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CLOSTRIDIA ASSOCIATED WITH FOAL DIARRHEA IN EGYPT

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ABSTRACT

Diarrhea is a common and important cause of suffering and loss in foals. The present work was to study the frequency of *Clostridium* infection in foal diarrhea and characterize *C. perfringens* toxins by multiplex polymerase chain reaction (PCR) and ELISA. By collection of fecal samples from both diarrheic (n=50) and apparently healthy (n=200) foals; *Clostridium* species were isolated with incidences of 30 and 7%, respectively. *C. perfringens* and *C. sordellii* were isolated alone from diarrheic cases with incidences of 8 and 2%, respectively and *C. perfringens* was isolated in a mixed form with *C. sporogenes, C. sordellii, C. butyricum, C. tertium and C. bifermentans* with the incidences of 2, 8, 2, 4 and 4%, respectively. The results of multiplex PCR confirmed the results of mice neutralization and dermonecrotic tests for typing *C. perfringens* toxins. ELISA results indicated that production of α-toxin among *C. perfringens* isolated from diarrheic foals was higher than apparently healthy foals.

KEYWORDS: ***Clostridium perfringens*, *Foal, Diarrhea, Multiplex PCR, ELISA***

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INTRODUCTION

Diarrhea in foals can vary from very mild to fatal. Several pathogens have been incriminated as cause of diarrhea in new born and suckling foals like *C. perfringens, C. difficile, Salmonella* species and *R. equi*. The common causative agents of diarrhea are *C. perfringens* and *C.difficile*[1].*Clostridium* species are Gram positive bacterial pathogens that widely propagate in the soil and the gastrointestinal tract of human and animals. They produce potent exotoxins that are responsible for a variety of intestinal diseases in domestic animals. Enteric diseases induced by *Clostridium* species are recognized more commonly during the early neonatal period[2]. *C. perfringens* is a pathogenic organism that produces 5 major exotoxins types A to E[3]. Although types A and C are the most common identified types affecting equines, but all 5 types have been reported[4].*C. difficile* is a serious enteric pathogen and it was associated with foal diarrhea in outbreak of enterocolitis. Uzal *et al*.[5] recorded that *C. perfringens* type C is one of the most important agents of diarrhea in newborn foals and *C. perfringens* infection may be acted as a predisposing factor for *C. difficile* or vice versa. Conventional methods for diagnosis of *Clostridium* species are time consuming and laborious. Multiplex PCR assay has been developed for rapid detection and toxin typing of *C. perfringens* toxin producing strains in feces[6]. The goal of the present study was to isolate *Clostridium* species from foals and detection of *C. perfringens* toxins by multiplex polymerase chain reaction (PCR). Also alpha toxin produced by *C. perfringens* type A isolated from apparently healthy and diarrheic foals was evaluated by ELISA.

MATERIALS AND METHODS

Samples

A total of 250 fecal samples were collected from foals with different age and sex. Fecal samples were collected from 50 diarrheic and 200 apparently healthy foals from El- Zahraa Stud (Ein Shams – Cairo) as shown in Table 1.

Table 1
Age, sex and number of the examined foals

Cases / Age	Diarrheic foals (n=50)		Apparently healthy foals (n=200)		Total (n=250)
	Male	Female	Male	Female	
Newly born foals up to 15 days	16	10	6	7	39
Foals > 15 up to 45 days	15	9	52	35	111
Foals > 45 day up to 4.5 months	0	0	67	33	100
Total	31	19	125	75	250

Isolation of Clostridium species[7]

Each sample was inoculated into cooked meat broth medium (Oxoid) and put in water bath at 80^{o}C for 10 minutes to eliminate non-spore forming microorganisms, then incubated anaerobically in an anaerobic gas pack jar with gas generating kits (Oxoid) for 24 h at 37^{o}C. The inoculated broth was streaked onto 5% sheep blood agar supplemented with neomycin sulphate. The plates were incubated anaerobically at 37^{o}Cfor 24 hours. Another plate was incubated aerobically.

Isolation of C. difficile[8]

One ml of liquid feces or 1 g of solid feces was homogenized in 1 ml of Indus trial alcohol and left for up to 30 minutes to kill off vegetative cells before plating on selective medium. Each sample was inoculated into sterile freshly prepared cooked meat broth (Oxoid) and

incubated anaerobically at 37°Cfor 24 hours. Then streaked onto *C. difficile* agar medium with *C. difficile* supplement (Himedia) ® and incubated anaerobically for 48 hours at 37°C. Another plate was incubated aerobically.

Identification of Clostridium species

The suspected isolates were subjected to detect hemolytic and biochemical activities according to Koneman *et al.*[9] and Quinn *et al.*[10].Nagler's test[11] was performed to identify *C. perfringens* isolates.

Typing of C. perfringens toxins

Using specific *C. perfringens* antisera types A, B, C, D and E (from Serum and Vaccines Production and Research Institute, Abbassia, Cairo, Egypt) , *C. perfringens* enterotoxins were typed after growing on toxin production medium[12] by mice neutralization test and dermonecrotic test[13].

Genotyping of C. perfringens toxins by multiplex PCR

DNA was extracted from *C. perfringens* isolates by heat block according to Sritharan and Barker[14]. Multiplex PCR procedure[15] was carried out using α/*cpa*, β */cpb* and ϵ /*etx* primers of Baums *et al.*[16]. Screening of multiplex PCR products by agarose gel electrophoresis in comparison with 100 bp – 1.5kb DNA ladder (Qiagen) was done[17].

Table 2

Oligonucleotide primers used for amplification of toxin genes among C. perfringens isolates[16].

Toxin/ Toxin gene	Primer designation	nucleotide Sequence	Amplified product size (bp)	Annealing temperature
α/*cpa*	CPA 5 L CPA 5 R	5' AGT'CTACGCTTGGGATGGAA 3´ 5' TTTCCTGGGTTGTCCATTTC 3'	900	56°C
β */cpb*	CPB L CPB R	5´TCCTTTCTTGAGGGAGGATAAA 3' 5' TGAACCTCCTATTTTGTATCCCA 3'	611	39°C
ϵ /*etx*	CPETX L CPETX R	5' TGGGAACTTCGATACAAGCA 3' 5' TTAACTCATCTCCCATAACTGCAC 3'	396	46°C

Evaluation of alpha toxin of C. perfringens type A by ELISA

Ten strains of *C. perfringens* type A were cultivated in cooked meat broth medium and incubated at 37°Cfor 48 hours anaerobically. One colony of each *C. perfringens* type A isolates was cultivated in 10 ml cooked meat broth and incubated for 24 hours anaerobically at 37°C. The culture was inoculated into a flask containing 250 ml of toxin production medium containing 1 – 2% glucose solution (60%) for production of toxins[18]. The extracted *C. perfringens* toxin was diluted 1/50, 1/100, 1/200, 1/400, 1/800 and 1/1600 in coated buffer. 100 µl / well of each diluted antigen were added in ELISA plate. According to Narayanan *et al.*[19] and Peterfy *et al.*[20] ELISA was carried out. The ELISA reading that equal to or higher than the double fold of ELISA reading of the negative control (saline) considered as positive[21]. The result was compared with toxin extracted from *C. perfringens* type A standard strain (positive control).

RESULTS

Incidence of Clostridium species among the examined foals

Fifteen out of 50 diarrheic foals (30%) and 14 out of 200 apparently healthy foals (7%) were harbored *Clostridium* species as shown in Tables 3 & 4. Table 3 showed that *Clostridium* infection were present in 23.1% and 37.5% of the examined fecal samples obtained from diarrheic newly born foals up to 15 days old and diarrheic foals > 15 up to 45 days old, respectively. Among the apparently healthy foals the infected cases with *Clostridium* species were 7.7, 8.1 and 6% in newly born foals up to 15 days old, > 15 up to 45 days old

and > 45 days up to 4.5 months old respectively. Table 2 showed that the diarrheic male foal was highly infected with clostridia (38.7%) in comparison to diarrheic female foal (15.8%).

Table 3
Relation between ages of the examined foals and the Clostridium infection:

Cases / Age	Diarrheic cases			Apparently healthy			Total		
	n	No.	%	N	No.	%	n	No.	%
Newly born up to 15 days	26	6	23.1	13	1	7.7	39	7	17.9
Over 15 days – 45 days Over 45 days -4.5 months	24	9	37.5	87	7	8.1	111	16	14.4
	0	0	0	100	6	6	100	6	6
Total	50	15	30	200	14	7	250	29	11.6

n: number of examined samples . No: number of positive cases.
%: was calculated according to the number of examined samples.

Table 4
Relation between sex of the examined foals and the Clostridium infection:

cases / Sex	Diarrheic cases			Apparently healthy			Total		
	n	No.	%	N	No.	%	n	No.	%
Male	31	12	38.7	125	7	5.6	156	19	12.2
Female	19	3	15.8	75	7	9.3	94	10	10.6
Total	50	15	30	200	14	7	250	29	11.6

n: number of examined samples .
No: number of positive cases.
%: was calculated according to the number of examined samples.

Incidence of C. perfringens among the examined foals

The frequency of distribution of *Clostridium* species obtained from the examined foals was isolated in single or in mixed form (Table 5). Among the diarrheic foals, *C. perfringens* and *C. sordellii* were isolated in a single infection (8% and 2%, respectively) and mixed infection of *C. perfringens* was detected with C. *sporogenes*, C. *sordellii, C. butyricum, C. tertium* and *C. bifermentans* (2, 8, 2, 4 and 4%, respectively). Among the apparently healthy foals, 13 cases infected with *C. perfringens* in a single form with an incidence of 6.5 % and 1 case had mixed infection of *C. perfringens* with *C. sporogenes* in an incidence of 0.5%.No *C. difficile* could be isolated among the examined foals.

Table 5

Prevalence of Clostridium species isolated from diarrheic and apparently healthy foals.

	cases	Diarrheic foals (50)		Apparently healthy foals (200)		Total (250)	
	Clostridium species	Positive cases		Positive cases		Positive cases	
		No.	%	No.	%	No.	%
Single form	*C. perfringens*	4	8	13	6.5	17	6.8
	C. sordellii	1	2	0	0	1	0.4
Mixed form	*C. perfringens* + *C. sporogenes*	1	2	1	0.5	2	0.8
	C. perfringens + *C. sordellii*	4	8	0	0	4	1.6
	C. perfringens + *C. butyricum*	1	2	0	0	1	0.4
	C. perfringens + *C. tertium*	2	4	0	0	2	0.8
	C. perfringens + *C. bifermentans*	2	4	0	0	2	0.8
	Total	**15**	**30**	**14**	**7**	**29**	**11.6**

%: was calculated according to the number of examined samples

Typing of toxin producing C. perfringens isolates by dermonecrotic and mice neutralization tests

Typing of *C. perfringens* isolates were done using mice neutralization and dermonecrotic tests. Table 6 shows that all *C. perfringens* isolated from diarrheic foals were typed as toxin producing strains. It is clear that, *C. perfringens* toxin type A was the most predominant type in an incidence of 64.3% followed by type C (21.4%) then type B and type D (7.1% each) among *C. perfringens* isolated from diarrheic foals. Among the apparently healthy foals 3 *C. perfringens* isolates were non toxin producing strains (21.4 %) while 42.9, 28.6% and 7.1%, of the isolates produced toxin A, D and B respectively.

Table 6

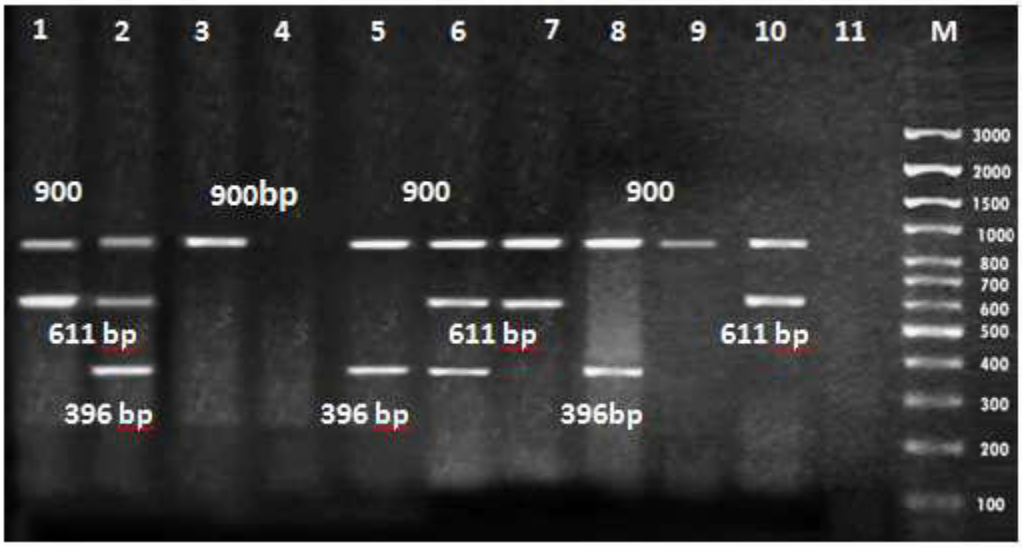
Toxin typing of C. perfringens isolated from fecal samples of diarrheic and apparently foals.

Types of *C. perfringens*	Diarrheic foals		Apparently healthy foals		Total	
	No.	%	No.	%	No.	%
Type (A)	9	64.3	6	42.9	15	53.6
Type (B)	1	7.1	1	7.1	2	7.1
Type (C)	3	21.4	0	0	3	10.7
Type (D)	1	7.1	4	28.6	5	17.9
Non toxigenic	0	0	3	21.4	3	10.7
Total	14	100	14	100	28	100

Detection of C. perfringens toxins by multiplex PCR

Figure1 shows that the 900 bp fragment of *cpa* gene was amplified from all *C. perfringens* isolates and the 369 bp fragment of *etx* gene was amplified from *C. perfringens* types B and D. While the amplified fragment of 611 bp of *cpb* gene was detected in *C. perfringens* types B and C.

Figure 1
Profile analysis of C. perfringens toxins using multiplex PCR.



Lane M: 100 bp marker (Qiagen). Lanes 1, 7 & 10: C. perfringens type C, Lanes 2 & 6: C. perfringens type B, Lanes 3 & 9: C. perfringens type A, Lanes 4 & 11: -ve control and Lanes 5 & 8 C. perfringens type D.

Titration of C. perfringens toxins using ELISA

The present study evaluated the production of alpha toxin among 10 *C. perfringens* isolates type A (7 isolates from diarrheic and 3 from apparently healthy foals) using ELISA. Large differences in the production of alpha toxin were detected, *C. perfringens* type A isolated from diarrheic foals produced higher amount of alpha toxin than *C. perfringens* isolated from apparently healthy foals.

DISCUSSION

Diarrhea in horses associated with high mortality. In severe cases, death may occur even before the onset of diarrhea and many of the clinical signs associated with acute diarrhea are indistinguishable. Clinical progression leads to severe dehydration and profound electrolyte disturbances[22]. Herholz *et al.*[23] showed a high rate of isolation of *C. perfringens* from horses with intestinal diseases. *C. perfringens* can cause gas gangrene and gastrointestinal illness in humans, necrotic enteritis in chickens and hemorrhagic enteritis in calves[24]. The objective of this study was to estimate the incidence of clostridia in diarrheic foals. A total number of 250 fecal samples were collected from diarrheic foals (50 cases) and apparently healthy foals (200 cases) for detection of *Clostridium* species. As shown in Tables 3&4, *Clostridium* species were isolated from diarrheic and apparently healthy foals with incidences of 30% and 7%, respectively. This result is in agreement with East *et al*.[4] who isolated clostridia from (26%) of examined neonatal foals suffer from enterocolitis; while Tariel et *al.*[25] isolated clostridia in an incidence of 70% among diarrheic horses. Tables 3&4 showed that *Clostridium* infections were present in 23.1% and 37.5% of the examined fecal samples obtained from diarrheic newly born foals up to 15 days old and from diarrheic foals > 15 up to 45 days old, respectively. *Clostridium* species could not be isolated from diarrheic foals > 45 days old. East *et al.*[4] isolated *C. perfringens* of diarrheic foals < 10 days old with an incidence of 80%, Terriwagner[26] isolated *C. perfringens* from diarrheic foals had ages of 2 days. Table 4 showed that male foal was highly infected with clostridia with an incidence of 38.7% in comparison to diarrheic female foal (15.8%). The bacterial isolation in diarrhea depends mainly on the age of animal and the

surrounding environment as well as weather, management which act as predisposing factor on animal for inducing diarrhea[22].

As shown in Table 5 C*. sordellii* was isolated singly and in mixed infection with *C. perfringens* in diarrheic foals with incidences of 2 and 8%, respectively. Other *Clostridium* species (*C. sporogenes*, *C. sordellii, C. butyricum, C. tertium* and *C. bifermentans*).were isolated from diarrheic and apparently healthy foals in mixed infection with *C. perfringens*. *C. perfringens* was isolated from the collected fecal samples of diarrheic and apparently healthy foals with incidences of 28 and 7%, respectively. In this concern Kanoe *et al.*[27]isolated *C. perfringens* from diarrheic and apparently healthy foals with incidences of 100 and 14%, respectively. Also *C. perfringens* were recorded from foals by Netherwood *et al.*[28]. *C. perfringens* produces numerous toxins responsible for severe diseases in humans and animals. Individual strains produce subsets of toxins, depending on the production of the four major toxins, alpha (α), beta (β), epsilon (Є) and iota (*i*), *C. perfringens* are divided into five toxigenic types, A, B, C, D and E[29]. The *C. perfringens* isolates were characterized for their toxins production using the classical typing method and multiplex PCR (Table 6 and Figure 1). All *C. perfringens* isolates recovered from diarrheic foals were toxin producing isolates. It is clear that C*. perfringens* type "A" was the most prevalent one. Foals with gastrointestinal diseases, type A represented 90% of the isolates of *C. perfringens*[27]. However, other studies showed that enterotoxigenic *C. perfringens* isolates do not play a role in intestinal disorders of horses[30]. As shown in Table 6 *C. perfringens* type "C" was detected from the examined diarrheic foals (21.4%). *C. perfringens* type A is the predominant isolates followed by *C. perfringens* type C from enteric cases of neonatal foals[31]. The common causative agents of infectious foal diarrhea were *C. perfringens* biotypes A and C[2].*C. perfringens* isolated by Donahue and Williams[32] from foals with enterocolitis were type A (60%) and 40% were type C. Regarding *C. perfringens* isolated from feces of apparently healthy foals, toxigenic and non toxigenic isolates of *C. perfringens* represented 78.6 and 21.4%, respectively. And of toxigenic isolates types A, B and D of were detected with incidences of 42.9, 7.1 and 28.6%, respectively.

Molecular genotyping of *C. perfringens* isolates using multiplex PCR (Figure 1) illustrated that all of the examined isolates had α toxin (900 bp). Alpha toxin is commonly produced by all five types and is a predominant product of *C. perfringens* type A[29].The *cpb* gene (611 bp) was amplified from *C. perfringens* types B and C. The β toxin is a major lethal toxin produced by types B and C and is known to play a major role in the pathogenesis of enterotoxemia and necrotic enteritis in humans and animals[29], β2-toxigenic *C .perfringens* not only may play an important role in the pathogenesis of equine typhlocolitis and other intestinal disorders of equines but also might be involved in the fatal progression of typhlocolitis in horses[23]. *C. perfringens* alpha toxin could be detected by Naylor *et al.*[33] from both the intestinal contents of animals died from suspected *C. perfringens* type A enterotoxemia and from culture supernatants of *C. perfringens* isolates using ELISA. ELISA was used to measure the concentration of alpha toxin production among *C. perfringens* type A isolated from both diarrheic and apparently healthy foals, the results revealed that alpha toxin was present in higher concentration among diarrheic cases than apparently healthy cases. It could be concluded that *C. perfringens* was isolated singly or in mixed infection from the examined foals. *C. perfringens* was more frequent in both diarrheic and apparently healthy foals with high frequency in diarrheic foals than apparently healthy one which reflects the importance role of *C. perfringens* in cause's diarrhea in foals. Multiplex PCR and ELISA tests are sensitive, rapid.

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