RESEARCH / INVESTIGACIÓN

Molecular characterization of *net*B and *tpe*L virulence factors and antimicrobial resistance genes of *Clostridium perfringens* isolated from herbs and spices

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Abstract: The present study aimed to determine some virulence-associated genes and antimicrobial multidrug resistance of *Clostridium perfringens* recovered from herbs and spices widely distributed in the Egyptian market. *C. perfringens* virulence and resistance factors were determined using PCR targeting the *net*B, *tpe*L, *erm*B, *bla* and *tetK* genes. Thirty three out of 392 samples (8.42%) from herbs and spices submitted to our laboratory for bacteriological screening were positive for presence *C. perfringens*. PCR results for the *tpe*L gene in isolated *C. perfringens* revealed 9 out of 33 (27.3 %) of isolates, while *net*B was not detected. The 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 genes mentioned above were selected to correspond to the ineffective antimicrobials; *erm*B for erythromycin, *tetK* for tetracycline, and *bla* for the remainder. PCR results for antibacterial resistant genes in isolated *C. perfringens* revealed their presence. From 33 isolates, *bla* gene was detected in 21 (63.4 %), *tetK* in 13 (39.4 %) and *erm*B in only one isolate (3.03 %). Sequencing analysis was done for the *bla* gene as an example for the detected genes as detected at the highest incidence (63.4%). No cross-relationship was detected upon comparing incidence data of both studied virulence genes and those of antimicrobial resistance. The present findings may explain the resistance of *C. perfringens* to the examined antibacterials and recommend avoiding the application of them to control the microbe. In addition, the authors recommend following strict hygienic procedures during the industry of herbs and spices to ensure their clearance from *Clostridium perfringens* before distributing the products as food additives into the markets.

Key words: *Clostridium perfringens*, Herbs, Spices, Antibiotic Susceptibility Test, Resistance, Genes.

Introduction

Among food additives, herbs and spices are considered as severe vectors for foodborne microorganisms, including *C. perfringens*[1,2]. Incidence of *C. perfringens* in herbs and spices has been checked worldwide, including India[3], Turkey[4], the United Kingdom[5], Italy[6], Lebanon[7], Saudi Arabia[8], and Egypt[9].

Abundant toxin production and multidrug resistance are considered as the two major problems caused by *Clostridium perfringens*. This microbe is Gram-positive eubacteria, a rod-shaped, spore-forming anaerobe widely distributed elsewhere in nature, including soil, surfaces, sewage, feces, foods, and food additives[10].

C. perfringens is a highly toxicogenic bacterium as it can produce various toxins (at least 17) that are considered to be its pathogenic virulence factors[11]. Four among these toxins are commonly categorized as significant as they may cause lethality; these are α-, ß-, £-, and i-toxins. Each strain with its particular toxins is associated with a particular disease in humans and animals[12]. For this, most studies are focused on looking at these toxins from bacterial isolates, including one carried out by our team (El-Tawab *et al.*, 2021, under publishing). On the other hand, some other genes were considered minor ones because they are thought not to be pivotal for *C. perfringens* pathogenesis. Among these genes are found *net*B and *tpe*L toxins. However, recently, these genes were suggested to contribute to disease pathogenesis. For many years, α-toxin was thought to be the major virulence factor involved in necrotic enteritis, but a clostridial strain cloned for deactivation of α -toxin still able to produce lesions in broilers. This study led to the discovery of the pathogenicity of a new toxin, NetB[13]. In addition, it was discovered that inoculation of broilers with strains positive for both *net*B and *tpe*L were associated with greater severity of gross lesions over strains with only *net*B[14], a fact that supports the idea of the pathogenicity of tpeL too.

Even though a relatively limited number of studies focused on investigating these two toxins and their driving genes, no study has looked at these two virulence factors in clostridial isolates from herbs and spices.

The second major problem of the studied microbe is its resistance to drugs. In a previous study, we have conducted the antibiotic susceptibility test and detected the resistance of clostridial isolates to Clindamycin, Vancomycin, tetracycline, and erythromycin. However, the underlying mechanism of this resistance was not investigated[9].

The present study aimed to investigate the *net*B and *tpe*L toxin/gene positivity of *C. perfringens* isolated from herbs and spices retailed all over the Egyptian markets and to determine the molecular mechanisms underlying the antimicrobial drug resistance by investigating the positivity to *bla*, *tet*K, and *erm*B genes. To fulfill this aim, PCR and sequencing, and phylogenetic analyses have been conducted on *C. perfringens* isolates from herbs and spices.

Methods

Samples and isolates

The study was applied to 392 samples obtained from the top herbs and spices suppliers in Egypt. The samples have been screened for the incidence of *C. perfringens*. Thirty-three *C. perfringens* isolates were obtained from these samples following ISO 6887-2, ISO 6887-3, ISO 6887-4, or ISO 8261[9]. The isolates were resistant to Clindamycin, Vancomycin, tetracycline, and erythromycin[9].

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DNA extraction

DNA was extracted from the pure isolated colonies using the QIAamp DNA Mini kit purchased from Qiagen® (Hilden, Germany) following the manufacturer's instructions with some modifications. The kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization; thus, the total hands-on preparation time is only 20 minutes. The DNA concentration was determined using NanoDrop ND-1000 Spectrophotometer from Thermo-scientific (Waltham, MA, USA) by reading absorbances at 260 and 280 nm. The extracted samples were stored at –20 °C until used as templates for PCR amplification.

PCR

The conventional PCR was applied to screen *net*B and *tpe*L toxin-encoding genes and those of resistance, namely, *bla*, *tet*K and *erm*B in the 33 isolates of *C. perfringens*, using Emerald Amp GT PCR Master Mix kit, Code No. RR310A was purchased from Takara Bio Inc.® (Shiga, Japan). The amplification process was performed according to the manufacturer's instructions, using specific primers from Midland® (TX, USA) and a thermal cycler from Biometra® (Jena, Germany). The thermal cycling conditions are briefly described in tables 1, 2, and 3.

Agarose gel electrophoresis

Thirty µl of each PCR test product, negative and positive controls, and 100-bp DNA ladder (purchased from Fermentas®, Massachusetts, USA; cat. no. SM0243) were loaded to agarose gel 1.5 % and the process was conducted according to (20) following instructions of the manufacturer. The power supply was adjusted between 1~5 volts/cm of the tank length. The run was stopped after about 30 min, and then the gel was transferred to the UV cabinet. A gel documentation system photographed the gel, and the data was analyzed through computer software.

Sequencing reaction of bla gene

Uniplex PCR products of five *C. perfringens* isolates positive of *bla* gene were taken randomly and purified using the QIAquik PCR product purification protocol (Qiagen®, Hilden, Germany) provided by the manufacturer. The purified PCR products were sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA), using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit, Cat. No. 4336817 (Perkin-Elmer / Applied Biosystems, Foster City, CA). The master mix using Big dye Terminator V3.1 cycle sequencing kit is described below (Table 4).

Toxin	Primer	Sequence	Amplified product	Reference
netB toxin	F	GCTGGTGCTGGAATAAATGC	560 bp	[15]
	R	TCGCCATTGAGTAGTTTCCC		
tpeL toxin	F	ATATAGAGTCAAGCAGTGGAG	466 bp	[16]
	R	GGAATACCACTTGATATACCTG		
bla	F	ATGAAAGAAGTTCAAAAATATTTAGAG	780 bp	[17]
	R	TTAGTGCCAATTGTTCATGATGG		
tetK	F	TTATGGTGGTTGTAGCTAGAAA	382 bp	[18]
	R	AAAGGGTTAGAAACTCTTGAAA		
ermB	F	GAA AAG GTA CTC AAC CAA ATA	638 bp	[19]
	R	AGT AAC GGT ACT TAA ATT GTT TAC		

Table 1. Oligonucleotide primers for the 5 targeted genes.

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x remix)	25 µl
PCR grade water	5 µl
Forward primer (20 pmol)	1 µl each
Reverse primer (20 pmol)	1 µl each
Template DNA	10 µl
Total	50 µl

Table 2. Preparation of 5 *Clostridium* genes uniplex PCR Master Mix.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
netB	94°C 5 min.	94°C 30.	58°C 45 sec.	72°C 45 sec.	35	72°C 10 min.
tpeL	94°C 5 min.	94°C 30.	55°C 45 sec.	72°C 45 sec.	35	72°C 10 min.
bla	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	35	72°C 10 min.
tetK	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
ermB	94°C 5 min.	94°C 30 sec.	57°C 45 sec.	72°C 45 sec.	35	72°C 10 min.

Table 3. Cycling conditions of the different primers for cPCR and RAPD.

Reagent	Amount
Big dye terminator v.3.1	2 µl
Primer	1 µl
Template according to quality of band and concentration of DNA	From 1 to 10 µl
Deionized water or PCR grade	Complete till to total volume become 20 µl
Total volume	20 µl (Well mixed and briefly spinned)

Table 4. Preparation of master mix using Bigdye Terminator V3.1 cycle sequencing kit.

Phylogenetic analysis

The nucleotide sequences of *bla* gene were compared with the sequences available at public domains using BLAST (Basic Local Alignment Search Tool) server to establish sequence identity to GenBank accessions[21]. A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, designed by (22) and phylogenetic analyses done using maximum likelihood, neighbor-joining, and maximum parsimony in MEGA6[23].

GenBank submission

The sequences of the bla gene have been deposited in the GenBank database under the following accession numbers: HY1 MT891107; HY2 MT891108; HY3 MT891109; HY4 MT891110; HY5 MT891111.

Data management

The obtained results were statistically analyzed using EXCEL® software version 16. The number of positive samples for each gene against the total number of examined samples was calculated as a percentage from the total.

Results and discussion

C. perfringens was first isolated by William Welch and George Nuttall at a Hospital in Baltimore, USA, following a postmortem autopsy from a dead patient and was termed as *Bacillus aerogenes capsulatus*[24]. The microbe can secrete various toxins of pore-forming nature by causing conformational change and barrel formation through the lipid bilayer of the affected host cells[25]. Among those toxins, 4 types are considered as significant, which are alpha, beta, epsilon, and iota; and according to the ability of a clostridial strain to secrete one or more of such significant toxins, *C. perfringens* was subtyped into five types, A, B, C, D and E[26].

Identification and characterization of *C. perfringens* isolated from various sources have been made by many researchers. In the present work, 33 positive isolates for the studied bacteria were obtained from herbs and spices (33 isolates/392 samples) commonly distributed in the Egyptian market[9]. Newer virulence factors, including *net*B and *tpe*L have been raised[13,27]. Relatively to the major ones (α-, ß-, £-, and i-toxins), these two factors have limited the number of studies on some source materials, especially poultry affected with necrotic enteritis. Moreover, no information is available about them from herbs and spices; therefore, we have encouraged to look at *net*B and *tpe*L from this source.

Irrational antimicrobial use has increased the antimicrobial resistance among bacterial pathogens, including *C. perfringens*. Moreover, an antimicrobial may also contribute positively or negatively to virulence factors of a bacterium[28].

In the present study, electrophoresis of the obtained multiplex PCR products confirmed the amplification of the target primer sequences for the positive controls of *net*B (560 bp) and *tpe*L (466 bp) gene fragments, while no bands were detected in the lanes of the negative controls. Bands corresponding to the *tpe*L fragments were detected in 27.3 % (9 out of 33) of the tested isolates, and no bands were detected for the *net*B fragments (Table 5 and Figures 1 & 2). The results may indicate the low prevalence of *tpe*L positive clostridial strains isolated from herbs and spices and the absence of those with *net*B genes. Although no similar studies were conducted on herbs and spices in Egypt, the absence of *net*B may be supported by the fact that this gene is only found in *C. perfringens* strains of poultry and exceptionally in one isolate recovered from a cow in the USA[29]. Comparatively, the presence of *tpe*L in about one-third of the obtained *C. perfringens* colonies may be partially consistent with (30), where was reported a 37 % of prevalence of clostridial strains positive for tpeL genes from ostriches. Low prevalence of both genes, netB, and *tpe*L in Alabama farms was also reported in (31). No studies have been conducted on herbs and spices regarding these genes to discuss. Generally, *net*B has a similar molecular size to beta-toxin, hence the name *net*B (necrotic enteritis toxin B-like), which has cytotoxic activity in chicken[13]. While *tpe*L is a member of the large clostridial toxins that is still poorly understood and needs further investigations, it also has cytotoxicity[27].

PCR data of the present study detected the presence of *bla*, *tet*K and *erm*B genes in *C. perfringens* isolates from herbs and spices. From 33 isolates, *bla* gene was detected in 21 (63.4 %), *tet*K in 13 (39.4 %) and *erm*B in only one isolate (3.03 %) (Table 5 and Figures 3, 4 & 5). This assay was aimed at exploring the genetic basis of our previous findings of antibiotic susceptibility test (AST) that found *C. perfringens* isolates 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[9]. The finding of *bla* may explain the resistance of *C. perfringens* to Clindamycin and Vancomycin based on its highest presence (about 64%), but the susceptibility to Penicillin-G (inhibition zone = 16.6 ± 1.16 mm) remains to be understood. The 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. These findings may be in partial consistency with (32) who reported zero % resistance of *C. perfringens* to Penicillin, Cefoxitin, Meropenem, and piperacillin with 3.8 % of resistance to Clindamycin. However, our finding of resistance to vancomycin may be inconsistent with that of (33,34) who reported that *C. perfringens* to vancomycin, is low (0–5.6 %) because of the limited use of this antibiotic in farms. 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

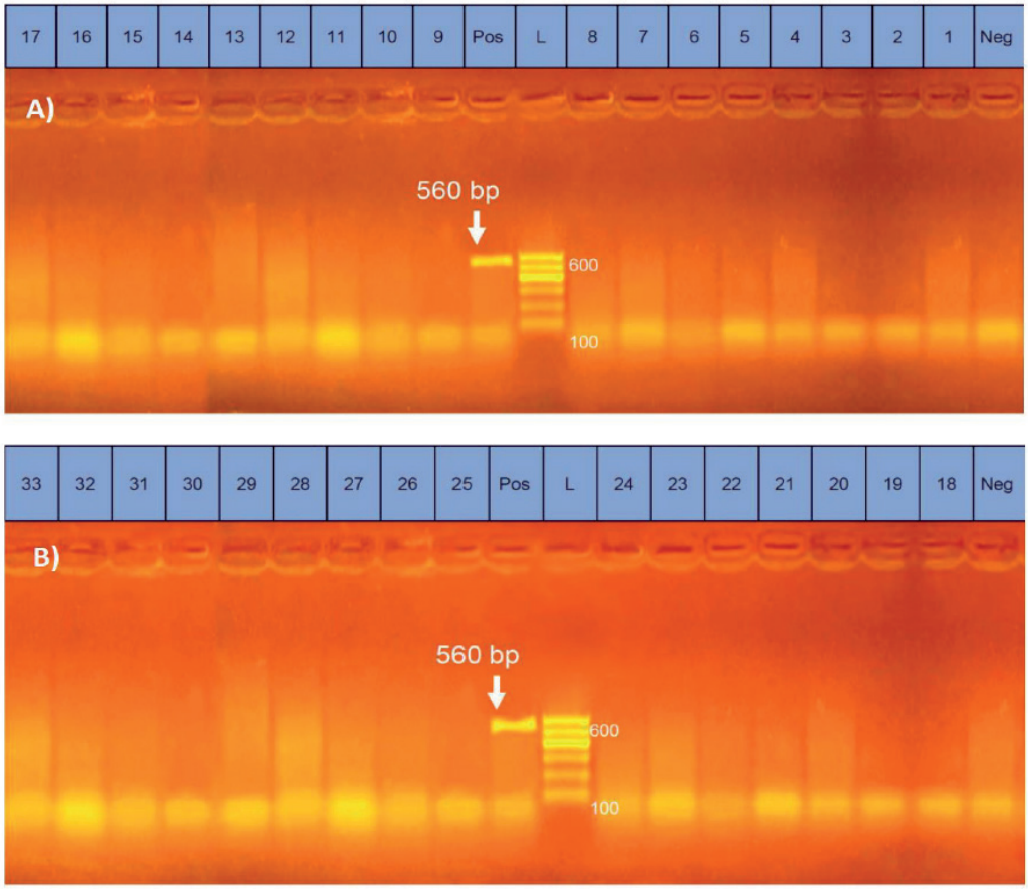

Figure 1. Uniplex PCR results of *net*B gene of *C. perfringens* isolated from herbs and spices in samples from 1~17 (A) and 18~33 (B); *net*B gene band was detected at 560 bp in control positive lane only.

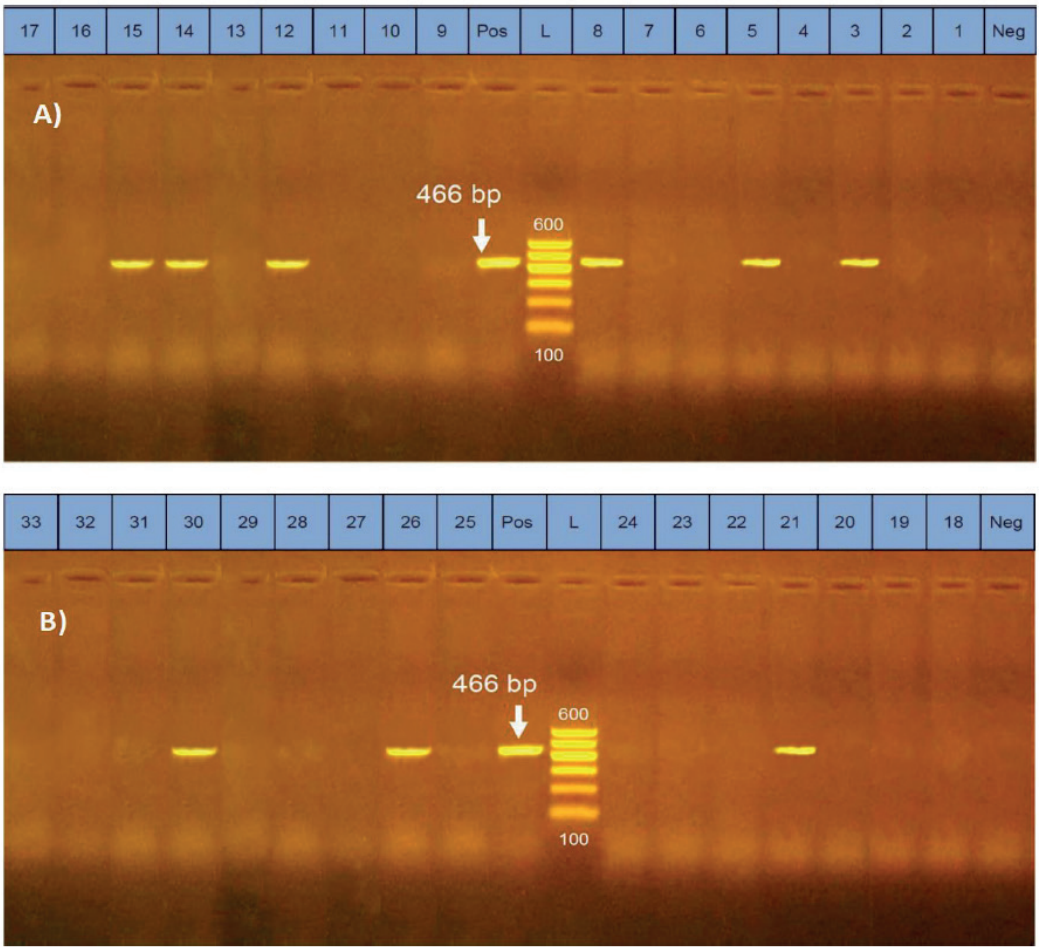

Figure 2. Uniplex PCR results of *tpe*L gene of *C. perfringens* isolated from herbs and spices in samples from 1~17 (A) and 18~33 (B); netB gene band was detected at 466 bp.

Sample #	Virulence genes		Resistance genes		
	netB	*tepL*	*bla*	*tetk*	*ermB*
1	-	-	+	+	-
2	-	-	-	+	-
3	-	+	+	+	-
4	-	-	+	+	-
5	-	+	+	+	-
6	-	-	+	+	-
7	-	-	+	+	-
8	-	+	+	-	-
9	-	-	-	-	-
10	-	-	+	-	-
11	-	-	+	-	-
12	-	+	-	+	+
13	-	-	-	-	-
14	-	+	+	-	-
15	-	+	+	-	-
16	-	-	+	-	-
17	-	-	+	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-
21	-	+	+	+	-
22	-	-	+	-	-
23	-	-	+	+	-
24	-	-	+	-	-
25	-	-	+	+	-
26	-	+	-	-	-
27	-	-	-	-	-
28	-	-	-	-	-
29	-	-	+	+	-
30	-	+	-	-	-
31	-	-	+	-	-
32	-	-	+	+	-
33	-	-	-	-	-

Table 5. Virulence (*net*B and *tpe*L) and multidrug resistance (*bla*, *tet*K and *erm*B) genes of *C. perfringens* isolated from herbs and spices.

explain the resistance of isolates to erythromycin (9.8 ± 0.7 mm inhibition zone). This might refer to the presence of other mechanisms exhibited by the bacterium for resistance against erythromycin. The findings of resistance to Tetracycline and Erythromycin are consistent with (35) who reported resistance of *C. perfringens* isolates to these two antibiotics by 50.8 and 29.2 %, respectively. However, our finding may not agree with 36, who reported that the most probable mechanism of resistance against macrolides is modifying a target site by a methylase encoded by the *erm*B gene. The authors added that the *erm*B genotype exhibits the highest level of resistance against all macrolides, the statement does not match our findings as the *erm*B gene was detected in only one isolate among all the resistant isolates.

Taken together, it could be speculated that *net*B toxin does not correlate with the resistance of *C. perfringens* to the tested antibiotics because it was absent in all resistant isolates. However, *tpe*L toxin might contribute to the antimicrobial resistance as it was detected in about 40 % of the resistant isolates. Although this hypothesis needs more in-depth investigations, it could be in agreement with (35) who reported that the *tpe*L gene was more common among *C. perfringens* isolates susceptible to tetracycline. From our previous studies, it could be stated that the antimicrobial resistance profiles of *C. perfringens* varies significantly according to sources, countries, other bacterial factors rather than the resistance-driving genes.

Despite its apparent minimal contribution to antimicrobial resistance, yet, *bla*- gene was detected in 21 out of 33 isolates (63.4 %) of *C. perfringens* isolated from herbs and spices; therefore, its sequencing analysis becomes essential to map the epidemiology of *C. perfringens* infections caused by herbs and spices or food containing them. In the present study, sequence analysis was done for bla gene to detect its genetic diversity. 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. Our data (Figures 6 A & B) show the sequence alignment of the first 80 nucleotides from a total of 770 and the deduced amino acids of the first bla fragment, firstly isolated from herbs and spices. The overall sequence has high similarity and conservation with little divergences compared with those of *bla*-gene sequence of other global strains listed in GenBank.

Figure 7 depicts the phylogenetic analysis of *bla*-gene sequence, which shows a great degree of sequence conservation with slight divergence. The tree showed that the sequences of our local strain from herbs and spices have the same ancestors. There are no other sequencing studies conducted on isolates from herbs and spices to discuss our results with them.

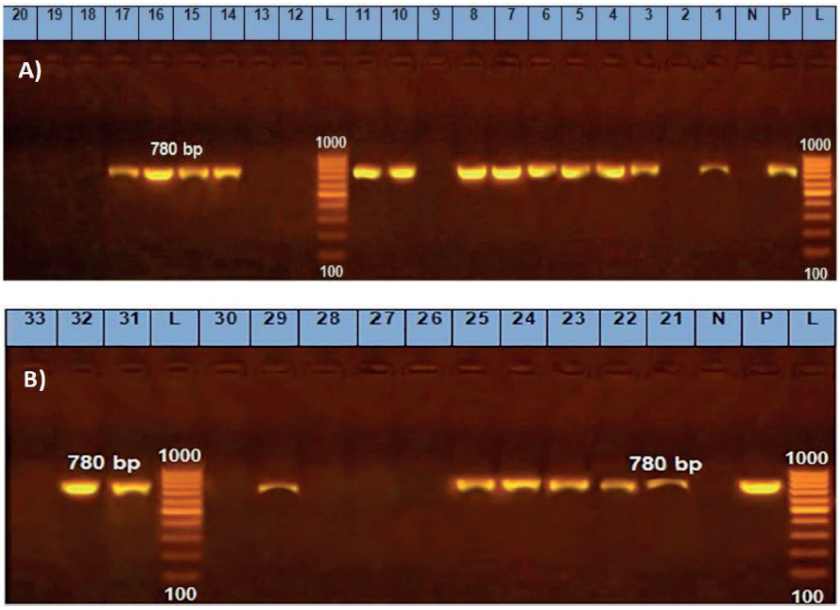

Figure 3. Uniplex PCR results of *bla* gene of *C. perfringens* isolated from herbs and spices in samples from 1~20 (A) and 21~33 (B); *bla* gene band was detected at 780 bp.

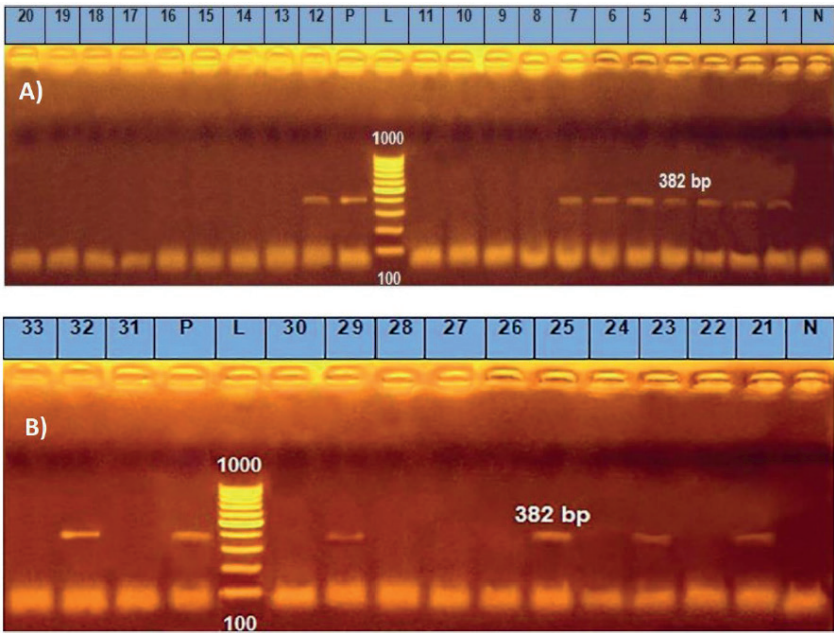

Figure 4. Uniplex PCR results of *tet*K gene of *C. perfringens* isolated from herbs and spices in samples from 1~20 (A) and 21~33 (B); *bla* gene band was detected at 382 bp.

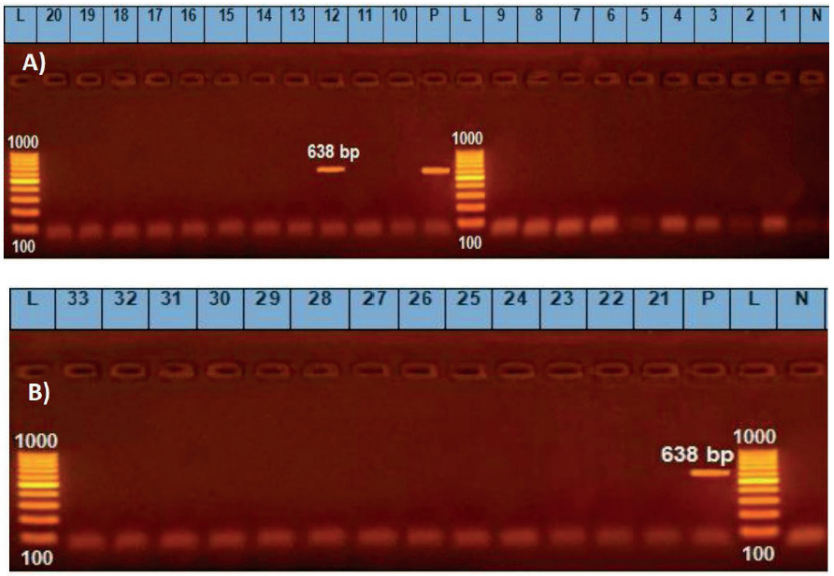

Figure 5. Uniplex PCR results of *erm*B gene of *C. perfringens* isolated from herbs and spices in samples from 1~20 (A) and 21~33 (B); *bla* gene band was detected at 638 bp.

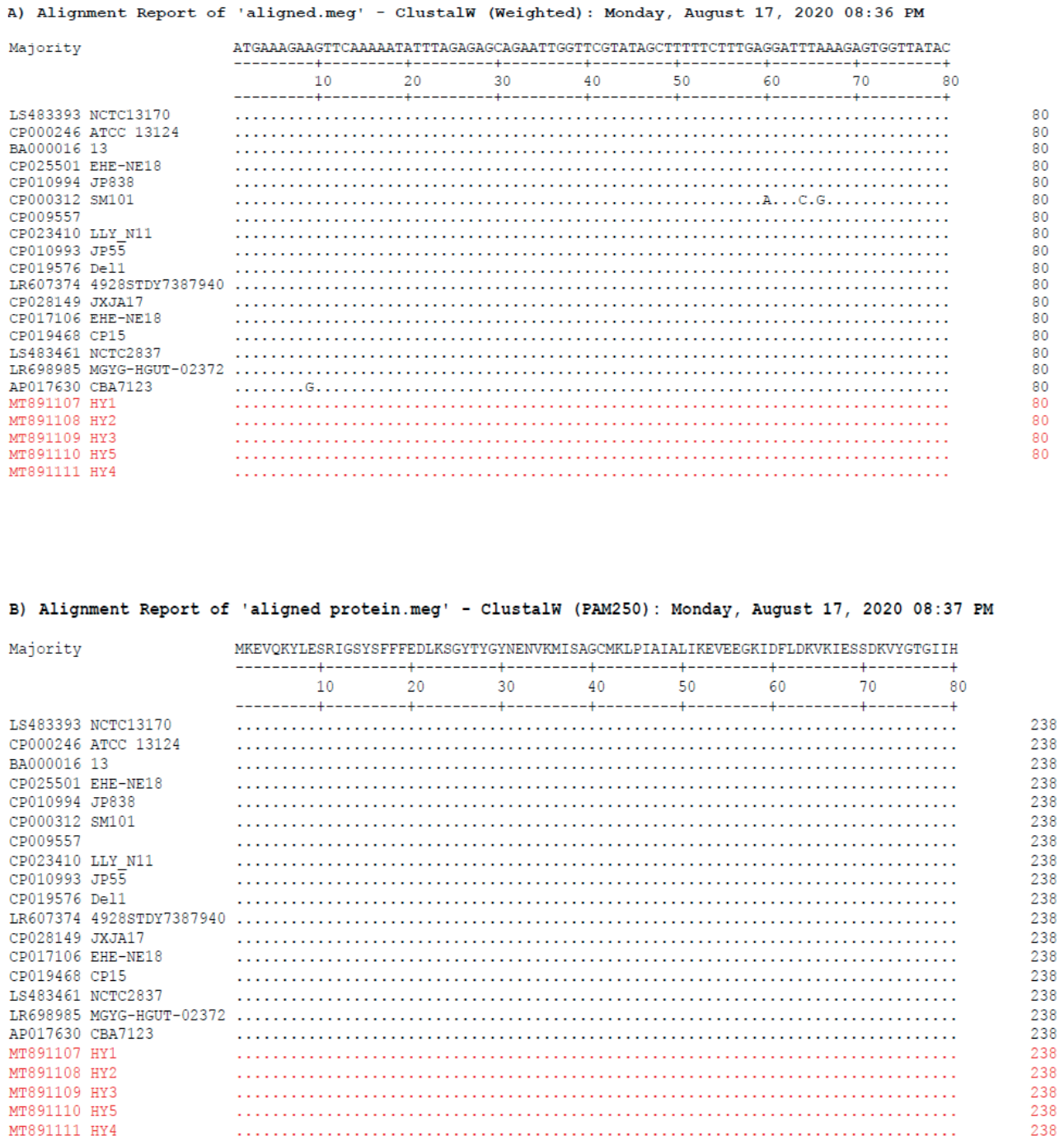

Figure 6. Nucleotides (A) and amino acid (B) graphic view of *bla* sequencing analysis showing similarities represented by dots and diversities represented by letters. Gene sequences submitted by the authors are colored red.

Conclusions

From the data presented above, it could be concluded that strains of *C. perfringens* isolated from herbs and spices retailed in the Egyptian market have a higher occurrence of *tpe*L but not *net*B toxin coding genes. Resistance of *C. perfringens* to tetracycline is partially dependent on the presence of *tet*K gene. At the same time, 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 the scientific basis for efficient prevention and intervention of *C. perfringens*-caused problems in man and animals.

Acknowledgments

Not applicable.

Declaration

Conflict of interest: There is no actual or potential conflict of interest concerning this article.

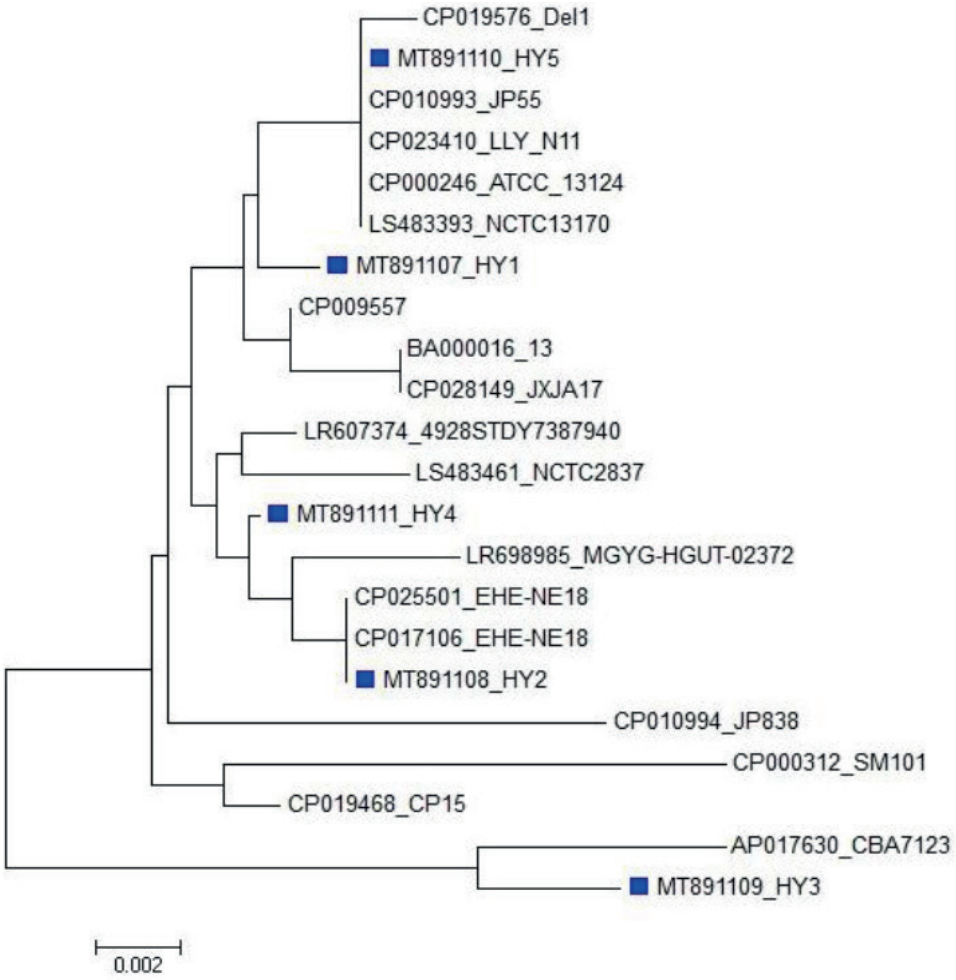

Figure 7. Phylogenetic analysis of nucleotide/amino acid sequences of *bla* coding gene of *C. perfringens* isolated from herbs and spices, the sequences of the present study are marked by blue squares among the sequences of GenBank.

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