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Restriction Enzyme Analysis and DNA Sequencing Comparison for α-toxin Gene among Different Types of *Clostridium perfringens*

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Abstract: α-toxin is produced by all types of *Clostridium perfringens*. The genes encoding α-toxin from the available five types of *Clostridium perfringens* [A (chicken strain), A (rabbit strain), B, C and D] were PCR amplified using specific primers and the PCR products were examined on 1.5% (w/v) agarose gel and demonstrated the same bands comparable to the published α-toxin gene. Restriction enzyme analysis using two sets of enzymes (one set known to have recognition sites; *Hinf*1, EcoRV and *Mse*1 and the other set known to lack recognition sites *Hind* III, *Pst*1 and *Bam*H1) were carried out. The first set of enzymes revealed the same cut specific for α-toxin gene. However, the second set of enzymes revealed no cut which is consistent to the published data. The PCR products of α-toxin gene from the five types were separately sequenced and aligned with all published α-toxin genes of *Clostridium perfringens*. Identities among all studied α-toxin gene sequences and with the published ones were nearly 96-98%. There are no any significant differences among these nucleotide sequences. It is concluded that α-toxin gene sequences among different types of *Clostridium perfringens* are similar and highly conserved.

Key words: *Clostridium perfringens*, α-toxin, phospholipase C, DNA sequencing, restriction enzyme analysis

INTRODUCTION

Clostridium perfringens, an anaerobic spore former rod is widely distributed in the environment and in the intestines of humans, animals and some wildlife (Nillo, 1993). *Clostridium perfringens* type A (α-toxin producer) is common in the intestinal tract of chicks, soil, dust contaminated feed and liter (Kalender and Ertafi, 2005). The α-toxin (α) the principal lethal toxin of *Clostridium perfringens* is a multifunctional phospholipase produced by nearly all isolates. The toxin is haemolytic, necrotizing and potently lethal. The hydrolytic action of the toxin on membrane phospholipid found in the erythrocytes, platelets, leukocytes, endothelial cells and muscle cells results in lysis or other forms of cytotoxicity (Songer, 1996). The α-toxin gene (cpa) has been cloned and sequenced and homologous genes have been found in other clostridia (Katayama *et al*., 1993). Phospholipases are enzymes that degrade phospholipids and their classification is based on the site of cleavage. Phospholipase C cleaves between glycerol and the phosphate moieties. Phospholipases are found in all types of cells and various subcellular locations within eukaryotic cells. Phospholipases may exert a direct biological effect on an animal since they can destroy the cell membrane phospholipids resulting in both cytotoxic and haemolytic effect. Moreover, subhaemolytic doses of phospholipases are capable of degenerating mast cells leading to local changes in vascular permeability and elevation in blood kinins and development of anaphlactoid syndrome. Phospholipases, exotoxins are virulence factors that damage the host, they hydrolyse phospholipids in the host cell membrane leading to its disruption and killing the host cells by lysing them and aiding the phagocytosed bacteria to escape the phagocytic vesicle and enter the host cell cytoplasm (Titball, 1993; Salyers and Whitt, 1994). The five types of *Clostridium perfringens* could not be differentiated reliably on the basis of cellular or colonial morphology, biochemical reactions or gas liquid chromatographic analyses of fatty and organic acids and products of metabolism. α-toxin of *Clostridium perfringens* a key virulence determinant was suggested to be a cause of necrotic entritis in chickens. Analysis of α-toxin of 25 chicken derived *Clostridium perfringens* strains demonstrated high homology to mammal derived strains rather than to the only avian *Clostridium perfringens* α-toxin sequence reported (Scott *et al*., 2004). α-toxin may be produced more by isolates from birds with necrotic entritis than by isolates from normal birds (Hofshagen and Stenwig, 1992). Molecular structure of *Clostridium perfringens* α-toxin revealed two domain protein; amino terminal domain containing phospholipase C active site and non-toxic carboxyterminal domain a paralogue of lipid binding domains (Titball *et al*., 1999).

α-toxin is produced by all types of *Clostridium perfringens* (Yoo *et al*., 1997; Effat *et al*., 2007). Genetic analysis of α-toxin gene among different types of

Clostridium perfringens is performed to check if it is conserved or not. There is no any published article dealt with the study of DNA sequence among different types of this organism and even inside the same type especially rabbit and chicken strains of *Clostridium perfringens* type A. This study looked for the presence of any difference of the α-toxin gene sequence among different types of *Clostridium perfringens*.

MATERIALS AND METHODS

Five reference types of *Clostridium perfringens* (A rabbit strain, A chicken strain, B, C and D used in this study, were provided by the Serum and Vaccine Research Institute, Abbassia, Egypt. Amplification of α-toxin gene among these 5 types was performed using the following:

The primer nucleotide sequences for α-toxin gene and the melting temperature (Tm) for each primer are as follows:

Forward primer: 5' GTT GAT AGC GCA GGA CAT GTT AAG 3' (Tm 61.0).

Reverse primer: 5'CAT GTA GTC ATC TGT TCC AGC ATC3' (Tm 61.0).

Primers used in this study were designed according to Yoo *et al.* (1997) and obtained from Metabion International AG, Germany.

Qiagen master mix was used to amplify the gene and the PCR solutions are: 25 μL master mix, 2.0 μL forward primer (10 pmol μL^{-1}), 2.0 μL reverse primer (10 pmol μL^{-1}), 11 μL distilled water and 10 μL template (heat blocked supernatant of each type of *Clostridium perfringens*).

PCR protocol: The PCR thermal cycler was programmed for *Clostridium perfringens*.

Initial dentauration for 5 min at 94°C then 30 cycles each consists of a dentauration step at 94°C for one minute, an annealing step at 55°C for 1 min and an extension step at 72°C for 1 min. The cycles were followed by a stage of final extension for 10 min at 72°C. The Programme was adjusted at a stage of 4°C (as pause for keeping PCR product refrigerated) after ending the cycles and the final elongation (Yoo *et al.*, 1997). Ten microliter from each PCR product were mixed with 2 μL loading buffer and analysed on 1.5% (w/v) agarose gel in 1X TBE buffer and ran along with 6 μL of 100 bp DNA ladder. Ethidium bromide was added to a final concentration of 1:20000 from a stock solution of 10 mg mL^{-1} (Maniatis *et al.*, 1982). The electric current volt was adjusted at 50. The gel was examined under UV transilluminator and the pictures were taken using digital Kodak camera.

A group of Restriction enzyme known to lack recognition sites inside α-toxin gene:

*Pst*1 5'...CTGCA▼G...3'
3'...G▲ACGTC...5'

*Hind*III 5'...A▼AGCTT...3'
3'...TTCGA▲A...5'

*Bam*HI 5'...G▼GATCC...3'
3'...CCTAG▲G...5'

Other group of enzymes which are known to have recognition sites inside α-toxin gene:

EcoRv 5'...GAT▼ATC...3'
3'...CTA▲TAG...5'

*Hin*fI 5'...G▼ANTC...3'
3'...CTNA▲G...5'

*Mse*I 5'...T▼TAA...3'
3'...AAT▲T...5'

Sequencing was done using an automated cycle sequencing ABI prism 310 cycler PCR amplification, restriction enzyme analysis and DNA sequencing were done at the National Research Centre, Dokki, Giza, Egypt during the summer of 2007.

RESULTS AND DISCUSSION

PCR amplification of α-toxin gene: Upon performing PCR amplification for α-toxin gene among different types of *Clostridium perfringens*, each type of *Clostridium perfringens* revealed an amplicon nearly around 400 bp (Fig. 1) which is consistent to that of α-toxin gene (Yoo *et al.*, 1997; Effat *et al.*, 2007).

Restriction enzyme analysis: Upon using two groups of enzymes we found that *Pst*1, *Hind*III and *Bam*HI known to lack recognition sites inside α-toxin gene gave no cut. However, on using the other group of enzymes *Hin*f1 (gave two cuts), EcoRV (gave one cut) and *Mse*1 (gave three cuts), we found that EcoRV cuts only one cut giving rise to two bands one band very close to 100 bp (110 bp) and one band below 300 bp band (about 290). However, *Hin*f1 cuts in two sites giving rise to three bands (200, 120 and 90). The two bands of 120 and 90 were very close to each other giving impression to be one band. *Mse*1 enzyme cuts in three sites giving rise to four bands, two of about 20 bp and were not noticed on the gel. However, other two bands of *Mse*1 were at 90 and 270 bp.

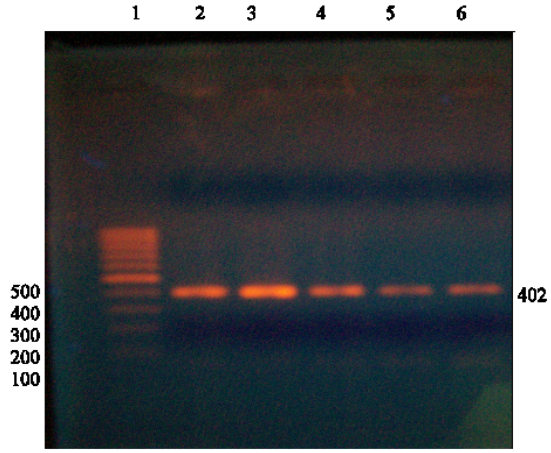

Fig. 1: Exhibits PCR product of α-toxin gene of different types of *Clostridium perfringens*, lane 1: 100 bp ladder, lane 2: *Clostridium perfringens* type A, rabbit strain, lane 3: *Clostridium perfringens* type A; chicken strain, lane 4: *Clostridium perfringens* type B, lane 5: *Clostridium perfringens* type C and lane 6: *Clostridium perfringens* type D

The results of the second set of enzymes were found to be the same as those published. No significant differences were found.

The identities of the nucleotide sequences between α-toxin gene of *C. perfringens* type A chicken strain and α-toxin gene of *C. perfringens* ATCC 13124 are very close to each other, nearly 98% which indicate high homology (Fig. 2).

The identities of the nucleotide sequences between α-toxin gene of *C. perfringens* type A rabbit strain and α-toxin gene of *C. perfringens* ATCC 13124 are very similar to each other, nearly 97% which indicate high homology (Fig. 3).

The identities of the nucleotide sequences between α-toxin gene of *C. perfringens* type B and α-toxin gene of *C. perfringens* ATCC 13124 are very close to each other, nearly 98% which indicate high homology (Fig. 4).

The identities of the nucleotide sequences between α-toxin gene of *C. perfringens* type C and α-toxin gene of *C. perfringens* ATCC 13124 are very close to each other, nearly 97% which indicate high homology (Fig. 5).

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TTTAGCTTTGCGAGGAAGAAACACAGTATAAAATAAACAGCAGGTTGCAAAACTAATGAGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAA
TGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGATTGGGATT
ATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTT
GGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGATGACTACATGTATTTTGG

Query  7      TTTGC-GAGG-AAG-AAA-CACAGTATAAAAT-AA-ACAGCAGGTTGCAAAACTAATGAG  60
              ||||| |||| ||| |||  |||||||||||| || |||||||||||||||||||||||
Sbjct  48906  TTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAG  48965

Query  61     GCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATATGCA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48966  GCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATATGCA  49025

Query  121    AGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49026  AGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGT  49085

Query  181    TGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAGCG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49086  TGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAGCG  49145

Query  241    GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGGAAAG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49146  GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGGAAAG  49205

Query  301    AATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGAT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49206  AATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGAT  49265

Query  361    GACTACATGTATTTTGG  377
              |||||||||||||||||
Sbjct  49266  GACTACATGTATTTTGG  49282
```

Fig. 2: Nucleotide sequence of α-toxin gene of *C. perfringens* type A chicken strain and its alignment with all published α-toxin gene of *C. perfringens* especially that of *Clostridium perfringens* ATCC 13124 (Rood and Cole, 1991)

```
TTTTGACTTTGCAGAGGAAGAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAGGATTTTTATGCTGATATCTTAAAAAACAAAGA
TTTTAATCGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGGAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGAT
TGGGATTATGCAGCAAAGGTAACTCTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCC
ATCAGTTGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAACAGATGACTACATGATTTTA

Query  5      GAC-TTTGCAGAGG-AAG-AAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  61
              ||| |||||||||| ||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  48902  GACTTTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  48961

Query  62     TGAGGATTTTTATGCTGATATCTTAAAAAACAAAGATTTTAATCGCATGGTCAAAAGAAT  121
              ||||| ||||||| |||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  48962  TGAGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAAT-GCATGGTCAAAAGAAT  49020

Query  122    ATGCAAGAGGTTTTGCTAAAACAGGGAAATCAATATACTATAGTCATGCTAGCATGAGTC  181
              |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  49021  ATGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTC  49080

Query  182    ATAGTTGGGATGATTGGGATTATGCAGCAAAGGTAACTCTAGCTAACTCTCAAAAAGGAA  241
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  49081  ATAGTTGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAA  49140

Query  242    CAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTG  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49141  CAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTG  49200

Query  302    GAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAA  361
              |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  49201  GAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAA  49260

Query  362    CAGATGACTACATG-ATTTT  380
              ||||||||||||| |||||
Sbjct  49261  CAGATGACTACATGTATTTT  49280
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Fig. 3: Nucleotide sequence of α-toxin gene of *C. perfringens* type A rabbit strain and its alignment with published α-toxin gene of *C. perfringens* especially that of *Clostridium perfringens* ATCC 13124 (Rood and Cole, 1991)

```
TTTGGCTTTTGCGAGGAAGAAAGACCGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAGGCTTTTTATACTGATATCTTAAAAAACAAAGATTT
TAATGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGATTGG
GATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATC
AGTTGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGATGACTACATGT

Query  6      CTTTTGC-GAGG-AAG-AAAG-ACCGTATAAAATAAACACAGCAGGTTGCAAAACTAATG  61
              ||||||| |||| ||| |||| || ||||||||||||||||||||||||||||||||||
Sbjct  48904  CTTTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAATG  48963

Query  62     AGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATATG  121
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48964  AGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATATG  49023

Query  122    CAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATA  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49024  CAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATA  49083

Query  182    GTTGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAG  241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49084  GTTGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAACAG  49143

Query  242    CGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGGAA  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49144  CGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGGAA  49203

Query  302    AGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAG  361
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49204  AGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAG  49263

Query  362    ATGACTACATGT  373
              ||||||||||||
Sbjct  49264  ATGACTACATGT  49275
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Fig. 4: Nucleotide sequence of α-toxin gene of *C. perfringens* type B and its alignment with published α-toxin gene of *C. perfringens* especially that of *Clostridium perfringens* ATCC 13124 (Rood and Cole, 1991)

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TTGACTTTGCGAGGAAGAAGACCAGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAGGATTTTTATGCTGATATCTTAAAAAACAAAGATTTTA
ATGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGGAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGATTGGGA
TTATGCAGCAAAGGTAACTCTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAG
TTGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAACAGATGACTACATGCCCCC

Query  3      GAC-TTTGC-GAGG-AAG--AAGACCAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  57
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Sbjct  48902  GACTTTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  48961

Query  58     TGAGGATTTTTATGCTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATA  117
              ||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48962  TGAGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATA  49021

Query  118    TGCAAGAGGTTTTGCTAAAACAGGGAAATCAATATACTATAGTCATGCTAGCATGAGTCA  177
              |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  49022  TGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCA  49081

Query  178    TAGTTGGGATGATTGGGATTATGCAGCAAAGGTAACTCTAGCTAACTCTCAAAAAGGAAC  237
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  49082  TAGTTGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGGAAC  49141

Query  238    AGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGG  297
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49142  AGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTGG  49201

Query  298    AAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAAC  357
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  49202  AAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAAC  49261

Query  358    AGATGACTACATG  370
              |||||||||||||
Sbjct  49262  AGATGACTACATG  49274
```

Fig. 5: Nucleotide sequence of α-toxin gene of *C. perfringens* type C and its alignment with published α-toxin gene of *C. perfringens* especially that of *Clostridium perfringens* ATCC 13124 (Rood and Cole, 1991)

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TGGACTTTTGCAGAGGAAAGAAAAGACAGTATAAAATAAACACAGCAGGTTGCAAAACTAATGAGGATTTTTATGCTGATATCTTAAAAAACAAGG
ATTTTAATGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCATAGTTGGGATGAT
TGGGACTATGCAGCAAAGGTAACTCTAGCTAACTCTCGAAAAGGGAACAGCAGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATC
CATCAGTTGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAACAGATGACTACATGATATTTT

Identities = 370/381 (97%)

Query  3      GACTTTTGCAGAGGAAAGAAAAG-ACAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  61
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Sbjct  48902  GACTTTTGCAGAGGAAAGAAAAGAACAGTATAAAATAAACACAGCAGGTTGCAAAACTAA  48961

Query  62     TGAGGATTTTTATGCTGATATCTTAAAAAACAAGGATTTTAATGCATGGTCAAAAGAATA  121
              ||||| ||||||| |||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  48962  TGAGGCTTTTTATACTGATATCTTAAAAAACAAAGATTTTAATGCATGGTCAAAAGAATA  49021

Query  122    TGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCA  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49022  TGCAAGAGGTTTTGCTAAAACAGGAAAATCAATATACTATAGTCATGCTAGCATGAGTCA  49081

Query  182    TAGTTGGGATGATTGGGACTATGCAGCAAAGGTAACTCTAGCTAACTCTCGAAAAGGGAA  241
              |||||||||||||||| |||||||||||||||| |||||||||| |||||| ||
Sbjct  49082  TAGTTGGGATGATTGGGATTATGCAGCAAAGGTAACTTTAGCTAACTCTCAAAAAGG-AA  49140

Query  242    CAGCAGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTG  301
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  49141  CAGCGGGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTTG  49200

Query  302    GAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAAAAAGATGCTGGAA  361
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  49201  GAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGGAA  49260

Query  362    CAGATGACTACATGATATTTT  382
              ||||||||||||| ||||||
Sbjct  49261  CAGATGACTACATG-TATTTT  49280
```

Fig. 6: Nucleotide sequence of α-toxin gene of *C. perfringens* type D and its alignment with published α-toxin gene of *C. perfringens* especially that of *Clostridium perfringens* ATCC 13124 (Rood and Cole, 1991)

The identities of the nucleotide sequences between α-toxin gene of *C. perfringens* type D and α- toxin gene of *C. perfringens* ATCC 13124 are very close to each other, nearly 97% which indicate high homology (Fig. 6).

α-toxin, a necrotizing toxin commonly produced by all five types of *C. perfringens*, is believed to be a major factor responsible for the organism tissue pathology and has been suggested to be a key virulence determinant and predominant product of *C. perfringens* type A (Yoo *et al*., 1997; Scott *et al*., 2004). Previous study revealed that α-toxin gene is found in all types of *Clostridium perfringens* (Effat *et al*., 2007). However, no articles are found dealt with studying the nucleotide sequences among different types of *Clostridium perfringens*. We here demonstrated the studying of nucleotide sequence among different types and different strains in the same type (A; rabbit and chicken strains). Similarly to the results obtained in this study, Scott *et al*. (2004) found that on applying sequencing for α-toxin of 25 chickens derived *C. perfringens*, all sequences demonstrated high homology to mammal derived strains rather than to the only avian derived strain.

CONCLUSIONS

α-toxin gene PCR amplification for the available 5 types of *Clostridium perfringens* was performed using specific one set of primers. Bands of PCR products for *Clostridium perfringens* types demonstrated the same as that published at nearly 402 bp (Yoo *et al*., 1997). Upon performing restriction enzyme analysis on the PCR products of α-toxin genes produced by five types of *Clostridium perfringens* using two groups of enzymes, we found that *Pst*1, *Hind*III and *Bam*HI known to lack recognition sites inside α-toxin gene gave no cut. Moreover, on using the other group of enzymes *Hin*f1 (two cuts), EcoRV (one cut) and *Mse*1 (three cuts), we found that EcoRV cuts only one cut giving rise to two bands one band very close to 100 bp (110 bp) and one band below 300 bp band (about 290). However, *Hin*f1 cuts in two sites giving rise to three bands (200, 120 and 90). *Mse*1 gave four bands, two of about 20 bp and were not seen on the gel. However, other two bands of *Mse*1 were at 90 and 270 bp. The results of the second set of enzymes were found to be the same as those published. No significant differences were found in the restriction enzyme analysis. DNA sequencing for α-toxin gene among the available 5 types of *Clostridium perfringens* was done for confirming the results of restriction enzyme study and ensures the absence of nucleotide sequence differences inside the α-toxin gene. No significant differences were found in DNA sequences for α-toxin gene among different types of *Clostridium perfringens* and also between different strains of the same type. We concluded that all studied α-toxin genes obtained by PCR from the five types of *Clostridium perfringens* are the same when performing restriction enzyme study and DNA sequencing thus α-toxin genes for the 5 studied types were highly conserved.

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