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POTENTIAL ROLE OF *CLOSTRIDIUM DIFFICILE* AND *CLOSTRIDIUM PERFRINGENS* AS A CAUSE OF DIARRHEA IN HORSES

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ABSTRACT

Diarrhea is a major clinical problem affecting foals and horses at different ages which can be fatal and cause great economic losses particularly in foals. The aim of this study was to identify *Clostridium perfringens* and *Clostridium difficile* involved in enteric infections and the associated virulence factors in diarrheic foals and horses. Thirty fecal samples were examined for identification of *C.perfringens* and *C.difficile* (bacteriological culturing, microscopical examination, count and molecular detection). *C.perfringens* was isolated from young horses less than 6 months age in an incidence of 46.7% with count ranged from 1.3×10^4- 5.6×10^8 cfu/g, while from horses of 6 months to one year, it was 20% with count ranged from 1.7×10^4- 1.9×10^6 cfu/g, on the other hand, 13.3% with count ranged from 1.4×10^3-2.5×10^4 cfu/g was the incidence of *C.perfringens* in horse more than one year. *C. difficile* was not detected in all examined samples. *C.perfringens* type A was the most predominant type detected in an incidence of 58.3% then type B (4.7%). All *C.perfringens* isolates were sensitive to penicillin, nitromedazole, fluramfenicole and ceftiofur and all are resistant to oxytetracycline. Based on the high count, eight *C.perfringens* isolates were chosen to be molecular characterized for the presence of some virulence factor genes which included ; *cpa*, *cpb2*, *cpe* and *netF* toxin genes. All examined isolates have cpa (alpha) and cpb2 (beta 2) toxin genes, 5/8 isolates were found to have *netF* gene while only 2 isolates harbored *cpe* (enterotoxin gene). *C. difficile tpi* gene was not detected in all examined samples (10). This study may throw the light on the synergistic effect of β2 and net F toxins together with alpha toxin on induction of enterocolitis in horse.

Key words: C.difficile , C.perfringens , Diarrhea, enteritis , horses , netF gene.

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INTRODUCTION

Diarrhea is one the most common causes of mortality of adult horses and foals, which results in economic losses worldwide (**Frederick *et al.*, 2009**). Until 6 months of life, up to 20% of foals have been reported to suffer from diarrhea caused by infectious agents .The etiology of diarrhea in neonatal foals is complex and involves infectious agents, management and facilities as well as nutritional environmental and physiological condition.

Infectious causes of horse diarrhea predominantly include bacteria, viruses, and parasitic agents (**Solvis, *et a.*, 2010**). Some studies about diarrhea in neonatal foals have focused on only one pathogen although it is usually caused by diverse combination of enteric infections (**Harris *et al.*,2012**.)

Horses are at risk of developing colitis when there has been disruption to the balance of the normal microbiota in the intestinal tract. Such disruptions include a rapid change in diet, anorexia due to disease , and stressful events (that also lead to diet changes)such as travel, competition,or hospitalization ,antibiotic administration can also significantly alter the intestinal microbiota of the intestinal tract . Certain bacteria can thrive and multiply in this alter condition, the most harmful examples being salmonella spp. and clostridium spp.(**Barr *et al.*,2012**).

Clostridium difficile is a Gram-positive anaerobic sporulating bacillus which has been associated with diarrhea and colitis in human and other mammals One of the most remarkable characteristics of this disease is that, in almost all cases, it occurs following antibiotic therapy. The pathogenesis of *C.*

difficile-associated disease (CDAD) is very particular and allows a better understanding of the circumstances in which the clinician should make a diagnosis. The very first condition to induce pathology is a disturbance of the normal intestinal flora.The gut flora act as a colonization barrier which, in a normal state, protecting against *C. difficile*. This barrier is compromised when the flora is disturbed. The main factor able to induce such a disturbance is antibiotic therapy **(Keel and Songer,2006**). The primary virulence factors of *C.difficille* are the two major toxins, toxin A (TcdA) and toxin B (TcdB) (Vedantam *et al.*, 2012).

Clostridium perfringens is a Gram positive anaerobic rod, which is ubiquitous in its environment, being found in the soil and decaying organic matter, and is also a member of the normal gut flora of many animals. The ability of the bacterium to form highly resistant endospores means that it is able to persist in the environment. However, if the microbiota has been disturbed and the conditions are right, the spores can transform into the active vegetative state multiply and potentially produce toxins. Types of *C. perfringens* are differentiated (five major types: A, B, C, D and E) based on the production of four major exotoxins: α, β, ε and i. All strains of the bacterium possess the gene encoding the α toxin (CPA). The differential possession of other toxinencoding genes is used to identify strains as biotypes A to E (**McDonel , 1986**). The role of type A *C. perfringens* in enteric diseases of horses is also not well understood. There is evidence that CPB2 toxin-producing *C. perfringens* play a role in the fatal progression of colitis in horses **(Herholz *et al.*, 1999) and (Bacciarini *et al.*, 2003).**

Vilei *et al.*, (2005) demonstrated that cpb2-positive equine disease isolates produced the CPB2 toxin when grown in sub-inhibitory concentrations of gentamicin. Anecdotally, there was an association between the isolation of cpb2-positive *C. perfringens* from cases of equine colitis and the use of gentamicin in hospitalized diarrheic horses, which ended when the antibiotic was stopped. They proposed a feasible direct role of these isolates in antibiotic-associated diarrhea in horses. Some *C.perfringens* isolates may have the gene known as *C. perfringens* enterotoxin (CPE), which is produced by sporulating cells in an alkaline environment and is released on lysis of these cells. It is resistant to proteolytic enzymes and will bind and insert on the brush border membrane causing pore formation in cells, leading ultimately to cell lysis **(Freedman *et al.*, 2016).**

More recently, associations with cpb_2 β2 toxin positive *C.perfringens* as well as *cpe* enterotoxin positive *C.perfringens* with equine enteritis and colitis has been described (**Weese *et al.*,2001)**.
A novel pore-forming toxin in type A *C.perfringens* named netF is associated with fatal foal necrotizing enterocolitis **(Diab *et al.*, 2013 a**). There was a highly significant association between the presence of netF with type A strains isolated from cases of foal necrotizing enterocolitis. **Mehdizadeh Gohari *et al.*,(2016)** investigated the role of both net F and enterotoxin in induction of enterocolitis in foals.

The aim of our study is to throw the light on the impacts of enteric clostridial (*Cperfringens* & *C.difficile*) co-infections in foals and adult horses with antibiotic associated diarrhea and identify their prevalence and virulent factors.

MATERIALS AND METHODS

Animals

This study was done on thirty horses at different ages and breed from different localities in Cairo and Giza governorates (brooks hospital, Vet. Clinics,clinics of Cairo Vet. Med. College and from sporadic cases in different stalls) suffering from persistent diarrhea with a case history of treatment with antibiotic (antibiotic associated diarrhea) with acute colitis or enterocolitis, as evidenced by the acute onset of diarrhea with multiple clinical signs of toxemia, such as fever,dehydration, tachycardia, tachypnea, and congested mucous membranes. Ten (10) of the thirty cases had a case history of intermittent diarrhea .

Fecal samples were obtained from horses during defecation over a seven months period (October 2017 to April 2018) each in a separate sterile bag and frozen at -80°C till used. Samples divided into three groups according to age (table 1)

Table 1: Groups of animals according to ages

Groups	No.
Group1(Less than 6 month)	14
Group2 (6-12 month)	6
Group3(More than12 month)	10
Total	30

Isolation and identification

According to **Quinn *et al.*, (2011) and Schoster *et al.*, (2012)**

1.*Clostridium difficile* detection

One gram (1 g) of fecal sample was inoculated in 9 mL of cooked meat broth (Oxoid,), and incubated

anaerobically at 37°C for 5 days. After incubation, 2 mL was transferred into a sterile test tube, mixed with an equal amount of absolute ethanol, and left at room temperature for one hour. Samples were then centrifuged at 3800g for 10 min. The supernatant was discarded and the resulting pellet was plated onto *Clostridium difficile* agar supplemented with cycloserine-cefoxitin and 7% horse blood and 0.1% sodium taurocholatec . After anaerobic incubation at 37°C for 72 h, all colonies with suggestive morphology of *Clostridium difficile* was identified on the basis of typical colonial characteristics, Gram-stain morphology.

2. Enumeration of *C.difficile* in the fecal samples

For quantitative culture ,1 g of feces was diluted in 9 ml of phosphate buffered saline at ph 7.2 and 10- fold serial dilutions (10^1-10^5) were prepared , One hundred microliter (100 µ L) aliquots of these dilutions were cultured on *Clostridium difficile* agar and incubated anaerobically (**Schoster *et al* 2012).**

3. *Clostridium perfringens* detection and enumeration

Feces were inoculated into PBS at a ratio of 1:9 (w/v) and serial ten dilutions prepared. One hundred microliter (100 µ L) aliquots of these dilutions were streaked on Shahidi Ferguson Perfringens (SFP) agar (Becton, Dickinson) containing 5% egg yolk emulsion (Oxoid) and *C. perfringens* selective supplement (Oxoid) and incubated under anaerobic conditions overnight at 37°C. Plates containing 20 to 200 lecithinase-positive colonies were selected for bacterial enumeration. To confirm the identity of the lecithinase-positive colonies, 5 presumptive *C. perfringens* colonies were sub-cultured onto 200 µg/ml neomycin blood agar plates, incubated anaerobically and examined for the large double zone of hemolysis , the microscopic morphology characteristic and biochemical tests of *C. perfringens.*

4. Typing of *Clostridium perfringens* isolates

Types and toxicity of *C.perfringens* isolates were detected by dermo necrotic reaction in laboratory animal (albino guinea pigs) according to **Stern and Batty(1975**).

5. Antimicrobial susceptibility test: (Koneman et al., 1992)

Disc diffusion test was performed to 24 *C.perfringens* isolates. The following antimicrobials were evaluated: penicillin, florfenicol, oxytetracycline, erythromycin, vancomycin, metronidazole, ciftiofur, epicoflosin and cefuroxime. The results were interpreted according to **NCCLS (2002).**

6. Genotyping of *C.perfringens* and *C. difficile* using PCR

Genotyping of *Clostridium perfringens* isolates: Eight *C. perfringens* isolates from samples of the highest count and sever toxicity to laboratory animals were subjected to multiplex polymerase chain reaction (PCR) for detection of: cpa (alpha toxin), cpb (beta toxin), cpb2 (beta2 toxin) consensus, cpe (enterotoxin), etx (epsilon toxin), and netf genes. Genotyping of *C.difficile* to detect tpi gene of ten fecal samples with a case history of intermittent diarrhea was also done. Genotyping was done using primers, probes, and conditions described in a table (3).

6.1. DNA extraction from isolates

Extraction from (8)*C. perfringens* isolates using PathoGene-SpinTM DNA/RNA Extraction kit iNtRON cat. No. 17154 Korea according to the instructions of the manufacturer. DNA extraction from(10) fecal samples for C.difficile tpi gene detection **.** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

6.2. PCR amplification

Oligonucleotide Primer used for *C.perfringens* in this study were synthesized by Sigma Company, (Germany), while Oligonucleotide Primer used for C.*difficile* were supplied from Metabion (Germany) all are listed in table (2). ***C. difficile*** primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. ***C. perfringens*** primers were performed in Gradient Thermal cycler (S 1000 Thermal cycler Bio-RAD USA).The reaction mixture (total volume of 50 µl) was 25 µl M.Mix (Cosmo PCR red Master Mix (2X) Willowfort W1020300x), England), 2 µl target DNA, 1 µl of each primers (containing 10 p mole/ µl) and the mixture was completed by water nuclease free to 50 µl .

6.3. Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each

gel slot. Gelpilot 100 bp (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 2: Target genes primers used in this study with their sequences

Organisms	Target gene	Primer sequence 5'-3'	Fragm bp	Reference
C. *perfringens*	*plc (cpa)*	F : GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324	**Ansari *et al.*, (2010)**
	cpb	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATCTTC	196	
	etx	F: GCGGTGATATCCATCTATTC R: CCACTTACTTGTCCTACTAAC	655	
	cpe	F: TTCAGTTGGATTTACTTCTG R: TGTCCAGTAGCTGTAATTGT	485	**Van Asten *et al., (*2009)**
	cbp2	F: AAATATGATCCTAACCAACAA R: CCAAATACTCTAATCGATGC	548	
	netF	F:AACAATATGTACAGGTATAACT R:TTGATAGGTATAATATGGTTCT	862	**Mehdizadeh. Gohari *et al.*, (2015)**
C. difficile	*tp*i	F:AAAGAAGCTACTAAGGGTACAAA R:CATAATATTGGGTCTATTCCTAC	230	**Rajabally *et al.*, (2016)**

Table 3: PCR cyclic conditions for genes detected in this study

Gene	Stage (ºC)	Temp.	Time	No. of cycle
Major toxins of *C. perfringens* (*cpa,cpb,etx)*	1ˢᵗ Denaturation	94	2	1
	2ⁿᵈ Denaturation	94	15 sec.	35
	Annealing	55	30 sec.	
	Extension	68	1 min.	
cpe	1ˢᵗ Denaturation	95	15 min.	1
	2ⁿᵈ Denaturation	94	30 sec.	40
	Annealing	53	90 sec.	
	Extension	72	90 sec.	
	Final extension	72	10 min	1
cpb2	1ˢᵗ Denaturation	95	15 min	1
	2ⁿᵈ Denaturation	94	30 sec.	40
	Annealing	53	1.5 min.	
	Extension	72	1.5 min.	
	Final extension	72	10 min.	1
netF	1ˢᵗ Denaturation	94	3 min.	1
	2ⁿᵈ Denaturation	94	30 sec.	30
	Annealing	45	30 sec	
	Extension	72	1 min.	
	Final extension	72	5 min	1
C. difficile tpi	1ˢᵗ Denaturation	94˚C	5 min	1
	2ⁿᵈ denaturation	94˚C	30 sec.	35
	Annealing	65-55˚C	30sec	
	Extension.	72˚C	30 sec	
	Final extension	72˚C	10 min	1

RESULTS

Atotal of 30 fecal samples were bacteriologically examined for presence of *C.perfringens* and *C.difficile* and their toxins. *C.perfringens* and *C.difficile* identified in the diarrheic foals and adult horses at different ages were as follows : All foals were positive for *C.perfringens;* 46.7% for foals less than 6 months and 20% for those between 6-12 months while animals more than one year harbored *C.perfringens* in an incidence of 13.3%. None of animals were positive for *C.difficile* (table 4).

Table 4: prevalence of *C. perfringens* and *C.difficile* in diarrheic foals and adult horses

Cases	No of animals/group	*C.perfringens*		*C.difficile*	
		No.	%	No	%*
Less than 6 months	14	14	46.7	0	0
6-12 months	6	6	20		
More than a year	10	4	13.3		
Total	30	24	80	0	0

* The percentage is calculated according the total no of samples

Among the 24 isolates of *C.perfringens* that tested by dermonecrotic reaction in Guinea pig, *C.perfringens* type A was identified in 58.3% of both diarrheic foals and adult horses, while (4.7%) was type B, on the other hand, 37.3% of the isolates were non toxigenic Table (5).

Table 5: Typing and toxigenicty of *C. perfringens* isolates

No of isolates	Toxigenic strain								Non Toxigenic strain	
	Type A		Type B		Type C		Type D			
	No	%	No	%	No	%	No	%	No	%*
24	14	58.3	1	4.2	0	0	0	0	9	37.5

*The percentage is calculated according the total no of isolates

Table 6: illustrates the numbers of toxigenic and non- toxigenic *C.perfringens* isolates according to ages of animals. Fourteen foals less than 6 months harbored *C.perfringens* type A in an incidence of 37.5% and type B in an incidence of 4.2%, while nontoxigenic isolates were (16.7%). Moreover, *C.perfringens* type A was isolated from foals between 6-12 months in an incidence of 20.8% and one isolate was nontoxigenic (4.2%). On the other hand, all *C.perfringens* isolatesfrom animals more than 12 months were nontoxigenic (16.7%).

Table (6): Toxigenicity of *C.perfringens* according to ages of animals

Animal age/ Number	Toxigenic isolates		Non toxigenic
	Type A (%)	Type B (%)	
Group 1: Less than 6 months(14)	9 (37.5)	1 (4.2)	4 (16.7%)
Group 2: 6-12 months(6)	5 (20.8)	-	1 (4.2%)
Group 3: More than 12 months(4)	-	-	4 (16.7%)
Total (24)	14(58%)	1(4.2%)	9 (37.5%)

* The percentage is calculated according the total no of isolates (24)

Table 7: showed the enumeration of *C.perfringens* in cfu/g and *C. difficile* of fecal samples of 30 diarrheic foals and adult horses. It illustrated the cases having high count of the organism for *C.perfringens* and cases having intermittent diarrhea for *C. difficile* which were consequently chosen to be examined genotypically.

Table 7: Enumeration of *C.perfringens* and *C. difficile* in fecal samples of horses with enterocolitis

Case NO	Groups	Age	*C.perfringens* cfu/g	*C.difficile*
1	Group 1	1w*	5.6×10^{6}	N
2		10D	8.0×10^{5}	N
3		2w*	3.4×10^{6}	N
4		2w*	1.3×10^{7}	N
5		3W*	1.9×10^{6}	N
6		1m	1.3×10^{4}	N
7		1m•	5.6×10^{5}	N
8		6w	1.8×10^{5}	N
9		6W*•	1.8×10^{6}	N
10		7w	1.4×10^{4}	N
11		8W•	4.5×10^{4}	N
12		2m*	1.6×10^{6}	N
13		3m	7.5×10^{5}	N
14		5m*•	2.8×10^{7}	N
15	Gr 2	9m	1.7×10^{4}	N

Case No	Groups	Age	*C.perfringens* cfu/g	*C.difficle*
16	Group 2	9m*•	1.9×10^{6}	N
17		10m•	3.0×10^{4}	N
18		10m	5.6×10^{5}	N
19		11m	1.8×10^{4}	N
20		12m•	7.1×10^{4}	N
21	Group 3	13m	ND	N
22		13m	ND	N
23		13m•	5.6×10^{5}	N
24		13m•	5.6×10^{5}	N
25		14m	ND	N
26		14m	ND	N
27		16m•	2.5×10^{4}	N
28		16m	2.2×10^{5}	N
29		17m	ND	N
30		18m	ND	N

*High count; N –Not detected; M- month; W-week; D- day N-Negative •intermittent diarrhea

The results of sensitivity test for 24 *C.perfringens* isolates were illustrated in table (8). All isolates were susceptible to penicillin, metronidazole, florfenicol and ceftiofur while 20 of 24 isolates were susceptible to

erythromycin and epicoflosin, meanwhile, 16 out of 24 were sensitive to cefuroxime and vancomycin. On the other hand, all isolates were resistant to oxytetracyclin.

Table 8: Antimicrobial susceptibility test of 24 *C.perfringens* isolates

Antibiotic	Symbols/potency	Classification		
		Susceptible	Intermediate	Resistant
Penicillin	P 10	24/24	0	0
Metronidazole	MTZ 10	24/24	0	0
Florfenicol	FFC 30	24/24	0	0
Ceftiofur	EFT 30	24/24	0	0
Erythromycin	E 30	20/24	0	0
Epicoflosin		20/24	0	0
Cefuroxime	CXM 30	0	16/24	0
Vancomycin	VA 10	0	16/24	0
Oxytetracyclin	OT 30	0	0	24/24

% calculated according to the No. of tested *C.perfringens* (24)

Eight isolates from samples of the highest count and sever toxicity on laboratory animals were tested for presence of beta 2 toxin (*cpb2*) ,enterotoxin (*cpe*) and *net F* toxin genes ,all of them harbor beta2 toxin encoding gene two isolates were positive for enterotoxin encoding gene. Meanwhile, five isolates were positive for net F encoding gene (table 9).

Table 9: Prevalence of cpa,beta2, cpe and netF toxins genes in eight *C.perfringens* isolates by PCR

Isolates	Animal age	Toxin genes			
		cpa	*cpb2*	*cpe*	*netF*
1	1w	+	+	-	+
2	2w	+	+	-	+
3	2w	+	+	-	+
4	3W	+	+	-	-
5	6W	+	+	+	-
6	2m	+	+	+	-
7	5m	+	+	-	+
8	9m	+	+	-	+

**cpa*: alpha toxin gene; *cpb2*:beta2 toxin gene; *cpe*: enterotoxin gene; *netF*: necrotic enteritis toxin gene F.

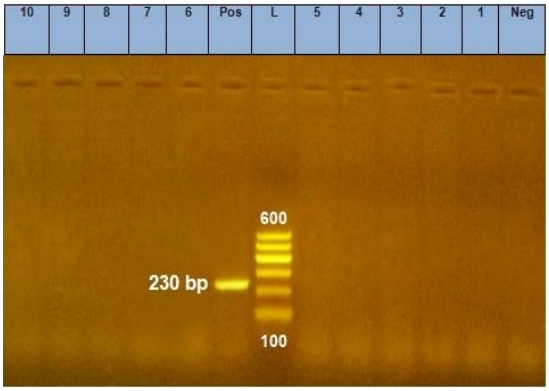

Photo 1. Agarose gel (1.5%) electrophoresis of *Clostridium difficile*
L: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)
Pos.:Control Positive.
Neg.: control Negative
Lane1-10: PCR product of fecal samples

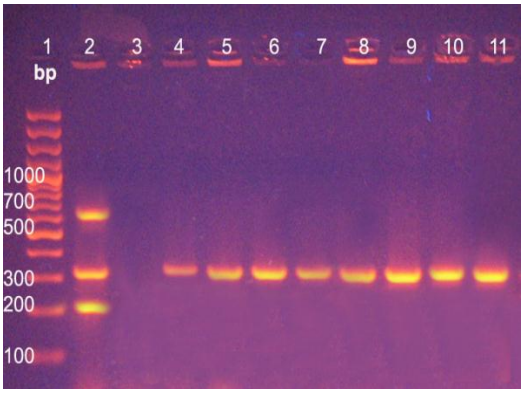

Photo 2. Agarose gel (1.5%) electrophoresis of multiplex PCR products obtained with various *Clostridium perfringens* toxin types.
Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)
Lane 2:Control Positive (mix of various toxin types).
Lane 3: control Negative
Lane4-11: PCR product of toxin from isolates

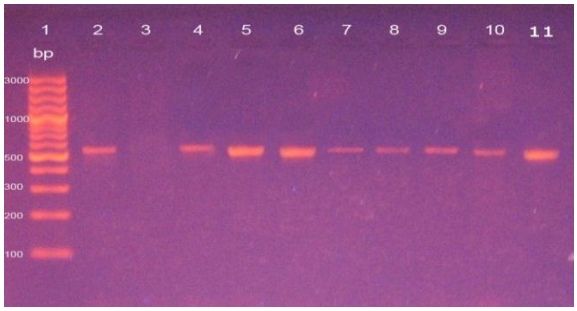

Photo3. Agarose gel (1.5%) electrophoresis of *Clostridium perfringens* of gene encoding for the beta2 toxin (cpb2)
Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)
Lane 2:Control Positive.
Lane 3: control Negative
Lane4-11: PCR product of cpb2 gene from isolates

Photo4. Agarose gel (1.5%) electrophoresis of *Clostridium perfringens* of enterotoxin
Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)
Lane 2:Control Positive.
Lane 3: control Negative
Lane4-11: PCR product of enterotoxin from isolates

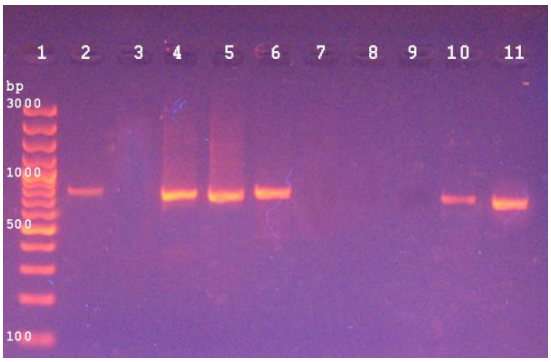

Photo 5. Agarose gel (1.5%) electrophoresis of Clostridium perfringens of Net F
Lane 1: DNA marker (GeneRuler 100 bp DNA Ladder, Fermentas)
Lane 2:Control Positive.
Lane 3: control Negative
Lane4-11: PCR product of Net F gene from isolates

DISCUSSION

Clostridium perfringens and *C. difficile* are considered frequent causes of colitis in human and livestock **(Radostitis *et al*.2007**). Enteric infections are prevalent in adult horses, neonatal foals, foals with antimicrobial associated diarrhea and equines affected by nosocomial outbreak in veterinary hospitals (**East *et al* 1998)** *Clostridium perfringens* commonly associated with necrotic enteritis in neonatal foals (**Frederick *et al* 2009**)

Twenty four out of thirty fecal samples (80%) were positive for *C.perfringens* while none of samples gave positive result for *C.difficile* (Table4) .In relation to ages, *C.perfringens* was detected from 46.7% of examined foals less than 6 months in age while 20% of 6 to 12 months age was positive for *C.perfringens* .On the other hand ,13.3 of horses more than a year harbored this organism .(Table 4).**Mehdizadeh Gohari *et al*., 2014** discussed bacterial causes of entero colitis in horses to improve understanding of these causes .They isolated *C.perfringens* and *C. difficile* in an incidence of 40% and 5.4% respectively in adult horses ,while in foals ,they detected *C.perfringens* in an incidence of 42% and no *C. difficile* was isolated .**Silva *et al.*, 2013**,detected *C.perfringens* and *C. difficile* in farm foals incidence of 31.8% (20/ 63) and 1.6 % (1/63) respectively.

On contrast of our result ,**Weese *et al*.,2001** contributed the cause on enterocolitis in 31 diarrheic foals to *C. difficile* which was isolated in an incidence of 35.5% .Furthermore , **Thean *et al.*, 2011** isolated *C. difficile* from diarrheic horses in an incidence of 23% .The author together with **Frederick *et al.*, 2009, and Diab *et al.*, (2013a)** stated that *C. difficile* rates in foals and adult horses with gastrointestinal diseases vary considerably among authors ranging from 5-63 %. This variability may reflect differences in the study designs, sensitivity and specificity of diagnostic tests, regional or temporal prevalence, sample collection , animal age, predisposing factors etc.

Clostridium difficile can be a difficult organism to isolate and is poorly aero- tolerant, this poor aero tolerance can result in false negative culture results particularly when there is a delay from sample collection to processing. Moreover, **Diab *et al.*, (2013b**) suggests that *C.difficile* shedding is transient and dynamic, that horses may harbor a strain for short periods of time. This may explain that none of the samples gave positive results for *C.difficile* neither on specific culture nor on PCR examination in our study (Table 4, photo1). *Clostridium perfringens* has been associated with enterocolitis in animals, including horses and humans (**Songer ,1996** and **Waggett *et al.*, 2010**) . This organism is mostly associated with colonic disease, but has been also identified in the small intestine of horses with gastrointestinal disease. **(Herholz *et al.*, 1999).**

In our study ,twenty four out of 30 fecal samples (80%) were positive for *C.perfringens* from which 58.3% were type A and 4.7% were type B, meanwhile 37.5% of isolates were non toxigenic as shown in Table (5). Type A *C.perfringens* is commonly found in the large intestine of animals including horses and is candidate as an agent for this often fatal disease. Considering the toxigenicity of *C.perfringens* in relation to animal ages ,foals less than 6 months showed the high percent of toxigenic pathogen in an incidence of 37.5% as type A and 4.2% as type B and 16.7% were nontoxigenic strains, while horses ranging from 6-12 months were found to harbor *C.perfringens* type A in an incidence of 20.8% and nontoxigenic *C.perfringens* strains was 4.2% , on the other hand, all isolates from adult horses more than one year were non toxigenic (16.7%) as shown in (Table 6). This result is more than that recovered by **Mehdizadeh Gohari,*et al.*, 2014** who isolated *C.perfringens* type A from foals and adult horses in an incidence of 17% and 14% respectivily.

Clostridium perfringens can be normal inhabitant of the intestine of most animals. Culture of this micro-organism from intestinal contents of animals has no diagnostic value unless a colony count is performed and large numbers (usually more than 10^4-10^7cfu/g) of *C.perfringens* are found. The most accepted criterion in establishing a definitive diagnosis of enterotoxaemia by *C.perfringens* is the detection of its toxins in intestinal contents **(Uzal, 2004).** Subsequently, enumeration of *C.perfringens* in the feces of horses at different ages was performed and the highest counts were chosen to be molecularly characterized.

The highest counts of *C.perfringens* were recorded in group 1 which threw the light on the susceptibility of foals to infection than adults. This result agreed with that achieved by **Barr *et al.*, 2013** who stated that foals are more prone to intestinal clostridial disease. At group 1 (less than 6 months), the count of *C.perfringens* ranged from $1.3x10^4$ to $5.6x10^8$ cfu/g while group 2 (6-12 months) ranged from $1.7x10^4$ to $1.9x10^6$ cfu/g .On the other hand, the count of *C.perfringens* In group 3 (more than a year) was ranging from $1.4x10^3$ to $2.5x10^4$ cfu/g. (Table 7).

Antimicrobial susceptibility test was performed to 24 *C.perfringens* isolates against 9 antibiotics. All isolates were susceptible to penicillin, metronidazole, florfenicol and ceftiofur corroborating the study of **Silva *et al.*, 2013,** meanwhile, resistance of *C.perfringens* isolates was reported against oxytetracyclin which is different to the finding of same author who reported 17% resistance of *C.perfringens* against oxytetracyclin. It was concluded that *Clostridium* species can carry tetracycline resistance genes that encode a ribosome-protecting cytoplasmic protein that mediates the active efflux of tetracycline from the cell **(Silva *et al.*, 2013).** The incidence of a new, yet unassigned toxin type of *C.perfringens* containing the genes for the alpha –toxin (*cpa*) and the recently described Beta –toxin in horses with intestinal disordered was reported. (**Herholz *et al.* ,1999).**

Since the alpha –toxin, which is produced by all types of *C.perfringens* including nonpathogenic, type A strains is not considered primary cause of digestive lesions (**Hathway,1990**), it was suggested that the β toxin when is present in this new type of *C.perfringens* play a rate in causing the digestive disease (**Gilbert *et al* ,1997**) In the present study, eight *C. perfringens* isolates were chosen according to their high count number from samples they isolated and their toxicity to laboratory animal to be examined for some toxin genes other than alpha toxin which is considered as major toxin found in all *C.perfringens* strains.

Recent studies discussed the role of Beta 2, enterotoxin and Net F in association with enterocolitis. The role of β2 toxin–producing *C. perfringens* in diarrheal illness in animals is not well defined, although there is evidence for a role in several species. For example, in human beings, antibiotic-associated *C.perfringens* diarrhea has been associated with strains carrying plasmids encoding both the enterotoxin gene (*cpe*) as well as *cpb2* gene. **Fisher *et al.*,2005.** Few studies have examined the role of beta-2 toxin as an additional virulence factor in *C. perfringens* infection in equines,(**Mehdizadeh Gohari,*et al*. 2014** and **Hazlett *et al* 2011)**. β2 toxin gene was identified in all tested C. *perfringens* isolates (Table 6) suggesting a possible causal relationship between *cpb2*-positive *C. perfringens* and the incidence of colitis.

Recently, a novel pore-forming toxin, NetF, has been strongly associated with foal necrotising enteritis and canine haemorrhagic gastroenteritis. **Mehdizadeh Gohari *et al*., (2015).**

Necrotizing enteritis in foals caused by *netF*-positive *C. perfringens* is most commonly a disease of neonatal foals, but little is known about its prevalence **Mehdizadeh Gohari *et al.*, (2015**) as they identified this type in 6.8% of 58 adult horses with undifferentiated severe enterocolitis but in none of the 11 foals with undifferentiated diarrheal illness. In our study, five isolates of eight tested ones harbor Net F coding gene. Pore-forming toxins could play an important role in the disease pattern of acute hemorrhagic diarrhea syndrome (AHDS) in dogs. Thus, the prevalence of *C. perfringens* gene encoding for in the diarrheic foals and horses should be determined and to evaluate any association between any clinical variables and the presence of these toxin genes **(Mehdizadeh Gohari *et al.*, 2015)**

To improve understanding of the cause of acute colitis in horses, **Mehdizadeh Gohari *et al*., 2014** hypothesized that *Clostridium perfringens* producing enterotoxin (*cpe*) and /or beta2 toxin *(cpb2*) genes which are common and important causes of severe colitis in horses and/or that *C. perfringens* producing an as-yet-undescribed cytotoxin may also cause colitis in horses. However, they identified *C. perfringens* in 42 of 233 diarrheic foals; of 24 isolates genotyped, all were type A but cpe was found in only 3. By contrast with the results of **Mehdizadeh Gohari *et al*., 2014**, **Weese *et al*., 2001** in Ontario detected CPE using ELIZA in 19% of 47 adult horses and 29% of 28 foals with colitis and diarrhea. In present study, genotyping of 8 isolates of *C. perfringens* showed only 2 isolates positive for cpe gene. Geographical differences, seasonal variations, as well as variation in detection approaches might all contribute to these differences in incidences.Additionally, **Mehdizadeh Gohari *et al*., 2014** detected cpb2 genes in 17.2% of examined cases of horses. The two cpe positive isolates identified in this study were both negative for Net F, this result agreed with that reported by **Finley *et al*., 2016.**

CONCLUSION

This study threw the lights on the need of more studies to clarify the role of *C.perfringens* type A as a primary agent of diarrhea in foals and horses confirming that alpha toxin of type A is a major virulence factor for enteric infections.A particularly high incidence of B2-toxigenic *C.perfringens* was found, this result indicated a correlation between the presence of β2 –toxigenic *C.perfringens* and enterocolitis.

The result also suggested that β2 –toxigenic *C.perfringens* might be particullay fatal in combination with antibiotic treatment. The study may throw the light on the synergistic effect of β2 and netF toxins together with alpha toxin in induction of enterocolitis in horse. The evaluation of some antimicrobial susceptibility of *C.perfringens* isolates might be useful in the treatment of enteric diseases associated with *C.perfringens.*The limitation of this study, including small number of animals assessed it is concluded that *C.perfringens* play an important role in enterocolitis in horses and negative result for *C.difficile* may form the basis of another work with larger number of samples .Overall improving the diagnosis, further studies are still needed to gain better understanding of pathogenesis.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

REFERENCES

ANSARI M.R., MOHAMMAD ABADI M.R. AND SHAMSADDINI M. B.2010. *Clostridium perfringens* isolate typing by multiplex PCR The Journal of Venomous Animals and Toxins including Tropical Diseases 16(4): 573-578

BACCIARINI, L.N, BOERLIN, P., STRAUB, R. 2003. Immuno-histochemical localization of *Clostridium perfringens* b2-toxin in the gastrointestinal tract of horses. Vet Pathol 40:376–381.

BARR,B.S.,WAIDRIDGE,B.M.,MORRESEY,P.R.,REED,S.M.,CLARK,C.,BEIGRAVE,R.,DONECKER,J.M.AND WEIGET,D.J. 2012. Antimicrobial –associated diarrhea in three equine referral practice. Equine Vetrinary Journal 45 .154-158

DIAB, S.S., SONGER, G., AND UZAL, F.A. 2013A. *Clostridium difficile* infection in horses, Vet. Microbiol. P (13): 378-1135.

DIAB, S.S., RODRIGUEZ, A., AND UZAL, F.A. 2013 B. Pathology and diagnostic criteria of *Clostridium difficile* enteric infection in horses.Vet. Pathol.,50(6),1028-1036

EAST, L.M., SAVAGE, C.J., TRAUB-DARGATZ, J.L., DICKINSON, C.E. AND ELLIS, R.P. 1998. Enterocolitis associated with *Clostridium perfringens* infection in neonatal foals. J. Am. vet. med. Ass. 212, 1751-1756.

FINLEY, A.; MEHDIZADEH GOHARI, I.; PARREIRA, V.R.; ABRAHAMS, M.; STAEMPFLI, H.R. AND PRESCOTT, J.F.2016. Prevalence of netF-positive *Clostridium perfringens* in foals in southwestern Ontario. Can.J.Vet. Res. 80: 242-244.

FISHER D.J, MIYAMOTO K.,AND HARRISON B. 2005. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol. Microbiol 56:747–762.

FREDERICK,J. GIGU`ERE, S. AND SANCHEZ, L. C. 2009. Infectious agents detected in the feces of diarrheic foals: a retrospective study of 233 cases (2003–2008)," Journal of Veterinary Internal Medicine, vol. 23, no. 6, pp. 1254–1260**.**

FREEDMAN, J.C.; SHRESTHA,A. AND MCCLANE, A.B. 2016. *Clostridium perfringens* Enterotoxin; Action, Genetics and Translational Applications. Toxins, 8(73): 1-16.

GILBERT,M., JOLIVET-REYNAUD,C.,AND POPOFF, M.R. (1997. Beta 2 toxin ,anovel toxin produced by *Clostridium perfringens* .Gene 203:65-73

HARRIS, R. SANKAR, K. SMALL, J.-A. SUEPAUL, R. STEWART-JOHNSON, A. AND ADESIYUN, A. 2012. Prevalence and characteristics of enteric pathogens detected in diarrhoeic and non-diarrhoeic foals in trinidad," *Veterinary Medicine International*, vol. Article ID 724959, 9 pages

HATHEWAY, C. 1990. Toxigenic clostridia. Clin Microbiol Rev.;3:66-76.

HAZLETT, M**. J. KIRCANSKI, J. SLAVIC, D. AND PRESCOTT, J. F. 2011.** "Beta 2 toxigenic *Clostridium perfringens* type A colitis in a three-day old foal," Journal of Veterinary Diagnostic Investigation, vol. 23, no. 2, pp. 373–376,

HERHOLZ C., MISEREZ R .,AND NICOLET J.1999. Prevalence of b2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. J. Clin .Microbiol. 37:358–361

KEEL,M.K.,AND SONGER,J.C.(2006): The comparative pathology of *Clostridium difficile* associated disease.Vet. Pathol.43.225-240

KONEMAN, E.W.; ALLEN,S.D.; DOWELL, V.R. AND SUMMERS, H.W.1992. Colour atlas and text book of diagnostic microbiology. 4th Ed.J.B.Lippincott, New York,London.

MCDONEL ,J.I. 1986. Toxins of *Clostridium perfringens* types A,B,C,D and E. Pharmacology of bacterial toxins, In F. Dorner and H. Drews. p. 477- 517, Pergamon press, Oxford, UK.

MEHDIZADEH GOHARI I., ARROYO L., MACINNES J.I., TIMONEY, J.F.,PARREIRA, V.T.,AND PRESCOTT , J.F. 2014. Characterization of *Clostridium perfringens* in the feces of adult horses and foals with acute enterocolitis.Can. J. Vet. Res.;78:1–7.

MEHDIZADEH GOHARI ,I., PARREIRA, V.R., NOWELL ,V.J.,NICHOLSON, V.M., OLIPHANT, K., AND PRESCOTT, J.F. 2015. A Novel Pore-Forming Toxin in Type A *Clostridium perfringens* Is Associated with Both Fatal Canine Hemorrhagic Gastroenteritis and Fatal Foal Necrotizing Enterocolitis. PLoS ONE 10(4):e0122684. doi:10.1371/journal.pone.0122684

MEHDIZADEH GOHARI, I., KROPINSKI ,A.M., AND WEESE, S.J.2016. Plasmid characterization and chromosome analysis of two netF+ *Clostridium perfringens* isolates associated with foal and canine necrotizing enteritis. PLoS One.;11(2):e0148344.

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS (NCCL)2002. GINOCCHIOCC. AM.J.HEALTH SYST.PHARM.

RAJABALLY, N.; KULLIN, B.; EBRAHIM, K.; BROCK, T.; WEINTRAUB, A.; WHITELAW, A.; BAMFORD, C.; WATERMEYER, G.; THOMSON, S.; ABRATT, V. AND REID, S. 2016. A comparison of *Clostridium difficile* diagnostic methods for identification of local strains in a South African centre.

RADOSTITS, O.M. GAY, C. C. HINCHCLIFF, K.W. AND CONSTABLE, P. D.2007. Acute diarrhea in suckling foals," in *Veterinary medicine Publishing Group-A Textbook of the diseases of Cattle, Horses, Sheep, Pigs, and Goats*, OM. Radostits, C. C. Gay, K.W. Hinchcliff, and P. D. Constable, Eds., pp. 274–277, Saunders, Philadelphia, Pa,
USA, 10th edition.

QUINN, P. J. MARKEY, B. K. DONNELLY, W. J. LEONARD, F. C. FANNING, S.AND MAGUIRE, D.2011. *Veterinary Microbiology and Microbial Disease*, Blackwell Publishing House Science LTDA, Oxford,UK.

SCHOSTER,A.;GUILLERMO,A.L.;STAEMPFLI,H.R.; SHEWEN,P.E.AND WEESE,J.S.2012. Presence and molecular characterization of *Clostridium difficile* and *Clostridium perfringens* in intestinal compartments of healthy horses.BMC Vetrinary Research ;8:94

SILVA, R.O.S.; RIBEIRO, M.G. AND PALHARES, M.S.2013. Detection of A/B toxin and isolation of *Clostridium difficle* and *Clostridium perfringens* from foals. Equine Vet. J. (45) 6:671-675.

SLOVIS, N. M. ELAM, J. ESTRADA, M. THAO, . M. F. AND LEUTENEGGER, C. M.2010. Comprehensive analysis of infectious agents associated with diarrhea in foals in central Kentucky, in Proceedings of the Annual Convention of the AAEP, Baltimore, Md, USA,

SONGER JG. (1996): Clostridial enteric diseases of domestic animals. Clin Microbiol Rev;9:216–234. 22

STERN ,M.AND BATTY,I.(1975): Pathogenic Clostridia Butter Worth, London Boston

THEAN, S., ELLIOTT, B.,AND RILEY,T.V. 2011. *Clostridium difficile* in horses in Australia--a preliminary study. Division of Microbiology and Infectious Diseases, J Med Microbiol. Aug; 60 (Pt 8):1188-92

UZAL,F.A .2004. Diagnosis of *C.perfringens* intestinal infection in sheep and goat **.**Anaerobe V(10) 2: 135-143

WAGGETT,B.E.,MCGORUM ,B.C. ,WERNERY ,V ,SHAW ,D.J. AND PIRIE ,R.S. 2010. prevalence of *Clostridium perfringens* in faces and ileal contents from grass sickness affected horses: comparisons with three control populations Equine Vet.J.,42 (6) :494-499

WEESE, J.S., STAEMPFLI, H.R., ANDPRESCOTT, J.F.(2001): A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhea. Equine ,Vet. J. 33:403–409.

VAN ASTEN, A.J.; VAN DER WIEL, C.W.; NIKOLAOU, G.; HOUWERS, D.J. AND GRÖNE, A.2009. A multiplex PCR for toxin

typing of *Clostridium perfringens* isolates. Vet Microbiol. 136(3-4):411-2.

VEDANTAM,C.,CLARK,A.,CHU,M.,MCQUADE,R.,MALLOZZI,M.,AND VISWANATHAN,V.K. 2012. *Clostridium difficile* infection :Toxins and non –toxin ,virulence factors and their contributions to disease establishment and most response, Gut Microbes 3,121-134

VILEI, E.M., SCHLATTER, Y., PERRETEN, V., STRAUB, R., POPOFF ,M.R., AND GIBERT, M.2005. Antibiotic-induced expression of a cryptic cpb2 gene in equine beta2-toxigenic *Clostridium perfringens*. Mol Microbiol 57: 1570–1581. PMID: 16135225

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