DIRECT DETECTION OF *Clostridium perfringens* BASED ALPHA TOXIN GENE FROM SHEEP AND CATTLE BY REAL TIME PCR TECHIQUE

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ABSTRACT

Real-time polymerase chain reaction assay was used for detection of *Clostridium perfringens* toxins genes alpha (cpa)directly from the fecal samples of cattle and sheep.Fecal samples from 20 clinically healthy cattle and 20 clinically healthy sheep were collected randomly from different farms located in AL-Diwanyia city ,All the strains of *Clostridium perfringens* were analyzed by Real-Time PCR using specific primers for alpha (cpa)toxin genes ,its reveald that (cpa)toxin gene of *Clostridium perfringens* ,were detected in cattles in 8(40%) and 14 (70%) in sheep Real time positive respectively samples ,This results indicate that *Clostridium perfringens* alpha toxin producing strains were prevalent in sheep and cattles and its possibly play an important role in the diarrheal disease caused by *Clostridium perfringens* .

INTRODUCTION

Clostridium perfringens is commonly causative agent of the diseases in the most animals including(sheep,goats,cattle ,horses and poultry (1)(2)

The toxins type that produce depend on the *C. perfringens* strain, and each type of this toxin induces a specific disease (3).

Clostridium perfringens has been classified into 5 types (A-E),these types produce several and lethal toxins (alpha ,beta, epsilon and iota) (4). these toxins are closely related to the virulence of *Clostridium perfringens* .

Clostridium Perfringens type A is responsible for haemorrhagic enteritis ,haemolytic disease and gas gangrene. (5).Also *C. perfringens* type A causes food poisoning in human, with diarrhoea and cramps (6)

The alpha toxin produce by all the types of *Clostridium perfringens* and it is has lecithinase activity (7), α- toxin,is also a necrotizing toxin, is believed to be a major factor responsible for the organism tissue pathology and has been suggested to be a key virulence determinant and product of *C perfringens*. type A (8).

Type A strains produce only alpha toxin, Some strains form additional toxins that important for the pathogenesis of intestinal syndromes in man and animals (9).

The alpha toxin is found most commonly in animals and is found in human frequently more than other toxins (10)

The objective of the present study was planned to detect of *Clostridium perfringens* from sheep and cattle based amplification of alpha toxin (cpa) in AL-Diwanyia city by real –time PCR.techique.

MATERIALS AND METHODS

Feces samples collection: Twenty feces samples from clinically healty sheep and cattle were collected from different field in Al-Diwanyia city. Samples were collected in 25ml sterile containers then transported to the laboratory .The samples were stored in a refrigerator in 4°C until use for genomic DNA extraction.

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

Real-Time PCR

Real-Time PCR technique was performed for direct detection of *Clostridium perfringens* based amplification of alpha toxin (cpa) gene. The primes were designed in this study by using NCBI-GenBankrecorded sequence for *Clostridium perfringens* alpha toxin (cpa) gene, GenBank: (DQ787190.1) and by using primer3

plus design online.The primers were provided by (Bioneer company. Korea) (table 1) .

Table(1)The nucleotide sequences of primers used for PCR amplification

Primer	Sequence		Amplicon
Cpa	F	GCTAGCATGAGTCATAGTTGGG	81bp
	R	TCCTGCTGTTCCTTTTTGAGAG	

The real-time PCR amplification reaction was done by using (AccuPower® GreenStarTM qPCR PreMix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to manifecture (table 2).

Table(2) The qPCR master mix components according to company instruction

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

These qPCR master mix component that mentioned in the 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, and placed in miniopticon real-time PCR system and applied the following thermocycler conditions (table 3).

Table (3) The thermocycler conditions in the Real-Time PCR system

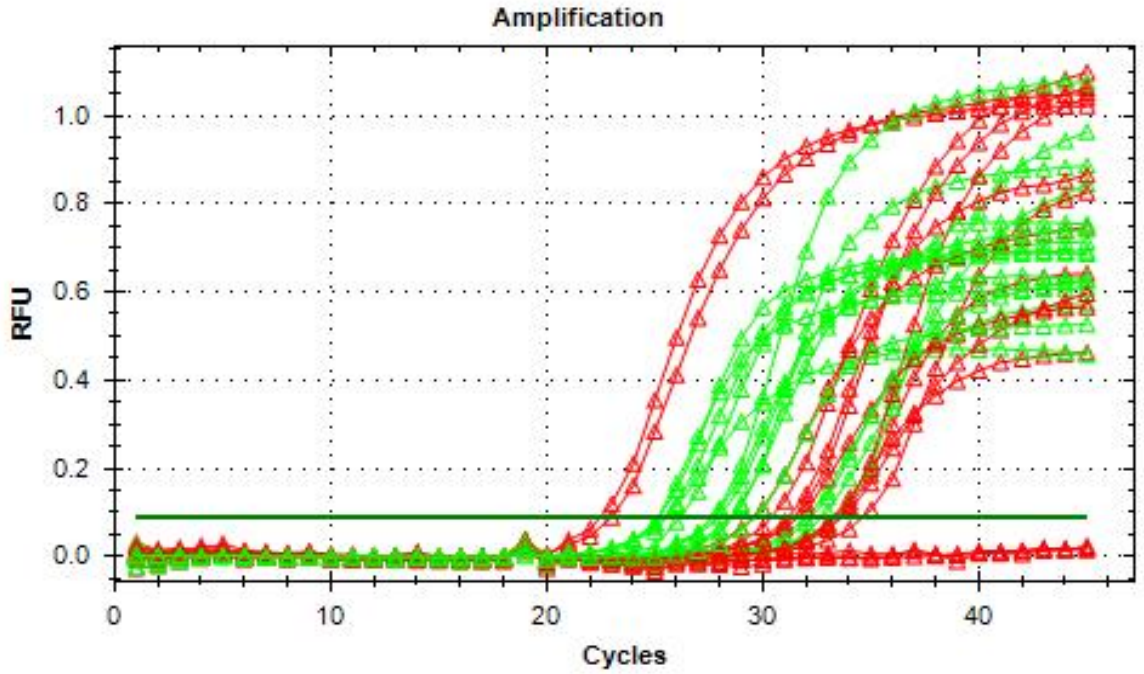
qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	3 minute	1
Denaturation	95 °C	10 sec	45
Annealing\Extension	60 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

RESULTS

The Real- time PCR assay revealed that (*cpa*)toxin gene were detected in cattle in 8(40%) and 14 (70%) in sheep Real time positive samples(table 4).

Table (4) Numbers of positive samples from sheep and cow and percent(%)

Sample	Positive	Percent (%)
Cow	8/20	40%
Sheep	14/20	70%



Fig(.1) Real-Time PCR amplification plots for cpa gene in *Clostridium perfringens* positive in cow and sheep feces samples that show threshold amplification cycle at 22 to 34 cycle. Cow: red plot and sheep: green plot.

Well	Fluor	Sample	End RFU	Call
A03	SYBR	Cow	0.453	(+) Positive
B03	SYBR	Cow	0.629	(+) Positive
C03	SYBR	Cow	1.04	(+) Positive
D02	SYBR	Cow	1.06	(+) Positive
D03	SYBR	Cow	1.02	(+) Positive
E02	SYBR	Cow	0.988	(+) Positive
E03	SYBR	Cow	0.554	(+) Positive
F02	SYBR	Cow	1.03	(+) Positive
A04	SYBR	Sheep	0.563	(+) Positive
A05	SYBR	Sheep	0.465	(+) Positive
B04	SYBR	Sheep	0.709	(+) Positive
C04	SYBR	Sheep	0.912	(+) Positive
C05	SYBR	Sheep	0.607	(+) Positive
D04	SYBR	Sheep	0.794	(+) Positive
D05	SYBR	Sheep	0.878	(+) Positive
E05	SYBR	Sheep	1.07	(+) Positive
F04	SYBR	Sheep	0.610	(+) Positive
F05	SYBR	Sheep	0.686	(+) Positive
G04	SYBR	Sheep	0.734	(+) Positive
G05	SYBR	Sheep	0.692	(+) Positive
H04	SYBR	Sheep	0.523	(+) Positive
H06	SYBR	Sheep	0.757	(+) Positive

Fig(2)Real-Time PCR endpoint data analysis of *Clostridium perfringens* positive in cow and sheep feces samples.

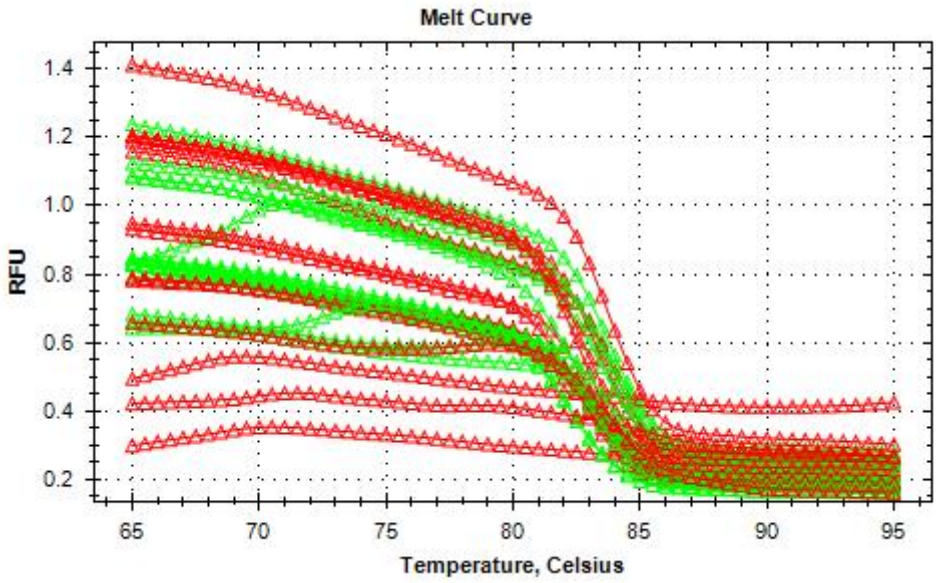


Fig (3) Real-Time PCR melt curve of *Clostridium perfringens* positive in cow and sheep feces samples

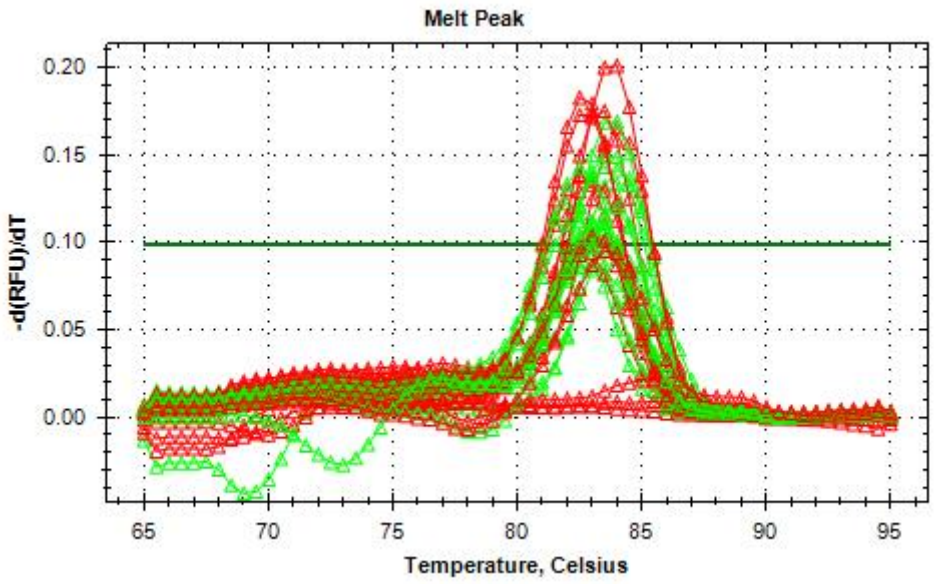


Fig (4) Real-Time PCR melt peak of *Clostridium perfringens* positive in cow and sheep samples.

DISCUSSION

In present study 8 out of 20 strains isolated from healthy cow (40%)and 14 out of 20 strains isolated from healthy sheep were type A.

The role of *Clostridium perfringens* type A in disease occurance is doubtful by some studies (11). The enteric pathogen including Clostridia are present in environment al samples such as feces in very low numbers (12). The results of our study similar with study occurs on healthy dairy cattle(13) .

They are 58 from 61 strains of *Clostridium perfringens* from healthy sheep were type A (95%). Also other studies in other countries reported that the most common type in sheep is (A)(15)thus we should be consider the Clostridium perfringens type A as zoonoses.

The highest percentage of alpha toxin genes from lambs. found that percentage of *Clostridium perfringens* from healthy sheep were(12%)were type A (6%).

35.1%prevalence of *Clostridium perfringens* type A genotype harboring cpa and cpb2 genes . These results suggest that *Clostridium perfringens* genotypes alpha and beta 2 toxin genes are distributed in animal species.

The presence of harbouring cpa belong to *Clostridium perfringens* strains in healthy animal may possible be an emerging to animal healthy if the physiological

equilibrium of the intestine and microflora is disturbed may be due to changes in feed or antibiotics treatment (19).

In conclusion ,our findings indicat that the Real- time PCR assay is good tool ,sensitive and rapid for detection of toxigenic *Clostridium perfringens* in fecal samples instead of conventional procedures . Other studies should be carried out to determine the other virulence factors of *Clostridium perfringens* strain from other diseased and healthy animals.

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الكشف المباشر عن المطثية الحاطمة المعتمدة على جينات سموم ألفا من الأغنام والماشية بتقنية الوقت الحقيقي لتفاعل سلسلة البلمرة

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الخلاصة

استخدم اختبار االوقت الحقيقي لتفاعل سلسلة البلمرة للكشف عن جينات جرثومة المطثيات الحاطمة المسؤولة عن افراز سموم الفا (cpa) مباشرة من عينات براز ل20 من الابقار السليمة سريريا و20 من الاغنام السليمة سريريا جمعت عشوائيا من حقول مختلفة تقع في مدينة الديوانية .جميع العزلات تم تحليلها بواسطة اختبار الوقت الحقيقي لتفاعل سلسلة البلمرة باستخدام بادئات متخصصة لجينات السموم نوع الفا (cpa)،تم من خلال الاختبار الكشف عن جينات سموم (cpa) لجرثومة المطثيات في 8 (40%) من الابقار و14 (70%)من الاغنام عينات موجبة على `التوالي .النتائج تدل على ان عتر جرثومة المطثيات المنتجة لسموم الفا موجودة في الابقار والاغنام ومن المحتمل انها تلعب دورا مهما في امراض الاسهال الناجم عن جرثومة المطثيات الحاطمة .

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