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Genotyping and Phylogenetic Analysis of *Clostridium perfringens* Isolated from Domesticated Ruminants in Duhok Governorate, Iraq

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Clostridium perfringens is a zoonotic pathogen causing various health problems like gas gangrene, food poisoning, and other enteric infections, especially in domestic livestock, leading to significant global economic losses. In present study, *C. perfringens* strains were isolated and identified depending on 16 rRNA and genotyping based on genes encoding alpha, beta, epsilon, iota, enterotoxin, NetB toxins, then sequencing of 16S rRNA and some main toxin-producing genes in 219 samples collected from domesticated ruminants in sheep, goats, and calves, during the period from August to December of 2020, in Duhok province/ Iraq. Out of a total 67, Forty-seven (28.8%) isolates of *C. perfringens* were obtained in 163 ruminal content samples from healthy sheep and goats, and out of 56 samples of calves (34 healthy and 22 diarrheic); 20 (35.7%) isolates (7 (20.5%) isolates from healthy and 13 (59%) isolates from diarrheic) were confirmed as *C. perfringens*. Of the 47 isolates from healthy sheep and goats, 39 (82.97%) were identified as type A, and 8 (17.03%) as type D. While all 20 isolates from healthy and diarrhetic calves were diagnosed as type A. None of the total 67 *C. perfringens* isolates were carrying genes encoding beta, iota, enterotoxin, and NetB toxins. Phylogenetic tree analysis revealed that all isolated strains were clustered in same group. *Clostridium perfringens* is one of the major pathogens with health concern in both humans and animals. Type A and D were the only genotypes obtained from the collected samples which were genetically similar.

Keywords: *Clostridium perfringens,* Phylogenetic tree, Alpha toxin, Epsilon.

Introduction

Clostridium perfringens is an anaerobic, gram-positive, spore-forming, bacillus bacterium [1]. It is a considerably versatile pathogen in both domesticated animals and humans, most often causes clostridial myonecrosis "gas gangrene" in infected wounds [2], food poisoning, diarrhea associated with antibiotics [3], and enteric necroticans which is a severe enteric infection (also called darmbrand or pigbel) [4]. In the animal industry, *C. perfringens* causes significant economic losses due to several animal diseases including lamb dysentery (necrohemorrhagic enteritis) in sheep [5], necrotic enteritis of poultry [6], and some newborn animals from various species (including cattle, sheep, and pigs), depending on the types of toxins produced by different strains [5].

However, not all *C. perfringens* strains synthesize all types of toxins, but among the clostridial group species, it is the most potent toxin-producing species due to its short generation time which can be less than 8 min in CPE-producing strains when cultured in autoclaved ground beef in temperatures between 41 °C and 46 °C, for instance [5]. Out of more than 20 toxins produced by *C.*

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perfringens, six major potent toxins (CPA, CPB, ETX, ITX, CPE, and NetB) are used to classify it into seven toxinotypes (from A to G), according to the recent toxinotyping system [7]. Based on this classification, type A strains of *C. perfringens* are mainly the causative agent of gas gangrene and do not cause food poisoning in humans, while type B, C, and D strains have clinical importance in the veterinary field by causing enteric diseases in animals. Type F strains, which produce CPE toxin, are implicated with food poisoning in humans and also antibiotic-associated diarrhea. Type G *C. perfringens* represents isolates that release NetB toxin and lead to necrotic enteritis in poultry [8].

In the veterinary field, *C. perfringens* is implicated in causing several GIT infections in most mammalians, which are called enterotoxaemia named after the toxins released by this species in the intestine which are then absorbed to reach the circulatory system [9]. In some cases, toxins only affect the intestine locally [6]. This microorganism can also cause infection in cutaneous, subcutaneous, and muscular tissues causing malignant edema and gas gangrene. One or more potent toxins contribute to the pathogenesis of all diseases associated with *C. perfringens* [1]. In poultry production, Avian specific strains of *C. perfringens* led to significant economic losses, particularly by causing necrotic enteritis, estimated to be $2 billion mainly by spending on control measures and losses in production. The number of cases is on the rise in some countries due to restrictions on using antibiotics as growth promoters, as a consequence, it increases the risk of human infections via the food chain [10].

The aim of this study was to isolate and genotype *C. perfringens* found in healthy sheep, goats, and healthy and diarrhetic calves in Duhok province/Iraq, in addition to investigate the 16S rRNA gene, α- toxins, and ε-toxins genes sequence variation.

Material and Methods

Sample Collection and Isolation of C. Perfringens

During the period from August to December 2020; 219 samples were taken randomly from ruminal contents and faecal sample from sheep, goats, and calves (diarrhetic and healthy) from slaughterhouses and some farms in Duhok city, Zakho, and Bardarash / Iraq. After sampling, the swabs were immediately put into tubes containing 10 ml of Fluid Thioglycollate Medium ((HiMedia, Mumbai, India) for selective enrichment, shaken sufficiently, and incubated 12-24hrs at 37°C. Then a loop of the enrichment broth was streaked on CHROMagarTM *C. perfringens* (CHROMagar, France), which was prepared according to the manufacturer's instructions, and incubated at 37 °C for 48 hours under anaerobic conditions using anaerobic jar (The 2.5-liter Oxoid AnaeroJar) and gas packs (Thermo Scientific™ Oxoid™ AnaeroGen™ 2.5L Sachet). All suspected *C. perfringens* colonies were then sub-cultured on CHROMagarTM *C. perfringens* and incubated at 37°C for 48 hours under anaerobic conditions [11] to obtain pure cultures. Suspected isolates were kept in 25% glycerol and brain heart infusion broth (BHIB) and stored at -20°C for further processing [12] .

DNA Extraction

DNA extraction was performed by boiling method [13], two to three colonies of *C. perfringens* were collected from CHROMagarTM *C. perfringens* agar plates, placed into microfuge tube, and resuspended in 100 µL of Nuclease-free water. The suspension was incubated at 100 °C for 10 min and immediately cooled on ice for 5 mins. The bacteria debris was removed by centrifugation (10000 rpm, 5 min), and the supernatant was transferred into a new tube, then stored at −20 °C until ready for use. The quantification and purity of DNA were measured using DS-11FX+ spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

PCR Characterization of 16S rRNA and Toxin Genes

Template DNA was obtained from cultures of the 104 suspected isolates growths on CHROMagarTM *C. perfringens* agar plates. A uniplex PCR was utilized to identify the isolates as *C. perfringens* through the amplification of 16S Rrna. Furthermore, uniplex and multiplex PCR were used to detect six toxin genes including alpha, beta, epsilon, iota, enterotoxin, and necrotic beta-like toxin (Table 1). The designed primers were blasted using (BLASTN, version 2.9.0+) https://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html website to recognize the reported BLAST sequences for the detected genes and the presence of significant homology with *C. perfringens* sequences. The 50 µL volume included 25 µL of 2x AddStart Taq Master (Addbio, Korea), 2 µL of each forward and reverse primer (10

μM) (Table 1), and 4 μL of template DNA. PCR cycling conditions were as follows; 2 min at 94ºC followed by (35) cycles of 94 ºC for 30 sec. (annealing, according to table 1 for each primer) for 30 sec, and at 72 ºC for 30 sec., and followed by 5 min at 72ºC for the final extension. Finally, the PCR product was characterized using a 2% (w/v) agarose gel prepared from 1X tris acetate ethylenediamineacetate (TAE) buffer containing Prime Safe Dye (GeNet Bio, Korea). A 100bp DNA marker (GeNet Bio, Korea) was used. The gel was allowed to run at 100 volts, 300 amperes for 40 mins in 1X TAE buffer using (Cleaver Scientific – UK) and was visualized under the UV transilluminator light.

Sequencing, Assembly, and Annotation Analysis

The 16S rRNA (8 isolates), Alpha (9 isolates), and Epsilon (5 isolates) were PCR amplified and Sanger sequenced to confirm the identity of the isolates. BioEdit software (Version: 7.2.5.) was used to analyse and visualize the nucleotide sequences. The 16S rRNA, Alpha, and Epsilon sequences have been deposited in the Gene Bank database under accession numbers. The 16S rRNA (8 sequences) OQ197472 to OQ197479. The CPA gene sequences (α-toxins) (9 sequences) from OQ409869 to OQ409877 and ETX gene (Epsilon toxin) (5 sequences) from OQ408129 to OQ408133. "The nucleotide Basic Local Alignment Search Tool (BLASTn; https://blast.ncbi.nlm.nih.gov/)" was utilized for finding the similarity with other sequences deposited in GenBank.

Phylogenetic Analysis

Phylogenetic analysis of submitted 16S rRNA, Alpha, and Epsilon sequences was performed using MEGA11. Dendrogram for *C. perfringens* was completed using bootstrap as phylogeny method. The neighbour joining phylogenetic tree was generated using maximum composite likelihood model and 1000 bootstrap replication. In dendrogram, current *C. perfringens* sequences were represented with accession numbers mentioned above.

Results

The samples were collected from domesticated ruminants for *C. perfringens* isolation and molecular characterization utilizing the selective CHROMAgarTM, and the PCR technique. The obtained results from the investigation of 219 samples, of which 163 ruminal content samples were collected from healthy sheep and goats, and 56 fecal samples from 34 healthy and 22 diarrheic calves from four slaughterhouses and 5 farms in Duhok province/ Iraq. Out of total 219 collected samples, 104 were culture positive on CHROMagarTM representing 47.5% (Figure 1), and from these culture positive isolates, 67 isolates were confirmed by PCR using 16S rRNA *C. perfringens* specific primers representing 64%. out of the 163 samples from healthy sheep and goats, *C. perfringens* were confirmed in 47 (28.8%) samples, and out of 56 samples from calves, 20 (35.7%) samples (7 (20.5%) from healthy and 13 (59%) from diarrhetic) were confirmed as *C. perfringens*. Among of 47 isolates from healthy

TABLE 1. The primers for detection of the main toxin's genes of *C. perfringens.*

Toxin (Target genes)	Primer Sequence5'-3'	Annealing temp.	Amplicon size bp	References
16S rRNA	F-TAACCTGCCTCATAGAGT R-TTTCACATCCCACTTAATC	53	481	[14]
Alpha (CPA)	F: GCTAATGTTACTGCCGTTGA R:CCTCTGATACATCGTGTAAGAATC	53	325	[15]
Beta (CPB)	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATCTTC	53	196	[8]
epsilon (ETX)	F: TGGGAACTTCGATACAAGCA R: AACTGCACTATAATTTCCTTTTCC	53	376	[16]
IAP	F: AATGGTCCTTTAAATAATCC R: TTAGCAAATGCACTCATATT	53	272	[16]
Enterotoxin (CPE)	F:GGAGATGGTTGGATATTAGG R: GGACCAGCAGTTGTAGATA	56	233	[17]
NETB	F: CAGGCCATTTCATTTTTCCGT R: ACCGCTTCACATAAAGGTTGGA	55	157	This study
Beta 2 toxin (cpb2)	F:AGTGCAAAAGAAATCGACGC R: CCCCAATTGCTTGTGCTAGTT	57	412	This study

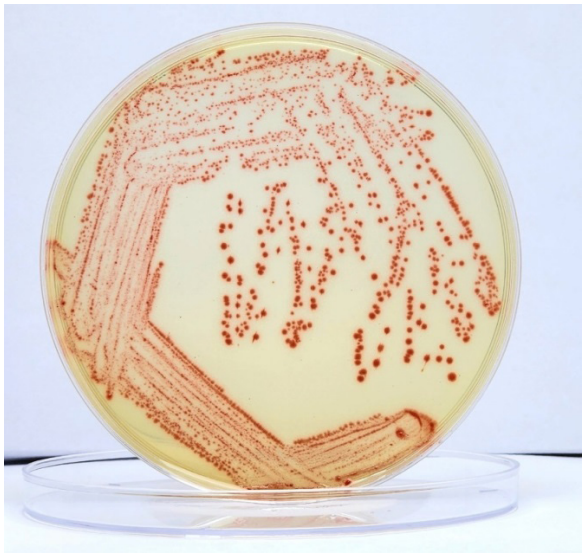

Fig.1. *Clostridium perfringens* colony on CHROMagar™.

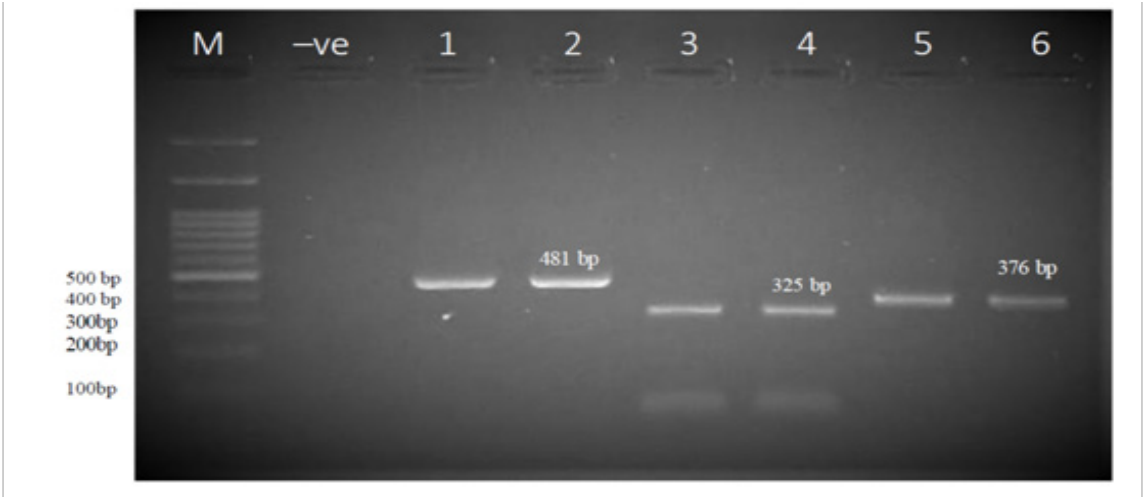

Fig.2. Simplex PCR for identification of *C. perfringens* and detection of toxin genes. M: marker (DNA ladder, 100 bp); (-ve) negative control for 16S rRNA, lane 1: positive control for 16S rRNA; lane 2: 16S rRNA gene; lane 3: positive control for CPA toxin encoding gene; lane 4: CPA toxin encoding gene; lane 5 positive control for ETX toxin encoding gene; lane 6: ETX toxin encoding gene.

sheep and goats, 39 (82.97%) were identified as type A as they only had the CPA gene, while 8 (17.03%) isolates carried CPA as well as ETX genes and were identified as type D (Figure 2). All 20 isolates from healthy and diarrhetic calves were diagnosed as type A as they carried only CPA gene from the six toxins genes investigated. None of the total 67 *C. perfringens* isolates were carrying genes encoding beta, iota, enterotoxin, and NetB toxins.

16S rRNA Gene Characterization

For the characterization of 16S rRNA, 8 sequences were analyzed. Sequences of 16S rRNA obtained in this study showed 100 % sequence identity to the sequences of 16S rRNA reported from ruminants from Pakistan (MT158886), China (ON715858, MW440587, MK156683, LC386311), India (OP872740), Japan (LC386311), USA (CP116430.1, CP116432.1). The phylogenetic tree was constructed based on 16S rRNA between *C. perfringens* strain AKB Duhok and *C. perfringens* strains included in the NCBI genome database. The dendrogram showed that All 16S rRNA partial gene sequences obtained were closely identical to that of the type strain of *C. perfringens* as in (Figure 3).

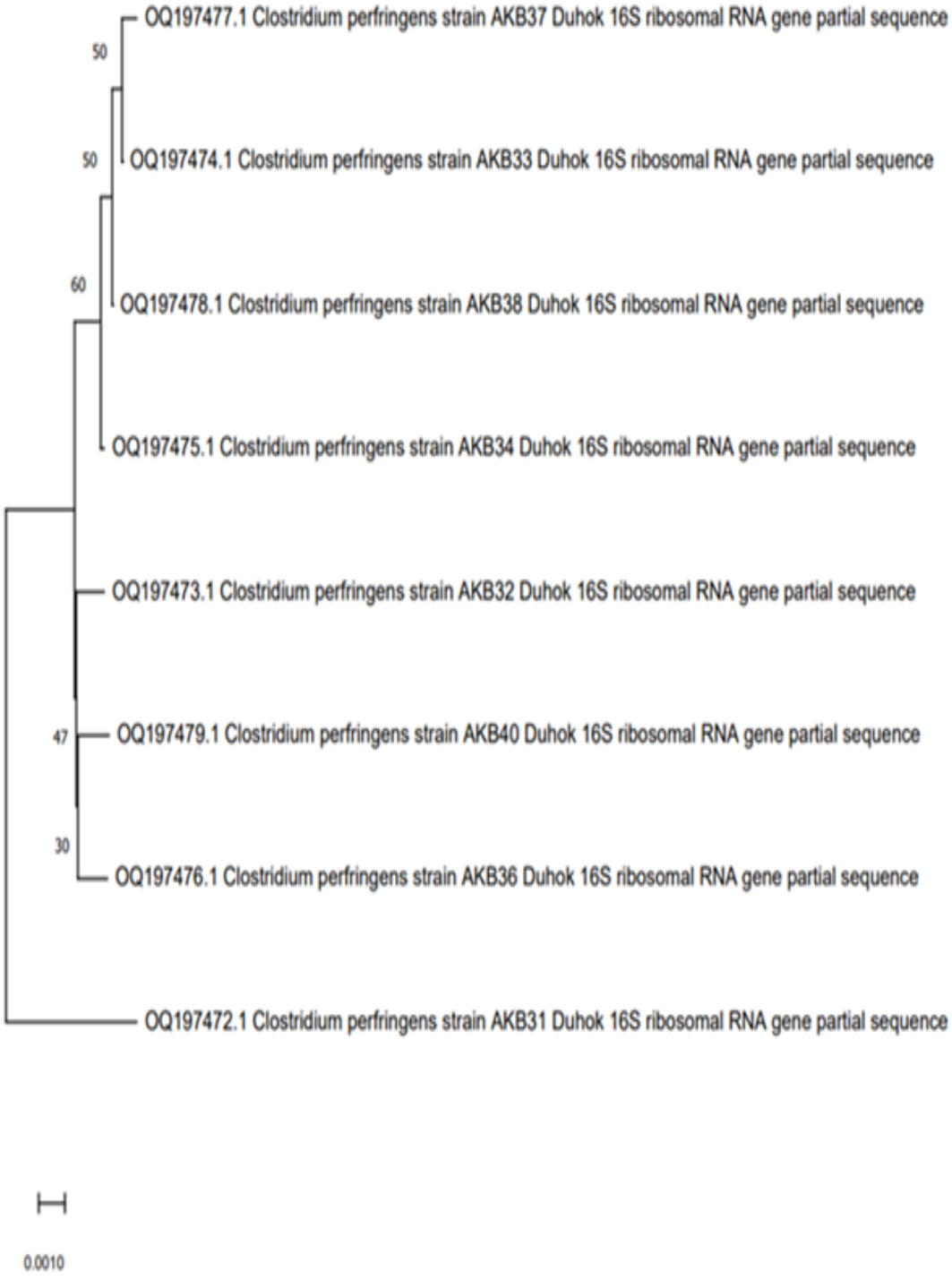

Fig. 3. Phylogenetic tree of *C. Perfringens* 16S rRNA gene and partial nucleotide sequences that was generated using neighbor joining in MEGA11. It showed clear clustering of the Duhok province / Iraq isolated strain, except AKB31 strain which is out branched maybe due to shorter sequence length.

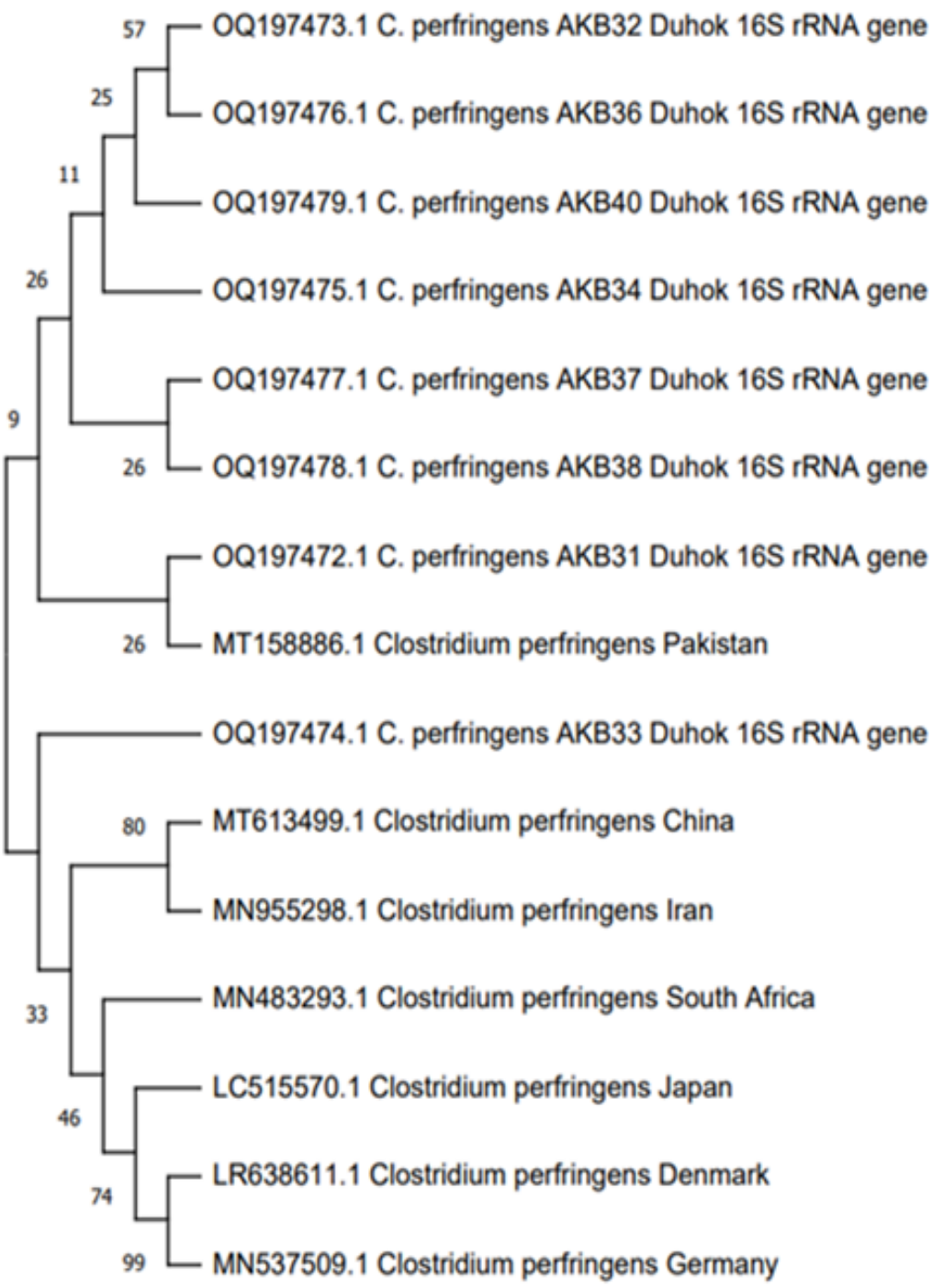

Fig. 4. Phylogenetic tree of *C. Perfringens* 16S rRNA gene and partial nucleotide sequences that was generated using neighbor joining in MEGA11. It showed clear clustering of the Duhok province / Iraq isolated strains and different *C. Perfringens* strains isolated from Pakistan, Iran, China, South Africa, Japan, Denmark and Germany uploaded from GenBank.

CPA Gene Characterization

CPA of 9 isolates illustrated significantly high sequence identity (100%) to each other and that of ruminant strains. The sequences from Duhok were scattered and clustered with sequences obtained from India (MK180784.1, Saudi Arabia (MN646321.1, MN646328.1, MN646332.1, MN646338.1, MN646361.1, MN646346.1, MN646344.1), India (KP163982.1), China (from MZ726104.1 to MZ726155.1), and Iran (JF298801.1, GU447317.1) (Figure 4).

ETX Gene Characterization

For the characterization of ETX gene, 5 sequences were analyzed. Sequences of ETX gene obtained in this study were OQ408129 to OQ408133. By BLAST querying against ETX sequence in NCBI database, we found 99.72% identity with ETX gene reported from ruminant from India (MF471362.1, KY938004.1, KY938005.1, KY938006.1, KY938007.1, KY466176.1, MF471361.1, AJ426474.1, MG600592.1, JX112705.1, JX033910.1), Pakistan

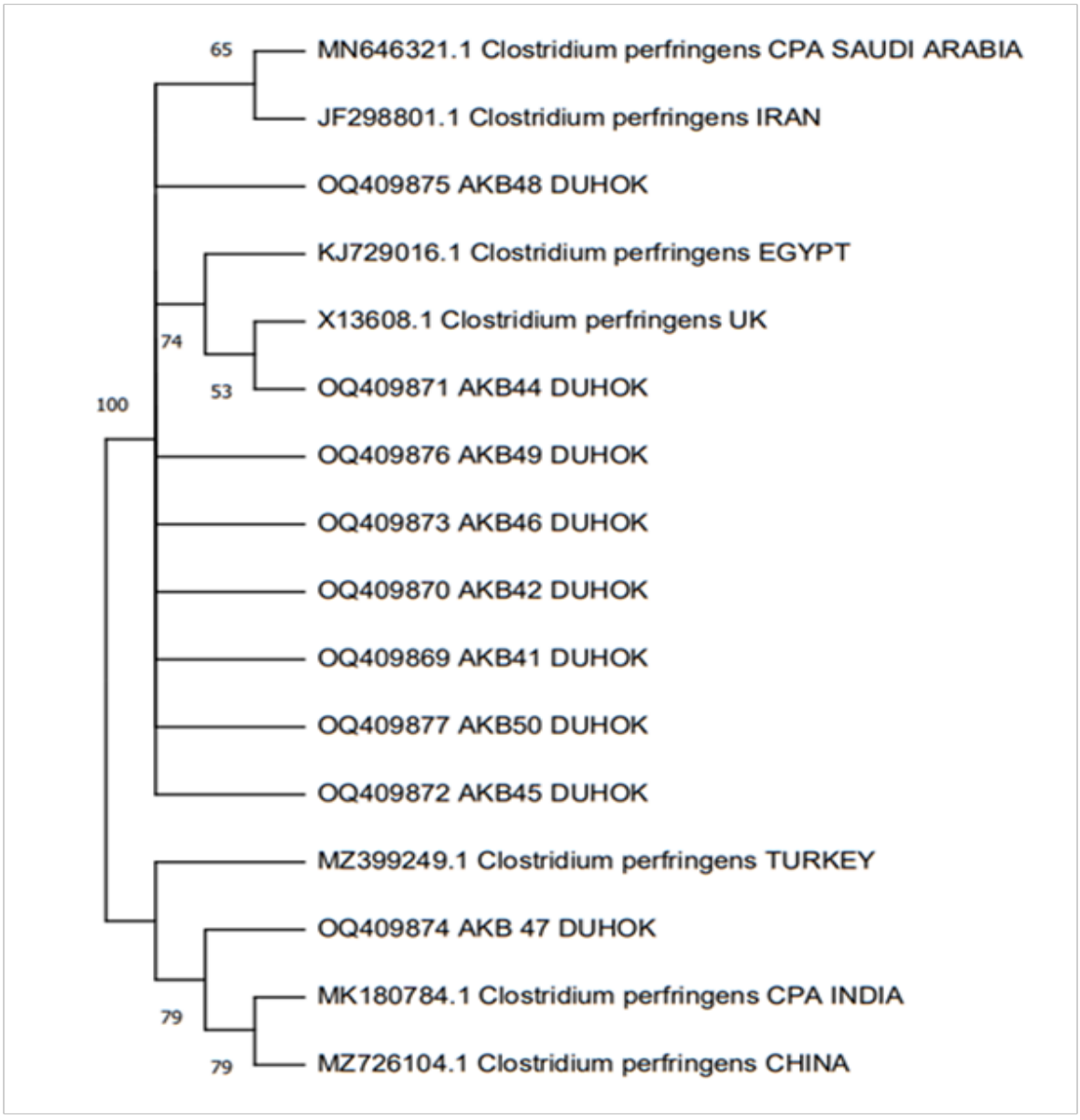

Fig. 5. Phylogenetic tree of C. Perfringens CPA toxin encoding gene and partial nucleotide sequences that was generated using neighbor joining in MEGA11. It showed clear clustering of the Duhok province / Iraq isolated strains and different C. Perfringens strains isolated from Saudi Arabia, India, China, UK, Egypt, Turkey, and Iran uploaded from GenBank.

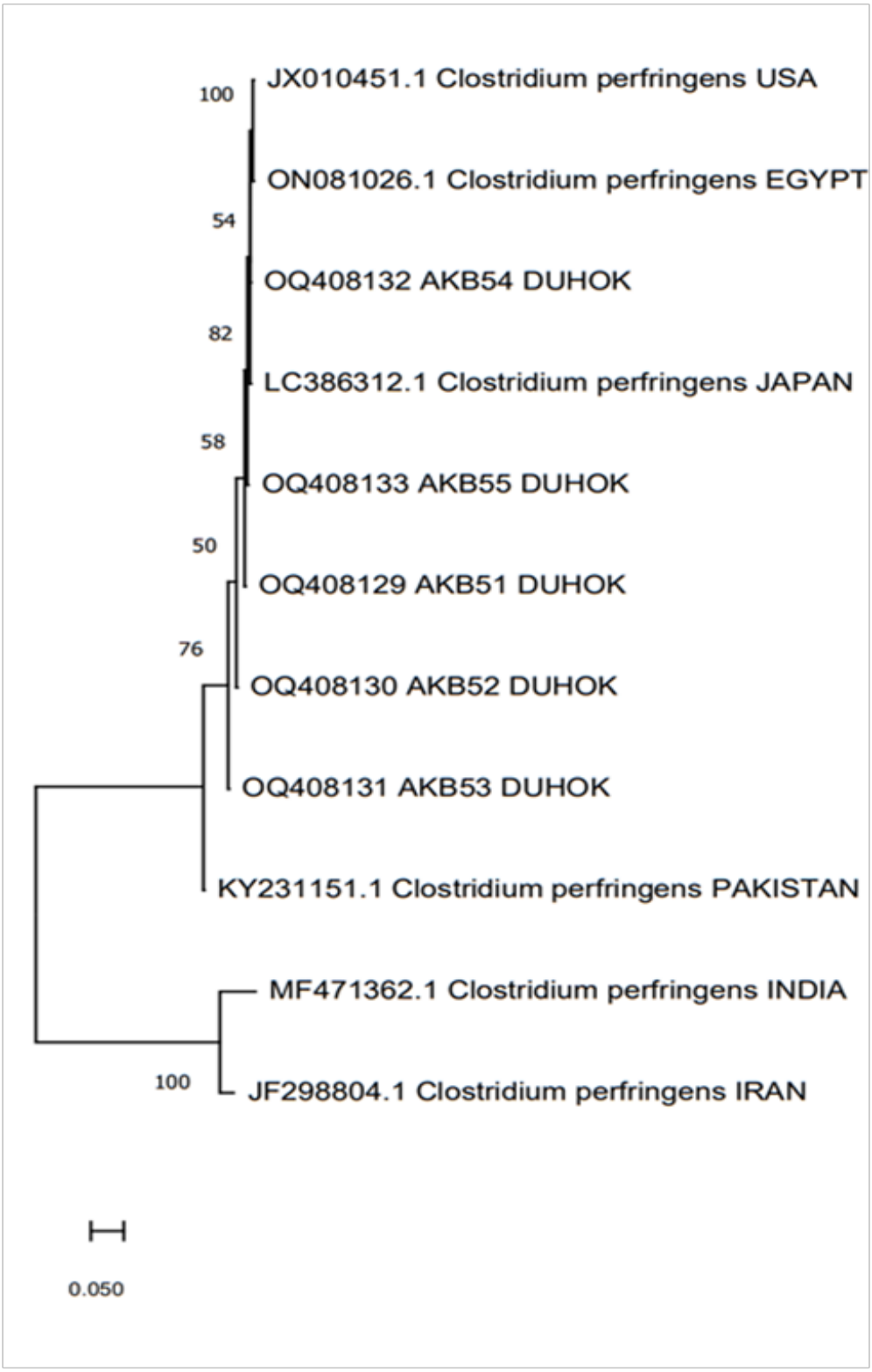

Fig. 6. Phylogenetic tree of *C. Perfringens* ETX toxin encoding gene and partial nucleotide sequences that was generated using neighbor joining in MEGA11. It showed clear clustering of the Duhok province / Iraq isolated strains and different *C. Perfringens* strains isolated from Iran, India, Pakistan, USA, Eygpt, and Japan uploaded from GenBank.

(KY231151.1, KY264126.1, MT946879.1, MT946876.1, MT946875.1, MT946874.1), China (CP119190.1), Japan (LC386312.1), USA (CP116437.1, CP116439.1, CP116429.1, CP116431.1, CP116433.1, JX010451.1), Egypt (ON081026.1, ON081027.1), Iran (JF298804.1, JF298805.1, OM417608.1). Neighbor joining phylogenetic tree generated revealed that ETX gene sequences from Duhok grouped with sequences obtained from ETX genes from ruminant species (Figures 5,6).

Discussion

Clostridium perfringens strains have been isolated from different sources including apparently healthy animals [18], which synthesize a number of potent toxins that cause health problems in humans and animals. Based on six major toxins, *C. perfringens* is classified into seven genotypes (toxinotypes) from A to G [19]. However, several minor toxins play a significant role in their pathogenicity and pathogenesis.

In present study, 67 isolates of *C. perfringens* were positive by both culture on CHROMAgarTM and PCR. Further molecular investigations revealed that 20 (100%) of the isolates obtained from both apparently healthy and diarrhetic calves, and 39 (82.97%) of the isolates from apparently healthy sheep and goats were type A strains carrying only alpha-toxin encoding gene, while 8 (17.03%) of the isolates were type D strains, carrying both alpha-toxin and epsilon toxin encoding genes. None of the other *C. perfringens* types (B, C, E, and F) were found by specific primers for the detection of toxins encoding genes utilized for its genotyping. This dominance by type A strain in sheep and goats is in agreement with previously conducted studies in Pakistan [20] and turkey [21], with 82% and 95%, followed by 18% and 5% of type D strains, respectively.

In the presence of *C. perfringens* type D in the intestinal tract, sudden alteration of diet to a high carbohydrate feed could be a predisposing factor for causing the infection and leads to a higher mortality rate compared to cases caused by type A of *C. perfringens* [22]. It is also reported that besides high carbohydrates and protein feed consumption, handling of the animals (for example; transportation) which causes stress, and sudden unexpected weather changes are also thought to be one of the predisposing factors for causing enterotoxemia in cattle [23]. Globally, it has been reported that the prevalence of enterotoxemia caused by type D is estimated between 24.13% and 100% [24]. Vaccination is required to reduce the risk of enterotoxemia [25] taking into consideration that a single dose may not provide enough protection and should be followed by a booster dose [26]. Phylogenetic analysis of the isolated strains using 16S rRNA, CPA, and ETX genes partial nucleotide sequences that was generated using neighbor joining in MEGA11 showed clear clustering of our strains and different *C. Perfringens* strains isolated from Pakistan, Iran, China, South Africa, Japan, Denmark and Germany downloaded from GenBank. The phylogenetic tree confirmed that the isolates were closely related, which indicate that the source of all bacterial strains may come from the same origin [27]

Conclusion

As only type A and D strains of *C. perfringens* were detected in ruminal content samples collected from healthy sheep and goats, and type A strains in fecal samples of healthy and diarrhetic calves, the diversity of *C. perfringens* was low. However, none of the isolates were carrying the enterotoxin encoding gene, which indicates that the mentioned animals are not the potential source of CPE-associated diseases in humans. These animals still shed *C. perfringens* to the environment and cause contamination of the water and soil, and as a consequence, vegetables and fruits during cultivation. Another potential risk is lacking good processing and hygiene in slaughterhouses may lead to the transmission of these bacteria through the food chain and cause health issues.

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Conflicts of interest

The authors declare no conflict of interest.

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النمط الجيني و تحليل شجرة المَحْتِد (الفيلوجينية) لبكتيريا *Clostridium perfringens* المعزولة من بعض الحيوانات الداجنة (المستأنسة) فى محافظة دهوك – العراق

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بكتيريا *Clostridium perfringens* هو من المسببات المرضية حيوانية المنشأ و يسبب مشاكل صحية متنوعة مثل الغَرغَرينَة الغازيّة و التسمم الغذائي و عُدى معوية اخرى , خصوصا في الحيوانات الداجنة و التي تؤدي الى خسائر اقتصادية حول العالم. في هذا البحث, تم عزل و تشخيص عترات من *Clostridium perfringens* عن طريق البيولوجيا الجزيئية باستخدام جين 16S rRNA و استخدام جينات مسئولة عن انتاج سموم (alpha, beta, epsilon, iota, enterotoxin, NetB) لتحديد النمط الجيني , و بعدها تحديد التتابع النووي ل جين 16S rRNA و جينات اخرى مسئولة عن تكوين السموم , من عينات تم جمعها من المعز و الخرفان و العجول خلال فترة من شهر اب الى الكانون الاول من سنة 2020 في محافظة دهوك – العراق. من مجموع 67 عزلة , سبع و اربعون عزلة (ما يعادل 28.8%) من العزلات تم عزلها من 163 عينة تم جمعها من محتويات الكرش للمعز و الخرفات التي لا تعاني "ضاهريا" من الامراض, و من مجموع 56 عينة تم جمعها من عجول (34 صحية و 22 تعاني من الاسهال), تم تشخيص 20 عزلة (35.7%) , منها 7 (20%) من الحيوانات الصحية ضاهريا و 13 (59%). و من مجموع 47 عزلة من المعز و الخرفان الصحية "ضاهريا" تم تصنيف 39 عزلة (82.9%) تحت النمط الجيني A و 8 (17.03%) كنوع D. بينما كانت جميل العزلات المعزولة من العجول سواء كانت صحية او تعاني من الاسهال من النمط الجيني A. جميع العزلات ال67 لم تكن تحوي الجينات المسئولة عن تكوين السموم (beta, iota, enterotoxin, and NetB). و أظهر تحليل شجرة المَحْتِد (الفيلوجينية) ان جميع العترات المعزولة تمحورت في مجموعة واحدة. بكتيريا *Clostridium perfringens* من المسببات المرضية الرئيسية في الشأن الصحي للبشر و الحيوانات. و الانماط الجينية AوD كانتا الوحديتان التان تم عزلهما من العيينات التي تم جمعها و كانت وراثيا متقاربة.

الكلمات الدالة: *clostridium perfringens* ، شجرة المَحْتِد (الفيلوجينية) ، سم الالفا ، ابسيلون .