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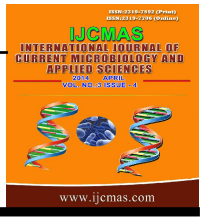

Original Research Article

Molecular Characterization and Genetic Diversity among *Clostridium perfringens* Isolates

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A B S T R A C T

Clostridium perfringens is serious bacteria causing enteric diseases in domestic animals and food poisoning in human. A total of 80 *C. perfringens* strains were isolated from different hosts (Sheep, goat, cows, buffalos and human) suffered from diarrhea with an incidence77. 77%, 83.33%, 75%, 57.69% and 60% respectively. Multiplex PCR was used in order to type *C. perfringens* by using four sets of primers specific for toxin -producing genes of *C. perfringens* (alpha, beta, epsilon and iota). The results of Multiplex PCR indicated that the most predominant type was *C. perfringens* type A with an incidence 87.5%. Sequence analysis of *C. perfringens* type A isolates obtained from different animal species and human in order to detect the genetic diversity of the alpha toxin gene was investigated.

Introduction

C. perfringens is one of the most important bacteria which cause diarrhea, it is a harmless member of the normal micro flora, but under certain conditions, it can multiply rapidly and secrete toxins and degradative enzymes that are associated with serious enteric disease (Songer, 1996). *Clostridium perfringens* is classified into five types; A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota (Yamagishi *et al*., 1997).

Alpha toxin is produced by almost all strains of this bacterium (Molly *et al*., 1976) and proposed to play a major role in both histotoxic infections as gas gangrene, and enteric infections as human food poisoning (McClane *et al*., 2006). The toxin has also been proposed to play an important role in several diseases of animals including enterotoxaemia in calves (Manteca *et al*., 2002), clostridial dysentery in lambs (Miserez *et al*., 1998),

hemorrhagic sudden death in veal calves during the feeding period (Singer *et al*., 2005) and in necrotic enteritis in chickens (Long and Truscott, 1976; Baba *et al*., 1992).

The gene encoding alpha toxin has been termed *plc* gene. The protein (*Cpa*) is known to comprise two domains; N-terminal domain possesses phospholipase C activity (Titball *et al*., 1991) and C-terminal domain lacks any detectable enzymatic or cytotoxic activity (Titball, 1993).

In addition to that, the *plc* gene is reported to be highly conserved in *C. perfringens* strains isolated from diseased chickens, with variations in only nine out of 397 deduced amino acid positions (Sheedy *et al*., 2004). Further knowledge about variations in the *plc* gene is important to obtain (Lone Abildgaard *et al*., 2009). The aim of our study is to investigate the molecular diversity between alpha toxin genes of *C. perfringens* type A isolated from diarrheic cases in Egypt.

Materials and Methods

Sample collection

Rectal swabs were collected from diarrhea cases (cows, sheep, goat, buffaloes and human) in Giza governors, Egypt, and then transferred directly to the laboratory. The total numbers of samples were illustrated in table (1)

Isolation and identification of C. *perfringens* isolates

Rectal swabs were inoculated onto a tube of sterile freshly prepared, cooked meat medium (Oxoid) according to Smith et al. (1968), and incubated anaerobically in an anaerobic jar using anaerobic gas generating kits at 37°C for 24-48 hrs.

A loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulfate (200 µg /ml). The plates were incubated anaerobically at 37°C for 24- 48 hrs. Colonies with hemolytic activity were transferred to anoxic reinforced clostridia medium (RCM) (Merck, Darmstadt, Germany) and incubated at 37°C for 24 hrs or until turbidity was clearly seen. The purity and the *C. perfringens identity* of the cultures was checked by phase-contrast microscopy and growth on Tryptose Sulfite Cycloserine (TSC) agar plates (Merck) supplemented with 4-methylbelliferyl phosphate (MUP) (Merck). The lecithinase activity of selected strains was tested on MUP supplemented TSC plates supplemented further with egg yolk emulsion (Oxoid).

DNA extraction, PCR and sequence of alpha toxin gene

Genomic DNA of *C. perfringens* was extracted by using an extraction kit (QIA amp mini kit, Qiagen). Specific oligonucleotide primers for the alpha (α), Beta (β), Epsilon (ε) and Iota (ι) toxin genes of *C. perfringens* were used as described in table (2).

The multiplex PCR assay was carried out in the PCR reaction mixture (25 µl) contained 1 µl of template DNA, 12.5 µl of master mix, 0.5 µl from each one of the primers (10 pmol/_l) and 10.5 µl of DNase free water. The PCR reaction mixtures were placed in a Biometra PCR thermal cycler. Following initial denaturation for 5 min at 94°C, the samples were subjected to 30 cycles of denaturation at 94°C for 1 min, annealing

at 55°C for 1 min and extension at 72°C for 1 min. After the last cycle, a final extension for 10 min at 72°C was performed according to Effat *et al*., (2007). The PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of 100-bp DNA ladder (Fermentas Life Science, EU) according to Sambrook *et al*., (1989).

PCR products of nine *C. perfringens* type A isolates obtained from different animal species and human was purified using the QIA quick quilt (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and sequenced at (Promega Lab. Technology) by using forward and reverse primers of alpha toxin genes as mentioned in Table (1).

Gene bank submission

The sequences of the alpha toxin gene have been deposited in the Gene Bank database under accession numbers kF383122 to kF383130

Sequence and phylogenetic analysis

Nucleotide (nt) sequences of the alpha toxin gene were compared with the sequences available in public domains using NCBI, BLAST server and the representative sequences of alpha toxin genes of *C. perfringens* of different animals and soil were obtained from Gene Bank, the accession number and sources of these sequences were described in the table (3).
Sequences were downloaded and imported into BIOEDIT version 7.0.1.4 for multiple alignments according to their deduced amino acid using the CLUSTAL W program of the BIOEDIT.

Phylogenetic analysis was performed with MEGA version 5.1 using the neighbor-joining approach. Bootstrap analysis was performed with 1000 resamplings.

Results and Discussion

Clostridium perfringens is a widespread enteropathogen, which responsible for many animal diseases (Ferrarezi *et al*., 2008). Complete identification of isolates confirmed that our isolates were *C. perfringens* as described by Quinn *et al*., (2002) where morphological and cultural identification revealed that all isolates were Gram-positive spore-forming bacilli, produced double zone of hemolysis on sheep blood agar with a total of 80 *C. perfringens* isolates obtained out from 115 examined samples with an incidence 69.56%.

The number and the incidence of *isolated C. perfringens* were illustrated in table (4). Results of the present work illustrated high rate of isolation of *C. perfringens* (69.56%), which indicated the role of this pathogen in causing disease in animal suffering from diarrhea and this result agreed with (Meer and Songer, 1997) who found that various forms of acute enteritis and fatal enterotoxaemia in animals have been attributed to *C. perfringens* and the pathogenicity of this organism is associated with exotoxins.

According to the present study, *C. perfringens* was isolated from sheep and goat with an incidence 77.77% and 83.33% respectively, these results nearly agreed with Rodriguez and Torres (1977), Cato *et al.*, (1986) and Mohamed (1991).The present work indicates incidence of *C. perfringens* in cattle and buffalos reach 75% and 57.69% respectively, these results nearly agreed with Manteca *et al*., 2002 and Das *et al*.,

(2012). These results indicated the presence of *C. perfringens* in all animal species with high prevalence and it is referred as the most widely distributed pathogenic microorganism in nature (Cato *et al*., 1986). In addition to that, isolation of *C. perfringens* from human in contact to animals showed high incidence (60%) is a clear cause of food poisoning outbreaks in humans; this result was in harmony with (Madoroba *et al*., 2010). The high levels of these bacterial toxins enter the bloodstream, leading to inflammation, shock, and cardiac arrest (Sherein *et al*., 2013).

Culturing and typing of *C. perfringens* by conventional methods such as dermonecrotic test is time-consuming ,expensive and requires the use of live animals, so PCR techniques have been developed to determine the *C. perfringens* toxinotypes Yoo *et al.,* (1997)**.**

PCR-based technology is considered to be a convenient and highly reliable tool for molecular detection of all the major toxin genes, such as α (*cpa*), β (*cpb1*), E (*etx*) and I (*iap*). According to multiplex PCR results, the given study proved that type A is the most frequently isolated genotype of *C. perfringens. A* total of 70 *C. perfringens* isolates were genotyped as type A with an incidence 100%, 80%, 91.66%, 80% and 83.33 % in sheep, goat, cow, buffalo and human respectively as described in table (5). These results were in harmony with Nahed *et al*., (2013) and Das *et al*., (2012). Alpha toxin is produced at high level in type A and is involved in pathogenesis of various diseases both in human and animal Petit *et al.,* (1999). It is the principal lethal toxin of *C. perfringens* which is a multifunctional phospholipase produced by nearly all isolates. The toxin is haemolytic, necrotizing and potently lethal Titball, (1993).

In the present study, all *C. perfringens* type A gave band with alpha toxin primers only at 402 bp as showed in photo (1). These results were agreed with the findings of El-Jakee *et al*., (2010) and Effat *et al*., (2007).

Sequence analysis of alpha toxin of *C. perfringens* type A is very important in order to understand the epidemiology of *C. perfringens* infections and may be helpful in the development of effective preventive measures. The concept of molecular variation between different *C. perfringens* toxin genes and within the same toxin gene is widely accepted and has been discussed in several studies (Ginter *et al*., 1996; Johansson *et al*., 2006; abildgaard *et al*., 2009).

Sequence analysis was performed on nine *C. perfringens* type isolates obtained from different animal species and human in order to detect genetic diversity of alpha toxin gene. The obtained amplicon sequences was first analyzed using BLAST n tool of Gene Bank, the BLAST result shows maximum identity ranging from 100% to 99% with *Clostridium perfringens* alpha toxin gene.

The given work, sequence alignment of 256 nt and deduced amino acid showed a higher percent of conservation exceeding 90% with some shared changes with the collected global strains and some unique mutations regarding our isolates suggesting an idea of co-evolution and/or molecular recombination which requires

Table.1 Number of collected samples according to species.

Species	No of samples
Sheep	9
Goat	12
Caprine	21
Cow	48
Buffalo	26
Bovine	74
Human in contact	20
Total	115

Table.2 Primer sequences for the alpha, beta, epsilon and iota toxin genes.

Primers target	Sequence	Amplified Product size	Reference
Alpha (α) toxin	F. GTTGATAGCGCAGGACATGTTAAG R.CATGTAGTCATCTGTTCCAGCATC	402bp	El-Jakee *et al*., 2010
Beta (β) toxin	F. ACTATACAGACAGATCATTCAACC R.TTAGGAGCAGTTAGAACTACAGAC	236bp	El-Jakee *et al*., 2010
Epsilon (ε) toxin	F. ACTGCAACTACTACTCATACTGTG R.CTGGTGCCTTAATAGAAAGACTCC	541bp	El-Jakee *et al*., 2010
Iota (ι) toxin	F. TTTTAACTAGTTCATTTCCTAGTTA R. TTTTTGTATTCTTTTTCTCTAGATT	298 bp	Effat *et al*., 2007.

further investigation (figure 1). Phylogenetic analysis showed that there are 2 distinct genetic groups as stated previously by Anders Johansson *et al*., (2006), despite the fact that samples had been collected from different animal species there were no species specific demarcation in the tree. One third of the study sequences 3/9 clustered with the sequence group showing greater degree of sequence conservation, the remaining two thirds 6/9 represented a divergent group with common evolutionary origin with a group of varied sequences.

The tree showed that the sequences of our local strains from (human, cow and goat) have the same ancestors, on the other side the soil strains on the gene bank and certain local strains have low diversion. This finding was supported by Youhanna and Songer, (2006) who mentioned that type A clinical isolates from different host species share common ancestor(s) or phylogenetic relationship(s) among themselves.

These given results also in harmony with Anders Johansson *et al*., (2006) who used PFGE to examine the genetic diversity of *C. perfringens* isolated from various animals, from food poisoning outbreaks and from sludge and found that the isolates from the same outbreak have very similar patterns while the genetic diversity is high in non-outbreak isolates and randomly selected isolates.

Table.3 Accession number of *C. perfringens* Strains.

Accession no.	Strain	Species	Country
kF383122	Zon-CU001	Buffalo	Egypt
kF383123	Zon-CU002	Cow	Egypt
kF383124	Zon-CU003	Human	Egypt
kF383125	Zon-CU004	Sheep	Egypt
kF383126	Zon-CU005	Sheep	Egypt
kF383127	Zon-CU006	Goat	Egypt
kF383122	Zon-CU007	Goat	Egypt
kF383129	Zon-CU008	Cow	Egypt
kF383130	Zon-CU009	Buffalo	Egypt
DQ180859	Strain 13	Pig	USA
JF298803	Strain CPC301	Sheep	Iran
JF298802	Strain CPB228	Sheep	Iran
JF298801	Strain CPA094	Sheep	Iran
DQ838702	Strain A18-Cp38	Goat	India
DQ838700	Strain A16-Cp34	Goat	India
AF477010	Alpha toxin	Chicken	Denmark
DQ787195	Strain CP33	Cattle	India
DQ787193	Strain CP29	Cattle	India
DQ787192	Strain CP27	Cattle	India
DQ787191	Strain CP25	Cattle	India
FR688011	Strain AL05-12	Soil	India
FR688010	Strain AG07-4	Soil	India
FR688009	Strain H2-3	Soil	India

Table.4 Incidence of *C. perfringens* in diarrheic samples

C. perfringens **Species**	**Positive isolates**		**Negative isolates**	
	No	**%**	**No**	**%**
Sheep	**7**	**77.77**	**2**	**22.22**
Goat	**10**	**83.33**	**2**	**16.66**
Caprine	**17**	**76.47**	**4**	**23.53**
Cow	**36**	**75.00**	**12**	**25.00**
Buffalo	**15**	**57.69**	**11**	**42.31**
Bovine	**51**	**68.92**	**23**	**31.08**
Human	**12**	**60**	**8**	**40**
Total	**80**	**65.16**	**35**	**34.84**

Table.5 Incidence of *C. perfringens* type A

Species	*C. perfringens*	*C. perfringens* Type A	
		No	%
Sheep	**7**	**7**	**100**
Goat	**10**	**8**	**80**
Caprine	**17**	**15**	**88.23**
Cow	**36**	**33**	**91.66**
Buffalo	**15**	**12**	**80**
Bovine	**51**	**45**	**88.23**
Human	**12**	**10**	**83.33**
Total	**80**	**70**	**87. 5**

Photo.1 Amplified PCR product of alpha toxin gene (402 bp). Lane M: 100bp ladder. Lane 1, 2: *C. perfringens* strains of Sheep. Lane 3, 4: Goat. Lane 5, 6: cow. Lane 7, 8: Buffalo & Lane 9:Human.

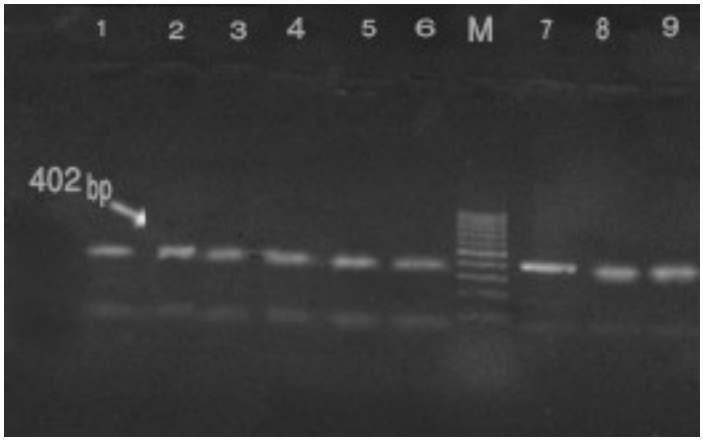


Figure.1 Amino acid graphic view showing similarities represented by dots and changes or differences represented by letters

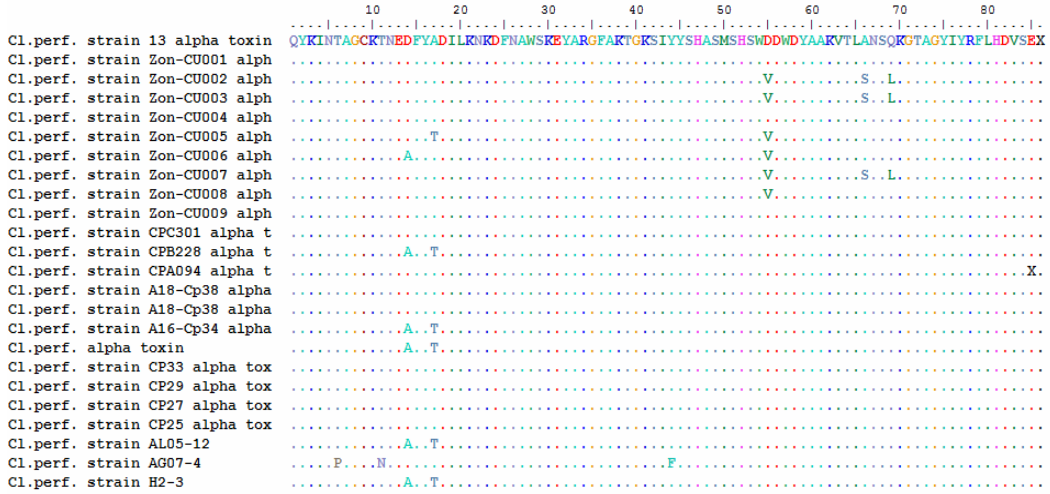
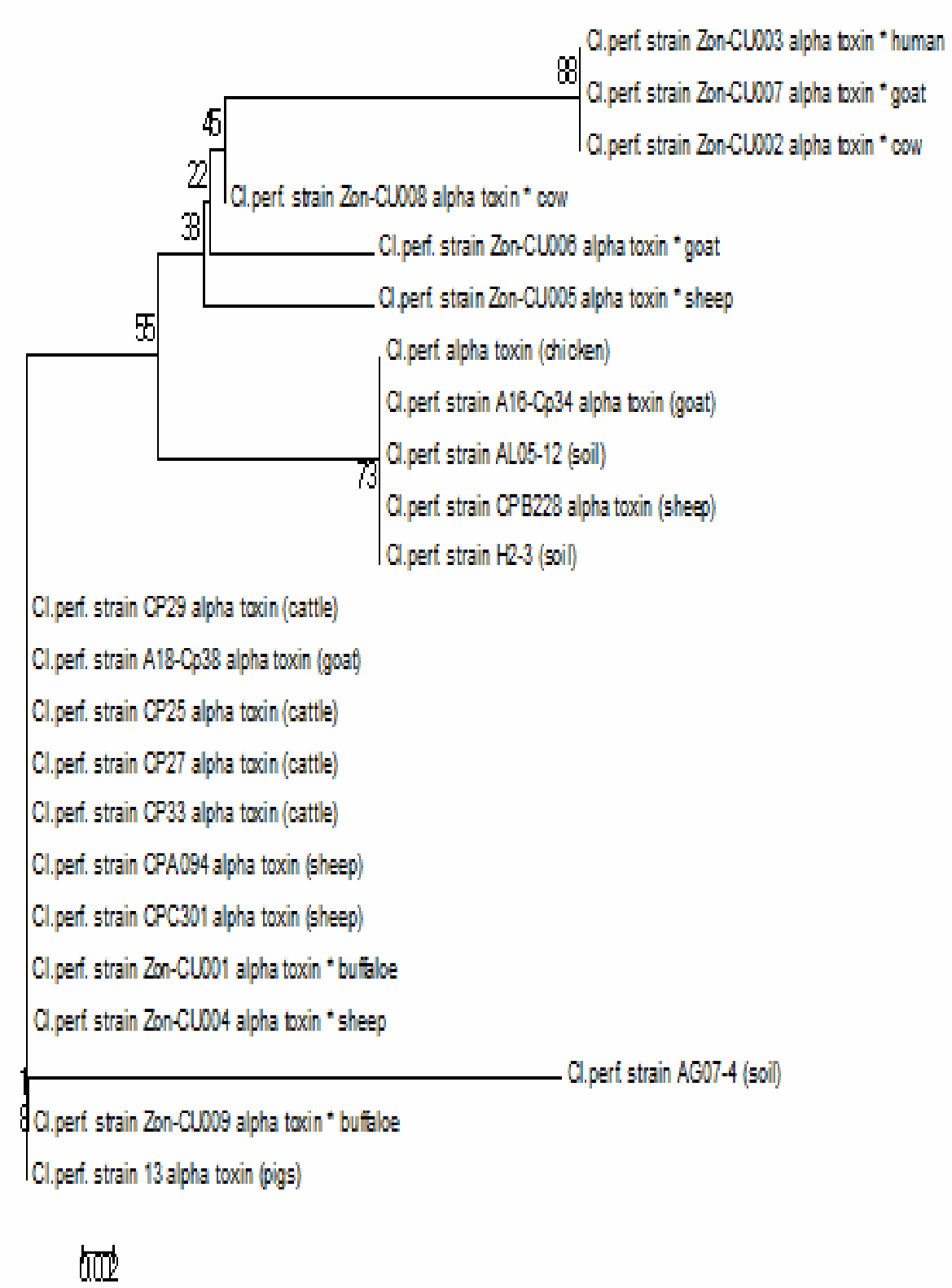


Figure.2 neighbor joining tree based on amino acid sequences, the source of the sequence of the present study represented by Asterisk, the source of the sequences of gene bank represented by brackets



The present study concluded that *C. perfringens* isolated from soil and animal species have a clear epidemiological relationship to human food poisoning. In addition it was indicated that *C. perfringens* strains have no species demarcation.

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