*Zag. Vet. J. (ISSN. 1110-1458) Vol. 42 No.2 (2014) pp. 124-130* 

# Isolation And Identification Of *Clostridium difficile* From Horses

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## ABSTRACT

Clostridium difficile is considered one of the most important causes of enterocolitis associated with diarrhea in horses especially foals, that may led to necrohaemorrhagic enterocolitis with high mortality rate. Diarrhea mostly develops while treating with antibiotics, however , it may develops without any history of antibiotics treatment. In this study, twenty faecal samples and seven rectal swabs from foals suffering from diarrhea, were collected and cultured onto Cefoxitin Fructose Agar (CFA) as specific growth medium . Isolates from twenty-one (77.7%) of these samples gave the characteristic microscopical examination and colony morphology as well as they gave negative results with catalase and oxidase tests also they gave fluorescence after exposure to long wave length ultraviolet light. Specific agglutination kit was used for confirmation, revealed nineteen isolates (70.4%) gave positive for agglutination, The A and B crude toxins were prepared from the isolates, toxogencity test in mice was done for the prepared toxins as preliminary test for toxogencity, nine (33.3%) isolates found to be lethal to mice, then, SDS-PAGE was done to the fifth isolate (as it gave the highest MLD, indicating its the most toxogenic isolate) where the toxins gave bands at molecular masses of 308 and 270 kDa, which resemble that of toxins A and B of *Clostridium difficile* respectively. Finally, the isolated *Clostridium difficile* recommended to be used in preparation of vaccine for controlling the severity of enterocolitis disease among horses.

## INTRODUCTION

*Clostridium difficile* has been firstly isolated in 1935 from human, and in 1984 from mature horses in a study of Potomaic horse fever *(1)* . It was previously named as *Bacillus difficilis* for its difficulty in isolation and studying. Laterally, it classified as belonging to the genus Clostridium as this bacterium found to be anaerobic endospore forming and Gram positive *(2)*.

In human, *C. difficile* accounts for about 20-25% of antibiotic-associated diarrheic cases and causes the majority of antibiotic associated colitis and pseudomembranous colitis *(3)*. Infants are very often asymptomatic carriers of *Clostridium difficile* even with toxigenic isolates *(4)*.

*Clostridium difficile* also appears to be the most cause of enteric disease in a wide variety of animal species, it has been isolated from poultry, sheep, pigs, chickens, goats, cattle and calves *(5)*.

The types of *Clostridium difficile* found in animals and humans are often indistinguishable *(6)*.

*Clostridium difficile* is an important cause of acute enterocolitis in horses with clinical signs ranging from mild self limiting disease to fluminant necrohaemorrhagic enterolcolitis with a high mortality rate.

Clinical signs of *C.difficile* diarrhoea not differ greatly from those of other diarrheic diseases, diarrhoea usually develops while the horses being treated with antibiotics, although it may begin several days after the treatment is withdrawn, even it may develop without history of antibiotics treatment. Foals and adult horses are equally susceptible to infection, the highly

resistant spore of *C. difficile* is the infectious unit of transmission that occur primarily via the faecal oral route *(7)*.

In foals, Clostridium *difficile* has shown to be associated with diarrhoea during several outbreaks as well as in sporadic cases. Almost one third of healthy normal foals younger than 14 days are asymptomatic carriers of *Clostridium difficile* .Many asymptomatic carriers were also found among non diarrheic foals of 1-3 months age with antibiotic treatment *(8)*.

Mostly, pathogenesis of *Clostridium difficile* started by antibiotic treatment, where there's an initial disruption of the normal colonic bacterial flora, allowing *Clostridium difficile* from endogenous or exogenous origins to establish itself in the colon and proliferate, toxins A (enterotoxin) and B (cytotoxin) are then produced simultaneously in most cases *(9)*.

The aim of this study is to isolate *Clostridium difficile* from from faecal samples or rectal swabs of foals and/or adult horses, using specific growth medium and identifying the isolates microscopically, culture characters, latex agglutination test specific to *Clostridium difficile* and by SDS-PAGE. The isolation of *Clostridium difficile* considered as primarily step for preparation of vaccine against acute enterocolitis in horses and foals from locally isolated strain .

## MATERIAL AND METHODS

Samples

Faecal samples from twenty Arabian foals at age 1- 4 months and seven rectal swabs from foals after birth to 13 days age, all suffering from diarrhoea and three from the later under course of antibiotic treatment.

All samples were kindly obtained from EAO ElZahraa Stud.

Isolation of *Clostridium difficile*

Faecal samples were shocked in alcohol *(10)*, then the pellets and rectal swabs obtained, were streaked on Cefoxitin Fructose Agar growth medium , prepared as mentioned before *(11)* and incubated 24 hrs. at 37 °C under anaerobic conditions. The colonies selected were stained with Gram stain, then these suspected colonies were harvested and inoculated on Cefoxitin Fructose broth ***(12)*** and incubated 24 hrs. at 37°C under anaerobic conditions.

Identification of *Clostridium difficile*

Microscopical examination , catalase test , oxidase test and culture characters of suspected colonies (including culture morphology, odour and effect of long wave length ultraviolet light) were carried out as previously mentioned *(13)*.

Agglutination test

The suspected colonies as well as the positive control provided (inactivated *Clostridium difficile* cell wall antigen) were tested by *Clostridium difficile* latex agglutination test using the specific provided reagent (latex particles coated with IgG antibodes specific for *Clostridium difficile* cell wall antigens) **(Oxoid Ltd., DR 1107),*(14)*.**

Toxin production

Proteose peptone broth was prepared and seeded with *C. difficile* suspected colonies and incubated anaerobically for 72 h., the supernatant obtained after centrifugation at 3500 rpm for 30 min. was seitz filtered and the crude toxin precipitated from the culture by slow addition of equal volume of 70% ammonium sulphate solution and dialysed against distilled water for 48 hrs and finally dissolved in 1/20 of original volume in sterile saline. Toxogenicity test was done by injecting the prepared crude toxins 0.2ml i.v.and double fold dilution from each toxin in mice and investigate their minimium lethal dose (MLD) *(13)*.

Finally, SDS-PAGE was applied for the prepared toxins according to ***(15)***.

## RESULTS

Out of twenty –seven samples (faecal samples and rectal swabs) from foals, twenty-one samples (77.7%) showed the characteristic morphological character of *Clostridium difficile* as greyish regular smooth colony with manure horse odour when cultivated on Cefoxitin Fructose Agar medium. Gram stain of these colonies revealed Gram positive large bacilli as in Fig.(1),

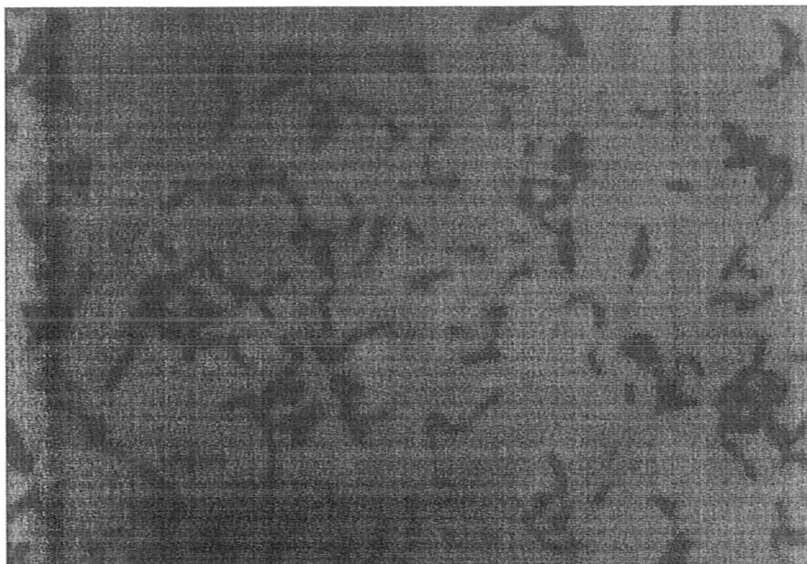

**Fig. 1. Gram stain of colonies on cefoxitin fructose agar medium**

The isolated colonies also produce a pale green fluorescence under long-wave length ultraviolet light.

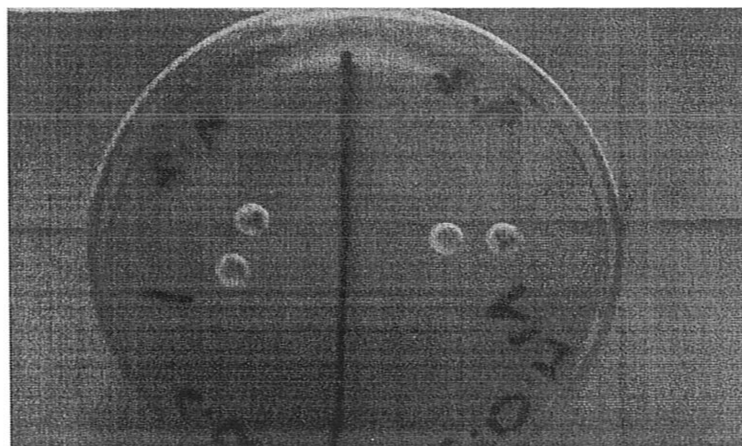

**Fig. 2. Pale green fluorescence colonies under long-wave length ultraviolet light on fructose agar medium with cefoxitin in form of antibiotic discs.**

The twenty one isolates also gave catalase negative using hydrogen peroxide (no bubbles of liberated oxygen formed) and oxidase negative when tetramethyl-p-phenylenediamine (TMPD) was added as no change in colour occurred. *Clostridium difficile* Latex agglutination test for the twenty-one colonies revealed that nineteen colonies(70.4%) gave positive agglutination in comparing to the positive control (fig. 3).

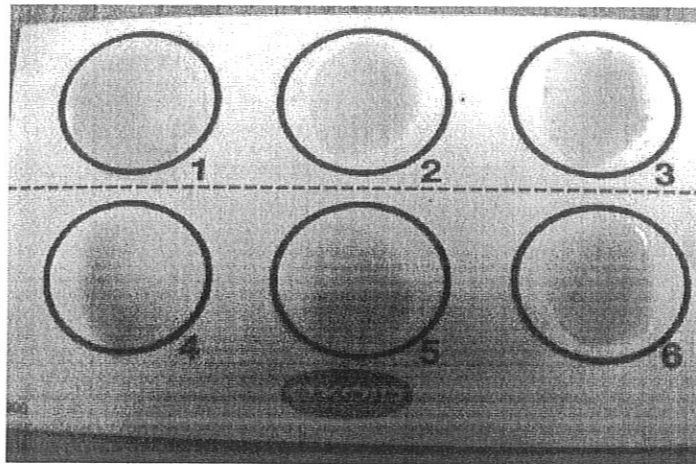


**Fig. 3. Latex agglutination test using *Clostridium difficile* agglutination kit 1,3,4,5 : positive faecal samples ; 2: negative faecal sample , 6 : Positive control**

Toxogenicity test for the nineteen isolates were done by injecting 3 mice 0.2ml i.v. of the prepared crude toxin from each isolate, and lethality investigated, where twelve isolates showed no death for mice for 48 hrs., the remaining seven isolates showing death for mice within 24 hrs, All the isolates gave 2 MLD except the fifth isolate which gave 4 MLD .SDS-Page were done for the crude toxin of the 5$^{th}$ isolate as it is the high toxogenic one according to the MLD (fig.4). it give two molecular bands at 270 and 308 Kda.in comparing to the protein marker.

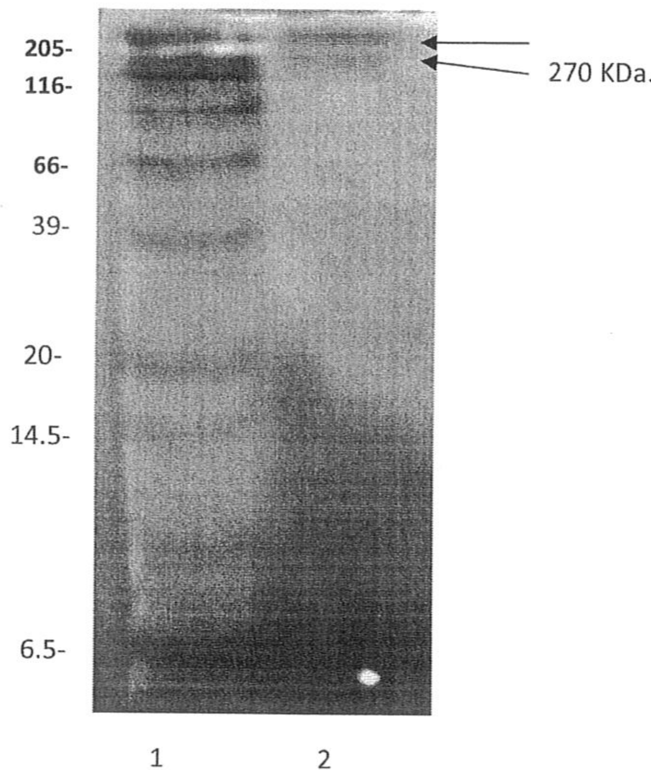


**Fig.4.SDS-PAGE for the prepared toxins from cultures *of C.difficile***
**Lane 1. protein marker, lane 2 . prepared toxin**

## DISCUSSION

*Clostridium difficile* has been implicated as an important cause of enterocolitis in a variety of species, including humans and horses ***(16)***. Although it is not considered as a normal constituent of the gastrointestinal flora, *C. difficile* can be isolated from faeces in a small percentage (0–4%) of normal horses and humans ***(17)***. Clinical disease ranges from mild and self-limiting diarrhoea to severe, necrotizing enterocolitis with high mortality rate follows by colonization and overgrowth by this organism with the concurrent production of two exotoxins: toxin A, an enterotoxin, and toxin B, a cytotoxin. Typically this overgrowth occurs after disruption of the normal protective gastrointestinal microflora, such as with antibiotic use ***(18)***.

In this study, twenty faecal samples and seven rectal swab from foals were alcohol shocked and the pellet cultivated on Cefoxitin Fructose Agar medium. Cefoxitin in the specific medium was added to inhibit the growth of the majority of Enterobacteriaceae, as well as *Streptococcus faecalis, staphylococci,* Gram-negative nonsporing anaerobic bacilli and *Clostridia* species. (except *Clostridium difficile*) which may be found in large numbers in faecal samples ***(19)***. Out of these samples, twenty one of them successfully show grey white coloured colonies with distinctive odour resemble horse manure odour and that fully agreed to what previously stated ***(12)***.Gram stain of these colonies revealed Gram positive large bacilli as in Fig.(1), this result agree with that mentioned by ***(20)*** who said that *Clostridium difficile* appear as Gram positive rod, measuring 0.5 x 3-6 µm and they added that these spores were never seen on Gram stain made from CFA. *Clostridium difficile* vary in shape from very short thick form to a large bacillus measuring (2.5-5.9) x (0.3-1. 5) µm ***(13)***.

The isolates gave negative results when tested with hydrogen peroxide and tetramethyl-p-phenylenediamine (TMPD) for catalase and oxidase tests respectively, that results fully agreed to what mentioned ***(13)*** as he said that Clostridium difficile is catalase and oxidase negative. The isolated colonies also produce a pale green fluorescence under long-wave length ultraviolet light as shown in Fig. (2). And it has stated before that one of the characteristics of *Clostridium difficile* colonies is the ability to produce pale green to chartreuse fluorescence when exposed to ultraviolet light ***(9)***.

The colonies from the twenty one samples (that gave the characteristic Gram stain , colony morphology, catalase and oxidase negative and fluorescence under long wave length ultraviolet) were inoculated separately on Cefoxitin Fructose Broth and incubated for 24 hrs at 37°C under anaerobic conditions. *Clostridium difficile* latex agglutination test **(Oxoid Ltd., Egypt)** were applied for each of them ***(21)*** .

The agglutination test revealed that nineteen of them (70.4%) gave positive agglutination in comparing to the positive control ***(6)*** (fig.3) and that confirmed that those isolates were *Clostridium difficile* .

Regarding toxogenicity test, the lethal effects of the prepared toxins from the positive isolates were investigated in mice, where 0.2 ml of toxin prepared from each isolate and its double fold dilution, injected intravenously in three mice, causing death of the mice after 24 hrs. where nine (33.3%) cause death for mice within 24 hrs. and gave 2 MLD, while only the fifth isolate gave 4 MLD and that agreed to what mentioned before ***(13)*** as they stated that 0.2-0.4 ml of crude toxin of Clostridium difficile when injected i.v. in mice could kill the mice within 72hrs.

SDS-PAGE applied on the most toxogenic isolate (the fifth isolate)., The SDS-PAGE revealed that the prepared crude toxins gave bands at approximate molecular weights of 308 and 270 KDa. fig. (4), and it has been mentioned before that pathogenic strains of *C. difficile* produce two potent toxins: enterotoxin A and cytotoxin B which are of major importance in clinical disease, they added that toxins A and B are both extremely large, having molecular masses of 308 and 270 kDa, respectively ***(22)***.

The result obtained from microscopical examination, colony morphology, effect of long wave ultraviolet light on colonies, specific agglutination test, toxogenicity test and SDS-PAGE could gave confirmation that the isolates are *Clostridium difficile*. Further studies are recommended to be done on the prepared toxins of *C.difficile* isolated for more confirmation for their toxogenicity. Also, locally isolated strain of *Clostridium difficle* could be used in the future for producing vaccine against antibiotic induced enterocolitis in equines for limiting the severity of the disease.

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## الملخص العربي

## عزل و تصنيف ميكروب الكلوستريديم ديفيسيل من الخيول

**مدحت محمد طه**
معهد بحوث الأمصال و اللقاحات البيطرية – العباسية

يعتبر ميكروب الكلوستريديم ديفيسيل من أحد اسباب الالتهاب المعوى المؤدى للاسهال فى الخيول و خاصة المهر وقد يصل الى التهاب غشاء القولون الكاذب مما يسبب ارتفاع فى معدل الوفيات. يرتبط هذا المرض فى الاغلب بالعلاج بالمضادات الحيويه .فى هذه الدراسه , تم عزل ميكروب الكلوستريديم ديفيسيل عن طريق جمع عشرون عينة براز وسبعة مسحات من المستقيم من مُهرات ، و تم زرعهما على بيئه غذائيه مخصصه .أحد و عشرون (٧٧,٧٪) من هذه العينات أعطت نتائج ايجابيه بالفحص المجهري و شكل النمو ، فضلاً عن أنها أعطت ايجابيه عند تعرضها لموجات الأشعة فوق البنفسجية و كانت سلبيه النتائج مع اختبارى الكاتالاز و الاوكسيديز, تم استخدام اختبار التلزن المُحدد لميكروب الكلوستريديم ديفيسيل للتأكد حيث أعطت تسعة عشر من المعزولات (٧٠,٤٪) نتائج ايجابيه. حُضرت السموم من المعزولات و تم عمل اختبار السميه فى الفئران ووجد ان هناك تسعة عينات (٣٣,٣٪) ادت الى وفاة الفئران و اعطت العينه الخامسه اعلى نسبه فى السميه فتم عمل اختبار اللطعى الكهربائى للسموم المحضره من هذه العتره حيث أعطت تفاعل عند وزن جزيئى ٣٨٠ و ٢٧٠ ك.د. و التى تطابق تلك عند السموم أ و ب على التوالى. اخيرا , يُنصح باستخدام ميكروب الكلوستريديم ديفيسيل المعزول لتحضير لقاح للحد من خطوره مرض الالتهاب المعوى القولونى بين الخيول .