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EPIDEMIOLOGICAL STUDIES AND MOLECULAR CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* IN SMALL RUMENIANT AT El-BEHERA GOVERNORATE, EGYPT

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Received: 31 December 2017; **Accepted:** 21 January 2018

ABSTRACT

Epidemiological factors associated with clostridium infection among sheep and goats. Through the collection of samples from suspected cases of sheep and goat, anaerobic isolation and identification of *C. perfringens* by using classical methods and application of multiplex PCR for molecular characterization of isolates. A total of 104 samples were collected from intestine, liver, kidney and spleen from sheep and goat either those showing signs of enterotoxaemia or suddenly dead from different farms and small holders at El- Behera Governorate for anaerobic bacteriological examination. Our study concluded that, the *C. perfringens* causes high mortality in sheep and goat as a result secretion of Alpha, Beta and Epislon toxins. Molecular characterization of *C. perfringens* by Multiplex PCR characterize *C. perfringenstype"A*" (alpha toxin) which gave a characteristic band at 402 bp.

Key words*: C.perfringens*, Enterotoxaemia, Epidemiology, Multiplex PCR, Sheep, Goats.

INTRODUCTION

Heterogeneous group of environmental bacteria can be found as pathogens in animals (Liu, 2011) one from this bacteria is the *C. perfringens* is a Gram-positive spore-forming anaerobic bacterium present in the intestinal flora of animals as well as in soil and water, where its presence might be indicative of fecal contamination (Jemal *et al.*,2016).

C. perfringens is classified into 5 toxin types (A, B, C, D, and E) according to the production of 4 toxins, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). Several other toxins (e.g. enterotoxin (CPE), beta 2 (CPB2) and perfringolysin O (PFO) can also be produced by some strains of all types of *C. perfringens*, but they are not currently used in the classification of this microorganism (Tabaran, 2017).

Alpha toxin is commonly produced by all five types and is a phospholipase C that can hydrolyze lecithin into phosphorylcholine and diglyceride and is believed to be a major factor responsible for the organism's tissue pathology (Rodriguez *et al.*, 2016).

C. perfringens is an anaerobic bacterium that produces several toxins. Of these, the alpha, beta, and epsilon toxins are responsible for causing the most severe *C. perfringens*-related diseases in farm animals (Moreira *et al.*, 2016).

The Multiplex PCR is the best method for identification of *C. perfringens* with determination of its molecular structure (Ashgan *et al.*, 2013) used a simple mPCR procedure to identify four toxitypes of *C. perfringens* collected from different origins by using genes of alpha, beta, epsilon and iota toxins. Also, Eman *et al.* (2013) performed PCR for molecular typing of *C. perfringens* was by using three sets of primers specific for toxin -producing genes of *C. perfringens* alpha (900bp), beta (236bp) and epsilon (541bp). Dean *et al.* (2011) concluded that, the PCR results is more accurate when used tissue samples than colony samples.

This study was carried to showepidemiological factors associated with clostridium infection and molecular characterization of *C. perfringens* in sheep.

MATERIALS AND METHODS

A total of 104 samples from intestine, liver, kidney and spleen were collected from sheep and goat either those showing signs of enterotoxaemia or recent suddenly dead. All samples were obtained from different farms and small holders at El-Behera Governorate. The samples were labeled and placed in sterile plastic bags and transported in ice box as soon as possible for anaerobic bacteriological examination.

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Table 1: Type and number of examined samples.

Animal species	Number of examined samples	Type of samples\ organs
Sheep	**83**	**Intestine , liver ,kidney and spleen**
Goat	**21**	**Intestine , liver ,kidney and spleen**
Total	**104**	

2. Enrichment and isolation of C. *perfringens* according to (Smith and Holdman, 1968). The samples were inoculated into tubes of freshly prepared and previously boiled cooked meat medium.

3. Identification of isolated bacteria according to (Koneman *et al.,* 1992), all suspected isolates that obtained were identified as follows:

a. Colonial appearance according to (Vaikosen and Muller, 2001).

b. Microscopicalappearance

c. Biochemical identification:
Suspected purified isolates were identified according to the schemes of (Koneman *et al.,* 1992) depending on the following tests catalase test, gelatin hydrolysis test, fermentation of sugars (glucose, lactose, sucrose, galactose, manitol, maltose xylose and mannose), indole test, H_2S production and lecithinase activity.

d. Extraction of DNA according to QIAamp DNA mini kit instructions.

e. Preparation of multiplex PCR Master mix for Alpha, beta, epsilon, Iota: **(Table2)**

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	**25*µl***
PCR grade water	**11 *µl***
Forward primer (20 pmol)	**1 *µl* each**
Reverse primer (20 pmol)	**1 *µl* each**
Template DNA	**8 *µl***
Total	**50 *µl***

Oligonucleotide primers used for conventional:
Source: Midland Certified Reagent Company_ oilgos (USA): **Table3**

Toxin	Primer	Sequence	Amplified product	Reference
Alpha toxin	*F*	GTTGATAGCGCAGGA CATGTTAAG	402bp	**YOO *et al*., 1997**
	R	CATGTAGTCATCTGTT CCAGCATC		
Beta toxin	*F*	ACTATACAGACAGAT CATTCAACC	236 bp	
	R	TTAGGAGCAGTTAGA ACTACAGAC		
Epsilon toxin	*F*	ACTGCAACTACTACT CATACTGTG	541 bp	
	R	CTGGTGCCTTAATAG AAAGACTCC		

f. Cycling conditions of the primers during cPCR.
g. Agarose gel electrophoreses (Sambrook *et al.,* 1989) with modification.

RESULTS

All isolates had abundant growth observed in cooked meat broth with significant gas formation, while the meat particles were pinkish and not digested with a sour odor. On 10% sheep blood agar medium: circular, convex, semi- translucent, smooth colonies with an entire edge were observed. Most of the isolates showed characteristic double zone of hemolysis the inner clear zone due to Beta toxin, and outer zone due to alpha toxin.

Micorscopica1 examination:, all isolates were central or sub terminal oval non bulging endospores.

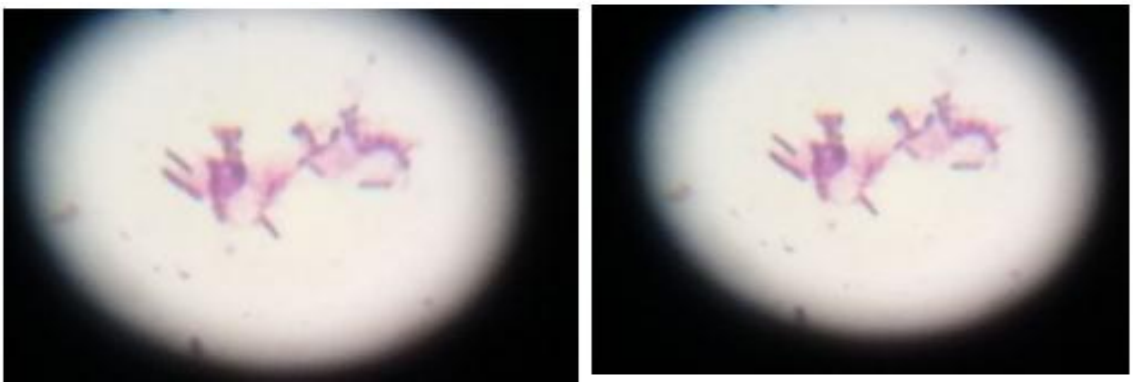

Photo (1): Gram positive bacilli of *C. perfringens.*

Morphologically, all isolates were central or sub terminal oval non bulging endospores

Biochemical characterization of *C. perfringens* indicated that, the isolates were +ve for Catalase, gelatin liquefaction, lactose, sucrose, maltose, mannose, H_2S production and Lecithinaseactivity. The isolates gave negative results for Catalase, indole, galactose, manitol and xylose.

The incidences of *C. perfringens* differ significantly ($P < 0.01$) among different types of the farm. The higher incidences of infection observed in closed farms (69.20 %) followed by semi closed farms (22.10 %) and the least infection observed in the open-yard farms (8.70) (Table4).

Table 4: Incidence of *C. perfringens* according to farm system and pasture.

Type of farm	Number	Percent
Closed	**72**	**69.2**
Semi closed	**23**	**22.1**
Open - yard	**9**	**8.7**
Total	**104**	**100**

$Chi^2 = 8.24$ ** = Significant at ($P < 0.01$)**

The significance ($P < 0.01$) differences of the incidences of *C. perfringens* among sheep and goats of different age (Table 5).The higher incidences observed in newly born kids up to 3 months of age (61.9 %), followed by newly born lamb up to 3 months of age (55.4 %), followed by its incidences in lambs from 3 months up to 6 months of age (28.90 %), while, in kids from 3 months up to 6 months of age (28.60 %). The lower incidences of *C. perfringens* observed in sheep that its age more than 6 months 15.70 and goats 9.5 %.

Table 5: Incidence of *C. perfringens* among sheep and goat of different ages.

Age	Sheep		Goat	
	Number	Percent	number	Percent
Newly born up to 3 months	**46**	**55.4**	**13**	**61.9**
From 3 months up to 6 months	**24**	**28.9**	**6**	**28.6**
More than 6 months	**13**	**15.7**	**2**	**9.5**
Total	**83**	**100**	**21**	**100**

$Chi^2 = 5.23$ ** = Significant at ($P < 0.01$)**

The significance differences of the incidences *C. perfringens* among different types of feeding regime. The higher incidences of *C. perfingens* observed in animals fed concentrates (77 %), followed by those fed hay and roughe (15.30 %) and the least incidences observed in mixed feed regimene (table6).

Table 6: Incidence of *C .perfringens* according to type of feeding.

Type of feeding	Number	Percent
Concentrates	80	77
Hay and barseem	16	15.3
Mixed	8	7.7

Chi^2 = 5.47 ** = Significant at (P < 0.01)**

The significance differences of the incidences of *C. perfringens* (P < 0.01) according to the previous vaccination against *C. perfringens*. The higher incidences of *C. perfringens* observed in the non-vaccinated group (71.20 %), followed by the vaccinated group (20.10 %) and the least incidences observed in the recently vaccinated group (8.70 %). The results recorded that about 21 case of the vaccinated animals were infected with *C. perfringens*, this is due to vaccination failure, there are 16 cases were vaccinated 8 months ago and 4 cases were vaccinated from 3-8 months ago and one case only were vaccinated less than 3 months ago (Table7).

Table 7: Incidence of *C. perfringens* among previous vaccinations against clostridia species.

Previous vaccinations	Number	Percent
Non- vaccinated	74	71.2
Recently vaccinated	9	8.7
Vaccinated	21	20.1
Total	104	100

Chi^2 = 9.24 ** = Significant at (P < 0.01)**

The incidences of *C. perfringens* differ significantly (P < 0.01) in relation to the incidences of the infection of the animals with other types of diseases. The higher incidences of *C. perfringens* observed in infection of the animals with viral diseases (46.10 %), followed by bacterial diseases (32.70 %) and the least incidences observed during the infection with mixed infection (21.10 %) (Table8).

Table 8: Incidence of *C. perfringens* in relation to diseases type.

Related disease	Number	Percent
Related to viral disease	48	46.1
Related to bacterial disease	34	32.7
Related to mixed infection	22	21.1
Total	104	100

Chi^2 = 7.22 ** = Significant at (P < 0.01)**

The incidences of *C. perfringens* differ significantly (P < 0.01) among different seasons.The higher incidences of *C. perfringens* observed in winter and autumn seasons (73 %) and the lower incidences observed in spring and summer seasons (27 %) (Table9).

Table 9: Incidence of C. perfringens according to seasons.

Season	Number	Percent
Winter and Autumn	76	73
Spring and Summer	28	27
Total	104	100

Chi2 = 5.33 ** = Significant at (P < 0.01)**

Molecular characterization of *C. perfringens* (Multiplex PCR)

The positive results showed a higher percentage (50 %) in Alph toxins, while, the beta and Epsilon toxins not detected in all examined samples. The negative samples observed in alpha toxins (50 %), beta-toxins (100 %) and Epsilon (100 %).

Table 10: Multiplex PCR for Molecular characterization of C. perfringens.

Sample	Results					
	Alpha		Beta		Epsilon	
	No	%	No	%	No	%
Positive	9	50	0	0	0	0
Negative	9	50	18	100	18	100
Total	18	100	18	100	18	100

Chi^2 = 6.78** ** = Significant at (P < 0.01)

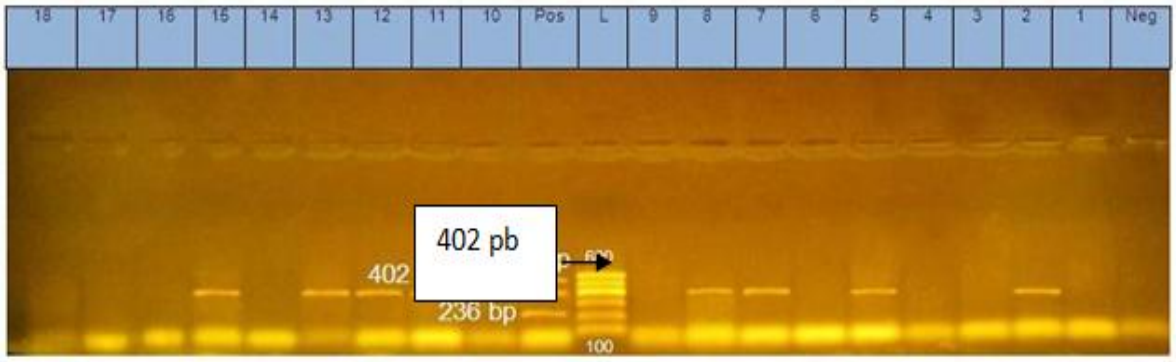

Photo 2: Multiplex PCR, *C. perfringens*type"A" (alpha toxin) which gave a characteristic band at 402 bp

Molecular characterization of *C. perfringens* from original tissue sample:

The samples were classified into two groups; the first group was (5 previously examined tissue give positive bacteriology and PCR positive results for C. perfringensstype "A" Alpha Positive and Beta and Epsilon Negative) and the second group was (5 previously examined tissue sample were bacteriologically and PCR negative in previously isolation were submitted for PCR examination from the original tissue:

The results revealed that; the first group givepositive bacteriology and PCR positive results for C. perfringens type "A" Alpha Positive and Beta and Epsilon Negative) and the second group (4) out of (5) examined negative tissue sample were PCR positive for C. *perfringens* type "A" Alpha Positive and Beta and Epsilon Negative) that conclude the importance of examination of tissue sample with PCR in addition to bacteriological examination. The result show that about 80% of negative sample bacteriologically revealed positive for PCR that could be turned to failure of isolation due to any reason or low and scanty isolates within examined tissue.

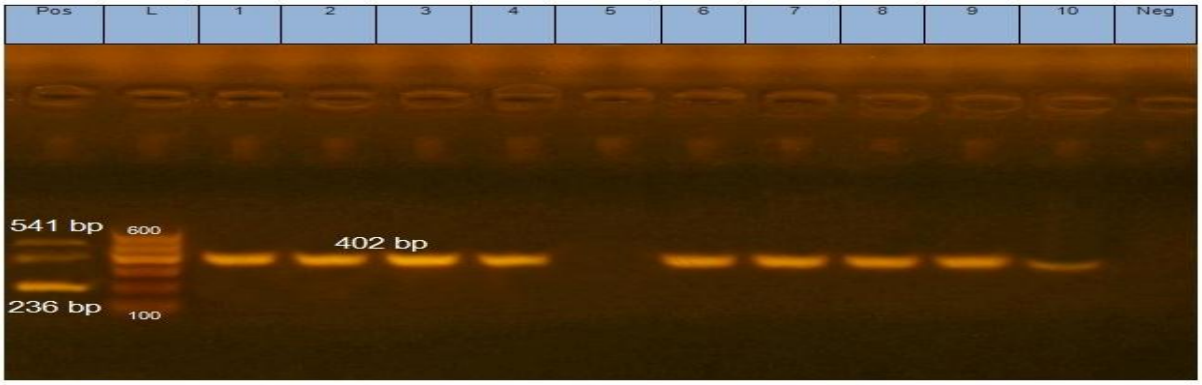

Photo 3: Multiplex PCR, C. perfringens type "A" (alpha toxin) which gave a characteristic band at 402 bp.

DISCUSSION

C. perfringens is spore-forming Gram-positive cocci that produce more than 17 toxins (Popoff and Bouvet, 2013). *C. perfringens* is classified into five main groups A to E in relation to the production of four lethal toxins alpha, beta, epsilon and iota toxins (Gurjar *et al.,* 2008). *C. perfringens* induced enterotoxaemia in sheep and goat represents a major economic obstacle facing developing countries attributable to the high fatality rate, decreased productivity, and increased treatment costs (Greco *et al.,* 2005).

Diarrhea, in appetence and depression were observed in sheep and goats infected with *C. perfringens* followed by collapse and death. Noteworthy, some animals suffered from progressive weakness with the development of nervous signs such as dullness, ataxia, in coordination and convulsive movement of the head with the neck rest laterally on the shoulder.

Regarding the necropsy finding, the data obtained showed that the rumen is full of ingesta, severely congested intestine and bloody fluids in the body cavities.

Our results on colonial character, in cooked meat broth, all isolates had abundant growth observed in cooked meat broth with significant gas formation, while the meat particles were pinkish and not digested with a sour odor. While, on 10% sheep blood agar medium: circular, convex, semi- translucent, smooth colonies with an entire edge were observed. Most of the isolates showed characteristic double zone of haemolysis the inner clear zone due to Beta toxin, and outer zone due to alpha toxin.

The results of micorscopica1 examination cleared that, all isolates were Gram positive bacilli, long rod, straight with parallel sides and rounded ends and rarely has central or sub terminal oval non bulging endospores.

Our results agreed with those reported by Elsify *et al.* (2016) where they reported that, the *C. perfringens* was isolated and characterized based on typical colony morphology on sheep blood agar with a characteristic double zone of hemolysis, while on *C. perfringens* agar medium supplemented with Dcycloserine and egg yolk emulsion, the micro-organism appeared as small black colonies surrounded by halo area due to lecithinase activity. Gram-stained smear from the colonies revealed the typical appearance of Gram-positive straight sided rods arranged singly or in pairs.

While, the results of biochemical identification of *C. perfringens* iso1ates, indicated that the isolates were positive for catalase, gelatin liquefaction, lactose, sucrose, maltose, mannose, H_2S production and lecithinase activity, and the isolates gave negative results for catalase, indole, galactose, manitol and xylose. These results agreed with those reported by Nasir *et al.* (2015) where they observed that, the main biochemical characters of C. perfringens were gas and acid production from glucose, fructose, lactose, sucrose and mannitol was observed. There was a double zone of hemolysis on blood agar. No growth was observed in the aerobic culture.

The incidence of *C. perfringens* according to age; Our results agreed with those reported by Bath *et al.* (2005) where they observed that, the young animals are most susceptible. Sudden and high mortality rates are concentrated in lambs and kids. Although adult animals are also susceptible to enterotoxemia, they develop immunity due to frequent exposure to these toxins.

The incidence of *C. perfringens* according to farm system and pasture cleared that, closed system is more infection with *C. perfringens* due to Accumulation of bacteria and Humidity. Our results in accordance with Mohiuddin *et al.* (2016), where they observed that, unsanitary conditions with increasing temperature and humidity facilitate the growth and infection with *C. perfringens*.

Our results on the incidence of *C. perfringens* according to type of feeding cleared that, the higher incidences of *C. perfingens* observed in animals fed concentrates (77 %), followed by those fed hay rougheand (15.30 %) and the least incidences observed in mixed feed regime.

Our results on the incidence of *C. perfringens* according to previous vaccinations against clostridia species in agreement with Haenlein (1996) where they found that the prevalence of *C. perfringenes* in healthy sheep and goat and those vaccinated against *C. perfringens* lower than its incidence in those diseased or non-vaccinated sheep and goats.

While, our results on the incidence of *C. perfringens* related to diseases cleared that, Our results agreed with those of, Habashy *et al.* (2009) failed to recover any C. perfringens isolates from apparently healthy sheep.

Osman (1993), who successfully isolated *C. perfringens* from 66.5%, 17.74% of samples collected from healthy sheep. While, in case of diseased sheep, the data presented in this study is lower in comparison with that obtained by Abd El-Moez *et al.* (2014), who showed that, *C. perfringens* was recovered from 77.8% of diseased sheep with no data regarding apparently healthy and soil. The existence of clostridia spores in soils plus in apparently healthy sheep can produce sporadic diseases episodes that are accountable for massive economic losses in animal

production through ingestion of the organism and then toxin release (Diego *et al.*, 2012).

While, our results on the incidence of *C. perfringens* according to seasons, cleared that, the infection with Clostridia is very high in winter and autumn due to high humidity and enteritis and changing of pasture.

the Multiplex PCR showed a higher percentage (50 %) in Alph toxins, while, the beta and Epsilon toxins not detected in all examined samples. were identified as *C. perfringens* type "A" (alpha toxin) which gave a characteristic band at 402 bp (Yoo *et al.,* 1997). The negative samples observed in alpha toxins (50 %), beta-toxins (100 %) and Epsilon (100 %).

This is consistent with the findings of Gerco *et al.* (2005), who showed that *C.perfringens* type A and D are the predominant causes of predominant causes of enterotoxaemia in very young lambs and kids.

Similarly, Abd El-Moez *et al.* (2014) showed that *C. perfringens* type A is the predominated type isolated from humans and animals. In contrast, previous studies showed that the main cause of sheep dysentery in UK, South Africa, and Greece was *C. perfringens* type B (Bueschel *et al.,* 2003).

Noteworthy, the finding that no C. perfringens type E strains were identified strongly advocate that *C. perfringens* type E is rare in lambs and kids (Greco *et al.,* 2005).

C. perfringens in general are associated with several forms of enteric diseases including fatal enterotoxemia in animals. *C. perfringens* type A is the main causative agent of gas gangrene (myonecrosis) and diarrhea (Hatheway, 1990), while type B and type D are the predominant causes of fatal enterotoxemia in domestic animals (Yamagishi *et al.,* 1997). C. perfringens type A is one of the major toxin producers among clostridia species; however, alpha is representing the main toxin type for this species (Popoff and Bouvet, 2013).

The genetic relationship between the various strains isolated need further investigation. Finally, the high positivity rate to *C. perfringens* type A toxins remarkably counsel counting of this strain in vaccine schedule in order to validate the ample guard to avert the disease in animals.

the Multiplex PCR of *C. perfringens* from original tissue sample show that about 80% of negative sample bacteriologically revealed positive for PCR that could be turned to failure of isolation due to any reason or low and scanty isolates within examined tissue this agreed with Dean *et al.* (2011).

Our study concluded that, *C. perfringens* causes severe economic losses in sheep and goats due to causes high mortality and losses among them through enterotoximia that resulted from secretion of Alpha, Beta and Epislon toxins, the higher incidences of *C. perfringens* observed in newly born kids up to 3 months of age, especially in closed farms, in animals fed concentrates, in non-vaccinated group. The higher incidences of *C. perfringens* observed in infection of the animals with viral diseases, followed by bacterial diseases and the least incidences observed during the infection with mixed infection, especially in winter and autumn seasons. The best method for molecular characterization of *C. perfringens* is the Multiplex PCR that characterize *C.perfringens* type "A" (alpha toxin) which gave a characteristic band at 402 bp.

Hence, further studies are requisite to authenticate the molecular association among *C.perfringens* isolated from soil and apparently healthy sheep in one hand and diseased sheep in the other to trace the source of infection.

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دراسات وبائية وخصائص جزيئية على الكلوستريديا المعوية فى المجترات الصغيرة بمحافظة البحيرة – مصر

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تم دراسة العوامل الوبائية المرتبطة بعدوى الكلوستريديا فى الاغنام والماعز عبر تجميع ١٠٤ عينة من الامعاء ، الكبد ، الكلية والطحال للاغنام والماعز التى وجد بها أعراض الانتيروتوكسيميا والتى نفقت فجأة او الاغنام التى ظهر عليها أعراض الاسهالات المعوية الشديدة من مزارع ذات انماط مختلفة للتربية بمحافظة البحيرة. وتم اجراء العزل البكتيرى اللاهوائي للكلوستريديم بيرفرينجيز بالطرق البكتيريولوجية التقليدية بالاضافة لاستخدام تفاعل البلمرة المتسلسل لتصنيف المعزولات. أوضح تحليل البلمرة المتسلسل أن أعلى نسبة اصابة هى الاصابة بـ الكلوستيريديم بيرفرنجيز-A والتى أعطت خطوط مميزة عند درجة ٤٠٢ bp. وهى المسبب الفعلى لارتفاع نسبة النفوق فى الاغنام .

الكلمات الدالة : الكولستريديم برفرنجينز ، انتيروتوكسيميا ، الوبائية تفاعل البلمرة المتسلسل ملتى بلكس اغنام ، ماعز