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Use of ELISA and polymerase chain reaction as rapid different methods in diagnosis of enterotoxemia in foals

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ABSTRACT

Necrotizing enterocolitis is a serious disease of sudden onset in foals associated with a high death rate, despite therapeutic intervention. Clinical manifestations of enterocolitis in foals vary from being found dead to spectrum clinical manifestations.

In this study, 50 fecal samples collected from foals aged 1-4 months and suspected clinically to be suffered from enterocolitis with main symptoms of profuse diarrhea, colic and abdominal pain. Rapid diagnosis was essential for controlling the disease instead of animal inoculation which is morally forbidden. Two rapid methods were used for diagnosis; ELISA and PCR tests. Out of 50 samples, 22 *Clostridium perfringens* isolates were detected (44%) and typed according to their major toxins and the presence of toxin genes using ELISA and PCR respectively. By PCR, 18 isolates were found to harbor *cpa* gene only (type A) and 4 isolates harbored *cpa* and *cpb* gene (type C), however, ELISA revealed that only 12 isolates have toxigenic activities for alpha toxin (type A), 6 isolates were non-toxigenic and 4 isolates gave alpha and beta toxins (type C) meaning that 6 isolates were not expressed by conventional PCR .

INTRODUCTION:

Diarrhea is one of the most common problems of foals, with up to 80% experiencing one or more episodes of diarrhea during the first 6 months of their life. Although often a transient and fairly being problem, diarrhea can be life-threatening and cause significant economic losses for breeding farms and individual horse owners. Causes of diarrhea can be divided into infectious and noninfectious etiology. Effective diagnosis, treatment and prevention depends on knowledge of the most common pathogens affecting certain age groups, the availability, specificity and sensitivity of diagnostic tests and interpretation of test results. The most frequently detected pathogens affecting foals at all age groups are *Clostridium* difficile, Clostridium perfringens types A and C and Salmonella spp **(Netherwood et al. 1996).**

Diarrhea has been claimed to occur in 70 to 80% in foals with the first 6 months of life, most frequently in the first week of life. Although the morbidity may be quite high, the mortality is very low. The majority of affected foals have a transient self-limiting diarrhea, but

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when it is not self-limiting or it is never, the fatality rate can be high **(Jonathan 1985)**.

Clostridium perfringens type A is commonly found in the environment of horse, whereas, type C is less commonly isolated. In a study, more than 60% of foals 8 to 12 hours of age and more than 90% of foals 3 days old had *C.perfringens* in their feces. It was noted that *C.perfringens* type A appears to be a normal inhabitant of the gastrointestinal tract of neonatal foals. It is not known why some foals develop enterocolitis due to *C.perfringens* and other foals do not (**Weese et al. 2001**).

C.perfringens Types A and C have the potential to cause a medical condition called enterocolitis in neonatal foals. The source of infection for a neonatal foal may be a cattier horse (possibly the mare) shedding the organism in feces or exposure to bacteria in the environment **(Sims et al.1985).**

Sever and often fatal infections can be seen in neonates less than 1 week of age.

C.perfringens is a Gram positive spore forming anaerobe which is categorized into A-E types based upon the expression of four major lethal toxins (α, β, ε and ί). Alpha- toxin is produced by all types of *C.perfringens*, but is not thought to be a significant enteric virulence factor. Beta toxin is produced by type C and D while β_2-toxin is produced by type A. **(Jones 2000)**. *C.perfringens* types A and C are the best described pathogens in foals **(East et al. 1998).**

Hadimil et al. (2011) discussed the using of the enzyme-linked immunosorbant assay (ELISA) in typing of *C.perfringens* and investigated the presence of the 4 major toxins genes (α,β,ε and ί) in the isolates by polymerase chain reaction (PCR).

This study aimed to detect the role of *Clostridium perfringens* in diarrhea in foals and to compare between ELISA and PCR as rapid methods for diagnosis of enterocolitis considering their sensitivities, specificities and time consuming factors.

MATERIALS AND METHODS:

Samples: fecal samples from 50 foals aged from 1-4 months and clinically suffer from profuse diarrhea of foul smelling (sometimes bloody), abdominal pain or colic, dehydration and depression from some equine center and veterinary clinics in Cairo and Giza governorates, were collected ,each in a separate sterile plastic bags and sent to the laboratory in an ice box within 6 hours for bacteriological examination.

Isolation and identification of *C.perfringens* from fecal samples: (Uzal 1996)

Each fecal sample was inoculated into tubes of freshly prepared cooked meat broth medium and incubated an aerobically at 37ºC for 24 hours. A loopful from each tube was then plated onto 200µl/ml neomycin sulphate sheep blood agar containing 5% defibrinated sheep blood and onto tryptose sulphate cycloserine agar (TSC) containing a selective supplement (D-cycloserine, Lab M Limited, UK) and they were incubated anaerobically for 24 hours at 37ºC. The suspected colonies were identified by Gram staining, cultural morphological and biochemical characters.

Detection of *C.perfringens* toxins (α,β and ε) from culture by ELISA (Hadimli et al. 2011):

A total of 22 *C.perfringens* culture isolates were used for detection of toxins production using commercial ELISA Kit (Bio-X Diagnostics, Belgium) according to the manufacturer's instructions.

Detection of *C.perfringens* toxins (α,β and ε) from culture by muliplex PCR:

DNA Extraction and PCR:

Total DNA was extracted from isolated strains (22) which were cultured on blood agar media using QIAamp DNA Mini Kit (Cat No. 51304).

Three specific primer sets corresponding to each toxin of *C. perfringens* (Table 1), according to (**Komoriya et al. 2007, van Asten et al. 2009)**. The PCR was performed in a Thermal Cycler® (Bio-Rad, USA) in a total reaction volume of 50 µL containing: 25 µL of 2x Master Mix (thermo scientific , 1µL of 10 pmol of

each primers and 5 µL of template DNA.

Amplification was obtained with 35 cycles following an initial denaturating step at 95°C for ten minutes. Each cycle comprised denaturation at 94°C for 45 seconds, annealing at C for 30 seconds, and synthesis at 72°C for 90 seconds. The final extension step occurred at 72°C for ten minutes. Then, 8 µL of the amplified product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

Table (1) Primers used in multiplex PCR.

Toxin	Gene	Sequence 5'-3'	Primer position (bp)	Amplicon (bp)
α	*plc (cpa)*	GCTAATGTTACTG CCGTTGA CCTCTGATA-CATCGTGTAAG	663-968	324
β	*cpb*	GCGAA-TATGCTGAATCAT CTA GCAGGAACATT-AGTATATCTTC	871-1045	196
ε	*etx*	GCGGTGA-TATCCATCTATTC CCACTTACTT-GTCCTACTAAC	267-862	655

RESULTS

Out of 50 diarrheic fecal samples, 22 were positive for *C.perfringens* by culturing on specific media in an incidence of 44%. Catalase -ve colonies with double zone of hemolysis on neomycin blood agar and black colonies with white hallow on TSC agar, were considered as presumptive *C.perfringens* colonies.

Table (2). Incidence of *C.perfringens* in the examined fecal samples of foals

No. of samples	Positive samples		Negative samples	
	No.	%	No.	%
50	22	44	28	56

% calculated according to No. of examined samples (50)

Typing of *C.perfringens* isolates using sandwich ELISA (Hadimli et al. 2011):

ELISA test is considered as a precise rapid test for detection of *C.perfringens* and its toxins. Twenty two *C.perfringens* isolates were examined using ELISA kit and they all were positive for *C.perfringens* organism in an incidence of 100%. Typing of the isolates revealed the presence of alpha toxin only in 12 isolates (type A) in an incidence of 54.5%, and alpha together with beta toxin in 4 isolates (type C) in an incidence of 18.2% and 6 isolates were non toxigenic (27.3%).

Table (3) Typing of *C.perfringens* isolates using sandwich ELISA:

No. of isolates	Types					
	Type A		Type C		Non toxigenic	
22	No.	%	No.	%	No.	%
	12	54.5	4	18.2	6	27.3

% calculated according to the total number of isolates (22)

Genoyping of *C.perfringens* isolates using muliplex PCR test:

When the twenty two isolates were tested using PCR test, 18 isolates were found to encode *cpa* gene only (type A) and 4 isolates encoded cpa and *cpb* gene (type C) as shown in photoes (1) and (2) and illustrated in table (3).

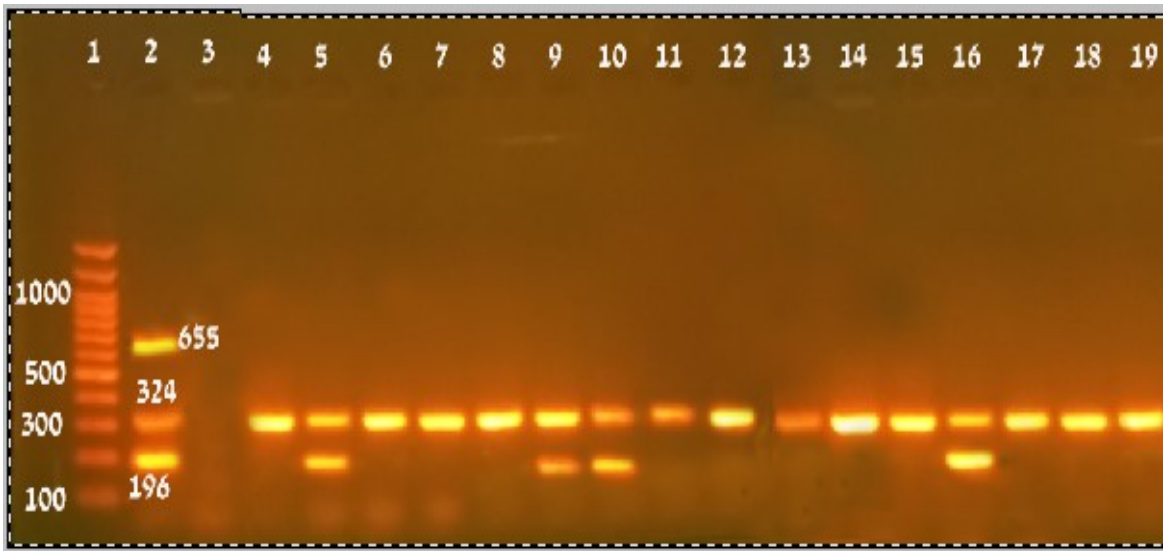

Photo (1). Agarose gel electrophoresis of PCR products obtained from *C. perfringens* isolated from sample.
Lane 1: marker (100 bp DNA ladder,)
Lane 2:control positive (655bp for epsilon toxin, 324bp for alpha toxin, 196 bp for beta toxin)
Lane 3: control Negative
Lanes: 5,9,10,16 : *C. perfringens* type C isolates (samples no. 2,6,7 and 13)
Lanes: 4,6-8,11-15,17-19 : *C. perfringens* type A isolates

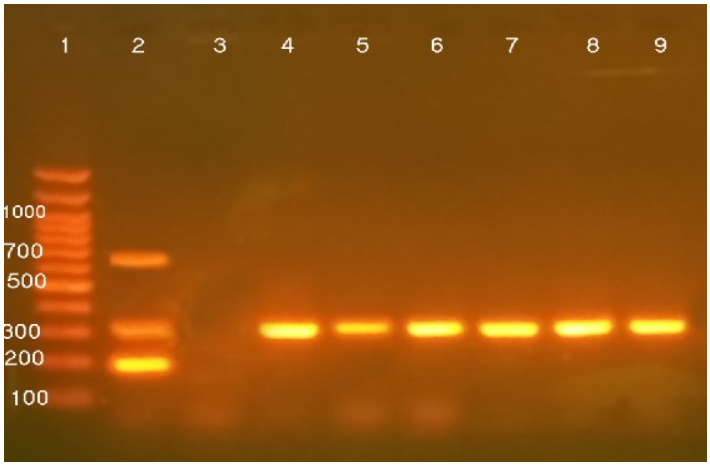

Photo (2). Agarose gel electrophoresis of PCR products obtained from *C. perfringens* isolated from sample.
Lane 1: Marker (100 bpDNA ladder,)
Lane 2: Control positive
Lane 3: Control Negative

Table (4) Genotyping of *C. perfringens* isolates using PCR test:

Strain No.	Toxic genes			Type
	cpa	cpb	etx	
1	+	-	-	A
2	+	+	-	C
3	+	-	-	A
4	+	-	-	A
5	+	-	-	A
6	+	+	-	C
7	+	+	-	C
8	+	-	-	A
9	+	-	-	A
10	+	-	-	A
11	+	-	-	A
12	+	-	-	A
13	+	+	-	C
14	+	-	-	A
15	+	-	-	A
16	+	+	-	A
17	+	-	-	A
18	+	-	-	A
19	+	-	-	A
20	+	-	-	A
21	+	-	-	A
22	+	-	-	A

Type A(*cpa*): No. (18), Type C (*cpa, cpb*): No. (4)

Table (4) showed the results of examination and typing of 22 *C. perfringens* isolates using PCR test and it revealed that, out of 22 isolates, 18 were found to harbor alpha toxin gene only (type A) and 4 isolates harbored alpha and beta toxin genes (type C) .

Table (5) Results obtained from ELISA test on 22 *C.perfringens* isolates

Strain No.	ELISA
1	NT
2	C
3	A
4	A
5	A
6	C
7	C
8	NT
9	A
10	A
11	NT
12	A
13	C
14	A
15	NT
16	A
17	A
18	A
19	NT
20	A
21	NT
22	A

NT: non toxigenic.

Table (5) showed the results obtained from ELISA test on 22 *Clostridium perfringens* isolates. The table illustrates that out of 22 *C. perfringens* isolates, 12 isolates only were type A and 4 isolates were type C, while 6 isolates were non-toxigenic. These results were unlike those obtained from PCR test which cannot detect the non –toxigenic isolates.

DISCUSSION

Clostridium perfringens is the most commonly isolated clostridial species worldwide and is part of the normal intestinal flora. Following acquisition of this bacterium by the foal from the mare's teats or the environment, the organism multiplies rapidly in the stomach and intestines.

C.perfringens numbers are reduced fairly quickly, so that by the time foals are several months of age, the organism is found in relatively low numbers within the large intestine. The almost universal presence of *C.perfringens* type A in the intestine of healthy young animals has complicated the understanding of its role in enterocolitis. Although most strains don't cause intestinal disease, there are two types that do so in foals. One of these, *C.perfringens* type C is a well established but relatively uncommon cause of necrotizing enteritis. The other is *C.perfringens* type A **(Sims et al. 1985)**.

As enterotoxemia is a complex intestinal illness, the disease may not only be related to the presence of toxins. The occurrence of illness depends on toxin synthesis and on the amount of bacteria (>10^4-10^5 cfu/ml), the presence of toxigenic strains and other conditions (environmental and microbiological) (**Uzal 1996) and (Manteca et al. 2001).**

Necrotizing enterocolitis (damage and death of cells in small intestine and colony) is a serious disease of sudden onset in foals less than 4 -6 days of age associated with a high death rate, despite therapeutic interventions. Clinical manifestations of enterocolitis in foals vary from being found dead to a spectrum of clinical manifestation. The most common of these include failure to suckle or nurse, fever, depression, sever colic and diarrhea **(Niilo and Chalmers 1982)**.

In this study, out of 50 fecal samples from diarrheic foals were examined for the inci-

dence of *C.perfringens,* 22 samples were found to harbor *C.perfringens* in an incidence of 44% which is nearly agree with **Eman *et al.*, 2019 who** isolated *C.perfringens* from foals less than 6 months suffering from diarrhea in an incidence of 46.7%. **(Alison 2013)** concluded that about 50% of foal diarrhea is attributed to *C. perfringens*

Typing of *C.perfringens* isolates in this study by ELISA revealed that, 12 out of 22 isolates were type A (54.5%) and 4 were type C (18.2%) while 6 isolates were non-toxigenic (27.3%).

Naylor et al. (1997) stated that the use of ELISA for the detection of α, β, and ε toxins allows the differential diagnosis of enterotoxemia cases caused by *C.perfringens* types A, B, C, and D from samples of intestinal content as well as the typing of *C.perfringens* cultures.

El- Idrissi and Ward (1992) reported that the comparison of ELISA with conventional assays (mouse assays and culturing of microorganisms) gave sensitivity and specificity rates of 90.5% and 89.2% respectively for β-toxin assays and 97.4% and 94.6% for ε-toxin assays respectively. **Vaikosen and Ikhatua (2005)** reported that out of 91 *Clostridium perfringens* isolates characterized by ELISA, types C and D were detected.

Although measurable levels of toxins did not fully reveal the cause of death, the ELISA kits used in the present study could be evaluated semi-quantitatively.

It was sensitive enough to detect as little as 1.0 ng/ml of purified beta and iota toxins and 0.1 ng/ml of purified epsilon toxin **(Nagahama et al. 1991).**

In this study, out of 22 *C.perfringens* isolates examined and typed with PCR technique, 18 isolates (81.8%) were shown to harbor alpha toxin gene which mean that they are type A, while 4 isolates (18.2%) were found to have alpha and beta toxin genes that mean that they are type C.

However, **Hamad et al. (2018)** examined 100 *C.perfringens* isolates that isolated from cases of enterotoxemia in sheep and typed them using multiplex PCR. The genotyping of these isolates indicated that all isolates were *C.perfringens* and 47% of them were type A (47%), 38 were type D (38%) and 15 were type C (15%) with obvious predominating for type A strains which deserve the attention to this type as a one of major causes of enterotoxemia. They stated that multiplex PCR technique can be used in diagnosis and typing of *C.perfringens* strains instead of convential procedures.

Moreover, (**Eman et al. 2019)** determined the types of isolates using multiplex PCR. Type A was the most predominant in an incidence of 58.3% then B (4.7%).

In addition, **(Miserez et al. 2002**) typed *Clostridium perfringens* isolates from sheep and goats with enterotoxemia using specific PCR assay for the detection of the alpha, beta, and epsilon toxin genes. They found that from 52 animals with pathological signs of enterotoxemia, alpha and epsilon toxin genes only were detected, while beta toxin gene was not detected. These strains could therefore be identified as type D.

Our results proved that, ELISA test showed more sensitivity in detecting *C. perfringens* toxins than PCR as it typed 6 non toxigenic isolates to be type A which may give an assumption that alpha toxin was secreted, while ELISA test gave negative result for alpha toxin of these 6 isolates.

Conclusion:

C. perfringens particularly its type A is a major and common cause of enterotoxemia in foals.

ELISA and PCR are rapid tests for detection and typing of *C. perfringens* isolates and conceder as alternative methods for animal inoculation which is morally forbidden.

It is preferable to use ELISA test for diagnosis of clinical cases of enterotoxemia in field as it detects the presence of toxins in microbial culture as well as intestinal content, while PCR detect the gene of the toxins which may not be expressed and subsequently secreted in the intestinal content.

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