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GENOMIC DIVERSITY OF THE *CLOSTRIDIUM PERFRINGENS* ISOLATES FROM HEALTHY AND ENTEROTOXEMIA AFFECTED LIVESTOCK USING MLVA METHOD

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Abstract

Clostridium perfringens is a component of the normal gut microbiota in humans and animals. However, it can become pathogenic under certain conditions, causing serious intestinal and systemic diseases. Enterotoxaemia is one of these diseases causing significant mortality in livestock annually. Analysis of genetic diversity or relatedness of isolates with molecular typing methods can be helpful for understanding the epidemiology of these infections and observing population structures of these bacteria. The aim of this study was to perform molecular typing of *Clostridium perfringens* isolates obtained from both healthy and clinically suspected enterotoxemic livestock in Fars Province using Multiple Locus Variable Tandem Repeat Analysis (MLVA) method. Collection of previously isolated and preserved *Clostridium perfringens* along with new collected isolates from healthy and diseased livestock were evaluated for toxin type and some virulence genes including cpe, tpeL, netB, and $\beta 2$. Genetic typing of isolates was done using specific primers for amplification of eight Variable Number Tandem Repeat (VNTR) loci. PCR was done and electrophoresis banding patterns of fragments were analyzed by BioNumerics software. The results of typ-

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ing revealed 71 distinct MLVA profiles from 74 strains. The strains analyzed included 27 clinical isolates, 43 non-clinical isolates, three vaccine strains, and the reference strain 13 obtained from the GenBank database. *Clostridium perfringens* strain 13 sequence was used as a reference strain and was evaluated for comparison of sequences for each locus. Clustering of isolates according to the minimum spanning tree showed 5 different clusters, excluding three orphan isolates. Clinical and non-clinical isolates were scattered in all clusters and no defined cluster related to health status, date of isolation, kind of host and place of isolation was detected. The total diversity index of our typing method was estimated to be 0.99. Overall MLVA molecular typing method shows high genetic diversity among *Clostridium perfringens* isolates.

Key words: *Clostridium perfringens*, sequence analysis, bacterial genomes, livestock, MLVA

UTVRĐIVANJE GENETIČKE RAZNOVRSNOSTI IZOLATA *CLOSTRIDIUM PERFRINGENS* KOD ZDRAVE I OBOLELE STOKE OD ENTEROTOKSEMIJE PUTEM MLVA METODE

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Kratak sadržaj

Clostridium perfringens je sastavni deo normalne crevne mikroflore kod ljudi i životinja. Međutim, pod određenim okolnostima može postati patogena i izazvati ozbiljne crevne i sistemske bolesti. Jedna od tih bolesti je i enterotoksemija. Ona svake godine izaziva značajan mortalitet kod stoke. Analiza genetske raznovrsnosti ili srodnosti izolata pomoću metode molekularnog tipiziranja može biti od velikog značaja za razumevanje epi-

miologije infekcija i proučavanje populacione strukture ovih bakterija. Cilj ovog rada je molekularno tipiziranje izolata vrste *Clostridium perfringens*, dobijenih od zdrave stoke i stoke sa kliničkom sumnjom na enterotoksemiju u provinciji Fars, korišćenjem "Multiple Locus Variable Tandem Repeat Analysis" (MLVA) Grupa ranije izolovanih i očuvanih sojeva *Clostridium perfringens*, zajedno sa novim izolatima prikupljenim od zdrave i obolele stoke, evaluirana je u pogledu tipa toksina i prisustva određenih gena virulencije, uključujući *cpe*, *tpeL*, *netB* i $\beta 2$. Genetsko tipiziranje izolata izvršeno je korišćenjem specifičnih prajmera za amplifikaciju osam regija "Variable Number Tandem Repeat" (VNTR). Izvršena je PCR analiza kao i elektroforeza pojavljivanja bendova fragmenata koji su analizirani putem BioNumerics softvera. Rezultati tipiziranja pokazali su da postoji 71 različit profil od 74 soja. Analizirani sojevi su obuhvatali 27 kliničkih izolata, 43 neklinička izolata, tri vakcinalna soja i referentni soj 13 dobijen iz GenBank baze podataka. Sekvenca soja *Clostridium perfringens* 13 korišćena je kao referentni soj i evaluirana je za poređenje sekvenci na svakom lokusu. Grupisanje izolata prema "minimum spanning tree" analizi pokazalo je pet različitih klastera, isključujući tri izolata. Klinički i neklinički izolati su bili raspoređeni po svim klasterima, pri čemu nije uočen nijedan klaster koji bi bio povezan sa zdravstvenim statusom, datumom izolacije, vrstom domaćina ili mestom izolacije. Procenjeno je da je indeks ukupne raznovrsnosti metode tipiziranja 0.99. Metoda MLVA metoda molekularnog tipiziranja pokazuje visoku genetsku raznovrsnost među izolatima *C. perfringens*.

Ključne reči: *Clostridium perfringens*, analiza sekvenci, bakterijski genomi, stoka, MLVA

INTRODUCTION

Clostridium perfringens is a causative agent of economically significant enteritis, enterotoxemia, and other major diseases affecting both livestock and humans. This spore-forming anaerobic bacterium is also considered a component of the intestinal microbiome (Sting, 2009; Rood et al., 2018). There are no distinct defined criteria to distinguish the pathogenic strains from normal flora of these bacteria. It is believed that the virulence of *C. perfringens* is related to production of toxins, and other factors, some of which have not been detected or well defined so far (Li et al., 2013; Freedman et al., 2015; Rood et al., 2018).

Many toxins are produced by *C. perfringens* which have major role in virulence, and most are plasmid born. Four major toxins, namely alpha, beta, epsilon and iota have been used for toxin typing of this bacterium. Recently two other toxins - enterotoxin and beta2 toxin - have been included in this typing. According to this new typing scheme, two toxin types of F and G were added to five previous types of A, B, C, D, and E. (Chan et al., 2012; Rood et al., 2018).

Molecular typing of pathogenic isolates can be helpful for epidemiological purposes and the study of ecology, evolution and phylogenetic relationship between different isolates from clinical and non-clinical sources (Lukinamaa et al., 2004; Van Belkum, 2007; Hassan et al., 2020). Some molecular typing techniques are introduced and applied for *C. perfringens* such as Restriction Fragment Length Polymorphism (RFLP), Pulsed Field Gel Electrophoresis (PFGE), Ribotyping, Repetitive Element PCR (REP), Random Amplified Polymorphic DNA (RAPD), Multilocus Sequence Typing (MLST) and Multiple-locus variable-number tandem-repeat analysis (MLVA), from which MLVA is considered as a low cost, rapid, reproducible with high discriminatory power and easy molecular typing method (Lindstedt et al., 2005; Johansson et al., 2006; Gelfand et al., 2007; Afshari et al., 2016; Wang et al., 2021).

This study was designed to apply the MLVA method for genetic subtyping of *C. perfringens* isolates in order to assess the phylogenetic relationships among strains collected from both healthy and enterotoxemic hosts in Fars Province, Iran.

MATERIAL AND METHODS

Bacterial isolation and characterization

Samples of intestinal contents, plural and peritoneal fluids from 15 cases of sudden death due to enterotoxemia and 30 samples of intestinal contents of healthy goats, and sheep from slaughterhouses were collected between 2019 and 2022. The samples were cultured for isolation of *C. perfringens*. Eight *C. perfringens* strains which were isolated from enterotoxemia cases were also provided by Veterinary Office of Fars province (seven sheep and one jebeer). In addition, about 37 more isolates preserved in our laboratory archive which were collected over a period of 10 years (2009-2018), seven of which were associated with confirmed cases of enterotoxemia (two sheep, four goats and one cattle) and the rest were from healthy animal hosts (16 sheep, seven goats, seven cattle). These isolates were also defrosted and included in this study along with the vaccine strains of type B, C and D (the data of all isolates are presented in supplementary Table S1). Neomycin-supplemented blood agar

(40 µg/mL) was used for the primary culture and isolation of the bacteria. Suspected colonies of *C. perfringens* were purified and confirmed by biochemical and PCR tests for 16SrRNA (Meer and Songer, 1997; MacFaddin et al., 2000). Toxin typing was done using related specific primers for alpha, beta, epsilon, iota, netB, enterotoxin, and the presence of other virulence factors including beta2, tpeL and tcpH for detection of conjugative plasmids were also evaluated (Table 1).

Table 1. Primers used for confirmation and evaluation of typing toxins and virulence genes of *Clostridium perfringens*

gene		primer sequence	Fragment size	Annealing temperature	references
16SrRNA	F	AAAGATGGCATCATCATTCAAC	279	55	Meer&Songer, 1997
	R	TACCGTCATTATCTTCCCCAAA			
cpa (alpha toxin)	F	GCTAATGTTACTGCCGTTGA	324	55	Meer&Songer, 1997
	R	CCTCTGATACATCGTGTAAG			
cpb (β-toxin)	F	GCGAATATGCTGAATCATCTA	196	55	Meer&Songer, 1997
	R	GCAGGAACATTAGTATATCTTC			
etx (ε-toxin)	F	TGGGAAGCTTCGATACAAGCA	376	55	Van Asten et al., 2010
	R	AACTGCACTATAATTTCTTTTCC			
iap (ι-toxin)	F	AATGGTCCTTTAAATAATCC	272	55	Meer&Songer, 1997
	R	TTAGCAAATGCACTCATATT			
cpe (enterotoxin)	F	TTCAGTTGGATTTACTTCTG	486	56	Van Asten et al., 2010
	R	TGTCCAGTAGCTGTAATTGT			
cpb2 (β2-toxin)	F	CAATACCCTTCACCAAATACTC	567	55	Bueschel et al., 2003
	R	AGATTTTAAATATGATCCTAACC			
tcpH	F	CATTAAGTGTCTTCTAAAGGC	931	55	Park et al., 2016
	R	GCGGAATAGTAATGATTAGTT			

gene	primer sequence		Frag- ment size	Anneal- ing tem- perature	references
<i>netB</i>	F	CGCTTCACATAAAGGTTGGAAGGC	316	60	Bailey et al., 2013
	R	TCCAGCACCAGCAGTTTTTCCT			
<i>tpeL</i>	F	ATATAGAGTCAAGCAGTGGAG	466	59	Keyburn et al., 2008
	R	GGAATACCACTTGATATACCTG			

Data analysis

The Chi-square (χ^2) test was performed to analyze significant differences between the studied virulence genes in two groups using SPSS software (ver. 27.0.1).

MLVA typing

For MLVA analysis, eight VNTR (07, 17, 24d, O1e, cp6, cp42, cp13, cp19) primer pairs were used (Table 2). The PCR reaction was performed in a total volume of 20 μ L per reaction containing 10 μ L of 2 $\times$ PCR master mix (Amplicon, Denmark), 1 μ L (20 pmol) of each forward and reverse primer (Metabion International AG[®], Germany), 6 μ L ddH₂O, and 2 μ L of genomic DNA as a template. PCR was conducted (Eppendorf thermocycler, Germany) with the following amplification conditions: Initial denaturation at 95 °C for 4 min, followed by 30 cycles at 94 °C for 1 minute, annealing at 58 °C for 45 s, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min.

Table 2. Primer pairs used for MLVA typing

VNTR locus	Primer sequence		reference
Cp6	F	GTAAAGATGATTGCTATTTAGAGATAA	Sawires and Songer, 2005
	R	TAAGGTATCATCAAAATCCACTCCAGG	
Cp13	F	AAGGAAGATGCTACTCAAGATG	Sawires and Songer, 2005
	R	GAAGCCATCATTACTACTCCTA	

VNTR locus		Primer sequence	reference
CP19	F	CTCAATCCAACAATATGTGCTGACTA	Sawires and Songer, 2005
	R	GTAGCAGCAATAAAACCAACCTAAA	
CP42	F	GATGGCCCAAGAAACAGAAC	Sawires and Songer, 2005
	R	GCTGGGAATAAAGGGTTTGA	
07	F	TTCAAAGCCAGGTACATTCAA	Chalmers et al.,2008
	R	TCTTCTGTTTCAAATTTAGTTTCAT	
17	F	TTAATGTCCATATTCGCCA	Chalmers et al.,2008
	R	TTGTGGACAATCTGTAGGAGGA	
O1e	F	ATGGGGTAAGGGGATCCAA	Chalmers et al.,2008
	R	GGGGTTAAACTTGATTTTCTGTG	
24d	F	TATAGTGTGGTTAAAAT- ATTAAATTATAGTT	Chalmers et al.,2008
	R	GAATTACTTTTCTACAATT- TATTTTCATAA	

The PCR products were analyzed by electrophoresis on 1.5% agarose gel, and the band sizes were recorded. Some PCR products as a representative of amplicon sizes in each VNTR were sequenced (Microsynth, Switzerland). The sequences were analyzed using NCBI BLAST search, and multiple alignments were done using Clustal Omega program; <https://www.ebi.ac.uk/jdispatcher/msa/clustalo> (Sievers et al. 2020). The sequence of *C. perfringens* strain 13 was also used as a reference for comparison of strains. The tandem repeats were evaluated by tandem repeat finder program; TRF (Benson, 1999).

The resulting banding patterns were analyzed using the BioNumerics software, Version 6.7 (Applied Maths, USA). Network creation method was Minimum Spanning Tree. MST was obtained based on the shortest mathematically possible distance between isolates (nodes). The first priority rules were set as maximum number of N-locus variants (N=1) and Weight: 10000 and the second one as maximum number of N-locus variants (N=2) and Weight: 10. For partitioning, Nodes closer than 2 were put into the same partition. Every partition contained at least one entry and one node. To generate the dendrogram, the unweighted pair group method with arithmetic mean (UPGMA) was employed, using the multistate categorical similarity coefficient (MCSC) with equal weighting for all loci.

The discriminatory power of individual VNTR was determined by calculating the Nei's diversity index (D) using the equation $D = 1 - \sum (\text{allele frequency})^2$

quency)². The allelic diversity (h) of the individual loci was calculated using the equation $h = n (1 - \sum x_i^2) / (n-1)$, where n is the number of strains and x_i is the frequency of the i^{th} allele at the related locus. The discriminatory power (Diversity Index = DI) of this typing system using combined eight loci was calculated according to Hunter and Gaston formula both individually and using online tool (http://insilico.ehu.es/mini_tools/discriminatory_power). The formula is as follows:

$$DI = 1 - 1 / N (N - 1) \sum_{j=1}^s N_j (N_j - 1)$$

where N in this equation is the total number of strains, s is the total number of genotypes, and N_j is the number of strains belonging to the j^{th} profile.

RESULTS

Culture, isolation and confirmation

Thirteen *C. perfringens* isolates were recovered from 30 intestinal content samples of healthy animals (three goats, 10 sheep) and 12 isolates from 15 intestinal content samples of livestock with suspected field cases of enterotoxemia (one goat and 11 sheep).

A total of 70 purified *C. perfringens* isolates showing a double zone of hemolysis - 43 from healthy animals (26 sheep, 10 goats, seven cattle) and 27 from clinical cases (20 sheep, five goats, one cattle and one jebeer) were evaluated and confirmed by biochemical tests including positive tests of sucrose, nitrate, gelatinase, lecithinase, lipase and negative tests of catalase, indole, motility, and lactose.

The results of PCR tests using 16SrRNA (figure 1-A) also showed 279 bp fragments indicating positive results for all of these confirmed colonies of *C. perfringens*.

Toxinotyping and other virulence genes

The results of toxin typing by PCR showed bands of 324, 196, 376 and 272 bp for alpha, beta, epsilon and iota toxins respectively (Figure 1). PCR results corresponding to identification of *cpe* and *netB* (487bp and 316bp) are shown in Figure 2. The number of different toxin types and related data are presented in Tables 3 and 4. Most of the isolates overall belonged to type A (53 isolates), followed by type D (14 isolates). In the healthy group, all isolates belonged to type A, except one, which was type D. In the clinical group, most of the isolates

were type D (13 isolates), followed by type A (11 isolates), two type G, and one type C. *C. perfringens* Type B, Type E, and type F were not present in our isolates except for a vaccine strain of type B. PCR results for screening the presence of *beta2*, *tpeL* and *tcpH* (567bp, 466bp and 931 bp respectively) are also shown in Figure 2.

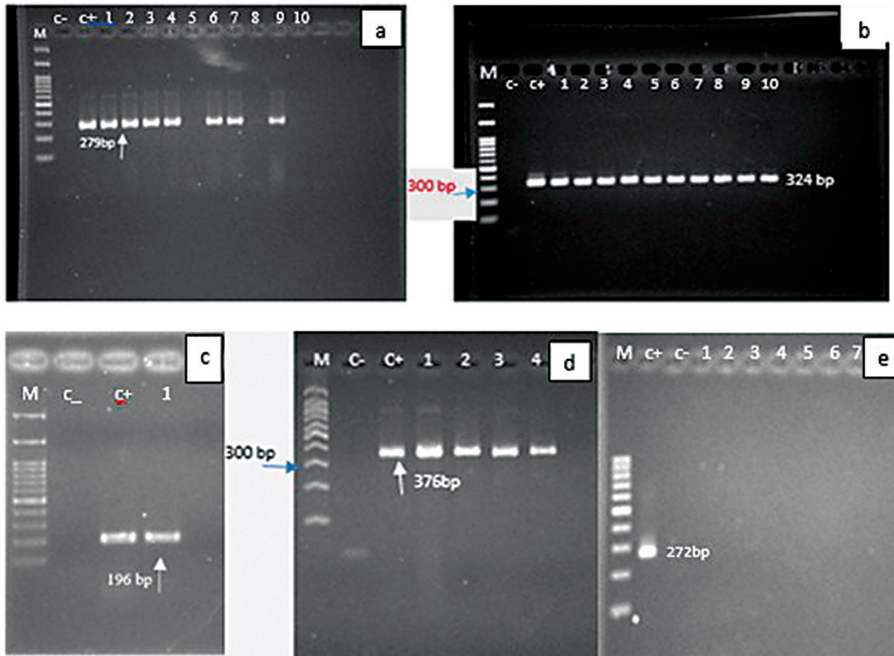


Figure 1. Agarose gel electrophoresis images of PCR products with specific primers for 16s rRNA (279 bp) [A]; *cpa* (324 bp) [B]; *cpb* (196 bp) [C]; *etx* (376 bp) [D]; and *iap* (272 bp) [E] genes. Lane 1 in each image is M: DNA marker 100 bp (SinaClon, Iran), c-: negative control, c+: positive control (type B vaccine strain for [A-D] and type E strain CN1241 [E]).

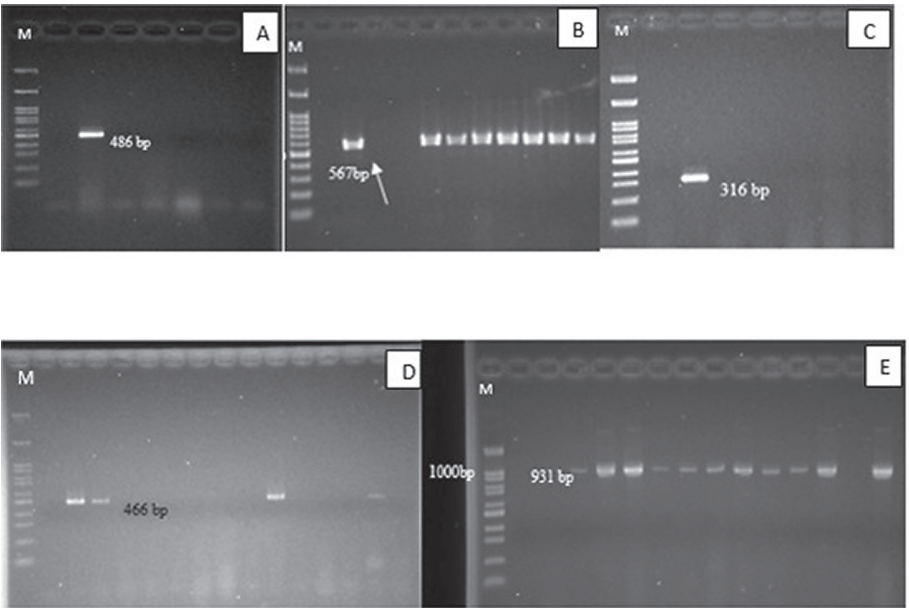


Figure 2. Agarose gel electrophoresis images of PCR for identification of *cpe* gene (487bp) [A]; *cpb2* (567bp) [B]; *netB* (316bp) [C]; *tpeL* (466bp) [D]; and *tcpH* locus (931bp) [E]. Lane 1 in each image is M: DNA marker 100 bp plus (SinaClon, Iran), Lane 2: negative control, Lane 3: positive control (strain Ba1/p, accession: OR725467 [A]; type B vaccine strain [B]; RTCC No: IRCPF-59 accession: PQ594181 [C]; type C vaccine strain [D]; type B vaccine strain [E]); Lane 4 and other lanes are different isolates.

The frequency of virulence genes in two groups is presented in Table 5. Statistical analysis of differences between the studied virulence genes in two groups using χ^2 test showed no significant difference except for beta2 which were higher among clinical group (92.5%) in comparison to non-clinical group (58%), ($p < 0.05$).

Table 3. Distribution of different toxin types in two groups of *C. perfringens* isolates

Toxin type	Healthy group		Clinical group		Total percent
	frequency	percent	frequency	percent	
A	42	97.67	11	40.74	75.71
B	0	0	0	0	0
C	0	0	1	3.70	1.43

Toxin type	Healthy group		Clinical group		Total percent
	frequency	percent	frequency	percent	
D	1	2.32	13	48.14	20
E	0	0	0	0	0
G	0	0	7.40	2	2.86
total	43	100	27	100	100

Table 4. Distribution of different toxin types of *C. perfringens* according to host and health status

Host source	Number in healthy group			Number in clinical group			
	Type D	Type C	Type A	Type D	type C	Type A	Type G
sheep	1	-	25	7	1	12	2
goats	-	-	10	5	-	-	0
cattle	-	-	7	1	-	-	0
Jebeer gazelle	-	-	-	-	-	1	0
total	1	-	42	13	1	13	2

Table 5. Frequency of virulence genes in *C. perfringens* in two clinical and non-clinical groups

Virulence genes	Non-clinical group			Clinical group					total	Total per-cent
	total	typeD	typeA	total	typeG	typeD	typeC	typeA		
<i>beta2</i>	25	1	24	25	2	12	1	10	50	71.42%
<i>cpe</i>	1	-	-	1	-	1	-	-	1	1.42%
<i>tpcL</i>	5	-	4	1	-	-	1	-	5	7.14%
<i>tcpH</i>	34	1	33	27	2	13	1	11	61	87.14%

MLVA typing

PCR using specific primers for eight VNTR loci produced 42 distinct banding patterns in total, excluding null alleles, which were observed

at all loci. These results are shown in Table 6. A representative agarose gel is shown in Figure 3. For all loci, selected PCR amplicons of varying sizes from different samples were submitted for sequencing (Bi-oneer Co., South Korea). The resulting sequences were compared using the online BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Table 6. VNTR characteristics obtained in this study.

Locus	Amplified frag- ment size	Number of different alleles	The most frequent al- leles in the related locus
Cp6	503-656	8	630
Cp13	225-296	5	296
Cp19	636-1600	9	1500
Cp42	220-1345	8	220
07	121	2	121
O1e	350-463	7	437
17	134-176	4	155
24d	144-194	5	159

C. perfringens strain 13 sequence was also used as a reference strain and was evaluated for comparison of sequences for each locus.

Analysis of sequences using tandem repeat finder program online (<https://tandem.bu.edu/trf/>) (Benson, 1999) showed that in most loci tandem repeats were polymorphic and not conserved. Observation of some various repeat units in different strains, presence of more than one TR and overlapping repeat sequences were common in these loci. For instance, the results of tandem repeats for locus cp6 in only one strain is shown in supplementary Table S2. Therefore, the MLVA scheme was based on the length of the resulting amplicons, measured in base pairs (bp).

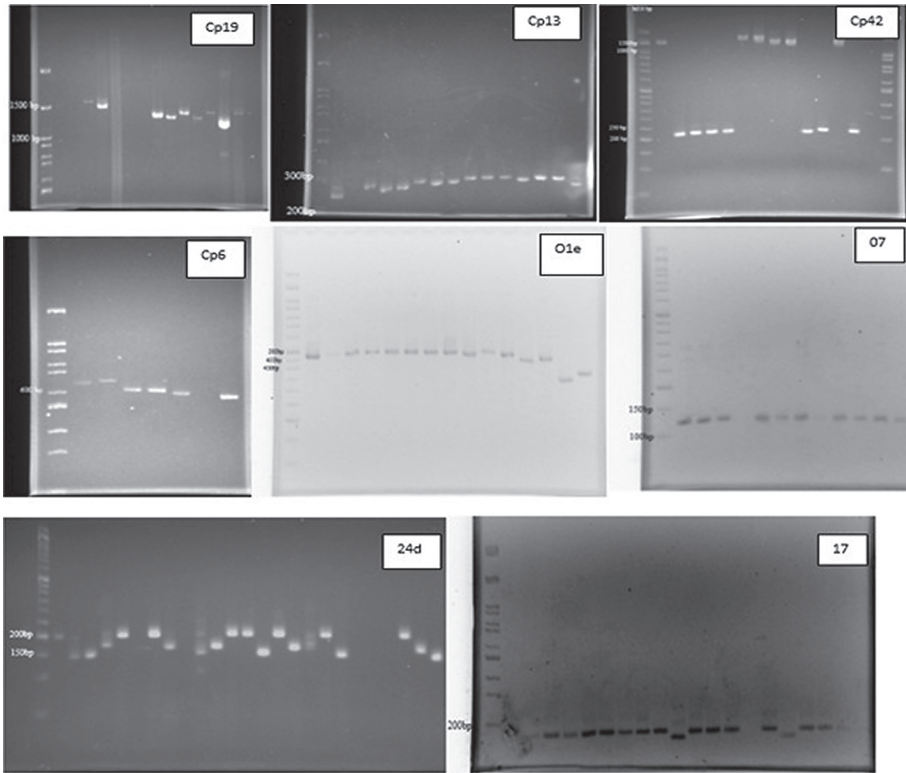


Figure 3. Agarose gel electrophoresis images of PCR products for eight loci.

The MLVA typing system used in this study classified the 74 *C. perfringens* strains into 71 MLVA distinct profiles. Figure 4. shows the minimum spanning tree (MST), created by Bionumerics software. As shown in MST, different clusters of strains are observed and the isolates have high genetic diversity. Six isolates exhibited identical profiles in pairs. Among them, two isolates shared the same profile despite differing in source (region and host), toxin type, and year of isolation. However, one pair - strains 54 and 55 - was isolated from enterotoxemic goats in the same region on the same date. Overall, five clusters (clonal complexes) and three singletons were identified.

Each cluster contained different toxin types, various hosts and isolation dates. Clustering is not related to toxin types i.e., type C isolate is more divergent from the vaccine strain of type C. There was no genetic relationship that separated the clinical isolates from the non-clinical ones.

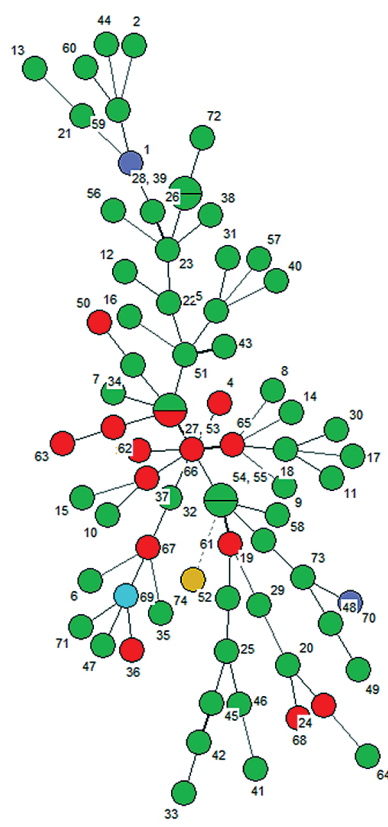


Figure 4. MST analysis based on 8 VNTR loci genotyping profiles of 73 *C. perfringens* strains plus strain 13 from GenBank as a reference strain. Circles represent genotypes, with the numbers beside them indicating the corresponding isolates. The color of each circle corresponds to a different toxin type: green for type A, red for type D, dark blue for type C, light (turquoise) blue for type B, and yellow for strain 13. Isolates numbered 59 and 60 are type G.

The discriminatory power of individual VNTR loci D (Nei's diversity index) was 0.09, 0.76, 0.25, 0.82, 0.79, 0.85, 0.8 and 0.84 for loci 7, 24d, 17, O1e, cp42, cp13, cp6 and cp19 respectively. Locus cp13 displayed the highest and locus 7 the lowest allelic diversity. The overall Diversity Index (DI) was calculated to be 0.99 for all eight loci.

The dendrogram based on the MLVA results is also shown in Figure 5. According to this dendrogram, eight clusters or clades are obtained. None of the clusters were associated with a specific characterization. *C. perfringens* strain 13 formed a closely related cluster, showing greater similarity to the type C

vaccine strain and 10 strains isolated from both healthy and clinical cases between 2015 and 2022. Nine isolates from cattle hosts were included in four cluster along with other strains. One cluster included merely 3 isolates from goats and the rest of 12 isolates from goats are included in 3 other clades with other strains. The isolates from sheep hosts are located on seven clusters.

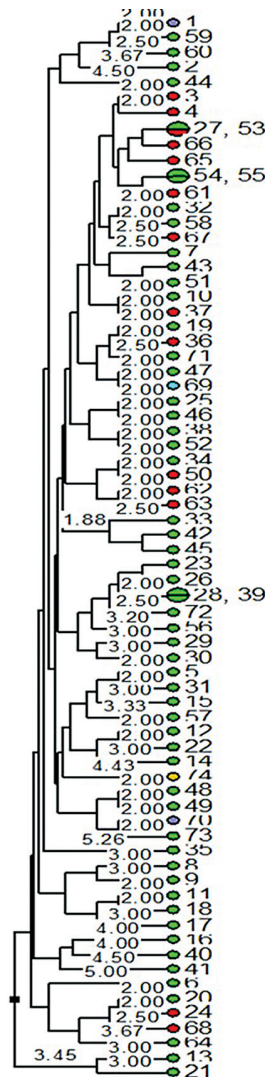


Figure 5. Dendrogram of the MLVA results for the *C. perfringens* strains in this study. The dendrogram was constructed by BioNumerics software using UPGMA and the Dice algorithm based on the band sizes on the electrophoresis gel.

DISCUSSION

The present study intended to determine the strain diversity of clinical and non-clinical *Clostridium perfringens* isolates, based on MLVA typing. *C. perfringens* isolates from different parts of Fars province of Iran isolated between 2009 and 2022 were used in this study. A total of 43 *C. perfringens* isolates were obtained from intestinal contents of healthy goats, sheep and cattle from slaughterhouses. In contrast, 27 isolates were recovered from confirmed enterotoxemia cases, either through field sampling or provided by the provincial veterinary office.

In addition to identifying the toxin types of our isolates, the presence of other virulence factors was also assessed. A majority of the isolates belonged to type A and D respectively, in both groups. According to the recent reclassification of *C. perfringens* by Rood et al., none of our type A strains carried the *cpe* gene to be classified as type F; however, one type D strain from the clinical group tested positive for this gene. The role of *CPE* *C. perfringens* isolates in the pathogenesis of enterotoxemia in livestock has not been precisely determined, although their contribution to enteritis and diarrhea in both animal hosts and humans is well established (Songer, 2010; Freedman et al., 2015; Suzuki et al., 2021; Alimolaei et al., 2023).

The other new reclassified type is type G, representing the presence of *netB* gene in type A strains. Although this virulence gene is associated with necrotic enteritis in poultry (Smyth et al., 2022), it was also found in two type A strains from the clinical group isolated from sheep, which were later reclassified as the new type G.

The majority of isolates in the two groups (a total of 87%) were positive for conjugative plasmids, as confirmed by amplification of the *tcpH* gene. Among the virulence genes *cpe*, *tpel*, $\beta 2$ and *netB*, evaluated in this study, beta2 gene was the only one showing statistically significant different result in two groups. The presence of this gene was 92% in clinical and 58% in non-clinical isolates.

This result aligns with previous findings (Manteca et al., 2002) but contradicts both our earlier study and those of other researchers (Sting et al., 2009; Razmyar et al., 2014; Hayati et al., 2021). The discrepancy between the two studies may be explained by the loss of viability in some archived *C. perfringens* strains, which prevented their inclusion in the present analysis.

The role of this gene in the pathogenesis of *C. perfringens* in livestock remains unclear, although the isolation of type A strains harboring the beta2 toxin from enterotoxemic calves has been documented (Manteca et al., 2002). It is well established that beta2 toxin exerts cytotoxic effects on CHO cells and

induces hemorrhagic necrosis in the guinea pig intestine, as well as enterocolitis and diarrheic disease in foals, piglets, dogs, and several other domestic, wild, and even human hosts (Fisher et al., 2005; Wasinski, 2007; Van Asten et al., 2010). The phylogenetic relationship between these *C. perfringens* isolates, obtained from different sources (healthy and enterotoxemic cases) was also assessed using MLVA method, as this method is considered a potential technique for genotyping a wide range of bacteria, including multiple *Clostridium* species (Lindstedt et al., 2005; Park et al., 2016)

In this study, MLVA analysis was performed using primer pairs targeting eight VNTR loci: 07, 17, 24d, O1e, cp6, cp42, cp13, and cp19. According to the investigations by Sawires and Songer (2005) and Chalmers (2008) on tandem repeats in the *C. perfringens* genome, five and six loci, respectively, were identified as high-priority targets among other TR sequences for use in MLVA typing of this species. They reported high typeability, reproducibility and Discrimination (DI = 0.99 and 0.97) of this technique for genotyping of their *C. perfringens* isolates (Sawires and Songer, 2005; Chalmers et al., 2008). Our results showed high Discrimination (DI = 0.99) as well. According to the criteria proposed by Hunter and Gaston (1988), a discriminatory index greater than 0.90 is considered desirable.

Considering allele frequency and tandem repeat length - and due to the absence of capillary electrophoresis - eight primers out of the eleven loci were selected to conduct this MLVA study. Four of these loci are located on protein coding sites on chromosome and the others on non-coding regions. Locus cp6 is located on collagen like protein sequence. The most frequent allele length recorded in the gene bank is 570 bp, whereas for our isolates from Fars province, Iran, it was 610 bp. The locus CP13 is located within the *nagK* gene, which encodes hyaluronidase. The most frequent allele in our study was 296 in two healthy and clinical groups. In the clinical group, 21 out of 27 isolates (77.7%) and in the healthy group, 20 out of 43 isolates (46.5%) exhibited a 296 bp band size for this locus, whereas the most frequent allele reported in Sawires and Songer' study was 282 bp. It has been suggested that variation in the hyaluronidase gene within a population may be linked to environmental adaptation, although further investigation is needed. Locus CP19 is related to a part of Iron transport protein B which has shown to have more null allele in both this and previous studies. Sequencing revealed that the polymorphism at this locus did not fully correspond to the number of VNTR repeat units. Locus CP42 is located partly on a hypothetical protein and partly on beta subunit of riboflavin synthase protein regions (Sawires and Songer, 2005). The most frequent allele of this locus was 220 bp in both clinical and nonclinical groups.

Other loci including O1e, 17, 07 and 24d were located on noncoding regions (Chalmers et al., 2008). The most frequent alleles of loci O1e, 17, 07 and 24d were 437 bp, 155 bp, 121 bp and (144 and 159 bp) respectively in both groups.

Forty-eight alleles with the average of six alleles per locus were detected using these eight loci in this study. Combining loci increased the discriminatory power for genotyping isolates; however, it failed to differentiate the clinical group from the non-clinical group, and clustering occurred independently of toxin type, health status, isolation date, region, or host. In addition, no single VNTR locus showed the specificity to reveal the adaptation of *C. perfringens* strains to the environment and virulence.

The extensive genetic diversity observed among our *C. perfringens* isolates is consistent with findings reported in other studies. This may be attributed to the high heterogeneity and variation of *C. perfringens* genome (Myers et al., 2006; Feng et al., 2020; Camargo et al., 2022). Although Johansson et al. (2006) showed a high genetic similarity among epidemiologically related isolates of *C. perfringens* and a wide genetic diversity among the isolates from different sources by PFGE.

The virulence of *C. perfringens* is primarily associated with its toxin production and the process of horizontal gene transfer (Van Asten et al., 2010; Gulliver et al., 2023). In addition, many of these toxin genes (major toxins related to typing and the rest ones) are present in *C. perfringens* isolates of intestinal microflora from healthy livestock (Freedman et al., 2015). It is believed that certain changes in the host and environment can trigger toxin production and contribute to disease development (Sawires and Songer, 2006; Uzal et al., 2014). Nevertheless, it is important to determine whether genetic differences exist between clinical and nonclinical isolates. Although phylogenetic analysis may not always effectively group these isolates, it remains an essential tool for tracking strains during an outbreak.

CONCLUSION

The VNTRs used for MLVA typing in this study revealed considerable genomic diversity among *C. perfringens* strains but did not demonstrate the expected close phylogenetic relationships among strains from the same source category or with similar virulence. It can be concluded that the limited number of VNTR loci examined (eight primers) and the lack of assessment of additional VNTRs across the genome may limit the representativeness of the results regarding overall genetic diversity and relatedness. Therefore, complementing MLVA with other phylogenetic methods is recommended for a more comprehensive analysis.

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Author's Contribution

S.S. collected the samples. M.M. and A.P. contributed to bacterial isolation. K.T. carried out the MLVA analysis using BioNumerics software. R.S., a colleague from the veterinary office, provided enterotoxemia-related isolates. Y.T. revised the manuscript. M.H. was involved in all aspects of the project, including experimental design, bacterial isolation, PCR, molecular analysis, data collection, and manuscript drafting. All authors read and approved the final version of the manuscript.

Competing interest

The authors declare that there are no commercial or financial relationships that could be seen as a potential conflict of interest.

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Supplementary Data

The supplementary material (Table S1) associated with this article is available online at the following permanent link:

<https://niv.ns.ac.rs/e-avm/index.php/e-avm/article/view/475/321>

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