STUDIES ON ENTEROTOXAEMIA IN CALVES

Nora, Mohamed Khalaf [1]; Ebeid, Mohamed Hassanin.[2]; Galila, Elsayed Moustafa[2]; El Seify, Ahmed[3]; Mustafa Abdel-Moneim Mohamed.[2] ; El- Meneisy, Alaa Abdel Fattah[1] .

[1]-Veterinary Serum Vaccine Research Institute, Abbasia. [2]-Animal Medicine Department, Faculty of Veterinary Medicine, Benha University. [3]- Animal Medicine Department, Faculty of Veterinary Medicine, El Sadat University.

ABSTRACT

This study was done on three hundred samples (fecal samples collected from200 diarrheic calves, intestinal samples from90 dead calves, intestinal samples from10 slaughtered calves). These samples were collected from Beheira, Kalubyia, El – Fayoum and Kafr El – Sheikh Governorates in Egypt. These were subjected to bacteriological anaerobic examination, dermonecrotic reaction, mice neutralization test and multiplex PCR. Most of the examined samples revealed the presence of Clostridium *perfringens* Type A and its toxin alpha toxin. One hundred and fifty out of two hundred fecal samples (78.9%) revealed toxigenic isolates and typed as *C. perfringens* type A. Eighty five out of ninety (94.4%) intestinal samples of dead calves were toxigenic isolates. Typing of toxin detected 88.8% alpha toxin, 3.3% beta toxin and 2.2% epsilon toxin. The results of 10 samples of slaughtered calves revealed four toxigenic isolates and typed as alpha toxin .The toxin typing was confirmed by multiplex PCR. So it could be concluded that the main cause of enterotoxaemia in young calves is *Clostridium perfringens* Type A and its toxin (alpha toxin) which lead to sudden death in young calves.

Key words: Calves**,** *C. perfringens* type A**,** Alpha toxin, PCR, Enterotoxaemia.

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1. INTRODUCTION:

Clostridial diseases are considered a constant threat to the health and productivity of all livestock species as sheep and goats (Green et al. 1987) and cattle and calves (Songer 1996). Clostridial diseases are characterized by sudden onset, short course disease and high fatality rate, which make the probability of treatment success become at minimal level. Enterotoxaemia caused by *Clostridium perfringens* is considered the worst killer disease in cattle, sheep and goats with serious economic losses (Manteca et al. 1999; Manteca et al. 2001; Uzal. 2004). Enterotoxaemia syndrome in calves is characterized by a high fatality rate, sudden death and lesions of hemorrhagic enteritis of the small intestine (Manteca et al. 2001) and sudden or very rapid death of calves with signs of colic, convulsion and nervous disorders. Suckling calves are the most frequently affected ones. The most frequently isolated bacteria in high number was *Clostridium perfringens* (Manteca et al. 1999). Calf mortality is one of the serious problems facing calf industry in many countries of the world, the importance of this problem emerges from the fact that calves play a major role in our future animal wealth as a source of animal protein, which is necessary to fulfill with the requirement of the rapidly increasing human population (Awad 1980). Therefore, this study was designed to investigate the following: a. Isolation and identification of *Clostridium perfringens* from feces of diarrheic calves and from the intestinal contents of dead and slaughtered

calves obtained from different governorates in Egypt to determine the main cause of enterotoxaemia in young calves. B. Toxin typing of the isolates using mice neutralization test and dermonecrotic reaction and confirmed this typing using multiplex PCR.

2. MATERIAL AND METHODS:

2.1. Samples:

- Fecal samples : A total number of 200 fecal samples were collected from diarrheic calves from different governorates in Egypt (table 1).
- Alimentary tract samples:

(1): From slaughtered diarrheic calves showing symptoms of enterotoxaemia: A total number of 10 samples collected from different parts of the alimentary tract (abomasums, duodenum, jejunum, ileum) of each animal. These were collected from both young (one month) and old calves (four month).

(2): From dead calves: A total number of 90 samples collected from parts of the alimentary tract (the same parts as in the slaughtered calves) of dead calves suspected to be suffering from enterotoxaemia.. The intestine with its contents were collected and legated from both ends and taken with its contents to a polythene bags. The samples were marked and kept in an icebox until examination in the laboratory.

Table (1): Total number of samples from different governorates in Egypt.

Egyptian governorates	No. of fecal samples from diarrheic calves	No. of intestinal samples	
		Dead calves	Slaughtered calves
Beheira	40	10	3
Kalubyia	40	10	0
El fayoum	70	20	5
Kafr el sheikh	50	50	2
total number	200	90	10

I- Cultivation of the collected samples:

All samples were inoculated into cooked meat; blood agar; neomycin sulphate blood agar; egg yolk agar and media for biochemical tests and toxin production and inoculated anaerobically at 37^0C. These media were prepared according to (Roberts 1970); (Cruickshank et al. 1975) and (Alaa Abdel Fattah 1990).

II-Antisera**:**

C. perfringens antitoxins sera were obtained from burrough welcome, research laboratories, Backengham, London.

III-Isolation and identification tests**:**

Isolation; identification and typing of toxigenic *C. perfringens* from different samples were carried out according to Schemes of (Smith 1975) and (Willis 1977).

IV-Toxigenicity test:

The content of each sample was mixed with equal volume of saline, centrifuged at 3000 rpm for 15 minutes, 0.3 ml of supernatant was injected in three mice I/V, if the mice died within 24 hours indicate presence of toxin.

V-Typing of the isolates:

A- By using seroneutralization test : *C. perfringens* isolates were typed by SNT in mice I/V and by dermonecrotic reaction in skin of Guinea pigs I/D with specific antisera of each *C. perfringens* type as performed by (Sterne and Batty 1975).

B- By multiplex polymerase chain reaction (multiplex PCR) assay according to (Yoo et al. 1997):
i- The nucleotide sequences of primers used for PCR amplification of genes for *Clostridium perfringens* toxins α, β, β_2, ε and ί were illustrated in the following table according to (Greco *et al*. 2005).

(Table 2): PCR primers used in this study:

Toxin/gene	Oligonucleotide sequences	Fragment length bp
α/cpa	5-TGC TAA TGTTAC TGC CGT TGA TAG-3 5-TGC TAA TGTTAC TGC CGT TGA TAG-3	247
β/cpb	5-AAC TTA ACT GGA TTT ATG TCT TCA-3 5-ATA GTA GAA AAA TCA GGT TGG ACA-3	317
ε/etx	5-ATT AAA ATC ACA ATC ATT CAC TTG-3 5-CTT GTG AAG GGA CAT TAT GAG TAA-3	206
ί/iap	5-TTT TAA CTA GTT CAT TTC CTA GTT A-3 5-TTT TTG TAT TCT TTT TCT CTA GGA TT-3	298

ii- Extraction of DNA: DNA was extracted from bacterial isolates according to (Sambrook et al. 1989).
iii- PCR protocol was performed according to (Kadra et al 1999).
iiii- Screening of PCR products: by using 1.5% agarose gel electrophoresis and then photographed according to (Sambrook et al. 1989).

3. RESULTS

Concerning to isolation , identification and typing of samples selected from different governorates as fecal sample of diarrheic calves as shown in table (3) from 190 fecal samples out of 200 (95%) were *C. perfringens*, by toxin typing of 190 fecal samples as shown in table (4) 150 isolates (78.9%) were toxigenic and typed as *C. perfringens* type A .Intestinal samples from dead calves as shown in table (5) from the 90 intestinal samples of dead calves and85 out of (94.4%) were toxigenic isolates, by typing(88.8%) were alpha toxin, 3.3% beta toxin and 2.2% epsilon toxin as shown in table (6) while ten samples of slaughtered calves as shown in table (7), the four toxigenic isolates were typed as alpha toxin .The toxin typing was confirmed by multiplex PCR as shown in Figure (2) *C. perfringens* type A appeared at amplified 247 bp.

Table 3: Prevalence of *C. perfringens* in fecal samples of diarrheic calves in different governorates and the percentage of the isolated positive samples for *C. perfringens*:

Egyptian governorates	No. of samples	positive samples for *C. perfringens*	
		No.	%
Beheira	40	38	95%
Kalubyia	40	39	97.5%
El fayoum	70	68	97.1%
Kafr el sheikh	50	45	90%
total number	200	190	95%

Table 4: Toxin typing of *C. perfringens* isolates from fecal samples using Dermonecrotic reaction:

Egyptian governorates	No. of *C. perfringens* toxigenic isolates	Type of toxigenic *C. perfringens* isolates	No. of Non toxigenic isolates
Beheira	30 (78.9%)	A	8 (21%)
Kalubyia	25 (64.1%)	A	14 (35.8%)
El fayoum	62 (91.1%)	A	6 (8.8%)
Kafr el sheikh	33 (73.3%)	A	12(26.6%)
Total number	150 (78.9%)		40(21.1%)

Table 5: Prevalence of *C. perfringens* in intestinal samples of dead calves in different governorates and percentage of intestinal samples positive for *C. perfringens* toxins:

Egyptian governorates	No. of samples	positive samples for *C. perfringens* toxin	
		No.	%
Beheira	10	10	100%
Kalubyia	10	9	90%
El fayoum	20	18	90%
Kafr el sheikh	50	48	96%
Total number	90	85	94.4%

Table 6: Toxin typing of *C. perfringens* isolates from intestinal samples of dead calves:

Egyptian governorates	No. of *C. perfringens* toxigenic isolates	Alpha toxin of *C. perfringens* type A	Beta toxin of *C. perfringens* type B	Epsilon toxin of *C. perfringens* type D	Iota toxin of *C. perfringens* type E
Beheira	10	9(90%)	-------	--------	-------
Kalubyia	9	8(88.8%)	-------	--------	-------
El fayoum	18	18(100%)	--------	--------	-------
Kafr el sheikh	48	45(93.7%)	3	2	--------
Total number	85	80(88.8%)	3(3.3%)	2(2.2%)	-------

Table 7: Prevalence of *C. perfringens* in intestinal samples of slaughtered calves in different governorates and toxin typing of toxins isolated from intestinal samples

Egyptian governorates	No. of samples	positive samples for *C. perfringens* toxin	
		No.	%
Beheira	3	1	33.3
Kalubyia	0	0	0
El fayoum	5	2	66.6
Kafr el sheikh	2	1	50
Total number	10	4	40%

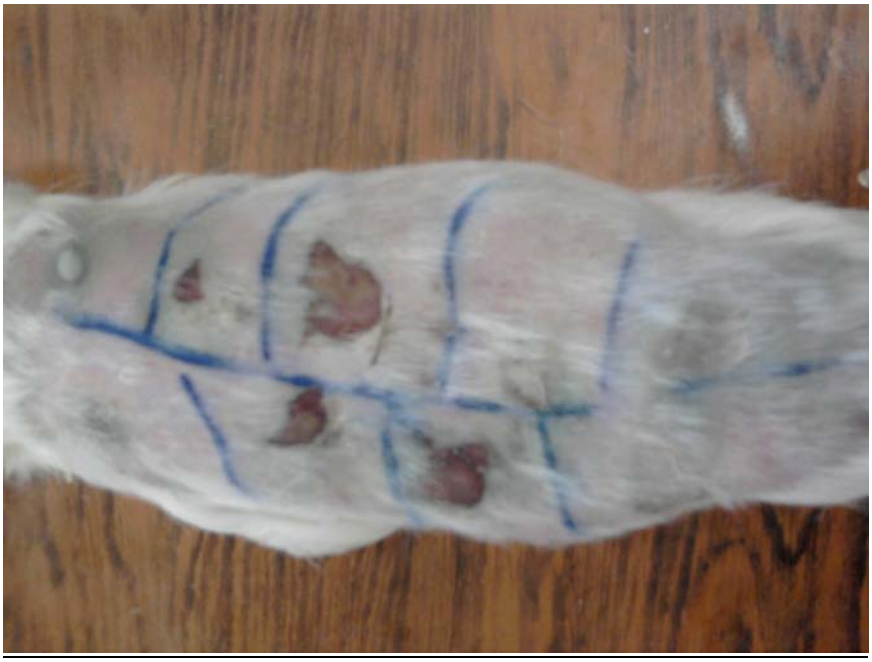
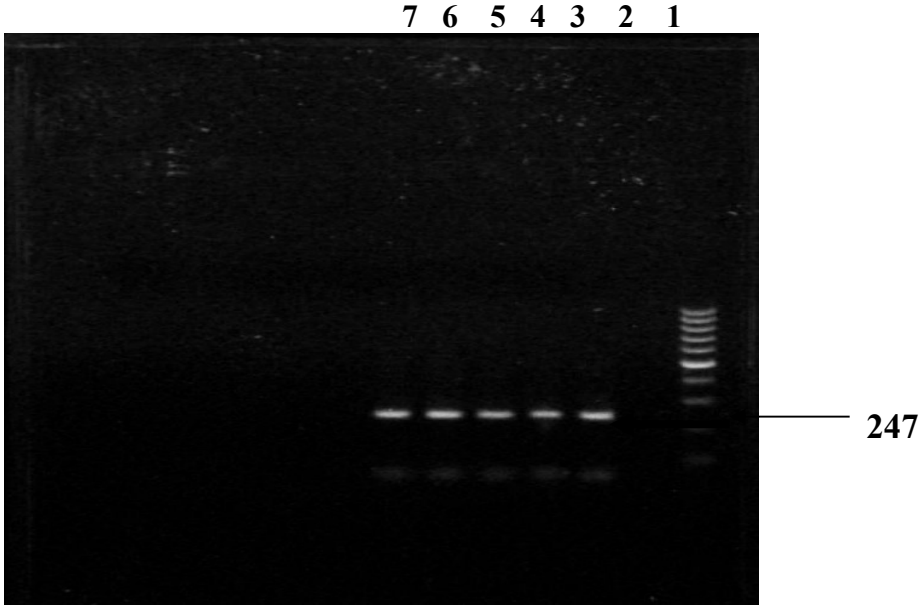


Figure (1): Showing dermonecrotic reaction in Guinea pigs after I/D injection of toxin of *C. perfringens* isolates.

Figure 2. Agarose gel electrophoresis of PCR amplified product from *C. perfringens* isolates:



Lane 1: molecular weight Marker .
Lane 2: - Ve control (200bp).
Lane 3: amplified 247 bp. of isolates of *C. perfringens* type A.
Lane 4: amplified 247 bp. of isolates of *C. perfringens* type A.
Lane 5: amplified 247 bp. of isolates of *C. perfringens* type A.
Lane 6: amplified 247 bp. of isolates of *C. perfringens* type A.
Lane 7: amplified 247 bp. of isolates of *C. perfringens* type A.

4. DISCUSSION

Enterotoxaemia caused by *C. perfringens* is probably the important cause of sudden death in young calves. Morbidity rate of enterotoxaemia is variable ; however, in general, it does not exceed 10% of the herd while its lethality is high and usually kills about 100% of the affected animals (Miyashiro et al. 2007). Concerning to isolation, identification and typing of isolates from fecal samples of diarrheic calves selected from different governorates (table 3) as well as from intestinal samples of dead calves from different governorates (table 5) besides samples of slaughtered calves from different governorates (table 7). Table (4) revealed that from the two hundred examined fecal samples collected from diarrheic calves harbored *C. perfringens* organisms (190) samples and it represent about 95%.These results were agreed with the results obtained by (Itodo et al. 1986) who isolated *C. perfringens* from two hundred fecal samples of cattle. Also (Ferrarezi et al. 2008) examined 141 fecal samples from diarrheic calves aged up to one month and all isolates were *C. perfringens*. On the other hand, results of (Niilo 1987) *C. perfringens* type C was isolated from cases of enterotoxaemic calves. One hundred and ninty of *C. perfringens* isolates, 150 (78.9%) were toxigenic strains and 40(21.1%) were non toxigenic strains (table 4). These findings nearly similar to that obtained previously by (Singh and Malik1968 and Alaa Abd-El Fattah 1990) where they found that the percentage of toxigenic *C. perfringens* isolates in fecal samples were 35% and 39.5% respectively and the percentage of non toxigenic *C. perfringens* isolates were 65% and 60.5%. On the basis of toxin typing on the skin of albino Guinea pig (Dermonecrotic reaction) as shown in Figure (1)and toxin neutralization test, it was found that out of the 150 of toxigenic strains of *C. perfringens, C. perfringens*

type A was the most predominant type as shown in table (4), these results in harmony with that recorded by (Itodo et al. 1986) who isolated 53% *C. perfringens* type A from two hundred fecal samples and (Ferrarezi et al. 2008) who examined 141diarrheic fecal samples of calves and they found that the most predominant isolated organism was *C. perfringens* type A. also (Alaa Abd-El Fattah 1990) found that most predominant isolates from fecal samples was *C. perfringens* type A with percentage 59.4% where types B, C,D were recorded in percentage of 3.7%, 2.5% and 13.9% respectively, in this respect (Manteca et al. 2001) isolated *C. perfringens* type A from most cases of calves suffered from enterotoxaemia. And (Savic et al. 2012) isolated *C. perfringens* type A from 9 month calves suffered from enterotoxaemia. Examination of one hundred intestinal filtrates: (90 samples from dead calves and 10 samples from slaughtered calves), as shown in table (5) and (7) revealed about 85 (94.4%) of 90 dead calves were positive for the presence of toxin while 5(5.5%) were non toxigenic and about 4(40%) from10 of slaughtered calves were positive for the presence of toxin, while 6(60%) were negative. The obtained result in table (6) using mice neutralization test for typing of toxins, it was found that from 85 positive samples of intestinal contents of dead calves 88.8% were alpha toxin, 3.3% beta toxin and 2.2% epsilon toxin and from 4 positive samples of intestinal contents of slaughtered calves were all positive for alpha toxin. These results were in agreement with results of (Senf 1983) who examined toxins of the intestinal contents of 365 suspected cases of enterotoxaemia and about 203 of samples were positive for toxin: alpha toxin revealed in 132 of cases (65%), beta toxin (8.8%) and epsilon toxin (4.9%), also these results similar to that of (Alaa Abd-El Fattah 1990) who isolated alpha toxin from dead goats with percentage 92.45% while beta toxin 1.88%. Meanwhile these results were disagreed with that (Niilo 1987)who isolated lethal ß toxin from cases of hemorrhagic enterotoxaemia in calves, and (Lulov and Angelov 1997) who examined intestinal samples of both slaughtered and dead calves and they found that α, ß and ε toxins of types A, C and D of *C. perfringens* caused calf enterotoxaemia. The PCR is a rapid and useful method for genotyping of *C. perfringens* (Meer and Songer 1997). Based on the recently published sequences of the major toxin genes.

In this study, The obtained results were confirmed by multiplex PCR as the toxin typing of *C.perfringens* isolates were determined by by (Yoo et al. 1997) as shown in (Figure 2) all isolates of *C. Perfringens* found in fecal and intestinal samples of diarrheic and dead calves carried only the cpa gene of *C. perfringens* type A. These results confirmed by (Aschfalk and Muller 2002) who examined fecal samples for occurrence of *C. perfringens* by PCR for the gene encoding, and all isolates were *C. perfringens* type A. also these results similar to that obtained by (Yoo et al. 1997) who used a multiplex PCR to investigate the most prevalent type of the organism in calves showing diarrhea, enterotoxaemia, only *Clostridium perfringens* type A was isolated from calves. These results confirm the advantages of multiplex PCR by (Laura and Maria 2010) as they found that the multiplex PCR provide a useful and reliable tool for *C. perfringens* genotyping in routine veterinary Diagnostics, providing the final piece of information needed to establish a diagnosis. This study proved that *C. perfringens* type A and its toxin (alpha toxin) was the predominant type of diarrheic and dead calves and have main role in the etiology of enterotoxaemia in calves, So immune prophylaxis of calves is needed as a control measure of paramount importance.

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دراسات عن التسمم المعوي الكلوستريدي في العجول

[1]نورا محمد خلف،[2]محمد حسنين عبيد،[2]السيد مصطفي جليلة،[3] أحمد الصيفي، [2]عبد المنعم محمد مصطفي، [1] علاء عبد الفتاح محمد المنيسي،

[1]معهد بحوث الامصال واللقاحات البيطرية-العباسية. [2] قسم الامراض المعدية-كلية الطب البيطري-جامعة بنها. [3] قسم الامراض المعدية-كلية الطب البيطري-جامعة السادات.

الملخص العربي

من مزارع العجول التي تم فحصها وجد انه من أجمالي 300 عينة (عينات براز من العجول الصغيرة التي تعاني من الاسهال؛ عينات امعاء من عجول ميتة؛ عينات امعاء من المجزر من عجول يشتبه بها التسمم المعوي الكلوستريدي). تم تجميعها من محافظات مصر المختلفة (البحيرة، القليوبية، الفيوم، كفر الشيخ). تم عزل وتصنيف وتحديد النوع لكلا من عينات الاسهال وعينات الامعاء باستخدام تفاعل الجلد التنكرزى وتفاعل البلمرة المتسلسل. تم عزل 190 معزولة كلوستريديم بيرفرنجينز بنسبة (95%) وبفحص العينات وجد ان هناك 150 معزولة من النوع الممرض بنسبة (78.9%) وتم تصنيفها الى الكلوستريديم بيرفرنجينز من النوع أ. من مزارع العجول التي تم فحصها من إجمالي 100 عينة امعاء من عجول ميتة تم عزل 90 معزولة من الكلوستريديم بيرفرنجينز منها 85 من النوع الممرض بنسبة 94,4%. ومن المجزر تم عزل 10 معزولات من الكلوستريديم بيرفرنجينز منها 4 عينات من النوع الممرض بنسبة 40% وحوالي 6 عينات من النوع الغير ممرض بنسبة 60%. ومن النتائج وجد ان السبب الرئيسي لحدوث التسمم المعوي الكلوستريدى في العجول الصغيرة هو سم الالفا الذي يفرزه ميكروب الكلوستريديم بيرفرنجينز النوع أ الذي يؤدى الي الموت المفاجئ في العجول الصغيرة.

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