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Conventional and molecular identification of Iranian Clostridia species associated with animal infection

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Abstract

Clostridial disease causes severe economic losses in livestock by rapidly killing ruminants. Therefore, implementing effective control approaches to prevent this fatal disease is of high importance. The causative agent of this disease is *Clostridium spp*. Accurate identification of this microorganism is crucial for effectively managing clostridial diseases in farm. There are conventional methods for detecting the disease including microbiological and biochemical tests and many of these tests are time-consuming and exhibit low sensitivity. Therefore, this study aimed to use conventional and molecular approaches to identify Iranian isolates associated with animal infections. To achieve this, 61 samples were collected from 1984 to 2024, cultured on liver media and subsequently subjected to microbiological and biochemical tests. For molecular identification, the DNA of isolates were extracted and confirmed by polymerase chain reaction (PCR) using specific primers. The results of the conventional analysis revealed that all Iranian isolates were identified as *Clostridium perfringens* and its type was determined using PCR assay. According to our findings, *C. perfringens* type A was the most prevalent strain in Iran which predominantly found in ostriches and bird samples followed by type D. This study underscored the presence of *C. perfringens* types across variety hosts and geographic locations in Iran. In conclusion, the combining conventional methods with PCR helped reliably detect *Clostridium spp*. This information holds the potential to significantly contribute to the development of preventive strategies against clostridial diseases in Iran.

Introduction

Clostridial diseases pose significant economic and welfare concerns worldwide. Clostridia are anaerobic Gram-positive rods and spore-forming bacterium.[1] It is a public health concern for both humans and animals resulting in several diseases including enterotoxemia, black disease and blackleg. Enterotoxemia is caused by *Clostridium perfringens* with high mortality rate in domesticated animals.[2] *Clostridium perfringens* alpha, beta, epsilon and iota toxins are major virulence factor associated with the pathogenesis.[3] Prompt and accurate diagnosis of *C. perfringens* are crucial for effectively managing fetal diseases. Several conventional methods, encompassing both microbiological and biochemical tests, are adopted for disease detection. The primary dependable approach for identifying the microorganism is through culturing.[4] The Mouse neutralization test (MNT) is a standard assay utilized by researchers for major toxin typing as was done previously.[5] However, MNT presents several disadvantages including time consuming (taking 24 - 72 hr) and notably ethical concerns.[6,7] This technique involves the utilization of numerous laboratory animals. An additional disadvantage stems from the fact that certain *C. perfringens* isolates may not generate detectable toxins consequently resulting in false-negative outcomes.[8] Thus, researchers are striving to introduce alternative methods for detection through *in-vivo* assays. Other method applied to detect *C. perfringens* toxin in intestinal fluids is counter immuno-electrophoresis, reversed passive latex agglutination and enzyme-linked immunosorbent assay.[5] Another rapid detection test for *C. perfringens* is polymerase chain reaction (PCR) which serves as a viable alternative to the MNT assay. The PCR assay was developed

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for typing of *C. perfringens* by Daube *et al.*[9] Diagnostic approaches for the detection of *C. perfringens* are either simplex PCRs[10] or multiplex PCRs.[11] Yoo *et al.* applied a multiplex PCR for toxinotyping of *C. perfringens* isolates. Yoo *et al*., suggested that the conventional SN method could be substituted with a PCR assay, and this approach could help in the development of epidemiological studies for disease prevention.[12] In Baum's study, they typed 186 isolates of *C. perfringens* from animal hosts with clinical symptoms.[11] The molecular approach is frequently used to distinguish between different species of *Clostridium* bacteria.[13,14] Clostridia diseases have been reported among domestic animals from decades ago. An outbreak of blackleg in 1938 among cattle in Iran was the first reported instance of clostridial infections in domestic animals.[15] Subsequent sporadic outbreaks have been documented from various areas of Iran with the most severe one occurring in cattle herd in Southern Iran in 1968.[16] In 1954, the first strain of *C. perfringens* type D was isolated from enterotoxaemia cases in sheep and lamb and later that year, type B of *C. Perfringens* was isolated from sheep and goats.[17] In Iran, the first case of isolation of *C. perfringens* type C from necrotic enteritis in piglets was reported in 1971 which was the cause of death of 900 out of 1,300 piglets.[18] It is believed that clostridial diseases have distributed across different geographical locations among sheep, goats and cattle. There are significant gaps in the different geographical locations among sheep, goat, cattle or other hosts. Therefore, this study described both microbiological, biochemical tests and PCR for the detection of *Clostridium spp.* among Iranian isolates associated with animal infection.

Materials and Methods

Sample collection. A total number of 61 suspicious samples including intestine samples (four cows, 28 sheep and lamb, four goats, one gazelle, 15 ostriches, and two hens) and feces (seven sheep) with a history of disease were included in the study from 1984 to 2024. The animal samples were collected from provinces of Iran including Mashhad, Karaj, Shahryar, Isfahan, Gorgan, Ahvaz, Tabriz, Lorestan, Kermanshah, Tehran, Borujerd, Zanjan, Esfarayen, Saveh, Kerman, Sirjan, Urmia and Neyshabur. The samples were transferred in a sterile plastic bag next to the ice, immediately transported to the laboratory and preserved at – 20.00 ˚C. Iranian isolate was inoculated in fresh liver media (synthetic media). After growth, microbiological and biochemical tests were done. In this study, *C. perfringens* type A, *C. perfringens* type B, *C. perfringens* type C, *C. perfringens* type D, *C. septicum*, *C. novyi* and *C. chauvoei* were also included as a control for confirmation. The current study was performed in compliance with the animal welfare act and regulations following the ARRIVE 2.0 guidelines. Before conducting the benefits of the study were clarified to the farmers and the permission was gained from cattle owners for the use of their animals.

Culturing of isolates. The sample was taken and then cultured into the fresh liver broth and streaked onto blood agar plates containing 10.00% sheep blood. For controls, each sample was cultured into blood agar, nutrient agar and nutrient broth and incubated aerobically. The cultured liver broth was incubated under anaerobic conditions for 24 hr (Mart Microbiology B.V, Drachten, The Netherlands). The suspected colonies of *C. perfringens* which were smooth, gray and with double hemolysis were picked, gram stained and subcultured onto blood agar plates. The turbid broth was also stained and cultured onto blood agar plates and incubated anaerobically. Pure colonies were analyzed based on colony morphology, hemolysis on blood agar plate, gram and spore staining, biochemical (catalase, lipase, lecithinase, and gelatinase) and microbiological tests (motility) as described in Bergey's manual.

Biochemical and microbiological tests. For motility test, a colony was stab-inoculated into sulphide indole motility medium (Himedia, Mumbai, India) and incubated under anaerobic conditions for 48 hr. After that, if growth away from the stab line was observed, motility test was considered motile, otherwise, it was considered non-motile. For indole test, a few drops of Coax reagent were poured on the surface of the sulphide indole motility medium. In positive cases red color appeared on the surface of the medium. For catalase test, a colony was placed on the slide and then a droplet of 3.00% hydrogen peroxide (Himedia) was added. The positive test was reported by the observation of bubble. For gelatinase test, two drops of a bacterial suspension inoculated into the nutrient gelatin tube. The tubes were then incubated for 48 hr under anaerobic conditions and checked for gelatine liquefaction by storing tube for 1 hr at 4.00 ˚C. Positive test was reported by the hydrolyzed gelatin in liquid form, otherwise, it was considered negative. Nitrate reduction test was performed by inoculation of sample into nitrate broth (Himedia) for 48 hr under anaerobic conditions. For confirmation of the reduction of nitrate, reagent A was added containing sulfanilic acid and acetic acid (Himedia), then reagent B was added containing 1-dimethyl-alpha-1-naphthalamine (Himedia) and acetic acid. The positive cases were checked for red color for 15 min, whereas, the negative tests remained yellow color. Zinc powder (Himedia) was then added and the medium checked for red color after 10 min. For sugar fermentation test, two drops of a bacterial suspension were inoculated into the sugar-based culture tube containing glucose 10.00%, maltose 10.00%, lactose 10.00%, sucrose 10.00%, mannitol 10.00% and salicin 10.00%, and incubated under anaerobic conditions for 48 hr. A change in color to yellow indicated a positive result, while reddish or pink colors were considered as a negative result. A loopful of bacterial

suspension was taken and cultured into egg yolk agar. The petri dish was then incubated under anaerobic condition for 48 hr. Cloudy turbidity encircled the bacterial colonies was regarded as a positive result for lecithinase test and clear zone encircled the colony, was reported as a positive result for lipase test, otherwise, it was reported as a negative result.[19]

Molecular typing. The genomic DNA from the sample was extracted using a combination of phenol and chloroform (SinaClon, Tehran, Iran) as follows: After harvesting the bacterial cells from liver media, they were centrifuged and treated with lysozyme at a concentration of 1.00 mg mL^{-1}, incubated at 37.00 ˚C. Subsequently, a mixture of 10.00% sodium dodecyl sulfate (Merck, Darmstadt, Germany) and RNase-A (10.00 mg mL-1; Thermo Fisher Scientific, Vilnius, Lithuania) was introduced and the solution was incubated on a shaker for 30 min. Proteinase K (50.00 mg mL^{-1}; Sigma, Osterode, Germany) was then added, followed by another incubation on a shaker for 1 hr. A phenol-chloroform solution in equal proportions was added to the mixture. After centrifugation, the aqueous phase, containing the DNA, was carefully transferred to a microtube. Genomic DNA was precipitated with sodium acetate (1/10 v/v; Merck) and isopropanol solution (1/1 v/v; Merck) and incubated at – 20.00 ˚C. Chromosomal DNA was then centrifuged, precipitated by ethanol 70.00%, and resuspended in distilled water.[20] The DNA quality and quantity was measured using 1.00% agarose gel (SinaClon) and Nanodrop spectrophotometer, respectively, and stored at –20.00 ˚C until use. The final volume of PCR reaction was 25.00 µL including 0.75 µL 50.00 mM $MgCl_2$ (SinaClon), 0.40 µL *Taq* DNA polymerase (5.00 U μL^{-1}; Fermentas, St Leon-Rot, Germany), 2.50 µL 10.00 X PCR buffer (Thermo Fisher Scientific), 0.50 µL 10.00 mM deoxynucleoside triphosphate (SinaClon), 1.00 µL DNA template (100 ng μL^{-1}), 1.00 µL forward and reverse primer (SinaClon) (10.00 pmol mL^{-1}) and 17.85 µL distilled water (SinaClon). Distilled water, *C. chauvoei*, and *C. septicum* were used as a negative control. The 16S rRNA, alpha toxin, beta toxin, epsilon toxin of *C. perfringens*, alpha toxin of *C. septicum*, flagellin gene of *C. chauvoei* and alpha toxin of *C. novyi* primers as well as the PCR conditions are listed in Table 1. The amplified products were visualized by electrophoresis using a 1.00% agarose gel under ultraviolet light after being stained with Ethidium Bromide (0.50 µg mL^{-1}).

Results

Microbiological and biochemical typing of *Clostridium.* Colonies on sheep blood agar were convex, smooth and slightly opaque with β- haemolytic. Gram staining showed gram-positive rod shape. Samples were positive for lecithinase and nitrate. Samples fermented sucrose, maltose, lactose and glucose but not salicin and mannitol. Catalase, motility, lipase and indol were negative. Briefly, the results of culture and biochemical tests for clostridial strains are listed in Table 2. *Clostridium perfringens* could be identified by these techniques and its type was determined by PCR assay.

Table 1. Primer sets for the detection of Clostridia species in this study.

Genes	Sequences (5'–3')	Product size (bp)	PCR conditions	References
16S rRNA	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	231	initial denaturation: 95.00 ˚C, 2 min 45 cycles: 95.00 ˚C, 20 sec, 63.00 ˚C, 30 sec, 72 .00 ˚C, 45 sec final extension: 72 .00 ˚C, 15 min	21
cpa	GCTAATGTTACTGCCGTTGA ATAATCCCAATCATCCCAACTATG	300	initial denaturation: 95.00 ˚C, 5 min 35 cycles: 95.00 ˚C, 1 min, 53.00 ˚C,1 min, 72 .00 ˚C, 1:30 min final extension: 72.00 ˚C, 15 min	22
etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	initial denaturation: 95.00 ˚C, 5 min 35 cycles: 95.00 ˚C, 1 min, 51.00 ˚C,1 min, 72.00 ˚C, 2:30 min final extension: 72.00 ˚C, 30 min	22
cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	initial denaturation: 95.00 ˚C, 5 min 35 cycles: 95.00 ˚C, 1 min, 52.00 ˚C,1 min, 72.00 ˚C, 1:30 min final extension: 72.00 ˚C, 10 min	22
csa	AATTCAGTGTGCGGCAGTAG CCTGCCCCAACTTCTCTTTT	270	initial denaturation: 94.00 ˚C, 5 min 35 cycles: 94.00 ˚C, 1 min, 55.00 ˚C,1 min, 72.00 ˚C, 1 min final extension: 72.00 ˚C, 5 min	23
flic	ATCGGAAACATGAGTGCTGC AGTCTTTATGCTTCCGCTAG	516	initial denaturation: 94.00 ˚C, 5 min 35 cycles: 94.00 ˚C, 1 min, 54.00 ˚C,1 min, 72.00 ˚C, 2:30 min final extension: 72.00 ˚C, 30 min	24
TcnA	AAA ATT ACT GGT GAG ACA TCA GTT ATT ACC AAC TAA TAT ACC TGC AAC AGG	609	Initial denaturation: 94.00 ˚C, 5 min, 30 cycles: 94.00 ˚C, 30 sec, 52.00 ˚C, 30 sec, 72.00 ˚C, 1 min, final extension: 72.00 ˚C, 10 min	24

cpa: alpha toxin of *C. perfringens*; *etx*: epsilon toxin of *C. perfringens*; *cpb*: beta toxin of *C. perfringens*; *csa*: alpha toxin of *C. septicum*; *TcnA:* alpha toxin of *C. novyi*

Table 2. Microbiological and biochemical tests for the detection of Clostridia species.

Species	Gram-staining	Morphology	Motility	Lecithinase/ Lipase	Salicin	Maltose/ Glucose	Sucrose	Manitol	Lactose	Gelatinase	Nitrate
C. perfringens	G positive rod shape	Convex, smooth, slightly opaque, β-hemolytic	-	+/-	-	+/+	+	-	+	+	+
C. septicum	G positive Straight, curved rod shape	Smooth, slightly opaque, β-hemolytic	+	-/-	+	+/+	-	-	+	+	+
C. chauvoei	G positive spindle, short rod	Convex, whitish β-hemolytic	-	-/-	-	+/+	+	-	+	+	+
C. novyi	G positive rod shape	Small, flat, rough, rhizoidal, translucent, β-hemolytic	+	+/+	-	+/+	-	-	-	+	-

Molecular typing. Polymerase chain reaction results showed that all isolates and *C. perfringens* types A, B and C, along with *C. perfringens* type D as a positive control were produced 231 bp fragment from 16SrRNA gene confirming them as *C. perfringens* strains.

The specific primers also successfully identified *C. perfringens* types A, B and C, along with *C. perfringens* type D, *C. septicum* and *C. chauvoei*, at fragment lengths of 300 bp, 655 bp, 196 bp, 270 bp, and 516 bp, respectively (Fig. 1).

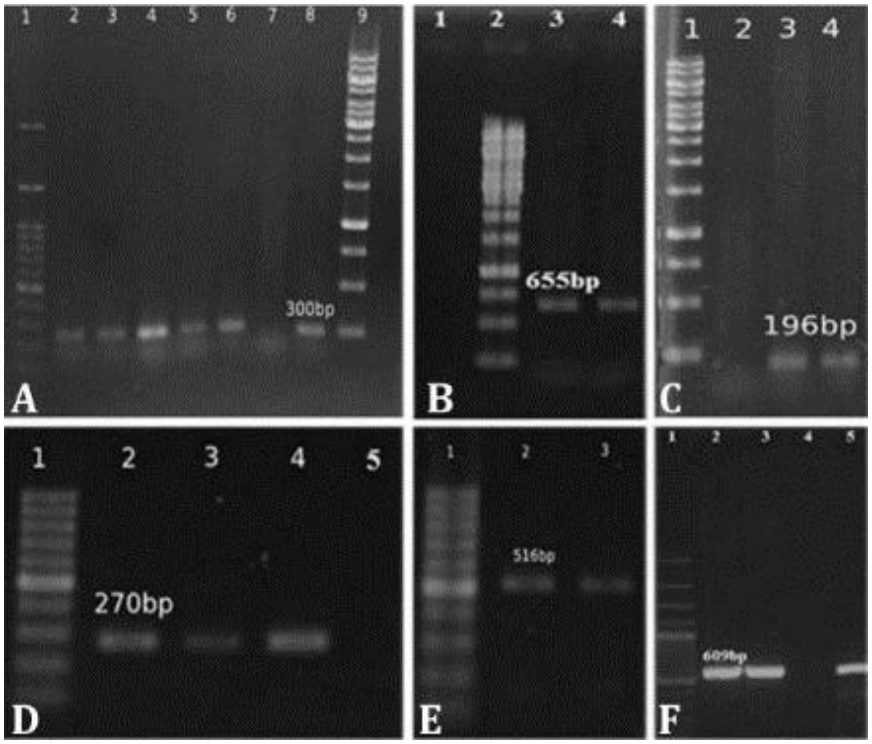

Fig. 1. A) Single polymerase chain reaction (PCR) for the detection of *Clostridium Perfringens* type A. Lane 1: 100 bp DNA size marker; lane 2: *C. Perfringens* type A (positive control); lanes 3 - 8: Iranian samples of *C. perfringens* type A (300 bp) Lane 9: 1.00 Kb marker; **B)** Single PCR for the detection of *C. perfringens* type D and *C. perfringens* type B: Lane 1: *C. perfringens* type C (CN 301; negative control); lane 2: 1.00 Kb DNA size marker; lane 3: *C. perfringens* type D (CN 409; positive control); lane 4: Iranian sample of *C. perfringens* type D (655bp); **C)** Single PCR for the detection of *C. perfringens* type B and *C. perfringens* type C: Lane 1: 1.00 Kb DNA size marker; lane 2: *C. C. perfringens* type D (CN 409; negative control); lane 3: *C. perfringens* type C (CN 301) (positive control); lane 4: Iranian sample of *C. perfringens* type C (196 bp); **D)** Single PCR for the detection of *C. septicum.* Lane 1: 100 bp DNA size marker; lanes 2: *C. septicum* (CN 913; positive control); lane 3: *C. septicum* (CN 912); lane 4: *C. septicum* (CN 901; 270 bp); lane 5 *C. perfringens* type D (CN 409; negative control); **E)** Single PCR for the detection of *C. chauvoei*. Lane 1: 100 bp DNA size marker; lanes 2: *C. chauvoei* (CN701; positive control); lane 3: *C. chauvoei* (CN710) (516 bp); **F)** Single PCR for the detection of *C. novyi*. Lane 1: 100 bp plus DNA size marker; lane 2: *C. novyi* (CN804; positive control); lane 3: *C. novyi* (CN813); lane 4: Iranian samples; lane 5: *C. novyi* (CN886; 609 bp).

In this study, a total number of 61 clostridial isolates were recovered, 53 out of the 61 examined cases yielded positive results. Among these isolates, 27 were identified as *C. perfringens* type A. These isolates were obtained from intestinal samples encompassing two cows, seven sheep and lambs, one gazelle, 15 ostriches, and two hens. Furthermore, 16 clostridia isolates were classified as *C. perfringens* type D. These were isolated from intestinal samples involving 15 sheep and lambs as well as one goat. Similarly, five clostridia isolates were identified as *C. perfringens* type C. These isolates were sourced from intestinal samples with three originating from sheep and lambs, while two were obtained from fecal samples of sheep. In the same way, four clostridia isolates were designated as *C. perfringens* type B. These isolates were derived from intestinal samples consisting of two sheep and one goat, and fecal sample of one sheep. Among the various isolates, *C. perfringens* type A was the predominant causative agent, isolated from ostrich and hens primarily in Tehran. The information about samples is shown in Table 3.

Discussion

The incidence of enterotoxaemia was notably high in Iran a few years ago with numerous cases of the disease reported among animals resulting in significant mortality rates.[18] Detecting *C. perfringens* is essential for disease control. Confirming clostridial isolates requires highly sensitive tests. Strain detection involves microbiological, biochemical, serological and molecular assays. While the culture remains the gold standard for detection,[4] isolating the causative agent is often difficult and time-consuming as it involves labor-intensive procedures and can only identify live bacteria.[14,25] Therefore, it is not applicable to perform identification on formalin-fixed samples. Microscopic assay is rapid and simple but differentiating between bacteria is challenging. While the biochemical method has conventionally been utilized as a test, it is incapable of distinguishing between different types of *C. perfringens*.[26] Another rapid toxinotyping method, the PCR system is based on detecting genes that usually encode major toxins. Kanakaraj *et al.* reported that PCR is a

Table 3. Information about origin, number of the samples, and type of *Clostridium perfringens.*

Locations	Host (sample) No.	Total sample	Type of *C. perfringens*
Mashhad	Sheep (Feces) 1	1	B (1)
Karaj	Sheep-lamb (Intestine) 9 Cattle (Intestine) 1 Hen (Intestine) 2 Sheep (Feces) 1 Goat (Intestine) 2	15	C (2) D (6) A (4) Non-typing (3)
Shahryar	Goat (Intestine) 1	1	D (1)
Isfahan	Sheep (Intestine) 2 Sheep (Feces) 2	4	A (2) Non-typing (2)
Gorgan	Sheep (Intestine) 1	1	A (1)
Ahvaz	Sheep (Intestine) 1	1	A (1)
Tabriz	Sheep (Intestine) 2	2	C (1); D (1)
Lorestan	Sheep (Intestine) 3	3	D (3)
Kermanshah	Sheep (Intestine) 3	3	D (2); A (1)
Tehran	Ostrich (Intestine) 15 Sheep (Feces) 1 Cattle (Intestine) 3 Sheep (Intestine) 1 Gazelle (Intestine) 1	21	A (18) C (1) Non-typing (2)
Borujerd	Sheep (Intestine) 1	1	B (1)
Zanjan	Sheep (Feces) 1	1	C (1)
Esfarayen	Sheep (Intestine) 1 Goat (Intestine) 1	2	B (2)
Saveh	Sheep (Intestine) 1	1	D (1)
Kerman	Sheep (Intestine) 1	1	D (1)
Sirjan	Sheep (Intestine) 1	1	D (1)
Urmia	Sheep (Intestine) 1	1	Non-typing (1)
Neyshabur	Sheep (Feces) 1	1	Non-typing (1)
Total	Sheep-lamb (Intestine) 28 Sheep (Feces) 7 Cattle (Intestine) 4 Hen (Intestine) 2 Goat (Intestine) 4 Ostrich (Intestine) 15 Gazelle (Intestine) 1	61	A (27) D (16) C (5) B (4) Non-typing (9)

valuable technique for swiftly detecting *C. perfringens* in field demonstrating both commendable sensitivity and specificity.[27] In the study of Kadra *et al.*, the PCR method exhibited exceptional specificity and sensitivity. This swift approach employs specific primers designed for *C. perfringens* ensuring no cross-interference with sequences from other strains. As a result, it stands out as a diagnostic tool for prompt detecting of clostridial diseases.[14] Nowadays, molecular approach are used frequently to distinguish clostridial spp[28,29] even those preserved in formalin-fixed tissue.[30,31] In this study, we used specific primers of the *C. perfringens* encoding toxin gene which did not interfere with other strain sequences similar to previous study.[32] Based on our result, the most toxinotypes was *C. perfringens* type A, similar to Ahsani *et al.'s* study in Kerman, Ezatkhah *et al.'s* study in Yazd, and Hayati and Tahamtan in Fars province.[33-35] Conversely, in another study, Ahsani *et al.* reported *C. perfringens* type C was the most and *C. perfringens* type A the least prevalent isolates among sheep population in Kerman province.[26] In another study, Alimolaei and Ezatkhah reported the most isotype was *C. perfringens* type D, followed by *C. perfringens* types A and F, equally among goat population in southeast Iran which highlighted the presence of *C. perfringens* type F isolates among goat with entero-toxaemia cases while it has not been mentioned in other studies in Iran.[36] Another important subject is *C. perfringens* type E that were not reported in previous studies,[33-36] similar to this study. Perhaps, toxinotype F has a high prevalence rate among goat population but it has been neglected so far. Furthermore, all cases of isolated from ostrich and hens were belong to *C. perfringens* type A, which was in accordance with previous studies.[37-40] Conversely, in Korea, Yoo *et al.* reported that all isolated cases were from cattle with enterotoxaemia and chickens with necrotic enteritis (100%), and some cases of piglets with enterotoxaemia or necrotic enteritis (85.71%) belonging to *C. perfringens* type A[12] which was different with Iran. Therefore, there are differences across different geographical locations among various hosts such as sheep, goats and cattle. The *C. perfringens* is a normal flora in ruminant intestine. Major toxins except alpha toxin genes are located on plasmids.[41] Therefore, they can be trans-ferred by others types by acquiring the toxin plasmids or vice versa.[42] Following this, *C. perfringens* type D emerged as the next common type which revealed high prevalence and critical role of these types in the infection of multiple hosts throughout the country. The lowest amount of prevalence is related to *C. perfringens* type C and *C. perfringens* type B. In this study, the lowest cases were observed in Mashhad, Gorgan, Ahvaz, Zanjan, Saveh, Kerman and Urmia. The majority of cases were observed in Tehran and Karaj. There are some reports of toxin typing of *C. perfringens* from different parts of Iran.[26,33-35,37-39] There are also some reports of *C. perfringens* typing from different parts of the world that were consistent with the results of previous study.[12,25,43-46] Recently rigorous techniques have only been applied to the identify clinical infections in animals specially ruminants. In the contemporary landscape, PCR has been emerged as an indispensable diagnostic technique with an extensive application.

Our findings underscored the efficacy of combining conventional methods with PCR regarding the accurate prevalence of clostridial infections in Iran. As mentioned, there were differences across different geographical locations among various hosts such as sheep, goats and cattle. Consequently, it becomes imperative to undertake further endeavors aimed at comprehending the disease epidemiology in specific geographical areas. Such insights are pivotal for devising effective control and preventive strategies against clostridial diseases in Iran.

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Conflict of interest

The authors declare that they have no conflict of interest.

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