. Alimolaei, RVSRI, Iran; Lane 2: *C. perfringens* type A reference strain (ATCC®$_{13124}$™; *cpa* gene positive) as the negative control; Lanes 3-5: *C. perfringens* type F field isolates are positive for *cpa* and *cpe* genes; Lanes 6: negative control (dH_2O)

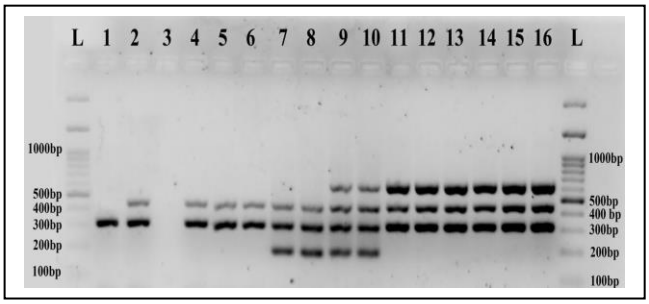

Figure 3. Multiplex PCR for *cpe*+ strains of different *C. perfringens* toxinotypes by *cpa* (324 bp), *cpb* (196 bp), *etx* (655 bp), and *cpe* (462 bp) gene primers. Lane L: 100 bp DNA ladder (Sinaclon, Iran, Cat. No.: PR911653); Lane 1: *C. perfringens* type A reference strain (ATCC®$_{13124}$™; *cpa* gene positive) as the negative control; Lane 2: *C. perfringens* type F control strain (IHP-MA1183; *cpa* and *cpe* genes positive); Lanes 3: negative control (dH_2O); Lanes 4-6: *C. perfringens* type F isolates that are positive for the *cpa* and *cpe* genes; Lanes 7 and 8: *C. perfringens* type C isolates that are positive for the *cpa*, *cpb*, and *cpe* genes; Lanes 9 and 10: *C. perfringens* type B isolates that are positive for the *cpa*, *cpb*, *etx*, and *cpe* genes (reported for the first time in sheep); Lanes 11-16: *C. perfringens* type D isolates that are positive for the *cpa*, *etx*, and *cpe* genes

Table 4. Genotyping of *C. perfringens* isolates from enterotoxemia-suspected sheep

Genotypes		Toxinotypes*										Total No. (%)
		A	**B**	**B^{cpe+}**	**C**	**C^{cpe+}**	**D**	**D^{cpe+}**	**E**	**F**	**G**	
		cpa+	***cpa+, cpb+, etx+***	***cpa+, cpb+, etx+, cpe+***	***cpa+, cpb+***	***cpa+, cpb+, cpe+***	***cpa+, etx+***	***cpa+, cpe+, etx+***	***cpa+, iap+***	***cpa+, cpe+***	***cpa+, netB+***	
Health status	Diarrhoeic	73	0	2	3	0	11	18	0	27	1	135 (71.4)
	Dead	14	0	0	1	2	7	4	0	4	2	34 (18.0)
	Healthy	12	0	0	2	0	2	0	0	4	0	20 (10.6)
	Sum.	99	0	2	6	2	20	22	0	35	3	189 (100)
Sex	Female	66	0	0	4	2	18	20	0	24	2	136 (71.9)
	Male	33	0	2	2	0	2	2	0	11	1	53 (28.1)
	Sum.	99	0	2	6	2	20	22	0	35	3	189 (100)
Age groups (month)	1-12	57	0	2	3	2	5	11	0	21	2	103 (54.5)
	13-24	17	0	0	2	0	10	8	0	4	0	41 (21.7)
	25-36	22	0	0	1	0	5	2	0	8	1	39 (20.6)
	37-48	0	0	0	0	0	0	0	0	2	0	2 (1.1)
	≥49	3	0	0	0	0	0	1	0	0	0	4 (2.1)
	Sum.	99	0	2	6	2	20	22	0	35	3	189 (100)
Seasons	Spring	50	0	2	3	2	11	14	0	25	2	109 (57.7)
	Summer	10	0	0	0	0	2	1	0	0	0	13 (6.9)
	Autumn	13	0	0	3	0	4	2	0	5	0	27 (14.3)
	Late winter	26	0	0	0	0	3	5	0	5	1	40 (21.1)
	Sum.	99	0	2	6	2	20	22	0	35	3	189 (100)

* A, B, C, D, E, F, and G refers to *C. perfringens* toxinotypes

4. Discussion

Sheep enterotoxaemia is caused by various *C. perfringens* toxinotypes, and the determination of different toxinotype prevalence supports the etiology of this disease. However, the role of the newly proposed types F and G (1) and the other *CPE+* toxinotypes in sheep enterotoxaemia has not been reported. In the present study, all *C. perfringens* types, except type E, were identified in the diarrhoeic sheep samples in southeast Iran. Types A, D, and F were the most predominant types that cause diarrhea in sheep, in that order.

According to the molecular investigations, about 28.6% (55/192) of the enterotoxemia-suspected sheep were positive for *C. perfringens*. This is in contrast with the results of previous studies performed in Iran by (5), Ahsani, Bafti (9), who reported 62.5% (25/40) and 54.0% (27/50) rates for *C. perfringens*, respectively. This contrast may be related to the low number of samples in those studies or local geographical differences. Similar to the present study, the same relative occurrence of 24.13% of *C. perfringens* in sheep has been recorded in Morocco (20).

In the present study, *C. perfringens* was isolated from both diarrhoeic and healthy cases. Noticeably, the isolation rate of *C. perfringens* from diarrhoeic sheep was much higher, compared to healthy ones (20 isolates out of 63 healthy sheep, compared to 169 isolates out of 129 diarrhoeic sheep), which was statistically significant (P<0.001). The result is consistent with those of the previous reports carried out in Iran (3, 5).

Although different *C. perfringens* types were isolated from sheep with enterotoxaemia, the most prevalent types in Iranian sheep are A (3, 5, 8), C (5, 9, 10), and D (3, 9) as the third most prevalent type after them. In the current study, *C. perfringens* type A was the most predominant toxinotype (52.4% of all *C. perfringens* isolates), which conforms with the earlier reports on the global dominance of type A (21).

Notably, most type A isolates (87.9%, 87/99) were recovered from diarrhoeic sheep. These results were in contrast with those of the previous studies performed in Iran by (9), Ahsani, Mohammadabadi (10) and Jabbari, Afshari (6), which reported types C and B as the dominant *C. perfringens* types in sheep, respectively. Ahsani, Mohammadabadi (10) reported that the most prevalent toxinotypes of *C. perfringens* in sheep-dung samples were types C and D (with rates of 34.8% and 26.1%, respectively) (10). They also reported that type C had the highest prevalence rate (40.7%) in the same samples of vaccinated sheep in Kerman, Iran, while type A had the lowest rate (14.8%) (9).

In the present study, eight *C. perfringens* isolates (4.2%) were confirmed as type C, while 42 isolates were identified as type D (22.2%). Results of the present research indicated that type D was prevalent in both healthy and diarrhoeic animals. In a study conducted by Jabbari, Afshari (6), the isolates were assigned to genotypes A (14.07 %), B (31.25%), C (26.56%), and D (28.12%). Based on these results, type B is the most prevalent type. However, *C. perfringens* type B was isolated sporadically during our survey (only 1.1% of all isolates).

These relative differences in *C. perfringens* type rates may be related to the sampling procedures, isolation protocols, season of sampling, or other unidentified factors. Following our results, several studies reported that type A is the most predominant type of *C. perfringens* in Iranian sheep (3, 5, 8) and even worldwide as reported in Pakistan (82.0%) (22) and Italy (93.0%) (23), whereas the other types were very limited.

The *C. perfringens* type E enterotoxemia in sheep was contemplated a rare occurrence (24), and scant information about it is available (24). Likewise, no type E strain was recovered in the present survey, which was in agreement with the previous data collected from Iran (3, 5, 6, 8-10), confirming that this type is rare in Iranian sheep.

Regarding the other *C. perfringens* isolates recovered in the present study, 35 and 3 were type F (18.5%) and G (1.6%). These toxinotypes were recently proposed as the new *C. perfringens* types (1). All type G and most type F isolates (77.1%) belonged to diarrhoeic sheep. It is noteworthy that type G (n=3) was identified in this survey from two very young lambs (<1-month-old) and one adult sheep (36 months old) that died due to enterotoxemia. Type G usually leads to necrotic enteritis in poultry (1) and is not found routinely in the intestine of animals. The isolation of the type G toxinotype from animals with enterotoxemia highlights its possible role in sheep enteritis, especially lamb dysentery.

In addition, the roles of the *CPE+ C. perfringens* isolates, especially the recently proposed type F, are not assessed precisely in the pathogenesis of enterotoxemia in sheep. In the present study, it was found that the *CPE* gene presented among 26.1% (35/134), 100% (2/2), 25.0% (2/8), 52.4% (22/42) of types A, B, C, and D, respectively. It should be noted that the *CPE* gene which is presented in type A has been reclassified as the newly proposed *C. perfringens* type F (1). Identification of type F in 9.4% of the diarrhoeic animals could be evidence of the possible role this type plays in the development of enteritis in sheep. This type is usually responsible for various human gastrointestinal illnesses, like food poisoning diarrhea, non-foodborne diarrhea, and enteritis necroticans (1, 25).

Moreover, the isolation of *CPE+* strains of toxinotypes B, C, and D from diarrheic or dead sheep significantly reinforces the hypothesis that these types may be associated with enterotoxemia in sheep. However, the zoonotic characteristics of *CPE+* strains should also be considered. The isolation of *CPE+* strains of *C. perfringens* type B from Iranian goats has also been reported previously (14). Isolation and identification of local circulating *C. perfringens* strains in Iranian sheep can be used to produce clostridial vaccines with higher potency and efficacy. The isolation of new *C. perfringens* toxinotypes make the subject even bolder as it needs to be given more attention.

Molecular characterization of *C. perfringens* toxinotypes from sheep with diarrhea showed the high presence of *C. perfringens* types A, D, and F in diarrhoeic and dead animals and highlighted the possible role of *CPE*-enterotoxigenic isolates. This study aimed to report the isolation of *C. perfringens* types F and G from diarrhoeic sheep for the first time. Finally, the findings indicated the importance of *CPE+* strains of *C. perfringens*, which suggests that the vaccines currently used for the prevention of clostridial diseases should be improved by including a CPE toxoid to confer adequate protection against enterotoxemia in small ruminants.

Authors' Contribution

Study concept and design: M. A.
Acquisition of data: M. A. and M. S.
Analysis and interpretation of data: M. A.
Drafting of the manuscript: M. A.
Critical revision of the manuscript for important intellectual content: M. A. and M. S.
Statistical analysis: M. A. and M. S.
Administrative, technical, and material support: M. A.

Ethics

The animal study was approved by the ethics committee of the Razi Vaccine and Serum Research Institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

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