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GENETIC CHARACTERIZATION OF TOXIGENIC *CLOSTRIDIUM perfringens* ISOLATED FROM HERBS AND SPICES IN EGYPT

A thesis submitted By

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6. SUMMARY

Molecular evaluation studies to determine the extent of pathogenicity of microbes are principal prerequisites to control or reduce safety hazards in food or feed or their additives. The International Organization for Standardization (ISO, Geneva, Switzerland) has regulated requirements for food safety hazards, including microbiology end point with final goal of human and animal safety and welfare.

From this concept, the present study was designed to investigate the toxicity of *Clostridium perfringens* isolated from herbs and spices delivered to and distributed all over the Egyptian markets. Fulfilling this aim required identification of different toxins produced by *C. perfringens* isolates, detection of their virulence and antimicrobial resistance genes, sequencing of the genes encoding such toxins and virulence factors.

C. perfringens is among microbes that cause foodborne illnesses and is a genetically diverse organism; therefore, it represents a focus for many microbiologists. For too many years, *C. perfringens* had been classified into five toxinotypes, A, B, C, D, and E, based on the production of four major toxins, namely, *CPA*, *CPB*, *ETX*, and *ITX*. Recently, an updated toxinotyping system based on the production of six major toxins, namely, *CPA*, *CPB*, *ETX*, *ITX*, *CPE*, and *net*B, has been proposed after incorporation of two new toxinotypes (F and G). In this new classification, *C. perfringens* type A strains are associated with gas gangrene but not with human food poisoning. *C. perfringens* type B, C, and D strains are often associated with enteric diseases in animals.

Identification and characterization of *C. perfringens* isolated from various sources have been done by many researchers, including us who found 33 positive isolates from herbs and spices commonly distributed in the Egyptian market.

PCR is considered as a rapid and useful method for genotyping of *C. perfringens*. In the present study, PCR was applied using primers for *alpha*, *beta*, *epsilon*, *iota* and enterotoxin toxin genes upon which identification and typing of *C. perfringens* depend. Results revealed that the 33 isolates from herbs and spices were positive for *alpha* toxin gene, 10 isolates were positive for *beta* toxin gene and only two isolates were positive for *epsilon* toxin gene, no

strains were positive for *iota* toxin-encoding gene, while five strains are positive for entero-toxin-encoding gene.

RAPD (Random Amplification of Polymorphic DNA) analysis is a simple type of PCR, using only a single short random primer (8–12 nucleotides). It is applied in the present study on twenty isolates. The results were confirmatory to those of conventional PCR. Similarity among RAPD analysis products from 20 isolates from herbs and spices was tested and revealed overall average coefficient of 0.26.

In a second run, PCR was applied to demonstrate *net*B and *tpe*L, which have lesser interest in most studies, virulence factors and *bla*, *tet*K and *erm*B antimicrobial resistance factors as well the link between these two virulence determinants and antimicrobial resistance in *C. perfringens* isolated from herbs and spices. The results indicated the low (27.3 %, 9 out of 33) incidence of *tpe*L positive clostridial strains isolated from herbs and spices and absence of those with *net*B gene.

The obtained multiplex PCR products indicated presence of *bla*, *tet*K and *erm*B genes in *C. perfringens* isolates from herbs and spices. *bla* gene was detected in 21 out of 33 isolates (63.63 %); *tet*K in 13 out of 33 (39.4 %); while *erm*B was detected in only one isolate out of the 33 (3 %).

This assay aimed at exploring the genetic basis of our previous findings of antibiotic susceptibility test (AST) that found *C. perfringens* isolates were resistant to Clindamycin, Vancomycin, Tetracycline and Erythromycin with inhibition zones of 6.28 ± 0.63, 8.78 ± 0.41, 9.63 ± 0.63 and 9.84 ± 0.66 mm, respectively. The finding of *bla* gene may explain the resistance of *C. perfringens* to Clindamycin and Vancomycin but the susceptibility to Penicillin-G (inhibition zone = 16.6 ± 1.16 mm) remains to be understood. Highest susceptibility of the microbe to Ampicillin-Salbactam (19.4 ± 0.98 mm) could be explained post-transcriptionally, where sulbactam inhibits beta-lactamase after its production from the bacterial cell.

The finding of amplified bands of *tet*K gene fragments in *C. perfringens* isolated from herbs (39.4 %) may partially explain and parallel with the recorded resistance of isolates to Tetracycline (8.8 ± 0.4 mm inhibition zone).

In contrast, the finding of only 3 % of *erm*B-positive strains is not parallel with and cannot explain the resistance of isolates to Erythromycin (9.8 ± 0.7 mm inhibition zone). This might refer to presence of other mechanisms exhibited by the bacterium for resistance against Erythromycin.

Sequence analysis was conducted upon the two genes with the highest incidence in the tested isolates, *viz alpha*-toxin encoding- and *bla* genes (100 & 63.63 %, respectively). The analyses were done on five *C. perfringens* type A isolates obtained from different representative source samples of herbs and spices in order to detect genetic diversity of alpha toxin-encoding gene. The obtained sequences were first analysed using BLAST tool of GenBank. The BLAST result showed maximum identity ranging from 100% to 96.3% with *C. perfringens alpha* toxin-encoding gene. Data of this study showed that sequence alignment of 402 nucleotides and deduced amino acids of *cpa* isolated from herbs and spices have high similarity and conservation with little diversities compared with those of *cpa* of other global strains.

Despite its apparent minimal contribution to antimicrobial resistance, yet, because of its high incidence in our tested isolates, its sequencing analysis was essential to map the epidemiology of *C. perfringens* infections caused by herbs and spices or food containing them and to detect genetic diversity of *bla*-gene. The analysis was done on random five *C. perfringens* isolates that was *bla*-positive obtained from different representative source samples of herbs and spices. The obtained sequences were analyzed using BLAST tool of GenBank. The BLAST result showed maximum identity ranging from 100% down to 97.3% with *C. perfringens bla*-gene. Data of this study showed that sequence alignment of first 80 nucleotides from total of 770 and the deduced amino acids of *bla*-gene first fragment isolated from herbs and spices. The overall sequence has high similarity and conservation with little diversities compared with those of *bla*-gene sequence of other global strains.

CONCLUSION:

From molecular data presented above, it could be concluded that, toxigenic strains of *C. perfringens* were detected in herbs and spices distributed in the Egyptian market with higher occurrence of *alpha* toxin-coding gene and absence of *iota* toxin. *C. perfringens* isolated from herbs and spices have a high similarity among global isolates of epidemiological concern. In

addition, *cpa* toxin-coding gene share common characterization with the sequences submitted to GenBank. Therefore, we strongly recommend making food professionals and organizations aware of this problem to take strict measures to avoid irrational use of herbs and spices whether fresh or dried.

Furthermore, strains of *C. perfringens* isolated from herbs and spices retailed in the Egyptian market have higher occurrence of *tpe*L but not *net*B toxin coding genes. Resistance of *C. perfringens* to tetracycline is partially dependent on presence of *tet*K gene, while sensitivity to Penicillin is evident despite the presence of *bla*-gene at a high rate among isolates that has a high similarity to the sequences submitted to GenBank among global isolates of epidemiological concern. These findings expand knowledge about *C. perfringens* isolated from herbs and spices as food additives, which provide scientific basis for efficient prevention and intervention of *C. perfringens*-caused problems in man and animals.