

## Real-Time PCR for direct detection of *Clostridium perfringens* from horse with enterocolitis infection

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### Abstract

*Clostridium perfringens* is one of the most important causative agents of enterocolitis infection in horses. This bacterium capable to produce potential enterotoxin that cause diarrhea in horses. This study was aimed to molecular identification of *Clostridium perfringens* from horses infected by diarrhea by using Real-Time PCR technique. The *Clostridium perfringens* type C was identified by using specific primers for enterotoxin (CPE) gene that designed in this study using NCBI-Genbank data base (Genbank code: GQ225719.1) and primer3 plus for primer design. The results show that prevalence of infection by *Clostridium perfringens* type C was (40%) of 12 positive samples out of 30 fecal samples. This study demonstrated that the direct Real-Time PCR technique is a highly specific and rapid tool for the detection *Clostridium perfringens*.

**Key word: Real-Time PCR, *Clostridium perfringens*, CPE toxin**

### الخلاصة

تعد بكتريا *Clostridium perfringens* واحدة من اهم مسببات الاضطرابات المعوية في الخيول وذلك لقدرة هذه البكتريا على انتاج اليفانات الداخلية القوية المفعول والذي يحدث الاسهال في الخيول . هدفت هذه الدراسة لتشخيص بكتريا *Clostridium perfringens* نوع C باستخدام تقانة Reat time PCR . بكتريا *Clostridium perfringens* نوع C شخصت باستعمال بادئ خاص بالذيفان الداخلي الجين (CPE) والذي تم تصميمه لغرض الدراسة بالاعتماد على (Genbank code: GQ225719.1) and primer3 plus لتصميم البادئ و اظهرت النتائج ان نسبة الاصابة كانت 40% أي بواقع 12 عينة موجبة من أصل 30 عينة براز التي تم جمعها من الخيول المصابة . أعتمدت هذه الدراسة استخدام تقانة Real time PCR والذي يعد فائق التخصصية والسرعة في تشخيص بكتريا *Clostridium perfringens* نوع C .

### Introduction

*Clostridium perfringens* is an important cause of enteritis and enterocolitis in adult horses and foals and lead to permanent dysfunction and may lead to death in foals (1). *C. perfringens* is classified into five types (A, B, C, D, and E) based on the production of major toxins: alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) as well as two other major

toxins, enterotoxin (CPE) and beta 2 (CPB2), can be produced by all types of *C. perfringens* (2,3,4). Although *C. perfringens* types A, B, and C have been associated with enterocolitis in foals, *C. perfringens* type C is the most commonly reported enteric pathogen in foals in North America (5). Enteritis, colitis, and enterocolitis apparent clinically by diarrhea and colic, are important causes of morbidity and

mortality in foals and adult horses. These conditions have been associated with various causes, including *Clostridium sp.*, *Ehrlichia risticii*, *Salmonella sp.*, *Aeromonas sp.*, *Lawsonia intracellularis*, *cantharidin toxicosis*, and *larval cyathostomiasis* and together *Salmonella sp.*, and clostridia, that including *Clostridium perfringens* and *Clostridium difficile*, are the most important agents of equine enterocolitis (6,7,8). *C. perfringens* type A produces major toxins that former a lecithinase (phospholipase C), which is considered the main virulence factor associated with myonecrosis in humans and animals. The importance of CPB toxin was considered responsible for the intestinal necrosis and systemic alterations in type C infections of several animal species, including horses (9). Enteric disease caused by *C. perfringens* type C in other many mammalian species, including humans, initiates when *C. perfringens* type C proliferate and produce toxins in the intestine (10). *C. perfringens* type C causes severe intestinal damage, but death is due to mainly from absorption of toxins from the intestine into the circulation. Therefore, type C infections are considered true enterotoxaemia (11,12). The presumptive diagnosis of *C. perfringens* infection can be based on clinical history (acute onset of diarrhea, colic, or sudden death) and gross and microscopic lesions (necrotizing enteritis or enterocolitis), because *C. perfringens* type C can be isolated from some healthy horses, definitive diagnosis should be based on detection of CPE toxin in intestinal contents. Therefore. this study was aimed to molecular identification of *Clostridium perfringens* by using Real-Time PCR technique.

## Materials and Methods

**Feces samples collection:** 30 feces samples from diarrheic horse were collected from different field in Al-Diwanyia city. The samples were collected in 25ml sterile containers transported into laboratory and stored in a refrigerator until use for genomic DNA extraction.

**Genomic DNA extraction:** Bacterial genomic DNA was extracted from stool by using (AccuPrep® stool DNA Extraction Kit. Bioneer. Korea). 200mg stool sample was placed in 1.5ml microcentrifuge tube and 20ul 10mg/ml Proteinase K and 400ul stool lysis buffer was added and mixed by vortex, then incubated at 60°C for 10 minutes. Then the tubes transferred in to centrifuged at 10000rpm for 5 min, After that, the supernatant was transferred in two new 1.5ml microcentrifuge tube and genomic DNA extraction was done according to company instruction. After that, the extracted DNA was checked by Nanodrop spectrophotometer, then store in -20°C at refrigerator until perform PCR assay.

## Real-Time PCR

Real-Time PCR technique was performed for direct detection *Clostridium perfringens* based amplification of enterotoxin (CPE) gene. The primes were designed in this study by using NCBI-GenBank recorded sequence for *Clostridium perfringens* strain CN1183 enterotoxin (cpe) gene, complete sequence, GenBank: (GQ225719.1) and by using primer3 plus design online. The primers were provided by (Bioneer company . Korea) as show in the following table:

Primer	Sequence		Amplicon
cpe	F	ATCCAATGGTGTTCGAAAATGC	142bp
	R	TCCCCTAATATCCAACCATCTCC	

The Real-Time PCR amplification reaction was done by using (AccuPower® GreenStar™ qPCR PreMix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table:

qPCR master mix	Volume
Genomic DNA template	2µL
CPE gene Forward primer (10pmol)	1µL
CPE gene Reverse primer (10pmol)	1µL
DEPC water	16µL
Total volume	20µL

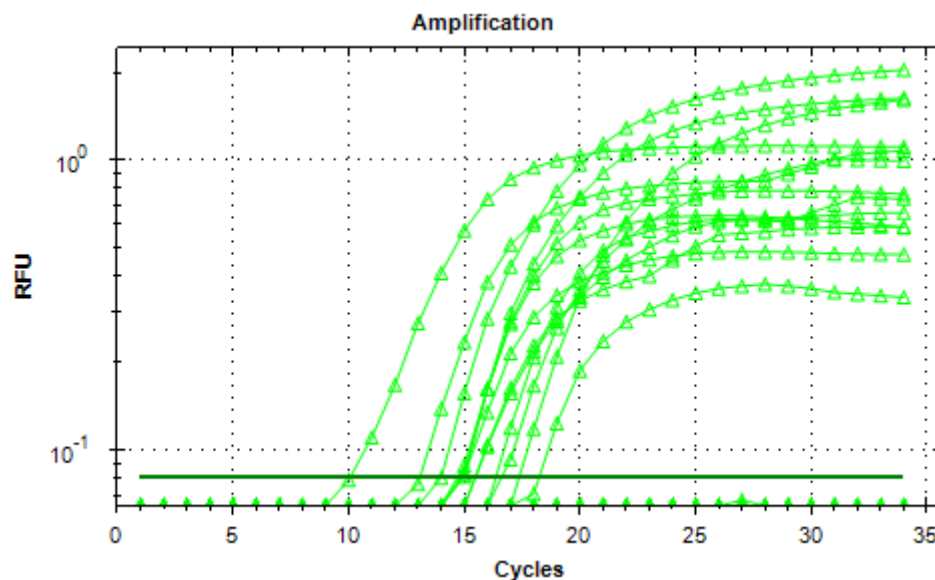
After that, these qPCR master mix component that mentioned in table above was transferred into Green star qPCR premix standard plate tubes that contain the SYBER green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, than placed in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following table:

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	10 sec	45
Annealing\ Extension	55 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

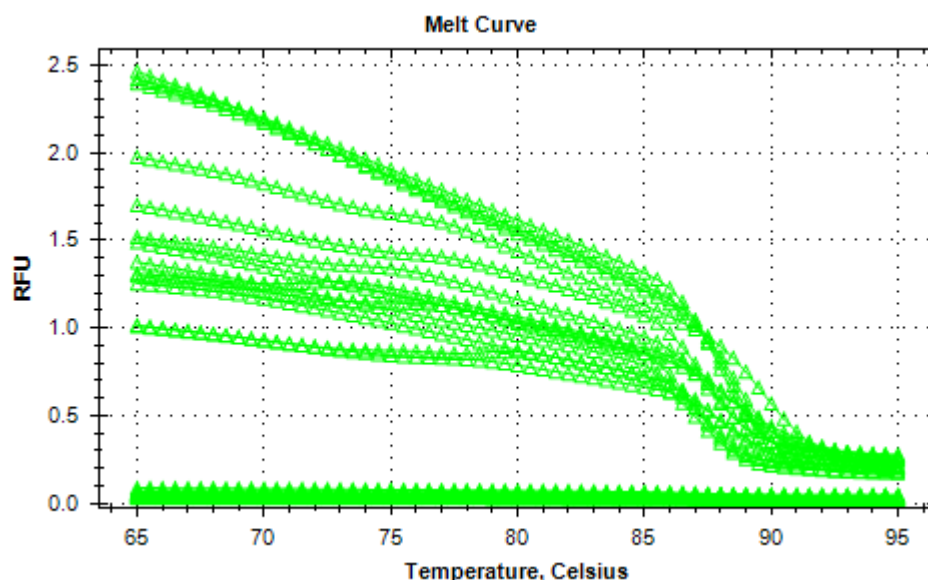
### Results and Discussion

The Real-Time PCR technique were show specific detection of *Clostridium perfringens* in total 12 positive out 30 fecal samples of horse infected by severe enterocolitis. The high prevalence of infection was show in foal have age from 1-2 month (8/12) positive samples at percent (26%). Whereas , less prevalence of infection was detect in camel in 4 positive out 30 fecal samples of stool samples at percent (13%). The Real-Time PCR technique based CPE toxin gene for direct

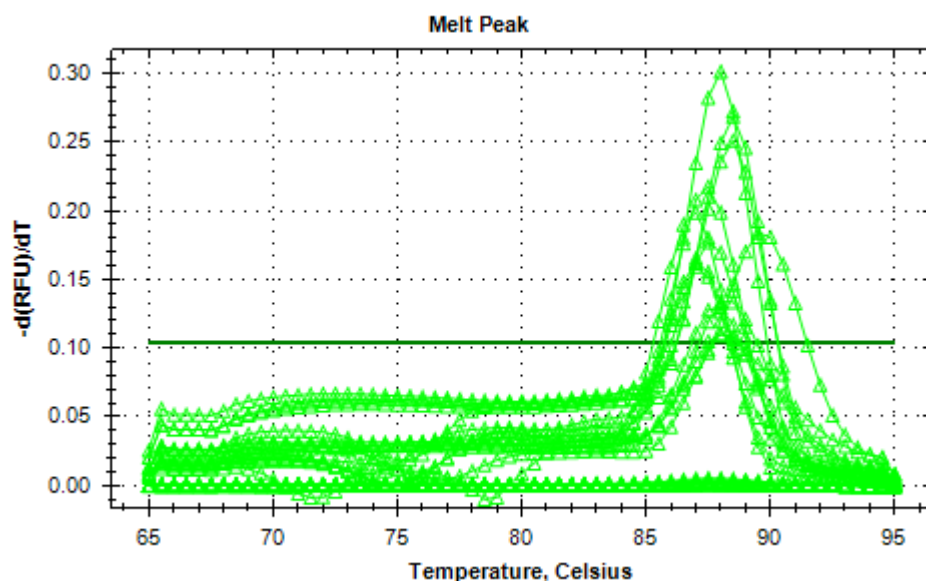
detection of pathogenic *Clostridium perfringens* were show good amplification in extracted DNA from fecal samples as shown in the following figures:



(Fig. 1): Real-Time PCR amplification plots for *cpe* gene in *Clostridium perfringens* positive in horse stool samples that show threshold amplification cycle at 10 to 18 cycle.



(Fig. 1): Real-Time PCR melt curve of *Clostridium perfringens* positive in horse stool samples



(Fig. 1): Real-Time PCR melt peak of *Clostridium perfringens* positive in horse stool samples that show primers specificity at approximately 88C melt peak.

This study was demonstrate that *C. perfringens* producing enterotoxin (CPE) is common and important causes of acute enterocolitis in horses, Despite this, the study has some limitations. Most importantly, this was not a case control study and the results were not compared to age-matched healthy horses. But our study focused on in detection of *C. perfringens* that producing enterotoxin (CPE) in only foal and adult horses that infection by enterocolitis and these limitations should be taken into account. The finding that *C. perfringens* was detected in half of the fecal samples was consistent with the detection of this bacterium in 54% in a previous study (13, 14). This study was support our hypothesis that cpe-positive and CPE-producing isolates of *C. perfringens* are common and important in equine enterocolitis. Enterotoxin (CPE) was detected 12 enterocolitis cases. Previous investigations study evaluating CPE in feces of horses with diarrhea and enterocolitis have had variable results. For example, *C.*

*perfringens* was identified in 42 of 233 diarrheic foals; of 24 isolates genotyped, all were type A but cpe was found in only 3. Subsequently, CPE was detected in only 2 of 17 cases with *C. perfringens* positive culture results (15). Weese and colleagues in Ontario detected CPE using ELISA in 19% of 47 adult horses and 29% of 28 foals with colitis and diarrhea (15). In Belgium, however, Van Baelen and Devriese did not find CPE in diarrheic feces from 9 adult horses (16). In another investigation, CPE was detected in 55% of foals with enteric diseases (17). Donaldson and Palmer found CPE in 9 of 57 diarrheic horses, but not in any of 57 control horses (18). In conclusion *C. perfringens* is important causes of enterocolitis infection in horse. whereas, the Real-Time PCR technique a rapid, sensitive, and specific method for the direct detection of *C. perfringens* in feces samples.

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