

The presence of toxin genes of *Clostridium perfringens* isolated from camels and humans in Egypt

Mohamed E. Mohamed^{1*}, Iman I. Suelam², and Mohamed A. Saleh³

¹Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Egypt

²Laboratory Animals Unit, Veterinary Hospital, Zagazig University, Egypt

³Animal Health Research Institute, Giza, Egypt

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ABSTRACT

Clostridium perfringens is a saprozoontic bacterium which causes food poisoning and wound infections in humans and enterotoxemia in animals. Camels are a common food animal in Egypt. There is a lack of available literature on genotypes of *C. perfringens* strains recovered from camels and humans in Egypt. So, the present study aims to detect some toxins genes in the circulating *C. perfringens* strains in man and camels. The multiplex PCR assay is effective and simpler and may be a useful alternative to standard in vivo typing methods. A total of 210 samples including 150 from camels (75 stool and 75 carcass swabs) and 60 human stools (40 diarrheic and 20 non-diarrheic) were collected from Zagazig Abattoir and Zagazig General Hospital, respectively. The samples were examined for isolation of *C. perfringens* then toxins genes were detected by multiplex PCR assay, using six pair primers to amplify the target genes. *C. perfringens* was isolated from camel stools, meat swabs, diarrheic and non-diarrheic human stool samples at the rate of 20/75 (26.7%), 2/75 (2.7%), 15/40 (37.5%) and 3/20 (15%), respectively. Multiplex PCR assay was performed to detect some toxin genes: alpha (*cpa*), beta (*cpb*), iota (*iap*), epsilon (*etx*) enterotoxin (*cpe*) and beta 2 (*cpb2*) in the isolates (No=40). The obtained results revealed that *C. perfringens* types A, B, C, D and E in the present study were detected at the rate of 26/40 (65.0%), 4/40 (10.0%), 1/40 (2.5%), 1/40 (2.5%) and 0/40 (0%), respectively. Eight *C. perfringens* isolates were negative to the toxin genes indicating that they are non-toxigenic. Two human enteritis strains of *C. perfringens* type A were positive to *cpe* and another two strains were *cpb2* positive. The present study indicates that *C. perfringens* harboring toxin genes are prevalent in camels and humans in Egypt. The most common circulating *C. perfringens* strains in camels and humans were *C. perfringens* type A. Further research is needed to study the molecular relationship between *C. perfringens* from camels and human strains to trace the source of infection.

Key words: toxin genes, PCR, zoonoses, camel, *Clostridium perfringens*, Egypt

*Corresponding author:

Dr. Mohamed Elsayed Mohamed, Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511 Egypt, Phone: +20 55 228 4283, Fax: +20 55 2283 683; E mail: bishet68@yahoo.de

Introduction

Clostridium perfringens is a gram positive, sporulating anaerobic bacterium responsible for a wide spectrum of anaerobic diseases in animals and humans (MacCLANE, 2001). Camels are a major source of animal protein in Egypt. Different *C. perfringens* biotypes are associated with various diseases in man and animals (GARMORY et al., 2000). *C. perfringens* Type A causes food poisoning in human, with diarrhoea and cramps (GARMORY et al., 2000; COLLIE and MacCLANE, 1998). Type C strains have been reported to cause enterotoxemia and necrotic enteritis in sheep, lambs, calves, piglets and fowl whilst type D strains cause lamb dysentery and pulpy kidney disease in sheep and lambs (HATHEWAY, 1990). *C. perfringens* has been classified into 5 toxigenic types (A-E) on the basis of major toxin production (CATO et al., 1986). Type A strains produce only alpha toxin, type B alpha, beta and epsilon toxin, type C alpha and beta toxin, type D alpha and epsilon and type E alpha and iota. Some strains form additional toxins that have been proposed to be important for the pathogenesis of intestinal disorders in man and animals (BAUMS et al., 2004). Genotyping of *C. perfringens* isolates solved the problems of enterotoxin detection which is only produced during sporulation (BAUMS et al., 2004). PCR is more accurate and faster than sero-neutralization with mice or guinea pigs. Multiplex PCR assays have been established to genotype *C. perfringens* with respect to the genes *cpa*, *cbp*, *etx*, *iap*, *cpe* and *cpb2*, encoding the alpha, beta, epsilon, iota, entero, and beta2 toxins, respectively (MEER and SONGER, 1997; GARMORY et al., 2000). Currently, there are no reports on the occurrence of toxin genes in *C. perfringens* isolated from camels and humans in Egypt. So, the present work was planned to study the occurrence of some toxin genes in *C. perfringens* isolates from camels and humans.

Materials and methods

A total of 210 samples including 150 from camels (75 stool and 75 carcass swabs) and 60 human stools (40 diarrhoeic and 20 non diarrhoeic) were collected from Zagazig abattoir and Zagazig General Hospital, respectively. The samples were collected during summer 2008. The age of the camels ranged from 3 to 5 years. None of the examined humans had a fever. Fecal specimens and camel carcass swabs were kept cool (4 °C) until processed.

Isolation of *C. perfringens* was performed (JORES et al., 2008) at the Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt. About one gram individual stool samples or carcass swabs were cultured anaerobically (AnaeroGen, Oxoid Ltd., England) at 40 °C for 24 hr in 10 mL cooked meat medium (Oxoid Ltd.). The crude cultures were sub-cultured onto blood glucose agar (blood agar base, Merck GmbH, Germany) containing 5% sheep blood and 1% glucose (Merck). After 24 hr at 40 °C, colonies with typical growth characteristics were sub-cultured and identified (KONEMAN et al., 1992).

Six reference *C. perfringens* strains used for multiplex PCR were of different genotypes (ATCC 13124, DSM 756, NCTC 4964, Stamm 585/00, NCTC 8346, NCIB 10748) (kindly provided from Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Zagazig University, Egypt). Forty *C. perfringens* isolates from camels and humans were tested for the prevalent toxin genes. Each isolate was cultured into 10 mL thioglycolate broth and incubated anaerobically overnight at 37 °C. One mL from each culture was centrifuged for 15 min at 3000×g at 4 °C and the pellet re-suspended in 1 mL containing 0.05 mol l⁻¹ Tris, pH 7.2, and kept frozen until DNA preparation.

DNA from the thawed suspension was prepared with the Easy DNA kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions.

The primers used for the detection of toxin genes in *C. perfringens* have been published by TITBALL et al. (1989), HUNTER et al. (1993), HUNTER et al. (1992), PERELLE et al. (1993), CZEZULIN et al. (1993), and GARMORY et al. (2000) as shown in Table 1.

Table 1. Primers sequences for some toxin genes of *Clostridium perfringens* in the present study.

Gene	Primer sequence 5' to 3'	Product size bp	Reference
<i>cpa</i>	GCTAATGTTACTGCCGTTGACC TCTGATACATCGTGTAAAG	324	Titball et al. (1989)
<i>cpb</i>	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	Hunter et al. (1993)
<i>etx</i>	GCGGTGATATCCATCTATTC CCACTTACTTGTCTACTAAC	655	Hunter et al. (1992)
<i>iap</i>	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	Perelle et al. (1993)
<i>cpe</i>	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233	Czczulin et al. (1993)
<i>cpb2</i>	AGATTTTAAATATGATCCTAACC CAATACCCTTACCAAATACTC	567	Garmory et al. (2000)

The multiplex PCR reactions (MEER and SONGER, 1997) each contained 50 ng *C. perfringens* template DNA, 62.5 pmol each *cpa* primer, 45 pmol each *cpb* primer, 55 pmol each *etx* primer, 70 pmol each *iap* primer, 45 pmol each *cpe* primer, 50 pmol each *cpb2* primer, dNTPs (PCR Dig Lab Mix; Boehringer Mannheim, Mannheim, Germany) to a final concentration of 0.1 mM, PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl₂), 0.1% Triton X-100, 2 units of Taq DNA polymerase (Pharmacia, Freiburg, Germany) and water to 50 µL DNA was denatured for 2 min at 95 °C and amplified for 35 cycles (1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C for denaturation, annealing and extension phases, respectively), and followed by an additional period of extension for 10

min at 72 °C (model 9600 GeneAmp PCR System; Perkin-Elmer). PCR products were separated by electrophoresis in a 2% (w/v) agarose gel with 0.5 µg of ethidium bromide/mL. Twenty µL PCR products were subjected to electrophoresis for 45-60 min at 80 V. Amplified bands were visualized and photographed under UV illumination.

Results

From the stools and meat of apparently healthy camel, 22 isolates of *Clostridium perfringens* were isolated and identified by biochemical tests.

Table 2. Occurrence of *Clostridium perfringens* in camels and humans

Source		Non examined	Positive	
			No.	%
Camel	Stool	75	20	26.7
	Meat	75	2	2.7
Human stool	Diarrhoeic	40	15	37.5
	Non diarrhoeic	20	3	15
Total		210	40	19

Concerning the control positive *Clostridium perfringens* reference strains, the corresponding amplified bands (data not shown) were detected as a quality control. In the obtained results (Table 3), the alpha toxin gene was amplified from 32/40 (80%) of *Clostridium perfringens* isolated from camel and humans. The results suggested that *C. perfringens* types A, AE, A β_2 , B, C, D, E and non toxigenic strains were genotyped with the following percentages: 55, 5.0, 5.0, 10, 2.5, 2.5, 0.0 and 20, respectively. The results suggested that the type A of *C. perfringens* was the most prevalent causative agent in samples collected from camels and humans in Egypt.

Table 3. Genotyping of *C. perfringens* isolated from camels and humans

<i>C. perfringens</i> type	Positive genes	Positive isolates		Camel		Human stool	
		No.	%	Stool	Meat	Diarrhoeic	Non-diarrhoeic
A	<i>cpa</i>	22	55	12	-	7	3
AE	<i>cpa</i> , enterotoxin	2	5.0	-	-	2	-
A β_2	<i>cpa</i> , <i>cpb2</i>	2	5.0	-	-	2	-
B	<i>cpa</i> , <i>cpb</i> , <i>etx</i>	4	10	4	-	-	-
C	<i>cpa</i> , <i>cpb</i> ,	1	2.5	1	-	-	-
D	<i>cpa</i> , <i>etx</i>	1	2.5	1	-	-	-
E	<i>cpa</i> , <i>iap</i>	-	0.0	-	-	-	-
Non toxigenic	negative	8	20	3	1	-	4
Total		40	100	21	1	11	7

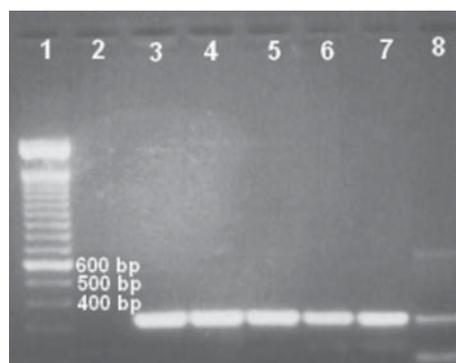


Fig. 1. Gel electrophoresis of PCR products (stained with ethidium bromide) obtained from the multiplex PCR genotyping assay of a representative *C. perfringens*. Lanes: 1, 100 bp marker; 2, control negative; 3-5, genotype A positive for *cpa*; 6&7, genotype AE positive for *cpa* and *cpe*; 8, genotype B positive for *cpa*, *cpb* and *etx*.

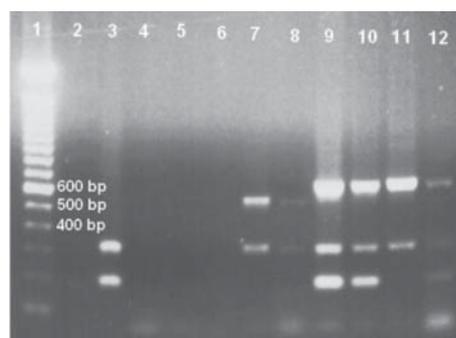


Fig. 2. Gel electrophoresis of PCR products (stained with ethidium bromide) obtained from the multiplex PCR genotyping assay of a representative *C. perfringens*. Lanes: 1, 100 bp marker; 2, control negative; 3, genotype C positive for *cpa* and *cpb*; 4-6, non toxicogenic; 7&8, genotype $A\beta_2$ positive for *cpa* and β_2 ; 9&10&12, genotype B positive for *cpa*, *cpb* and *etx*; 11, genotype D positive for *cpa* and *etx*.

Control *Clostridium perfringens* isolates (six different strains, A, Ae, $A\beta_2$, B, C, D and E) were tested for the reliability of the PCR results in the present study (data not shown because the amplified bands are similar to the tested strains). The amplicon band size of the reference strains matched that of the tested isolates.

The data shown in Figs. 1 and 2 are representative (No. = 16 field isolated) to the forty examined *Clostridium perfringens* strains.

Discussion

Clostridium perfringens is generally found in the gastrointestinal tracts of humans and animals. Humans are a reservoir for different genotypes of *cpe*-positive *C. perfringens* type A (HEIKINHEIMO et al., 2006). Nevertheless, little is known about the role of camels as a source of *C. perfringens* genotypes in Egypt. The pathogenicity of etiological agents could be tested by detection of toxin genes harbouring. Bacteriology and animal based typing assays are being replaced by conventional PCR-based confirmation techniques to identify the five toxin-types of *C. perfringens* (A-E) (SONGER, 1996). Table 2 shows that out of 150 analyzed samples of camel (75 each stool and carcass swabs), 20 (26.7%) and 2 (2.7%) *C. perfringens* strains were isolated, respectively. The incidence of *C. perfringens* is lower than that obtained by NASR et al. (2008) who reported 21/31 (67.74%) diarrheic and 6/11 (54.6%) apparently healthy camel calves. The variation may be attributed to the age of the examined animal which in the present study ranged from 3 to 5 years. Most of the camel isolates (12/22) were genotype A (Table 3).

The determination of genotype and sub genotype are of great importance in epidemiological studies of etiological agents and may help in tracing the source of infection. Of the 22 strains of *C. perfringens* isolated from camels in this study, 12 were type A (carrying only alpha genes). This value is similar to that found by NASR et al. (2008) who found that the incidence of type A (71.43 and 50.0%) was the most prevalent in diarrheic and apparently healthy camel calves, respectively, followed by type D (19.05% and 16.67%, respectively). In the present study, the PCR method was used for typing the *C. perfringens* isolate but NASR et al. (2008) used the biologic assay method. *C. perfringens* type A caused two outbreaks of enterotoxemia in racing camels (WERNERY et al., 1991). In the present study, other genotypes were detected in camel as type B (4/22), C (1/22) and D (1/22), but type E was not found. Moreover, 4 isolates were non toxigenic. The public health significance of the detected genes from camels is the burden by contamination of human food of camel origin. ALBINI et al. (2008) found that the correlation between toxin expression and PCR detection of the major toxin genes was 99% in cases of disease (MEER and SONGER, 1997) with almost no silent genes found. The current study reveals the potential role of camels as a potential reservoir for toxin gene positive *C. perfringens* strains and its role in food chain. It is noticed in Fig. 2 that the amplified band of the alpha toxin gene is weaker in intensity than other genes, which may be due to the fact that the chromosomal alpha toxin DNA is present in lower copy numbers than the plasmid- borne toxin genes (ALBINI et al., 2008).

Regarding the occurrence of *C. perfringens* in the examined human stools shown in Table 2, out of 60 examined human (40 diarrheic and 20 non-diarrheic), 15 (37.5) and 3 (15) *C. perfringens* strains were isolated, respectively. A higher result (28%) was found by CAMACHO et al. (2008) in antibiotic-associated diarrhoea cases in Costa Rica.

Concerning the isolated *C. perfringens* genotypes from humans (Table 3), only genotypes A, Ae and A β_2 were isolated with the rate of 10/18, 2/18 and 2/18, respectively. *C. perfringens* type A is associated with 5-20% cases of antibiotic-associated diarrhoea (VAISHNAVI and KAURS, 2008). The obtained result is near to that found by SONGER and MEER (1996) who reported that *cpe*-positive isolates represent only a very small fraction of the global *C. perfringens* population. Moreover, higher results (71.4% and 68.8%) for the enterotoxin gene (*cpe*) were found by KOBAYASHI et al. (2008) in *C. perfringens* isolates from two diarrhoea clusters in geriatrics. This variation may refer to the age factors and the immunocompromised nature of geriatrics. Also, LAHTI et al. (2008) reported higher results (91%) of *C. perfringens* carried *cpe* which were isolated from food poisoning outbreaks. CAMACHO et al. (2008) reported that all faecal *C. perfringens* isolates (No.=29) from human diarrhoea cases were classified by PCR as genotype A, one of which was positive to enterotoxins (3%). WATANABE et al. (2008) concluded that *C. perfringens* caused nosocomial diarrhoea one-tenth as frequently as *C. difficile* did in Japan, and that strains causing nosocomial diarrhoea might have already been acquired in the community.

It could be concluded from the present study that *C. perfringens* harboring toxin genes are prevalent in camels and humans in Egypt. The most common circulating *C. perfringens* strains in camels and humans were *C. perfringens* type A. Moreover, the obtained data may be important for understanding *C. perfringens* epidemiology and a control strategy plan. Further research is needed to study the molecular relationship between *C. perfringens* from camels and human strains to trace the source of infection.

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MOHAMED, M. E., I. I. SUELAM, M. A. SALEH: Prisutnost gena za toksine bakterije *Clostridium perfringens* izdvojene iz deva i ljudi u Egiptu. Vet. arhiv 80, 383-392, 2010.

SAŽETAK

Clostridium perfringens je saprozoonska bakterija koja uzrokuje otrovanje hranom i infekcije rana u ljudi te enterotoksemije u životinja. Uobičajeno je da se deve u Egiptu rabe za prehranu ljudi. U literaturi nema podataka o genotipovima sojeva bakterije *C. perfringens* izdvojenima iz deva i ljudi u Egiptu pa je ovo istraživanje usredotočeno na neke gene za toksine u sojeva te bakterije koji kruže u ljudi i deva. Višestruka lančana reakcija polimerazom učinkovita je i jednostavna metoda koja se može rabiti umjesto standardnih metoda tipizacije *in vivo*. Ukupno je bilo prikupljeno 210 uzoraka, od čega 150 iz deva (75 uzoraka fecesa i 75 obrisaka iz trupala) na klaonici u Zalazigu te 60 uzoraka stolice ljudi (40 s proljevom i 20 bez proljeva) u Općoj bolnici u Zalazigu. Uzorci su bili pretraženi na prisutnost vrste *C. perfringens*, a u izolatima su dokazivani geni višestrukom lančanom reakcijom polimerazom upotrebom parnih početnica specifičnih za određeni gen. *C. perfringens* je bi izdvojen iz 20/75 (26,7%) uzoraka fecesa deva, 2/75 (2,7%) uzoraka mesa, 15/40 (37,5%) uzoraka stolice ljudi s proljevom i 3/20 (15%) uzoraka stolice ljudi bez proljeva. Višestruka lančana

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reakcija polimerazom rabljena je za dokaz gena za neke toksine: alfa (*cpa*), beta (*cpb*), jota (*iap*), epsilon (*cpe*) enterotoksin (*etx*) i beta 2 (*cpb2*) u ukupno 40 izolata. Rezultati pokazuju da je tip A bakterije *C. perfringens* bio dokazan u 26/40 (65,0%) uzoraka, tip B u 4/40 (10,0%), tip C u 1/40 (2,5%), tip D u 1/40 (2,5%), a tip E nije bio ustanovljen ni u jednom uzorku. Osam izolata je bilo negativno na toksinske gene što znači da nisu bili toksigeni. Dva soja izdvojena iz ljudi s enteritisom bila su pozitivna za gen *cpe*, a druga dva za *cpb2*. Istraživanje pokazuje da se *C. perfringens* nositelj toksinskih gena javlja u deva i ljudi u Egiptu. Najčešće dokazani sojevi u ljudi i deva bili su oni tipa A. Daljnja istraživanja potrebna su za određivanje srodnosti između sojeva bakterije *C. perfringens* iz deva i ljudi da bi se mogao ustanoviti izvor zaraze.

Ključne riječi: toksinski geni, lančana reakcija polimerazom, zoonoze, deva, *Clostridium perfringens*, Egipt
