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DOMINANCE OF *CLOSTRIDIUM PERFRINGENS* TOXINOTYPES A AND D IN ENTEROTOXEMIA CASES AMONG BASRAH GOVERNORATE SHEEP

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ABSTRACT

Clostridium perfringens is a Gram-positive, anaerobic bacterium linked to various diseases in humans and animals, particularly necrotic enteritis in ovine and caprine species. Typically, researchers use its toxins to systematically classify isolates into seven toxinotypes (A-G) based on their presence or absence. Using microbial culture, microscopic examination, and specific primers, this study was conducted to identify major and secondary toxins, their isolation sources, and their clinical association with sheep necrotic enteritis. Between September 2023 and June 2024, 85 isolates of *C. perfringens* bacteria were isolated from 250 sheep samples. The PCR results revealed that the *cpa* and *plc* genes were present in all samples, and the *cpe* and *etx* genes were present in 20 bacterial isolates, accounting for 23.53%. The major toxinotypes in local clinical cases were Type A (65/85, 76.5%) and type D (20/85, 23.53%). Type A cases comprised 20 lambs and 45 adult sheep, whereas all type D cases were from adult animals. The sequence analysis of partial *16S rRNA* sequences demonstrated different degrees of similarity with sequences of other *C. perfringens* strains recorded in GenBank from Iraq and other countries. However, the local isolates clustered together, indicating a strong ancestral connection. These findings suggest the necessity for the integration of toxinotypes A and D into sheep vaccination programs and the pursuit of comprehensive epidemiological studies on the toxinotypes in humans and animals.

Keywords: *Clostridium perfringens*, polymerase chain reaction (PCR), *cpa* gene.

INTRODUCTION

C. perfringens is a bacterium that can be found in the digestive systems of both humans and animals, as well as in soil and wastewater (Narayan *et al*., 2023; Garofalo *et al.*, 2024). Some *C. perfringes* types, such as type A, can be found in animals and humans alike and is known to cause illnesses and intestinal diseases in individuals of both species (McClane *et al.*, 2006). The bacterium is strict anaerobe and creates spores to survive in the environments, which allow them to spread among susceptible animals and hinder the control measures (Liggins *et al.*, 2023).

C. perfringes can produce more than 20 toxin types, which makes it a strong opportunistic pathogen among humans and

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animals (Grenda *et al.*, 2023). However, toxins production varies markedly across strains of *C. perfringens*. Of these main toxins are the alpha (CPA, *cpa*/*plc* gene), beta (CPB, *cpb* gene), epsilon (ETX, *etx* gene), iota (ITX, *iap* gene), and enterotoxin (CPE, *cpe* gene), with each toxin having an impact on causing diseases (Freedman *et al*., 2015). The existence of these toxin genes serves as an identification system for *C. perfringens* strains. This identification system includes seven toxinotypes (A, B, C, D, E, F, G), determined by the presence of genes encoding for CPA, CPB, ETX, and ITX toxins, in addition to enterotoxin CPE and necrotic enteritis B-like toxin (NetB) as described in Table (1) (Rood *et al*., 2018). There is still little information available on *C. perfringens* toxinotypes in southern Iraq, particularly the Basrah Governorate, despite the fact that they have a major influence on animal health and the economy. This emphasizes the need for thorough epidemiological investigations that cover a wide variety of human and animal hosts. The goal of this study was to identify major and secondary *C. perfringens* toxins, their isolation sources, and their clinical association with sheep necrotic enteritis (enterotoxemia cases) in Basrah Governorate.

Table 1: Classification of *Clostridium perfringens* toxin-based typing (Rood *et al*., 2018).

***C. perfrigens* Type**	**Typing Toxins**					
	α-toxin CPA (*plc* or *cpa*)	**CPB (*cpb*)**	**ETX (*etx*)**	**ITX (*iap*)**	**CPE (*cpe*)**	**NetB (*netB*)**
A	+	−	−	−	−/+	−
B	+	+	+	−	−/+	−
C	+	+	−	−	−/+	−
D	+	−	+	−	−/+	−
E	+	−	−	+	−/+	−
F	+	−	−	−	+	−
G	+	−	−	−	−	+

Alpha toxin (CPA, *cpa*/*plc* gene); Beta toxin (CPB, *cpb* gene); Epsilon toxin (ETX, *etx* gene); Iota toxin (ITX, *iap* gene); Enterotoxin (CPE, *cpe* gene); Necrotic enteritis B-like toxin (NetB, *netB* gene).

MATERIALS AND METHOD

1. Sample collection:

Between September 2023 and June 2024, A total of 250 samples were collected. They comprised fecal material (150) and intestinal contents (100) from animals suspected to have died due to enterotoxemia.

The samples were collected from organized sheep farms and unorganized sectors, such as local farmers rearing sheep. Samples were chosen from suspected cases from various regions in Basrah Governorate. This included animals that died suddenly without any prior clinical signs, as well as sick animals suspected of intestinal poisoning. These animals displayed symptoms such as diarrhea, lethargy, arched backs, respiratory distress, and occasionally bloating, while others were in advanced stages of coma. The samples were collected within 2 hours after the death of the animals. The samples were collected in sterile tubes containing thioglycolate broth to provide anaerobic conditions (Al-Farhan *et al*., 2019) and were transported to the laboratory within two hours.

Culture and growth conditions:

Thioglycolate broth tubes inoculated with the samples were incubated at 37°C for 24-48 hours under anaerobic conditions using an anaerobic jar and anaerobic CO2 gas bags (CampyGen™, Thermo Scientific USA). After that, using sterile cotton swaps, thioglycolate samples were spread on chrome agar medium (CHROMagar™, France), then placed in the anaerobic jar

with anaerobic bags, and incubated at a temperature of 37°C for 48–72 hours. Suspected colonies were chosen for additional phenotypic investigation, including colony morphology, Gram staining, spore staining, and growth on blood agar (Cappuccino and Welsh, 2018; Shaker *et al*., 2018; Jaber *et al*., 2021).

DNA extraction:

The genomic DNA extraction was conducted utilizing the Wizard Genomic isolation kit from Promega, USA, according to the directions provided by the manufacturer. After DNA extraction, the concentration and purity of the isolated DNA were evaluated using a nanodrop spectrophotometer. Bacterial DNA was stored at -20 °C until utilized for PCR experiments.

Molecular and phylogenetic characterization

C. perfringens species-specific PCR was done using primers that target *16S rRNA* gene as described in Table 2 (Wu *et al.*, 2009).

Detection of toxin genes was performed using PCR primers that target the *cpa*, *plc*, *cpb*, *etx*, *iap*, *cpe*, and *netB* genes (Rood *et al*., 2018; Forti *et al*., 2020). The primer sequences and corresponding lengths of the amplicons are shown in Table (2).

Table 2: The genes, primers, and PCR programs used in this study

Gene	Primers	Sequence (5'-3')	size	Amplification(35 cycles)			Ref.
				Denaturation	Annealing	Extension	
16S rRNA	C._perfring16S-F C._perfring16S-R	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279bp	94°C 1min	50°C 1min	72°C 1min	(Wu *et al*,.2009)
cpa	CPA-F CPA-R	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402bp	95°C 20s	56.2°C 20s	72°C 1min	(Forti *et al.*, 2020)
netB	JRP6656 JRP6655	CTTCTAGTGATACCGCTTCAC CGTTATATTCACTTGTTGACGAAAG	738 bp	95°C 20s	51.5°C 20s	72°C 1min	(Rood *et al* .,2018)
etx	JRP4234 JRP4235	CCACTTACTTGTCCTACTAAC GCGGTGATATCCATCTATTC	656 bp	95°C 20s	50°C 20s	72°C 1min	(Rood *et al* .,2018)
iap	JRP5507 JRP5508	GGAAAAGAAAATTATAGTGATTGG CCTGCATAACCTGGAATGGC	461 bp	95°C 20s	50°C 20s	72°C 1min	(Rood *et al* .,2018)
plc	JRP4232 JRP4233	GCTAATGTTACTGCCGTTGACC CCTCTGATACATCGTGTAAG	324 bp	95°C 20s	50°C 20s	72°C 1min	(Rood *et al* .,2018)
cpe	JRP5179 JRP5180	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233 bp	95°C 20s	51.5°C 20s	72°C 1min	(Rood *et al* .,2018)
cpb	JRP5181 JRP5182	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196 bp	95°C 20s	50°C 20s	72°C 1min	(Rood *et al* .,2018)

PCR of all target genes was performed in a total reaction volume of 50 µl, including 25 µl of **GoTaq® Green Master Mix** (Promega Corporation, USA), 3 µl of purified genomic DNA (100-150 ng total), 1.5 µl of each primer (0.3 µM), and 19 µl of sterile distilled water. The amplification programs used included an initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (the cycling steps are listed in Table 2). All the PCR programs included a final extension step of 5 min at 72°C.

The PCR products of the *16S rRNA* amplicons for five samples were sequenced using forward and reverse primers. A neighbor-joining phylogenetic tree for five local *Clostridium perfringens* was constructed using the MEGA X program version 11.0.13 (Tamura *et al*., 2021). The local *C. perfringens* isolates were contrasted to other closely related ones from China, Iraq, Saudi Arabia, France, Poland, Sweden, India, and the United Kingdom.

Gel electrophoresis:
The 1.5% electrophoresis gel was prepared by dissolving 1.5 gm of the agarose granules in 100 ml of TBE buffer. After loading samples and gel electrophoresis, the gel was then transferred to a UV transilluminator for product verification. Images were subsequently captured using a digital camera. The PCR bands were visualized against a DNA marker (100-3000 base pairs, Geneaid - China) and (100-1500 base pairs, Promega - USA).

Statistical analysis
Descriptive statistics were used to summarize the data, presenting the results as frequencies and percentages. The percentage of positive samples was calculated as the ratio of the total sample [positive samples/Total samples] multiplied by 100.

RESULTS

Growth on Chrom agar
Out of the 250 samples from suspected cases tested in this study, 85 (35 from feces and 50 from the intestine) showed positive colonies for *C. perfringens* on Chrom agar and typical Gram-positive rod-shaped bacilli under the microscope (Figures 1 and 2). This represents 23.33 % from fecal samples and 50% from the intestinal samples.

Figure 1: Red-colored colonies on Chromagar *C. perfringens*.

Figure 2: Gram staining demonstrating rod-shaped (bacilli) organisms with a purple coloration.

The molecular diagnosis of *C. perfringens* using the *16S rRNA*:
The positive samples on the plates were identified using conventional specific PCR for the presence of *16S rRNA* for *C. perfringens*. All tested samples (85, 100%) showed a positive *16S rRNA* PCR band of 279 base pairs and were represented by a single band in the corresponding region of the DNA ladder **(Figure 3)**.

***C. perfringens* toxin genes detection:**
The toxinotyping of the positive *C. perfringens* strains demonstrated the presence of toxins (CPA, PLC, ETX, and CPE) among the 85 *C. perfringens* local isolates. The toxins detection combination indicated that Type A (65/85, 76.5 %) and

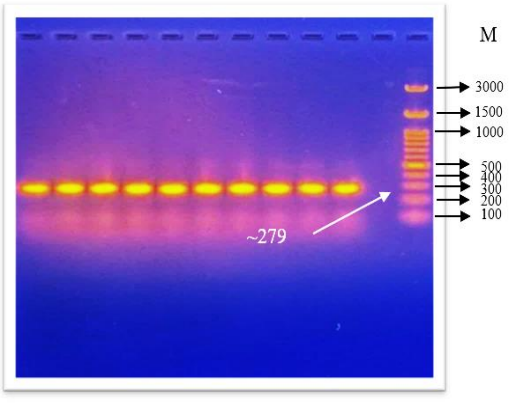

Figure 3: Agarose gel electrophoresis (1.5%) reveals the characteristic amplicon of the 16*S rRNA* gene, which is species-specific for *C. perfringens*, giving a PCR product of 279bp. Line (M) depicts the DNA Marker 100-3000 base pair ladder.

Type D (20/85, 23.53 %) as described in Table (3) were the predominant toxino-types among the local clinical cases. Type A cases comprised 20 lambs and 45 adult sheep, whereas all type D cases were from adult animals.

***cpa* gene detection:**

A total of 85 bacterial isolates were identified using a conventional PCR assay for the presence of the *cpa* with all 85 (100%) isolates showing positive results. The size of the *cpa* band was 402 bp, **(Figure 4).**

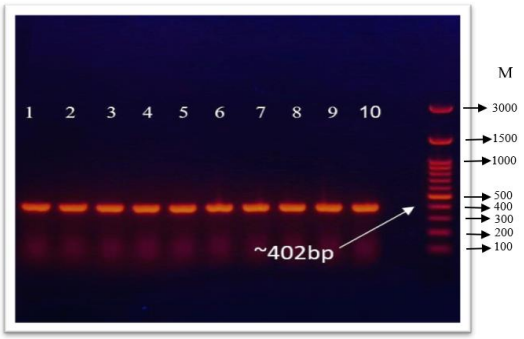

Figure 4: Agarose gel electrophoresis (1.5%) showing the amplicon of the *cpa* gene PCR product of 402bp. Lane (M) shows DNA marker 100-3000 bp ladder.

***plc* gene detection:**

A total of 85 bacterial isolates were identified using a conventional PCR assay for the presence of the *plc* with all 85 (100%) isolates showing positive results. The size of the *plc* band was 324 bp, **(Figure 5).**

***cpe* gene detection:**

Using the standard PCR technique, a total of 20 bacterial isolates representing (23.53 %) were detected by a standard PCR technique for the detection of *cpe*. The *cpe* band measured 233 bp **(Figure 6).**

***etx* gene detection:**

Using the standard PCR technique, a total of 20 bacterial isolates representing (23.53 %) were detected by a standard PCR technique for the detection of *etx* gene. The size of the *etx* band was 656 bp, **(Figure 7).**

Figure 5: Agarose gel electrophoresis (1.5%) showing the amplicon of the *plc* gene PCR product of 324bp. Lane (M) shows DNA marker 100-3000 bp ladder.

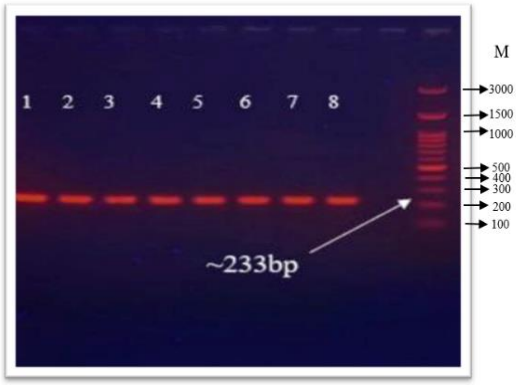

Figure 6: Agarose gel electrophoresis (1.5%) showing the amplicon of the *cpe* gene PCR product for *C. perfringens* of 233bp. Lane (M) shows DNA marker 100-3000 bp ladder.

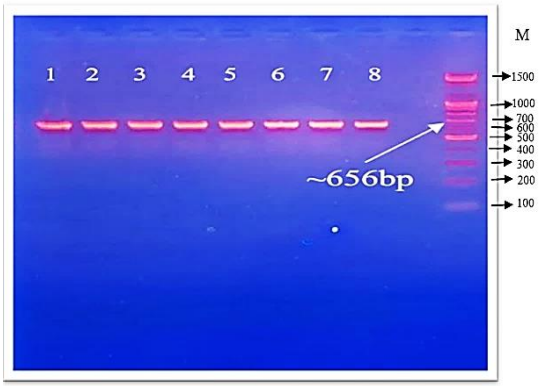

Figure 7: Agarose gel electrophoresis (1.5%) showing the amplicon of the *etx* gene PCR product of 656bp. Line (M) shows DNA marker 100-1500 bp ladder.

Table 3: *C. perfrigens* toxins typing

C. perfrigens Type	Typing Toxins					
	α-toxin (PLC or CPA)	**CPB**	**ETX**	**ITX**	**CPE**	**NetB**
A 76.5 % (65/85)	65	–	–	–	–	–
D 23.53 % (20/85)	20	–	20	–	20	–

Sequencing of PCR products and phylogenetic analysis:

The NCBI BLAST analysis results showed that all PCR reactions were specific to *C. perfringens.* We deposited these sequences in the NCBI database (Table 4). A neighbor-joining phylogenetic tree for five local *Clostridium perfringens* was constructed using the MEGA X program version 11.0.13. The local *C. perfringens* isolates were contrasted with other closely related ones from China, Iraq, Saudi Arabia, France, Poland, Sweden, India, and the United Kingdom **(Figure 8).** The local isolates showed a close relationship and phylogenetic relatedness to other global isolates.

Table 4: Accession number of 16S rRNA gene sequence

List	Accession numbers	Name of isolation Gen Bank	size
1	PQ202458.1	C. perfringens strain AJBC1	279
2	PQ202459.1	C.perfringens strain AJBC2	279
3	PQ202460.1	C.perfringens strain AJBC3	279
4	PQ202461.1	C.perfringens strain AJBC4	279
5	PQ202462.1	C.perfringens strain AJBC5	279

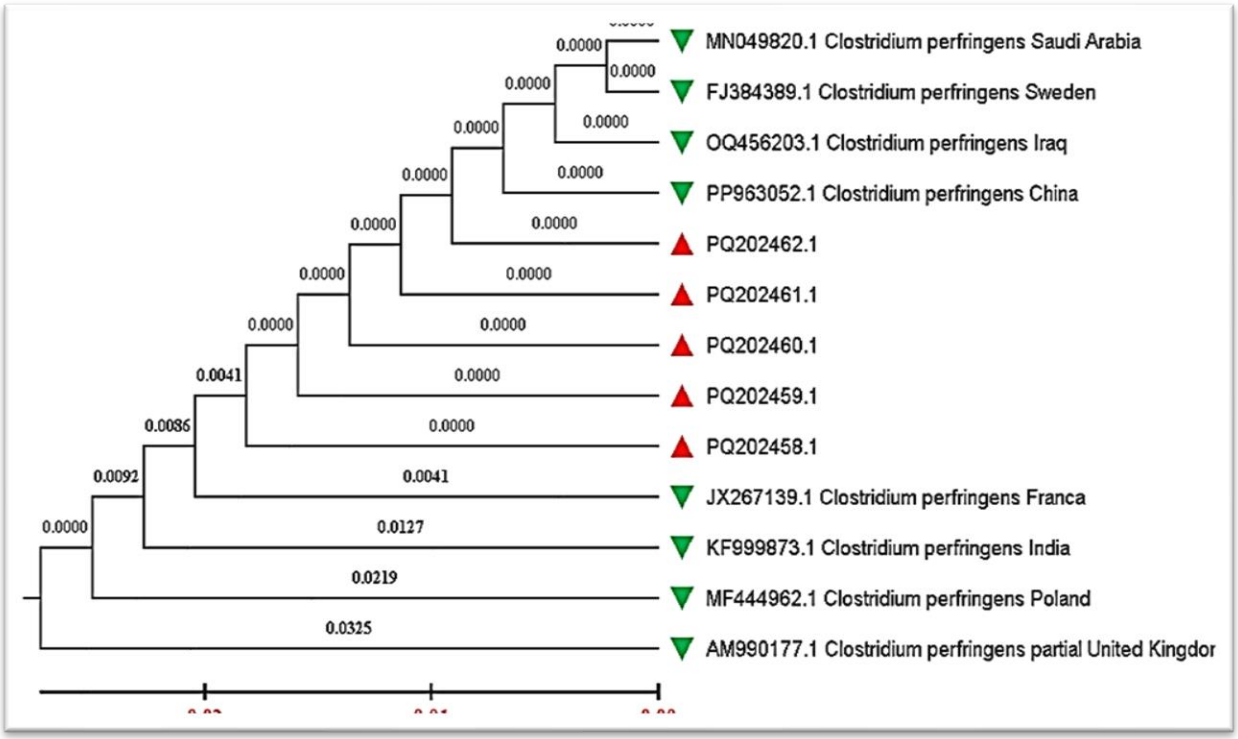

Figure 8: Phylogenetic tree including 16s rRNA of five local *Clostridium perfringens* isolates sequences (Red triangles) and 8 isolates from China, Iraq, Saudi Arabia, France, Poland, Sweden, India and United Kingdom isolates (Green triangles). The neighbor-joining tree was constructed using MEGA X program version 11.0.13.

DISCUSSION

C. perfringens is an important pathogen that causes illness among sheep and other animals. It's known for its ability to form spores and produce a variety of toxins, which can lead to severe diseases in animals and humans (Heikinheimo *et al*., 2006). The classification into groups A - E toxinotypes is based on the production of four major toxins: alpha, beta, epsilon, and iota (Rood *et al*., 2018). These toxins are responsible for different types of infections and diseases, including enterotoxaemia in sheep and goats, which is a major concern in developing countries due to its high fatality rate and economic impact (Fu, Alenezi, & Sun, 2022).

In this study, 85 isolates from 250 suspected cases were identified using CHROMAgar™ and PCR. Cultural and molecular characteristics were used to identify the isolates and their toxins genes. The Chrom agar was very efficient in selecting the positive *C. perfringens* and inhibiting other forms of bacteria. The microscopic examination results were consistent with the typical characteristics of Gram-positive *C. perfringens* bacilli. In the present study, the isolation results were similar to previously reported findings, with a few exceptions (Hustá *et al*., 2020; Mamsin *et al*., 2023). In our study, few isolates showed variation in morphological and microscopic appearance, which we believe is due to genomic variations and mutations in some related genes, which was also noticed in other studies (Lacey *et al*., 2018; Camargo *et al*., 2022). Nevertheless, these variants were positive for Clostridium *perfringens* PCR verification and sequencing. The PCR technique was efficient in the diagnosis and classification of Clostridium *perfringens* isolates, and this notion is consistent with other studies (Mohiuddin *et al*., 2020; Mamsin *et al*., 2023). Therefore, we suggest utilizing this technique as a faster diagnostic method for enterotoxemic cases by detecting the major toxins directly during the infection course in fecal samples. However, this has to be further characterized in wider-scale studies that involve more samples.

Our further molecular investigations revealed that the positive *C. perfringens* strains contain toxins (CPA, PLC, ETX, and CPE). The toxin detection analysis revealed that Type A (65/85, 76.5%) and Type D (20/85, 23.52%) are the major toxinotypes in local clinical cases. Type A cases comprised 20 lambs and 45 adult sheep, whereas all type D cases were from adult animals. None of the other *C. perfringens* types (B, C, E, and F) were found in our study. These results are consistent with a study in Duhok Governorate, where 83% of the isolates from apparently healthy sheep and goats were type A strains and carrying only alpha-toxin encoding genes, while 17% of the isolates were type D strains and carrying both alpha- and epsilon toxin encoding genes (Mamsin *et al*., 2023). Consistent with this, the dominance of type A followed by type D among sheep and goats has been reported among healthy and diseased ovine and caprine populations, in the Punjab region of Pakistan, where the primary isolates were categorized as type A (82%), whereas type D constituted 18% (Mohiuddin *et al*., 2020). Genotyping of 52 strains from infected sheep in Turkey indicated that 33 (64%) were classified as type A, 11 (21%) as type D, and 8 (15%) as type C (Kalender *et al*., 2005).

Yellow lamb disease, an infrequent disease in young sheep, has been associated with type A *C. perfringens*. The condition is marked by hemolysis and is believed to result from alpha toxin-producing *C. perfringens* type A (Uzal *et al*., 2022). This may elucidate the findings in our study about type A in young sheep, which included 20 lambs and 45 adult sheep.

Type D isolates generate alpha and epsilon (ETX) toxins. A study suggested ETX is essential for type D isolates to cause illness,

underscoring the toxin's critical function in the pathogenesis of type D disease (Garcia *et al*., 2013). The epsilon toxin generated by *C. perfringens* Type D results in vasogenic edema that can impact different organs and possibly cause fatal consequences, for animals like sheep and goats (Finnie and Uzal, 2024). *Clostridium perfringens* type D causes infection in sheep, goats, and several other ruminants (Ilyas *et al.*, 2024).

The phylogenetic analysis revealed a clustering of *C. perfringens* compared to isolates from other countries. The fact that they differ in their toxinotyping, yet share close ancestral relationships, suggests a complex genomic variation among local isolates. Studies have proposed that the prevalence of *C. perfringens* toxinotypes may vary geographically and may be influenced by animal species in such areas. A study in dairy farms has revealed a diversity of *C. perfringens* toxin-genotypes and suggested that cattle strains may specifically affect cattle compared to other animals (Fohler *et al*., 2016; Camargo *et al*., 2022). In our study, we only focused on sheep, and therefore a wider investigation may help understand the relation of toxinotypes among different animal species.

There are limited studies about *C. perfringens* toxinotypes in the south of Iraq, including the Basrah governorate, highlighting the need for in-depth epidemiological studies that include a wide range of animal hosts and humans.

CONCLUSION

The present investigation determined the distribution of *C. perfringens* toxin types in sheep populations related to enterotoxemia cases. *C. perfringens* type A and type D are the most common toxinotypes responsible for enterotoxemia in sheep in Basrah governorate. The prevalence of the alpha toxin gene (*C. perfringens* type A) and the occurrence of the Type D strain (notably in adult animals) align with findings from different countries, indicating the necessity to integrate these strains into the existing vaccine for *C. perfringens*. This study is the first study to categorize toxin genes in the feces of endotoxemic sheep cases in Basrah.

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سيطرة النوعين A وD من سموم *Clostridium perfringens* في حالات التسمم المعوي بين أغنام محافظة البصرة

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تُعتبر بكتريا *Clostridium perfringens* من البكتيريا إيجابية الجرام اللاهوائية، وهي مرتبطة بمختلف الأمراض لدى البشر والحيوانات، وخصوصًا التهاب الأمعاء النخري في الأغنام والماعز. يُصنِّف الباحثون العزلات البكتيرية لهذه الأنواع بشكل منهجي إلى سبعة أنواع سمومية (A-G) اعتمادًا على وجود أو غياب سموم معينة . في هذه الدراسة, أجريت باستخدام الزرع الميكروبي، والفحص المجهري، والبادئات الجينية المحددة، تم تحديد السموم الرئيسية والثانوية، ومصادر عزل البكتيريا، وعلاقتها السريرية بالتهاب الأمعاء النخري في الأغنام. بين سبتمبر ٢٠٢٣ ويونيو ٢٠٢٤، تم عزل ٨٥ عزلة من بكتيريا C. perfringens من ٢٥٠ عينة أُخذت من الأغنام . أظهرت نتائج تفاعل البوليميراز المتسلسل (PCR) وجود جيني *cpa** و*plc** في جميع العزلات. كما تم الكشف عن جيني *cpe** و*etx** في ٢٠ عزلة بنسبة ٢٣,٥٣٪. وبالنسبة لتصنيف الأنواع السمومية، كانت النسبة الأعلى من العزلات تنتمي إلى النوع A بنسبة ٧٦,٥٪ (٦٥ عزلة من أصل ٨٥)، في حين شكّل النوع D نسبة ٢٣,٥٣٪ (٢٠ عزلة). وقد شملت حالات النوع A إصابة ٢٠ حملًا و٤٥ شاة بالغة، بينما اقتصرت حالات النوع D على الأغنام البالغة. عند تحليل تسلسلات الجينات16S rRNA، أظهرت العزلات درجات متفاوتة من التشابه مع تسلسلات مسجلة لبكتيريا *C. perfringens* في بنك الجينات، سواء من العراق أو دول أخرى. ومع ذلك، تجمعت العزلات المحلية في مجموعة واحدة، مما يشير إلى وجود صلة وراثية قوية بينها .
تخلص نتائج الدراسة إلى ضرورة دمج الأنواع المنتجة للسموم للنوعين A و Dضمن برامج تطعيم الأغنام. كما توصي بإجراء دراسات وبائية واسعة النطاق حول الأنواع السمومية للبكتيريا وتأثيرها على البشر والحيوانات لتحسين استراتيجيات الوقاية والعلاج.