Alexandria Journal of Veterinary Sciences 2015, 45: 132-138
ISSN 1110-2047, www.alexjvs.com
DOI: 10.5455/ajvs.179782

Studies on Diarrhea in Equine Associated with *Clostridium Difficile* and *Clostridium Perfringens* Infection

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Key Words:
Diarrhea, Equine, *Clostridium Difficile*, *Clostridium Perfringens*

ABSTRACT:

Clostridium difficile and *Clostridium perfringens* play a significant role in diarrhea affecting equine.This study was designed to determine the role of *Clostridium difficile* and *Clostridium perfringens* in equine diarrhea, 380 animals were examined clicically, where 65 were suffering from diarrhea. Fecal samples were collected from diarrheic animals, and 10 samples from apparently healthy animals.

Clostridium difficile and *Clostridium perfringens* were isolated from 19 and 11 samples respectively, while the two bacteria were isolated from 5 samples simultaneously. Moreover 4 samples from apparent clinical healthy animals had *Clostridium difficile* infection, indicating the high prevelance of theses organisms in equine.

Toxins of *Clostridium difficile* wasn't detected in diarrheic samples or culture of *Clostridium difficile* isolates by ELISA and PCR-technique, while α and β2 toxins of clostridium perfringens were detected in culture of clostridium.

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1. INTRODUCTION

Diarrhea is an important cause of mortality of adult horses and understanding of this syndrome is limited by the complex and dynamic nature of the gastrointestinal flora. Equine colitis has been associated with a variety of pathogens. Recently, *Clostridium difficile and perfringens* have been associated with enterocolitis in adult horses and foals (Donaldson and Palmer 1999). Many reports have implicated *Clostridium difficile* in cases of sporadic, antibiotic – induced and nosocomial colitis in adult horses and foals (Ed Kane, 2012)., although the full extent of its role in equine disease is still un clear. *Clostridium difficile* produces at least 5 toxins (Borriello 1998), although the effect of only toxin A and B were well understood. Isolation of *Clostridium difficile* was designated as toxigenic or non- toxigenic based on the production of these 2 toxins.

Toxin A is apotent enterotoxin with slight cytotoxic activity (Henry, 2012). Which unlike toxin B which cause fluid accumulation in animal intestinal models (Borroello, 1998). Toxin B is a potent cytotoxic with up to 1000 times the cytotoxicity of toxin A (Henry, 2012) but no demonstrable effect on intestinal permeability, migration of neutrophils in the intestinal lumen or intestinal morphology (lima et al., 1988). Clinical signs of *Clostridium difficile*-associated disease (CDAD) can be variable and range from mild enteritis to fulminant necrotizing hemorrhagic enteritis. Diagnosis of CDAD has been hampered in the gut due to the fastidious nature of the organism and requirement for complicated labolatory procedures to detect toxins. The ready availability of selective culture media and ELISAs for the detection of toxins has led an increased understanding the role of the organism in equine colitis.(Diab, et al., 2013).

The role of *Clostridium perfringens* in equine colitis is less clear. Equine enterocolitis has been associated with C. perfringens Types A (Bueschel et al., 1998) and Types C (East et al., 1998). The production of Clostridium perfringens enterotoxin (CPE) is most commonly associated with Type A strains, but can occur with other types (Songer., 1996). CPE production is co-regulated with, sporulation and is released upon lysis of vegetative cells (Songer, 1996). *Clostridium perfringens* was significantly associated with diarrhoea in foals (Netherwood et aI., 1996), while isolation of *Clostridium* perfringens was not associated with diarrhoea in foals (Browning et al., 1991). This study was designed to determine the role of *Clostridium difficile* and clostridium perfringens as etiologic agents of diarrhea in foals and adult horses.

2. MATERIAL AND METHODS

Animals:

This study was done on 380 animals from different localities at alexandria gavernorate, animals were clinically examined for diarrhea according to Kelly, (2000).

Collection and preparation of samples

A total of 75 fecal samples were collected from foals and adult horses. 65 samples were from animals suffering from diarrhea, while the remained 10 samples were from apparent clinicaly healthy horses. Samples were collected directly from the rectum then loaded in plastic bags and transported directly to the laboratory. Samples were stored at – 20° c (Ba˚ verud et al., 2003).

Isolation and identification of Clostridium difficile

Under complete aseptic conditions the fecal samples were transferred into cooked meat broth supplemented with D-cycloserine to inhibite the growth of microorganisms other than *Clostridium* species then incubated anaerobically for 5-7 days at 37 °c in anaerobic condition. The enriched samples were treated with heat shock in water bath at 80 °c for 10 minutes then streaked on blood agar and incubated anaerobically for 48 hrs at 37 °c in macintosh jar (ArulkumarThangamani and Saravanan Subramanian, 2012).Typical colonies on blood agar were picked up and purified on cooked meat broth then incubated anaerobically for 48 hrs at 37 °c and further used for biochemical confirmation tests (Johnson et al, 2007).

Isolation and identification of Clostridium perfringens:

The enriched samples streaked on *Clostridium perfringes* agar base supplemented with egg yolk emulsion (50 ml / littre) and D-cycloserine (2 vials / littre) (DespinaKotsanas et al., 2010) and also streaked on blood agar medium **(Johnson et al, 2007)** then incubated anaerobically at 37 °c for 48 hr.Typical colonies from the cultivated *Clostridium perfringens* agar medium and from blood agar medium were picked up and purified on cooked meat broth for further biochemical confirmation tests. (Rhodehamel. et al, 1995) .

PCR-techniques :

a. identification of *Clostridium difficile*

PCR identification of *Clostridium difficile* strain from the isolated colonies by amplification of *tpi* gene by using specific primer (5′AAGAAGCTACTAAGGGTACAAA-3′), and (5′CATAATATTGGGTCTATTCCTAC-3′) according to **(LudovicLemee, et al. 2004)**.

b. Detection of *Clostridium difficile* toxines

Examination of feces and colonies for *Clostridium difficile* toxin A and B was done using primers (5′-GATGCTAATAATGAATCTAAAATGGTAAC-3′), (5′-ACCACCAGCTGCAGCCATA-3′) and (5′GATGCTAATAATGAATCTAAAATGGTAAC-3′), (5′ACCACCAGCTGCAGCCATA-3′), respectively acoording to (**Charles Darkoh, et al. 2011**), by using Multiplex -PCR technique .

c. Detection of *Clostridium perfringens* toxins

Two primers were used for detection of *Clostridium perfringens* α toxin and β2-toxin by multiplex PCR technique using specific primers (5AAGAACTAGTAGCTTACATATCAACTAGTGGTG-3). (5-TTTCCTGGGTTGTCCATTTCC-3) and (5′GATGCTAATAATGAATCTAAAATGGTAAC-3′). (5′-ACCACCAGCTGCAGCCATA-3′) respectively, (Albini, et al. 2008).

ELISA for *Clostridium difficile* A and B Toxins identification:

ELISA kits used for qualitative determination of toxin A and B from *Clostridium difficile* in stool samples (RIDASCREEN, 2011).

Table (1) Prevalence of diarrhea among examined equine

Area	Total no.of animals	No.of diarrheic animals	%
El- giad club	100	6	6%
Smoha club	80	5	6.25%
Max-marine farm	60	16	26.6%
El-hadara stable	20	1	5%
Carmoze stable	40	12	30%
Abo-keer stable	80	25	31.25%
Total	380	65	17.1 %

Table 2. Prevalence of *Clostridium Dificille* and *Clostridium perfringens* isolation from diarrheic cases

Total	*Samples No.*	*Clostridium difficile*		*Clostridium perfringens*		*Mixed infection*	
		No.	**%**	**No.**	**%**	**No.**	**%**
Diarrheic cases	**65**	**15**	**23**	**11**	**16.9**	**5**	**7.6**
Apparently normal	**10**	**4**	**40**	**0**	**0**	**0**	**0**
Total	**75**	**19**	**25.3**	**11**	**16.9**	**5**	**7.6**

3. RESULTS

As showed in table (1). From 380 animals examined, diarrhea was observed in 65 equine (17.1 %) . the highest prevalence was in Abo-keer stable and lowest was in El-hadara stable ,10 control samples were taken from apparently healthy animals

Clinical signs observed on diarrheic cases:

Clinical signs not differ greatly between equine age groups that ranged from normal temperature to 39 °c. consistency of diarrhea range from watery, soft to semiformed diarrhea. Some diarrheic horses were treated with anthelmintic drugs (banminth, duramectine) or antibiotics (panstrep, tetracycline, erythromycin), before incidence of diarrhea

Prevelance of *Clostridium difficile* and *Clostridium perfringens:*

75 samples were streaked on blood agar, 19 samples were found to be *Clostridium difficile* (15 samples from diarrheic animals and 4 samples from apparently halthy animals). 11 samples were found to be *Clostridium perfringens* (TSC), 5 have mixed infection with both clostridium difficile and clostridium infection(table 2).

Results of PCR applied on *Clostridium difficile* isolates: 19 clostridium difficile isolates were examined by convential PCR technique using specific primers for universal gene of *Clostridium difficile* species,19 samples were confirmed as *Clostridium difficile* by PCR, figure (1)

Detection of *Clostridium difficile* toxins by ELISA

ELISA examination for toxins A and B of *Clostridium dificille* directly from fecal samples, or bacterial culture was negative

Detection of *Clostridium difficile* toxins by PCR

19 positive fecal samples for *Clostridium difficile* culture were examined by PCR technique for detection of toxin DNA and no toxin DNA was detected, figure (2)

***Clostridium perfringens* toxin detection:** 11 positive isolates of *Clostridium perfingens* detected by bacterial culture were tested by multiplex- PCR for detection of toxins by using specific primers and results revealed that 11 sample were positive for α and/or β2 toxins, figure (3)

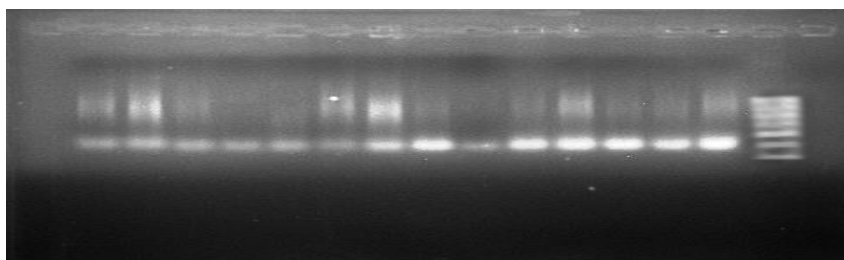

Figure (1): electrophoretic analysis of the convential PCR products obtained from DNA extracts of *Clostridium difficile* colonies , by amplification of *tpi*gene (230 bp)

- Lane 1 marker
- Lane 2- 14(14 positive representive sample for *tpi* gene 320bp)

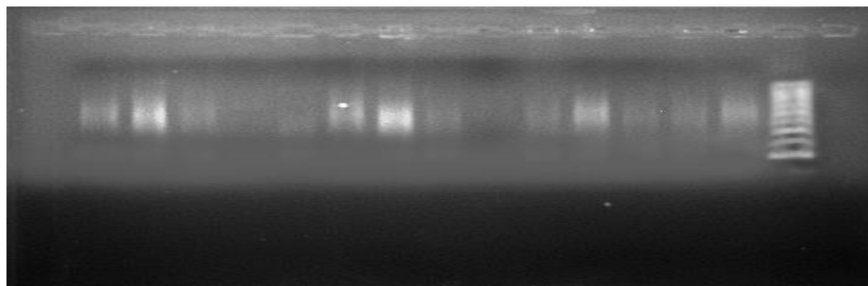

Figure (2): electrophoretic analysis of multiplex- PCR products obtained from DNA extracts of *Clostridium difficile* colonies , by amplification of *toxin A* and *B* gene (- ve results)

- Lane 1 marker
- Lane 2- 14 (14 negative samples for toxin A and B)

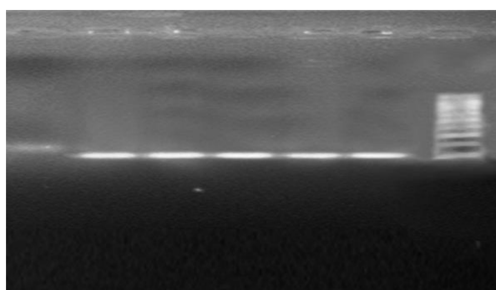

Figure (3): electophoretic analysis of multiplex- PCR products obtained from DNA extracts of *Clostridium perfrinfens* colonies , by amplification of α, and β2 toxin geneswhich revealed positive for *α*-toxin (124bp) and *β2* toxin (127bp)

- Lane 1 marker
- Lane 2-6 5 positive representive samples for *α*- toxin (124), *β2* (127)

4- DISCUSSION

Diarrhea is a common problem in equines and causes significant losses in foals and adult horses. (Lyerly et al ., 1998), it has been associated with a variety of pathogens, including *Salmonella* (Stewart et al. 1995; Cohen and Divers 1998) *Ehrlichiaristicii* (Stewart et al. 1995;Cohen and Divers 1998) *Aeromonas spp*., (Browning et al.1991, Hathcock et al., 1999*) Lawsonia intercellularis* (Brees et al., 1999) and *larval schitosomiasis* (cohen and Diver 1998).more recently , *Clostridium difficile and perfringens* have been associated with enterocolitis in adult horses and foals (Jones et al. 1987; Traub-Dargatz and jones 1993) Madewell et al. 1995; Baverud et al., 1998 ;Donaldson and Palmer 1999).

This study was designed to throw light on the role of *Clostridium difficile* and *Clostridium perfringens* in diarrhea in equine

In this study, 65 out of 380 horses from different localities of Alexandria governorate were diarrheic (table 1), mostly affected animal were from Abo-keer stable (25 out from 70) and lowest prevalence were from El-hadara stable (1 out from 20).

As shown in table 2, *Clostridium diffcile* was isolated from 15 diarrheic animals (23%). In similar previous studies (Thean et al., 2011) isolated *Clostridium difficile* from 23% of diarrheal animals, and high isolation rate reported by (Ba°verud at al., 2003) isolated *clostridium difficile* from 42% diarrheic horses horse, while low isolation rate was reported by (Weese, et al., 2001) which was 12% of horses with colitis was posiyive for *Clostridium difficile*.

Clostridium difficile was isolated from 4 of 10 non diarrheic horses (40%) (table 2).. This support the results of (Ba° verud, et al., 2003), Who isolated *Clostridium difficile* from normal non diarrheic horses, while (Weese, et al., 2001) suggested that

Clostridium difficile is uncommonly isolated from healthy foals (13%). On the other hand *(Gustafsson* et al., 2004) reported that *Clostridium difficile* is not considered part of the normal flora of the equine adult gastrointestinal tract and is uncommonly isolated from normal mature horses. The isolation rate may increase in asymptomatic horses being treated with antimicrobials. However, up to 42% of horses that develop acute colitis during treatment with antimicrobials can have *clostridium difficile* isolated, and this confirmed by what reported by (Butterworth et al., 1998). As clostridial diarrhea mainly induced due to disturbance of number of normal inhabitant microflora due to administration of antimicrobials

Convential PCR-technique was used to confirm *Clostridium difficile* isolation by targeting species-specific internal fragment of the *tpi* (figure 1), as PCR-technique used for identification more specific than bacteriological cultivation for confirming the strain isolated (LudovicLemee et al., 2004). These results indicate that PCR may be used to overcome the cumbersome and time-consuming of biochemical tests. Also PCR allows the direct detection of infectious agents in stool samples when PCR was used (Gouvea, et al., 1990). Also these results may be in accordance with some reports revealed that PCR –technique is rapid, sensitive and requires minimum specimen preparation. Results could be obtained within 3 h of primary isolation and PCR was successful even in mixed culture with *Clostridium sporogenes* (Donaldson and Palmer, 1999 and Henry, 2012)

Clostridium difficile induce diarrhea through the production of toxins. The bacteria produce 2 toxins (A and B), Toxin A is potent enterotoxin with slight cytotoxic activity (Henry, 2012). Which unlike toxin B which cause fluid accumulation in animal intestinal models (Borroello 1998). Toxin B is a potent cytotoxic with up to 1000 times the cytotoxicity of toxin A (Henry, 2012) .Isolation of *Clostridium difficile* was designated as toxigenic or non- toxigenic based on the production of these 2 toxins.

In this study we could not detect toxin A or B from feces of diarrheic equines or from isolated *Clostridium difficile* by ELISA or PCR. These results may indicate that the isolated *Clostridium difficile* was not toxigenic strains and that there are other causes of diarrhea and this is supported also by our results of isolation of *Clostridium difficile* from normal non diarrheic horses. However the carrier rate of *Clostridium difficile* is nil or low in asymptomatic foals and adult horses. The rate of isolation from clinically normal adults is between 0 and 4.3%, whereas normal foals are generally reported to be culture negative (Baverud et al., 1997; Jones et al., 1987; Madewell et al., 1995; Weese et al., 2001). Therefore, isolation of this microorganism from the intestinal tract of horses with gastrointestinal disease is considered by some authors as highly suggestive of *Clostridium difficile* -associated disease.

Clostridium perfringens was first implicated as a cause of antibiotic-associated diarrhea (AAD) in 1984 and may be diagnosed by detection of enterotoxin (CPEnt) in faces. *Clostridium perfringens* affecting foals less than 7 days of age and especially day old foals , they colonize the gut some produce toxin that cause gut damage that allow toxin or even bacteria to enter to blood stream leading to foal septicemia, the diarrhea is bloody and can be diagnosed by detecting toxins in feces, the most common types are type A and type C , type A elaborate an enterotoxin (CPE) which is released during sporulation and stimulate intestinal epithelial cell to secret excess fluid into the lumen. Type A has been isolated in >90 % of feces of healthy neonatal foals, so the number of bacteria and phase of growth predispose to this type of diarrhea, while type C infection (Clostridium associated enterocolitis) this type may associated with *Clostridium perfringens* type A (Tillotson et al., 2002).

Type C is rarely found in the feces of normal foals and horses and results in more severe diarrhea than type A. Toxins produced by *Clostridium perfringens* are type α and β and β2 toxins (Hickey et al., 2008 , Bryant et al., 2003)

In this study *Clostridium perfringens* was isolated from horses in a frequency of (16.9%) from diarrheic horses as showed in table (2) which nearly similar to that reported by (Herholz et al., 1999). However, it differs greatly from (Tillotson et al., 2002), who reported that *Clostridium perfringens* considered a part of normal flora that has high incidence rate in young age and decreased prevalence with advanced age.

The toxins secreted by *Clostridium perfringens* were identified by multiplex-PCR technique by using specific primers for α and β2 toxins that revealed positive results showed in figure (3) which indicates that *Clostridium perfringens* toxins were the cause of diarrhea in horses affected with this organism as showed at table (2), this agree with that reported by(Alec and Louise, 2001). Also agree with (Wilcox, 2000) who reported that Although *Clostridium difficile* is the most commonly identified pathogen in horses who acquired diarrhea through hospitalization , the

cause(s) of the majority of the cases, in some series up to 80%, currently remain undiagnosed. Staphylococcus aureus and *Clostridium perfringens* are the most frequently cited alternative causes of AAD.

Mixed infection by *Clostridium difficile* and *Clostridium perfringens* was observed in 5 diarrheic cases (table 2) which indicate that the causative agent of diarrhea was *Clostridium perfringens* not *Clostridium difficile* as *Clostridium difficile* was non-toxigenic strain, while *clostridium perfringens* toxins was confirmed by multiplex-PCR technique . Mixed infection by both clostridia was reported in diarrheic horses by (Uzal et al., 2012) suggests a possible synergism of *Clostridium perfringens* type C and *Clostridium difficile* in foal enterocolitis. Because none of the foals had received antibiotic therapy, the predisposing factor, if any, for the *Clostridium difficile* infection remains undetermined; it is possible that the *Clostridium perfringens* infection acted as a predisposing factor for *Clostridium difficile* and/or vice versa. The presence of mixed infection cases in this study stresses the need to perform a complete diagnostic workup in all cases of horses with diarrhea.

5- CONCLUSION

Toxigenic *Clostridium prfringens* play a significant rol as a causative agent of diarrhea in horses. Mixed infection in both *Clostridium perfringrns* and clostridium difficile may indicate synergestic action of them or that *Clostridium perfringens* predispose for *Clostridium difficile* action

Isolation of *Clostridium difficile* from diarrheic horses not enough for confirming its role in diarrhea

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