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Typing of *Clostridium perfringens* Isolated From Small Ruminants with Enterotoxemia by Serological and Molecular Tests

Afnan Adel Elnabawy[1], Ahmed Orabi Hassan[1], Eman Fathy Ahmed[2], Eman M. Elghazaly[3] and Jakeen Kamal El Jakee[1]

[1]*Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.*

[2]*Anaerobic unit, department of bacteriology, Animal Health Research Institute, Dokki, Giza, Egypt.*

[3] *Department of Microbiology, Faculty of Veterinary Medicine, Matrouh University, Matrouh, Egypt.*

Abstract

ENTEROTOXEMIA that is caused by *Clostridium perfringens* is one of the most important diseases in small ruminants. This study is aimed to reveal the prevalence, toxinotyping, and antibiotic resistance profiles of *C. perfringens* in the localities under study as Matrouh Governorate. A total of 150 internal samples (intestine and internal: liver, kidney, heart, and lung) were collected from the suddenly dead sheep and goats and investigated for the presence of *C. perfringens*. Out of 90 sheep and 60 goats, *C. perfringens* was isolated from 59 sheep (65.6%) and 40 goats (66.7%), respectively. According to PCR analysis, the most prevalent type was *C. perfringens* type A, which was represented 70.8% of the total toxigenic isolate types, while *C. perfringens* types B and D were represented 12.5% and 16.7%, respectively, but no *C. perfringens* type C or E was detected. By using ELISA, the prevalence of *C. perfringens* types A, B, and D was 62.5%, 8.3%, and 8.3%, respectively. The antimicrobial sensitivity test reveals that all isolates showed 100% resistance to carbenicillin and aztreonam, 83.3% and 66.7% to kanamycin and erythromycin, respectively. On the other hand, 66.7% of the isolates were showed sensitivity to cefadroxil, lomefloxacin, and oxytetracycline, 50% to cephradin and epicoflocin, and only 33.3% to erythromycin. It was concluded that the mortality rate due to *C. perfringens* type A was significantly higher than that of *C. perfringens* types B and D. PCR and ELISA are the most effective methods for toxinotyping *C. perfringens*.

Keywords: Antibiotic sensitivity test, ELISA, Goat, Sheep, PCR.

Introduction

Clostridium is a normal inhabitant of the gastrointestinal tract of animals; it is usually harmless unless it is given the chance to multiply due to dietary changes or stress, which can lead to the production of toxins [1]. *C. perfringens* is classified into 5 toxin types (A, B, C, D, and E) according to the production of 4 major toxins, namely alpha, beta, epsilon and iota. The Multiplex PCR is the best method for identification of *C. perfringens* toxins. Both humans and animals are susceptible to a number of enteric clostridial infections [2]. *Clostridium* species were isolated from fecal samples of diarrheal and apparently healthy foals with percentages of 30% and 7%, respectively [3].

In Matrouh Governorate, the small ruminants, including sheep and goats, are usually kept for milk and meat purposes. Small ruminants, especially sheep and goats, are a common source of mutton, wool, milk, and leather and are important to the nation's economy and farmers' livelihoods. *Clostridium perfringens* produces a disease in goats, sheep, and other animal species known as enterotoxemia [4].

C. perfringens is associated with different enteric and systemic diseases inanimals and humans, such as food poisoning, gas gangrene, enterocolitis, and non-foodborne diarrhea [5]. Worldwide, *C. perfringens* is a common cause of death for domestic animals [6]. Enterotoxaemia, primarily in small ruminants, is a severe and peracute disease caused by *C. perfringens*

*Corresponding authors: Eman M. Elghazaly, E-mail: emanmoneer1@gmail.com Tel.: 01097003383
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that results in high mortality and significanteconomic losses [7].

The main objectives of this research are to study the prevalence of *C. perfringens* in suddenly dead sheep and goats in the localities under study, such as Matrouh Governorate, determine the types of its toxins by PCR, ELISA, and dermonecrotic test, and study its antibiotic resistance profile.

Materials and Methods

Animals and sampling

A total of 90 sheep and 60 goats, two weeks to two years of age, were included in this study. The animals belonged to forty distinct herds, ranging in size from 150 to 1000 animals, from different regions of the Matrouh Governorate. Samples were taken within six hours of the animals' deaths following necropsy. A total of 150 internal samples (intestine and internal: liver, kidney, heart, and lung) were collected from the suddenly dead sheep and goats and investigated for the presence of *C. perfringens*.

Isolation and Identification of C. perfringens:

According to Smith and Holdman [8, 9], the collected samples were placed into tubes containing freshly prepared Robertson's cooked meat medium, boiled and cooled (Oxoid), and incubated anaerobically at 37°C for 24 hours. A loopful of inoculated fluid medium was streaked onto sheep blood agar plates supplemented with neomycin sulfate [10, 11]. The streaked plates were incubated anaerobically for 48 hours at 37 °C using a Gaspak anaerobic jar [12]. The catalase-negative colonies were picked up and subjected to morphological, cultural, and biochemical analyses, according to Koneman et al. [13]. Two plates of sheep blood agar and egg yolk agar were used to cultivate suspected *C. perfringens* colonies. One plate from each medium was incubated anaerobically, and the other plate was incubated aerobically. The colonies that produced lecithinase, grew only in anaerobic conditions, and displayed a double zone of hemolysis on blood agar were selected and purified for identification tests [10, 14].

Typing of C. perfringens toxins by dermonecrotic reaction

Typing of *C. perfringens* isolates was characterized by dermonecrotic reactions in Albino Guinea pigs, which performed according to Stern and Batty [15] and Nagler's test [8].

Antimicrobial sensitivity test of the isolates

The isolates were tested for susceptibility to 9 different antimicrobial agents (oxytetracycline (OT 30), cephradine (CRD 30), cefadroxil (CDX 30), epicoflocin (OFX 5), lomefloxacin (Lom 10), erythromycin (E 15), amikacin (AK 30), carbenicillin (CB100), aztreonam (ATM 30), and kanamycin (K 30)) according to Finegold and Marten [16] using the disk diffusion method on Mueller-Hinton agar (Merck, Darmstadt, Germany). After inoculating the agar plates with *C. perfringens* cultures, an antimicrobial paper disk was applied. After that, the plates were incubated for 24 hours at 37 °C in an anaerobic environment, and by using a digital caliper, the diameters of the zones of inhibition surrounding the disk were measured. The result was interpreted according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [17]. The antimicrobial drugs utilized in the antimicrobial susceptibility testing were chosen based on their application in veterinary medicine and the resistance profiles of *C. perfringens* isolates, as mentioned in previous investigations [18] and [19].

Multiplex PCR to detect toxinogenic C. perfringens:

Template DNAs for PCR were prepared from 24 isolates (sheep n = 12 and goat n = 12) according to QIAamp DNA mini kit instructions. As indicated in Table 1, specific oligonucleotide primers were chosen based on known sequences [20] for the toxin genes alpha (α), beta (β), and epsilon (ε) of *C. perfringens* toxins. The PCR result was examined in accordance with Sambrook *et al.* [21] using ethidium bromide in 1.5% agarose gel electrophoresis and a DNA molecular weight marker of a 100-1000 base pair ladder (Bioron GmbH) (Jena Bioscience, Germany).

Evaluation of C. perfringens toxins by ELISA

C. perfringens isolates were grown in cooked meat broth medium and incubated anaerobically for 48 hours at 37 °C. For the production of toxins, the culture was inoculated into a 250-ml flask of toxin production media that contained a 1-2% glucose solution (60%) [22]. The extracted *C. perfringens* toxin was diluted 1/50, 1/100, 1/200, 1/400, 1/800, and 1/1600 in coated buffer on the basis of commercial sandwich ELISA kit (Bio-Rad, Thermo Fisher) According to manufacturer's instructions. In an ELISA plate, 100 µl of each diluted antigen was applied to each well. ELISA testing was perfo|rmed. A positive ELISA reading is one that is at least twice as high as the ELISA reading of the saline negative control. The result was contrasted with the toxin extracted from the standard strain of *C. perfringens* type A (positive control).

Results

Prevalence of C. perfringens among the collected samples

Necropsy revealed the presence of pericardial and peritoneal bloody effusions; enteritis ranged between catarrhal and hemorrhagic in the small and large intestine; a pale, friable liver; and congested,

oedematous lungs. Ecchymotic hemorrhages were found on the myocardium and kidney cortex.

Out of 90 sheep and 60 goats, *C. perfringens* was isolated in pure culture from 59 sheep (65.6%) and 40 goats (66.7%), respectively, as shown in Table 2. The 24 *C. perfringens* strains produced the α-toxin and were positive for Nagler's and dermonecrotic reactions. *C. perfringens* was found in sheep aged 2 weeks–3 months and 3 months–8 months with percentages of 64.3% (18/ 28) and 65.8% (25/ 38), respectively. In goats aged one month–6 months and 6 months–2 years, it was found with percentages of 65.7% (23/ 35) and 68.0% (17/ 25), respectively (Table 2).

Multiplex PCR toxin genotyping

The most prevalent type of *C. perfringens* was *C. perfringens* type A, which represented 70.8% of the total toxigenic isolate types, while *C. perfringens* types B and D represented 12.5% and 16.7%, respectively, but no *C. perfringens* type C or E was detected by PCR (Table 3 and Fig. 1).

ELISA

C. perfringens types A, B, and D were detected by ELISA in percentages of 62.5%, 8.3%, and 8.3%, respectively, in both sheep and goats (Table 4).

Antimicrobial sensitivity test

The antimicrobial sensitivity test among 24 identified *C. perfringens* isolates by PCR and ELISA revealed that all isolates (100%) showed resistance to carbenicillin and aztreonam, 83.3% and 66.7% of isolates showed resistance to kanamycin and erythromycin, respectively, 50% showed resistance to amikacin, cephradin, and epicoflocin, but only 33.3% showed resistance to cephadroxil, lomefloxacin, and oxytetracycline. Only 16.7% of isolates showed intermediate sensitivity to kanamycin. On the other hand, 66.7 percent of isolates showed sensitivity to cefadroxil, lomefloxacin, and oxytetracycline; 50% showed sensitivity to cephradin and epicoflocin; and only 33.3% showed sensitivity to erythromycin, as shown in Table 5.

Discussion

Clostridium is the primary cause of mortality in a variety of animals, including sheep and goats. Because it can create a wide range of toxins, *Clostridium perfringens* is one of the most prevalent and significant infections in livestock [23]. Worldwide occurrences of enterotoxemia are linked to *Clostridium perfringens*.

The abomasum of the tested lambs and goat kids was found to be significantly loaded with gas and caseated fluid during necropsy, and there were various degrees of hemorrhagic lesions in the abomasum mucosa [24].

The most well-recognized standard for making a conclusive diagnosis of enterotoxemia is the presence of *C. perfringens* toxins in the intestinal contents [4]. In the present study, out of 90 sheep and 60 goats, *C. perfringens* was isolated in pure culture from 59 sheep (65.6%) and 40 goats (66.7%). Enterotoxemia caused 87.58, 75.81, and 76.11% of the morbidity, mortality, and case fatality in Makhi Cheeni, Beetal, and Teddy goats, respectively [7]. A lower prevalence (51.5%) was recorded by Moustafa et al. [23].

C. perfringens was found in sheep aged 2 weeks–3 months and 3 months–8 months with percentages of 64.3% and 65.8%, respectively, while in goats aged one month–6 months and 6 months–2 years, it was found with percentages of 65.7% and 68.0%, respectively. In the Hussain et al. [7] study, there was no discernible variation in morbidity, mortality, or case fatality depending on age or sex. According to published reports, the disease is common in sheep and goats, with acute cases occurring in animals between the ages of 3 and 10 weeks [25]. However, acute and chronic enterotoxemia can strike animals at any age [26].

On the basis of the production of four major toxins (epsilon, alpha, beta, and iota), Clostridium perfringens is classified into seven toxinotypes (A, B, C, D, E, F, and G). Using the dermonecrotic test and PCR, El Jakee et al. [27] classified toxigenic isolates; 107 (68.2%) of the 157 isolates produced toxins. In the current work, multiplex PCR was performed to identify toxin types using certain primers. The sensitivity of PCR for the recognition of *C. perfringens* types was compared with the traditional culture method among fecal samples contaminated with *C. perfringens* types [28]. Moreover, the serological typing of 24 *C. perfringens* isolates was investigated using ELISA kits.

Three serotypes of *C. perfringens* were identified from lamb: *C. perfringens* type A (54.2%), *C. perfringens* type B (28.8%), and *C. perfringens* type D (16.9%) [23].

Although there is speculation that the entero-pathogenicity of *C. perfringens* type A may be linked to high levels of α-toxin expression, the exact involvement of alpha-toxin and *C. perfringens* type A in enteric illnesses remains unclear [29]. The α-toxin was produced by the 24 *C. perfringens* isolates found in our study, as demonstrated by the double zone of hemolysis in blood agar and the evaluation of lecithinase activity on agar. According to the current study's findings, enterotoxemia in very small ruminants is primarily caused by *C. perfringens* type A (70.8%).

Using PCR, 66.7% and 75.0% of sheep and goat isolates, respectively, produce toxin type A. Using ELISA, 66.7% and 58.3% of sheep and goat isolates,

respectively, produce toxin type A. In humans, *C. perfringens* type A causes gas gangrene, food poisoning, and diarrhea; in many domestic and wild animals, it causes enterotoxemia and hemorrhagic gastroenteritis [29]. *C. perfringens* type A was the most commonly found species (86.84%) either alone or in combination with other Clostridium species, according to the analysis of swab samples by culture and PCR. Three of the *C. perfringens* type A positive sample also included the beta2 toxin gene (cpb2) [24]. Enteric diseases in numerous animal mammalian species are often linked to *C. perfringens* type A strains that encode the alpha toxin [2].

Necro-hemorrhagic enteritis in sheep is primarily caused by *C. perfringens* type B, which encodes the toxins CPA, beta (CPB), and epsilon (ETX). It has been proposed recently that these strains may also be linked to multiple sclerosis in humans [2].

In the present study, *C. perfringens* type B was detected at 12.5% and 8.3% using PCR and ELISA, respectively. 16.7% from sheep and 8.3% from goats by PCR, but 8.3% from both by ELISA. This is in stark contrast to the importance of *C. perfringens* type B as the primary cause of dysentery in lambs under two weeks of age in the UK, South Africa, and Greece [30; Bueschel et al., 2003], where type B infection is widespread.Sudden death or prolonged diarrhea in sheep and goats can be attributed to exotoxemia, which is caused by *C. perfringens* type D [4].

Using PCR, *C. perfringens* type D was detected in sheep and goats (16.7% each). While it was detected in goats only (16.7%) using ELISA. It has previously been documented that *C. perfringens* type D isolation is uncommon [30; 31]. Sheep and goats younger than two weeks' old are typically affected by enterotoxemia caused by Clostridium perfringens type D [32]. Because colostrum has a trypsin-inhibitory effect on intestinal content and lower trypsin activity, it was thought that younger animals are not impacted by type D enterotoxemia [32]. Meanwhile, Hussain et al. [7] recorded that deaths from *C. perfringens* type D (68.10%) were substantially greater than those from

C. perfringens type A (34. 90%). In line with earlier findings [29], no *C. perfringens* type C or type E was found during the current examination, indicating that *C. perfringens* type E is uncommon in lambs and young.

Over the years, several antimicrobial drugs, including metronidazole, ampicillin, bacitracin, lincomycin, tetracycline, chloramphenicol, and imipenem, have been utilized in the prophylaxis and treatment of infections caused by *C. perfringens* [18; 33]. According to numerous studies, there has been a noticeable rise in antimicrobial resistance in recent years to erythromycin, tetracycline, lincomycin, or chloramphenicol [34]. Clostridium difficile is a major cause of antibiotic-associated diarrhea, followed by Clostridium perfringens [35].

In the present study, the antimicrobial sensitivity test among 24 identified *C. perfringens* isolates by PCR and ELISA revealed that all isolates (100%) showed resistance to carbenicillin and aztreonam, 83.3% and 66.7% of isolates showed resistance to kanamycin and erythromycin, respectively, 50% showed resistance to amikacin, cephradin, and epicoflocin, but only 33.3% showed resistance to cephadroxil, lomefloxacin, and oxytetracycline. Only 16.7% of isolates showed intermediate sensitivity to kanamycin. On the other hand, 66.7% of isolates showed sensitivity to cefadroxil, lomefloxacin, and oxytetracycline; 50% showed sensitivity to cephradin and epicoflocin; and only 33.3% showed sensitivity to erythromycin. Beres et al.'s findings

Also,[36] showed that while resistance to tetracycline (71.4%), penicillin (64.2%), erythromycin (42.8%), and enrofloxacin (35.7%) was also noted, all recovered *C. perfringens* isolates are sensitive to vancomycin, rifampicin, and lincomycin. The increasing antibiotic resistance of *C. perfringens* strains is a serious problem [5, 37].

Conclusion

The results of this investigation verify the existence of enterotoxmia among sheep and goats in the localities under study as Matrouh Govenorate, producing financial losses. The mortality rate due to *C. perfringens* type A was significantly higher when compared with *C. perfringens* types B and D. The best techniques for toxinotyping *C. perfringens* are PCR and ELISA. Utilizing the proper immunization schedule is strongly advised, particularly for protection against *C. perfringens* types A, B, and D.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Funding statement

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Ethical of approval

All experiments were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects and/or their legal guardian(s). This study protocol was approved by the Institutional Animal Care and Use Committee, Vet. CU. IACUC, Cairo University, Egypt (Vet CU 18042024909).

Author's contribution

All authors contributed equally according to their tasks and approved the final manuscript.

TABLE 1. Target genes, Primer sequences, amplicon sizes, cycling conditions of *C. perfringens* toxin genes.

Target genes	primer Sequence	Amplicon size (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
Alpha toxin	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402 bp	94˚C 5 min.	94˚C 45 sec	50 ˚C	72 ˚C	72 ˚C	Yoo *et al.* (1997) [20]
Beta toxin	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236 bp			45 sec	45 sec	10 min	
Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541 bp						
IOTA toxin	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	317 bp						

TABLE 2. Prevalence of *C. perfringens* among the examined samples

Source of the isolates	Age	The examined number	Positive number	%
Sheep	**2 weeks - 3 months**	14	9	64.3%
	3 months - 8 months	76	50	65.8%
Total sheep		**90**	**59**	**65.6%**
Goat	**Month - 6 months**	35	23	65.7%
	6 months - 2 years	25	17	68.0%
Total goat		**60**	**40**	**66.7%**
Total all		**150**	**99**	**66%**

TABLE 3. Typing of *C. perfringens* toxins using PCR

Source of the isolates	The examined number	Toxigenic isolate types									
		A		B		C		D		E	
		No.	%	No.	%	No.	%	No.	%	No.	%
Sheep	**12**	**8**	**66.7**	**2**	**16.7**	**-**	**-**	**2**	**16.7**	**-**	**-**
Goat	**12**	**9**	**75.0**	**1**	**8.3**	**-**	**-**	**2**	**16.7**	**-**	**-**
Total	**24**	**17**	**70.8**	**3**	**12.5**	**-**	**-**	**4**	**16.7**		

%: according to the number of examined samples.

TABLE 4. Typing of *C. perfringens* toxins using ELISA

Samples	The examined number	ELISA							
		A		B		D		Non toxigenic	
		No.	%	No.	%	No.	%	No.	%
Sheep	**12**	8	66.7	**1**	8.3	**-**	**-**	**3**	**25**
Goat	**12**	7	58.3	1	8.3	**2**	16.7	2	16.7
Total	**24**	**15**	62.5	**2**	8.3	**2**	8.3	5	20.8

%: according to the number of examined samples.

TABLE 5. Antibacterial sensitivity against *C. perfringens* isolates

Antimicrobial agents	Total NO. of tested isolates	Sensitive		Intermediate		Resistant	
		n=	%	n=	%	n=	%
Amikacin (AK 30)	24	-	-	12	50	12	50
Aztreonam (ATM 30)		-	-	-	-	24	100
Carbenicillin (CB100)		-	-	-	-	24	100
Cefadroxil (CDX 30)		16	66.7	-	-	8	33.3
Cephradine (CRD 30)		12	50	-	-	12	50
Epicoflocin (OFX 5)		12	50	-	-	12	50
Erythromycin (E 15)		8	33.3	-	-	16	66.7
Kanamycin (K 30)		-	-	4	16.7	20	83.3
Lomefloxacin (lom 10)		16	66.7	-	-	8	33.3
Oxytetracycline (OT 30)		16	66.7	-	-	8	33.3

%: according to total No. of tested isolates.

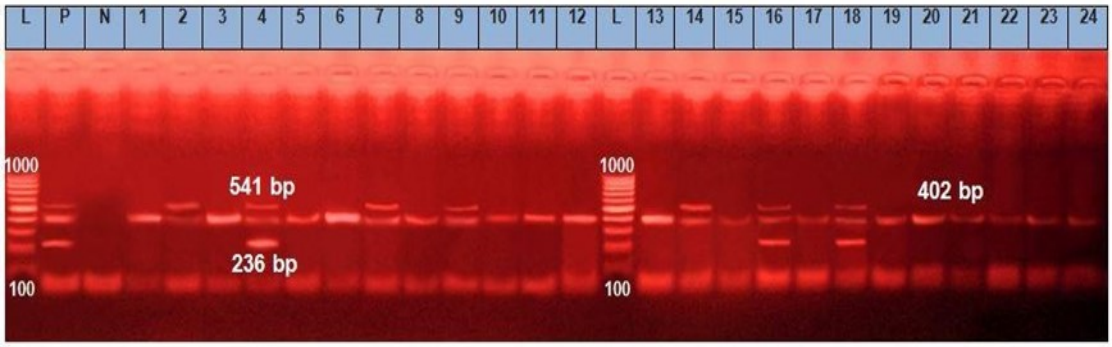

Fig. 1. Amplified PCR products of *C. perfringens* isolates toxins by using the specific primers
Lane L: 100 bp DNA marker (Fermentas, Germany), Lane P: positive control and Lane N: negative control, Lanes 1, 3, 5, 6, 8, 10, 11, 12, 13, 15, 17, 19, 20. 21, 22, 23, and 24: *C. perfringens* type A, Lanes 2, 7, 9 and 14: *C. perfringens* type D, Lanes 4, 16 and 18: *C. perfringens* type B

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دراسات على تسمم الدم المعوي الكلوستريديوم بيرفرينجنز في المجترات الصغيرة بمحافظة مطروح

افنان عادل النبوي[1]، احمد عرابي حسن[1]، ايمان فتحي احمد[2*]، ايمان منير الغزالي[3] و جكين كمال الجاكي[1]

[1] قسم الميكروبيولوجيا، كلية الطب البيطري، جامعة القاهرة، القاهرة، مصر.

[2] وحدة اللاهوائيات، قسم البكتريولوجيا، معهد بحوث صحة الحيوان، الدقي، الجيزة، مصر.

[3] قسم الميكروبيولوجيا، كلية الطب البيطري، جامعة مطروح، مطروح، مصر.

الملخص

يعد التسمم المعوي الناجم عن ا لكلوستريديوم بيرفرينجنز من أهم الأمراض التي تصيب المجترات الصغيرة. هدفت هذه الدراسة إلى دراسة مدى انتشار بكتيريا كلوستريديوم بيرفرينجنز ونمط سميتها ومقاومتها للمضادات الحيوية في المحليات محل الدراسة مثل محافظة مطروح.من بين 90 خروفًا و60 ماعزًا، تم عزل كلوستريديوم بيرفرينجنز في مزرعة نقية من 59 خروفًا (65.6%) و40 ماعزًا (66.7%) على التوالي. وفقًا لتفاعل البلمرة المتسلسل، كان النوع الأكثر انتشارًا هو type A *C. perfringens*، والذي يمثل 70.8% من إجمالي أنواع العزلات المسببة للسموم، في حين يمثل النوع D, 12.5% Bو16.7% على التوالي، ولكن لا يوجد النوع C and E. باستخدام الاليزا، كان معدل انتشار الكلوستريديوم بيرفرينجنز بأنواعها A وB وD 62.5% و8.3% و8.3% على التوالي. أظهر اختبار الحساسية للمضادات الميكروبية أن جميع العزلات أظهرت مقاومة بنسبة 100% للكاربنيسيلين والأزتريونام، و83.3% و66.7% للكانامايسين والإريثرومايسين، على التوالي.من ناحية أخرى، أظهرت 66.7% من العزلات حساسية للسيفادروكسيل، اللوميفلوكساسين، والأوكسيتتراسيكلين، 50% للسفرادين والإبيكوفلوسين، و33.3% فقط للاريثروميسين. تم التوصل إلى أن معدل الوفيات بسبب الكلوستريديوم بيرفرينجنزمن النوع A كان أعلى بكثير من معدل الوفيات الناجمة عن الكلوستريديوم بيرفرينجنز من النوع B وD. يعد تفاعل البوليميراز المتسلسل والاليزا أكثر الطرق فاعليةللتعرف على الكلوستريديوم بيرفرينجنز

الكلمات الدالة: اختبار الحساسية للمضادات الحيوية،الاليزا، ماعز، اغنام، تفاعل البلمرة المتسلسل.