Multiplex PCR for Detection and Genotyping of *C. botulinum* Types A, B, E and F Neurotoxin Genes in Some Egyptian Food Products

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Abstract: *Clostridium botulinum* [*C. botulinum*] is an obligatory anaerobic, Gram positive, endospore forming bacterium. It produces lethal botulinum neurotoxin (BoNT) that causes life threatening neuroparalytic illness known as botulism. Many cases of foodborne and infant botulism were reported worldwide. This study determined the prevalence of *C. botulinum* and its spores in some Egyptian food products using conventional methods and Multiplex PCR.

A total number of 250 food samples (50 honey, 100 fish products and 100 meat products) were randomly collected from apiaries, fish shops, butcher shops, retail stores and supermarkets in Assiut City. They were examined for the presence of *C. botulinum* and its spores using conventional methods. Multiplex PCR was done for genotyping of isolated *C. botulinum* for neurotoxin genes types (A, B, E and F).

The total number of positive samples for *C. botulinum* was 13 (26 %) in honey, 19 (19%) in fish products and 21 (21%) in meat products. Supernatant filtration (SF) method detected *C. botulinum* spores in honey more efficiently than dilution centrifugation (DC) method. Genotyping using Multiplex PCR was successful. It revealed that *C. botulinum* type A was 10% in honey and 6% in meat products; *C. botulinum* type B was 20% in honey, 5% in fish and 11% in meat products; *C. botulinum* type E was 14% in fish and 4% in meat products. While, *C. botulinum* type F was 0%.

The present study revealed that some food products in Assiut City may present a potential hazard for foodborne and infant botulism.

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1. Introduction:

C. botulinum is an obligatory anaerobic, Gram positive, endospore- forming bacterium that produces a lethal neurotoxin called botulinum neurotoxin (BoNT). BoNT is the most potent biological substance known ***(Shunji et al., (1998)***. It causes a fatal neuroparalytic condition known as botulism. Botulism comes from the Latin word "botulus," meaning sausage. When botulism was first recognized in Europe, many cases were caused by home-fermented sausages. BoNT acts presynaptically at the neuromuscular junction by blocking acetylcholine release, thereby leading to muscle weakness, paralysis, respiratory arrest and death ***(Ornella et al., 2001)***.

The spores of *C. botulinum* are commonly found in soil and marine sediments throughout the world. They also colonize the gastrointestinal tract of fishes, birds and mammals ***(CDC, 2004)***.

C. botulinum strains are divided into four distinct phenotypic groups (I-IV) based on cultural, biochemical, and physiological characteristics ***(Bremer et al., 2003).*** The composition of these groups are as following: group I include type A , type B and type F proteolytic strains; group II include type E, type B and type F non-proteolytic strains; group III include type C and type D strains and group IV include type G strains. Based on its phenotypic and genetic traits, group IV would be named *Clostridium argentinense* ***(Sagua et al., 2009).***

The pathogenecity of *C. botulinum* is associated with the production of 7 serologically distinct (BoNTs), designated (A, B, C [C_1, C_2], D, E, F, and G). The strains causing the majority of human botulism cases belong to groups I and II; while group III strains are the most frequent causes of botulism in animals. *C. botulinum* type C and D toxins have been questioned as the causative agent of human botulism. However; it is suspected that these toxins are not readily absorbed in human intestine ***(BAM, 2001)***.

There are four naturally occurring clinical categories of botulism, depending on the mode of acquisition of botulinum toxin: 1) foodborne botulism; 2) infant botulism; 3) wound botulism and 4) adult infectious botulism ***(Peck, 2009)***. Other forms of human botulism are recently considered.

These forms are inhalation botulism and iatrogenic botulism. Inhalation botulism results from an accidental or a deliberate release of aerosolized BoNT. Iatrogenic botulism occurs as a complication in the treatment with Botox A for therapeutic or cosmetic use ***(Sobel, 2005)***.

Foodborne botulism results from ingestion of food containing preformed botulinum neurotoxin. The toxin is heat labile and produced by *C. botulinum* in foods that have not been properly handled or canned as canned fish, meat and vegetables ***(Ahsan et al., 2005)***. Foodborne botulism doesn't spread from person to person. Home canned products, especially low acid food products attribute to most of the cases of foodborne botulism ***(Braconnier et al., 2001)***.

Infant botulism is a novel form of human botulism. In this type of botulism ingested spores of *C. botulinum* colonize and grow in the infant's large intestine and produce botulinum neurotoxin. It doesn't occur due to ingestion of preformed botulinum neurotoxin in food. *C. botulinum* spores can germinate in the gut of infants younger than 1 year because of: 1) their relative lack of gastric acid; 2) decreased levels of normal flora (is insufficient to competitively inhibit the growth of *C. botulinum* spores); and 3) immature immune systems (i.e. specifically lacking secretory immunoglobulin A). This environment is conducive to toxin production; therefore, infant botulism can arise from eating the spores present in uncooked foods ***(Arnon, 2004)***.

Infants are susceptible to infant botulism in the first year of life, with more than 90% of cases occurring in infants younger than six months ***(Underwood et al., 2007)***. To date, all inhabited continents except Africa have reported cases of infant botulism. Recognition of cases seem directly related to physician awareness and clinical suspicion ***(Lucia and Fabrizio, 2009)***. About 60% of infant botulism cases in Europe had a history of honey consumption ***(Aureli et al., 2002)***.

In the laboratory *C. botulinum* is very specific in the growth requirements. It requires strict anaerobic condition for growth. This could be achieved by several commercial kits as anaerobic Gas Pak System. The optimal temperature for growth of group I, II, III and IV is 35-40, 26-30, 40, and 37 ºC, respectively. A value of a_w above 0.93 is required to support growth and toxin production of *C. botulinum.* Optimal pH for growth of *C. botulinum* is from 7.0-7.2. However, their growth limiting pH is 4.5 ***(Vu, 2006)***.

The most widely used media for enrichment of *C. botulinum* are non-selective broths, such as Robertson's cooked meat medium, or trypticase -peptone-glucose-yeast extract (TPGY) broth. After enrichment the cultures are then streaked on a non-selective agar, such as blood agar, egg yolk agar (EYA), modified McClung-Toabe EYA plates or brain heart agar. *C. botulinum* is lipase positive on egg yolk containing media and produces β-haemolysis on blood agar ***Solomon et al., (2001)***.

The mouse bioassay has remained the standard test for the detection of botulinum neurotoxins. However, several problems are associated with it; in addition to being ethically debatable, it is laborious, expensive and time-consuming. Moreover, it requires specially trained personnel and appropriate facilities for the animals. Detection of neurotoxin gene fragments by PCR is a rapid alternative method for detection and typing of BoNT-producing Clostridia ***(Dario et al., 2009)***.

The aim of this study was: 1) to determine the prevalence of *C. botulinum* in some Egyptian food products in Assiut City; 2) to evaluate two methods, supernatant filtration (SF) and dilution centrifugation (DC), used for detection of *C. botulinum* spores in honey; 3) to isolate and identify *C. botulinum* using conventional methods 4) to genotype the isolated *C. botulinum* for neurotoxin genes types (A, B, E and F) using Multiple PCR.

2. Material and methods:

Safety precautions:

Safety precautions for handling *C. botulinum* in the laboratory were performed according to the Bacteriological analytical manual ***(BAM, 2001)***.

Sample collection:

A total number of 250 food samples (50 honey, 100 fish products and 100 meat products) were randomly collected from apiaries, fish shops, butcher shops, retail stores and supermarkets in Assiut City. The samples were collected from 2009 to 2011. Faseikh and moloha are traditional foods in Egypt and were collected during Sham Elnessim season (beginning of the spring).

a) Honey samples:

A total number of 50 honey samples (10 Orange honey, 10 Medicinal plants honey, 10 Clover honey, 10 Nabak honey and 10 Black cumin honey) were randomly collected from apiaries, retail stores and supermarkets in Assiut City. Five samples from each honey type were collected from apiaries and the rest five samples were retail jars collected from retail stores and supermarkets.

b) Fish products samples:

A total number of 100 fish products (20 canned sardine- 20 canned tuna- 20 faseikh - 20 moloha - 20

packaged smoked fish) were randomly collected from fish shops, retail stores and supermarkets in Assiut City.

c) Meat products samples:

A total number of 100 meat products (20 canned sausage –20 canned luncheon - 20 Egyptian fresh sausage - 20 basturma and 20 packaged smoked beef) were randomly collected from butcher shops, retail stores and supermarkets in Assiut City.

- Ten of the canned fish and meat products were with normal appearance while, the rest 10 samples were with signs of internal spoilage such as swollen, flipper, springer and leaker.

Sample preparation:

a) Honey samples:

In the present study two methods for detection of *C. botulinum* spores in honey were evaluated. They were dilution centrifugation (DC) and supernatant filtration (SF) methods ***(Özlem et al., 2006)***.

1) Dilution centrifugation (DC) method:

Each honey sample (25 gram) was diluted in 100 ml of 1% Tween 80 solution and kept in a water bath at 65 ºC for 30 minutes to inactivate other non sporeforming microorganisms then centrifuged for 30 minutes at 9000 xg. The precipitates were transferred into 9 ml Trypticase Peptone Glucose Yeast (TPGY) broth and 9 ml cooked meat medium (CMM). Before inoculation dissolved oxygen was removed from enrichment medium by heating in a boiling water bath for 15 minutes then followed by rapid cooling to room temperature in cold water without agitation. The inoculated TPGY broth and CMM were incubated under anaerobic condition using AnaeroGen™ gas generating kit (Oxoid) for 7-10 days. TPGY broth was incubated at 28 ºC for isolation of non proteolytic strains of *C. botulinum* and CMM was incubated at 37 ºC for isolation of proteolytic strains of *C. botulinum*. After 7 days of incubation, each culture was examined for turbidity, gas production and proteolytic activity. Culture pH was measured using pH paper strips (Edmund scientific) to detect acids production.

2) Supernatant filtration (SF) method:

The (SF) method resembles DC method but in (SF) method; centrifugation was combined with membrane filtration and the supernatant was used instead of precipitate. Spores were captured from the supernatant by filtration through two 0.45 µm cellulose acetate membrane filters (Sartorius) using filtration apparatus (Nuclepore). One filter was inoculated into 9 ml TPGY broth and the other was inoculated in 9 ml CMM.

b) Fish and meat products samples: *(John and Greg 2009)*

All samples were refrigerated until being tested, except unopened canned foods, which didn't need to be refrigerated. However, badly swollen canned food which were in danger of bursting, were refrigerated. Before testing, the product designation, manufacturer's name or home canner, source of sample, type of container and its size, labeling, manufacturer's batch, lot or production code, and condition of container were recorded. The samples were marked with laboratory identification codes.

The surface of the cans were cleaned, dried and the top was covered with 96% ethanol and left to stand for 2 minutes. The residual alcohol was decanted and flamed off. The can was placed in a large plastic bag to avoid the spread of aerosols and was opened with a sterile can opener.

In a sterile mortar 20 g portion of each fish and meat product was aseptically placed with 10 ml sterile 0.1% peptone water then blended for 2 minutes. Mortar was placed in a large plastic bag to avoid the spread of aerosols.

Two grams of the prepared solid food samples were inoculated into two screw capped bottles one contained 15 ml TPGY broth and the other contained 15 ml CMM. The inoculated media were incubated under anaerobic condition using AnaeroGen™ gas generating kit (Oxoid) for 7-10 days. TPGY broth was incubated at 28 ºC and CMM was incubated at 37 ºC. Each culture was examined for turbidity, acidity, gas production and proteolytic activity. Enrichment cultures with no significant signs of growth were considered negative after 10 days. Subcultures were done on TPGY broth at 30 ºC for 16 hours for purification. Bacterial suspension in this overnight culture was used for isolation of *C. botulinum* and to prepare DNA template for Multiplex PCR.

DNA extraction:

DNA extraction was done using DNeasy Tissue and Blood Kit (Qiagen, Cat. No. 69504).

Isolation and identification of *C. botulinum* using conventional methods *(Solomon et al., 2001)*:

a) Isolation of pure culture of *C. botulinum*:

Isolation of *C. botulinum* in pure culture was greatly improved by adding equal volume of absolute ethanol to 2 ml of enrichment culture. The mixture was incubated at room temperature for 1 hour. A loopfull of this treated enrichment culture of each sample was streaked by plating out method on two plates of Clostridial agar (Himedia). One of the streaked plates was incubated at 37 ºC for isolation of Group I (proteolytic strains) of *C. botulinum* types

(A, B and F). The other plate was incubated at 28 ºC for isolation of Group II (non proteolytic strains) of *C. botulinum* types (B, E and F). The streaked plates were incubated for 3-5 days under anaerobic condition using AnaeroGen™ gas generating kit (Oxoid). All cultured plates were inspected after 3-5 days for typical colonies of *C. botulinum*.

b) Identification of *C. botulinum* isolates:

Typical colonies of *C. botulinum* were strict anaerobic, large (3 mm), irregularly circular, smooth, grayish, and translucent with spreading fibrillar edge as shown in Photo (1). Films stained with Gram's stain were made from the isolated colonies. *C. botulinum* is Gram positive straight or slightly curved bacilli with oval sub terminal spores as shown in Photo (2). *C. botulinum* produced narrow zone of β- haemolysis on blood agar containing 10% sheep blood as shown in Photo (3). Pure colonies of *C. botulinum* were examined for catalase, lipase and proteolytic activity. They were catalase negative; no evolution of gas bubbles occurred upon adding a drop of 3% hydrogen peroxide onto *C. botulinum* colony. Lipase positive colonies produced a surface iridescence which often referred to as pearly layer when examined by oblique light as shown in Photo (4). *C. botulinum* was examined for proteolytic activity by inoculation into cooked meat medium anareobically at 42 ºC. Proteolytic strains of *C. botulinum* (Type A, B and F) in cooked meat media caused blackening of the meat, decomposing it, reducing its volume with the formation of foul smelling.

Multiplex PCR for detection and genotyping of *C. botulinum* types (A, B, E and F) neurotoxin genes:

a- Primers:

Four pairs of primers were used for detection and genotyping of *C. botulinum* types (A, B, E and F) neurotoxin genes as described by ***Dario et al., (2009)***, their sequences are illustrated in Table (1).

b- Multiplex PCR components:

Multiplex PCR was performed with a 50 µl mixture contained 2x Multiplex PCR master mix (Qiagen), 0.3 µM of each primer (Bioneer) and 3 µl of purified DNA template.

c- Multiplex PCR amplification program:

The amplification program was conducted in a programmable thermal cycler (Biometra, USA). The reaction mixture was heated at 95°C for 15 min. to activate the HotStarTaq DNA Polymerase and then subjected to 35 cycles each consists of denaturation at 95°C for 30 second, annealing at 51°C for 30 second and extension at 72°C for 90 second. This was followed by a final extension at 72°C for 7 min.

d- Detection of Multiplex PCR products by agarose gel electrophoresis:

PCR products were analyzed by 2% agarose gel electrophoresis at 90 V for 70 min, and were examined under ultraviolet transilluminator. The size of the Multiplex PCR products was compared with a standard DNA ladder (100 bp Nippon genetics) as shown in Photo (5).

Statistical analysis:

Data entry and analysis were carried out via Statistical Package of Social Science (SPSS) version 16 for windows. A plan for data analysis was established based upon the objective of the study and the conceptual framework. Data analysis began by obtaining frequency distribution and descriptive statistics for most variables. Chi- square test and Fisher Exact test were used to measure the significant difference in all groups. A P value <0.05 was considered significant.

3. Results:

In the present study the conventional methods and Multiplex PCR revealed the same number of positive samples for *C. botulinum* in some food products collected from Assiut City. However, Multiplex PCR was more rapid, saved time and labor and detected the type of *C. botulinum* neurotoxin genes.

The total number of positive samples for *C. botulinum* in honey samples was 13 (26 %), (2 (20%) in Orange honey, 1 (10%) in Medicinal plants honey, 5 (50%) in Clover honey, 3 (30%) in Nabak honey and 2 (20%) in Black cumin honey as shown in Table (2).

It is clear from the results in Table (3) and Figure (2) that 10 isolates (20%) of *C. botulinum* were isolated from samples collected from apiaries however, only 3 isolates (6%) were detected from samples collected from retail stores and supermarkets in Assiut City. They were 2 (20%) isolates from Orange honey, 1 (10%) isolate from Medicinal plants honey, 3 (30%) isolates from Clover honey, 2 isolates from Nabak honey and 2 (20%) isolates from Black cumin honey collected from apiaries. On the other hand, only 2 (20%) isolates from Clover honey and 1 (10%) isolate from Nabak honey were detected from retail jars honey samples.

It is evident from Table (4) and Figure (3) that 13 isolates were obtained using SF method while, only 7 isolates were obtained using DC method. They were 2 (20%) isolates from Orange honey, 1 (10%) isolate from Medicinal plants honey, 5 (50%) isolates from Clover honey, 3 (30%) isolates from Nabak honey and 2 (20 %) isolates from Black cumin honey by SF method. While, only 1 (10%)

isolate from Medicinal plants honey, 3 (30%) isolates from Clover honey, 1 (10%) isolate from Nabak honey and 2 (20 %) isolates from Black cumin honey were detected by using DC method. No positive result was detected from Orange honey using DC method.

It could be observed from Table (2) and Figure (1) that by using Multiplex PCR; the total number of isolated strains were 5 for *C. botulinum* type A (1 (10%) in Orange honey, 2 (20%) in Clover honey, 1 (10%) in Nabak honey and 1 (10%) in Black cumin honey) and 10 for *C. botulinum* type B (2 (20%) in Orange honey, 1 (10%) in Medicinal plants honey, 3 (30%) in Clover honey, 3 (30%) in Nabak honey and 1 (10%) in Black cumin honey). On the other hand, the number of isolated strains of *C. botulinum* type E and *C. botulinum* type F were 0. Moreover, 2 honey samples (1 orange honey and the other was from Nabak honey) were positive for both types of *C. botulinum* (type A and type B). *C. botulinum* type B strains that were isolated from honey samples were all proteolytic but variable in the proteolytic activity.

The total number of positive samples for *C. botulinum* in fish products samples was 19 in a percentage of (19 %) as shown in Table (5).

Data presented in Table (5) and figure (4) revealed that the number of isolated *C. botulinum* was 4 in canned sardine, 3 in canned tuna, 7 in faseikh, 1 in moloha and 4 in packaged smoked fish in a percentage of (20%), (15%), (35%), (5%) and (20%) respectively.

The recorded results in Table (5) and Figure (4) pointed out the number and type of *C. botulinum* neurotoxin genes that were genotyped by Multiplex PCR in fish products samples. The number of isolated strains were 5 for *C. botulinum* type B (1 (5%) in canned sardine, 1(5%) in canned tuna, 2 (10%) in faseikh, 1 (5%) in packaged smoked fish) and 14 for *C. botulinum* type E (3 (15%) in canned sardine, 2 (10%) in canned tuna, 5 (25%) in faseikh, 1 (5%) moloha and 3 (15%) in packaged smoked fish). On the other hand, the number of isolated strains of *C. botulinum* type A and *C. botulinum* type F were 0. Isolated strains of *C. botulinum* type B from fish products samples were non proteolytic strains belongs to Group II, as they grew on TPGY broth at 28 ºC while, no positive result was obtained on CMM at 42 ºC.

The total number of positive samples for *C. botulinum* in meat products samples was 21 in a percentage of (21 %) as shown in Table (6).

The recorded result in Table (6) revealed that the number of isolated *C. botulinum* were 3 in canned sausage, 4 in canned luncheon , 8 in Egyptian fresh sausage, 0 in basturma and 6 in packaged smoked beef in a percentage of (15%), (20%), (40%), (0%) and (30%) respectively.

It is clearly evident from Table (6) and Figure (5) the number and type of *C. botulinum* neurotoxin genes that was genotyped by Multiplex PCR in meat products samples. They were 6 for *C. botulinum* type A (1 (5%) in canned sausage, 2 (10%) in canned luncheon, 1 (5%) in Egyptian fresh sausage and 2 (10%) in packaged smoked beef), 11 for *C. botulinum* type B (2 (10%) in canned sausage, 1 (5%) in canned luncheon, 5 (25%) in Egyptian fresh sausage and 3 (15%) in packaged smoked beef) and 4 for *C. botulinum* type E (1 (5%) in canned luncheon, 2 (10%) in Egyptian fresh sausage and 1 (5%) in packaged smoked beef). While, the number of isolated strains of *C. botulinum* type F were 0.

Eight isolates of *C. botulinum* type B that were isolated from meat products samples were proteolytic belongs to Group I, as they grew on CMM at 42 ºC and caused blackening of the meat, decomposing it, reducing its volume with the formation of foul smelling. The degree of proteolysis in CMM was variable. However, 3 isolated *C. botulinum* type B strains from packaged smoked beef were non proteolytic belong to Group II.

Concerning the results in Table (7) it is evident that the total number of positive samples for *C. botulinum* in normally appeared canned fish and meat products was 2 (2.5%). While, the total number of positive samples for *C. botulinum* in canned fish and meat products with signs of internal spoilage was 12 (15%).The number of the isolated strains was 2 (10%) in canned sardine with normal appearance. No positive samples were found in canned tuna, canned sausage & canned luncheon with normal appearance.

Also, it is apparent from Table (7) and Figure (6) that the number of the isolated strains was 3 (15%) in canned sausage, 4 (20%) in canned luncheon, 2 (10%) in canned sardine and 3 (15%) in canned tuna that showed signs of internal spoilage.

Table (1): Primers used for detection and genotyping of *C. botulinum* types (A, B, E and F) neurotoxin genes *(Dario et al., 2009)*

Toxin type	Primers	Sequence (5'- 3')	Product size (bp)
A	IOA_f	GGG CCT AGA GGT AGC GTA CTG	101
	IOA_r	TCT TGA TTT CCA GAA GCA TAT TTT	
B	$CBMLB_f$	CAG GAG AAG TGG AGC GAA AA	205
	$CBMLB_r$	CTT GCG CCT TTG TTT TCT TG	
E	$CBMLE_f$	CCA AGA TTT TCA TCC GCC TA	389
	$CBMLE_r$	GCT ATT GAT CCA AAA CGG TGA	
F	$CBMLF_f$	CGG CTT CAT TAG AGA ACG GA	543
	$CBMLF_r$	TAA CTC CCC TAG CCC CGT AT	

Table (2): Prevalence of *C. botulinum* types (A, B, E and F) in honey samples

Types of honey	No. of samples analyzed	Total Isolates		*C. botulinum* type							
				A		B		E		F	
		No.	%	No.	%	No.	%	No.	%	No.	%
Orange honey*	10	2	20.0	1	10.0	2	20.0	0	0.0	0	0.0
Medicinal plants honey	10	1	10.0	0	0.0	1	10.0	0	0.0	0	0.0
Clover honey	10	5	50.0	2	20.0	3	30.0	0	0.0	0	0.0
Nabak honey*	10	3	30.0	1	10.0	3	30.0	0	0.0	0	0.0
Black cumin honey	10	2	20.0	1	10.0	1	10.0	0	0.0	0	0.0

-Total number of positive isolates in honey samples = 13 (26%)
-Two honey samples (one Orange honey and the other was Nabak honey were positive for *C. botulinum* type A and type B

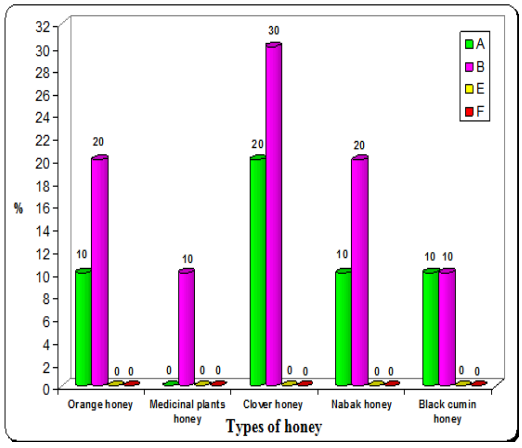


Figure (1): Prevalence of *C. botulinum* types (A, B, E and F) in honey samples

A= *C. botulinum* type A **B= *C. botulinum* type B**
E= *C. botulinum* type E **F= *C. botulinum* type F**

Table (3): Prevalence of *C. botulinum* in honey samples collected from apiaries and retail jars

Types of honey	No. of samples analyzed	Source			
		Apiaries		Retail jars	
		No.	%	No.	%
Orange honey	10	2	20.0	0	0.0
Medicinal plants honey	10	1	10.0	0	0.0
Clover honey	10	3	30.0	2	20.0
Nabak honey	10	2	20.0	1	10.0
Black cumin honey	10	2	10.0	0	0.0
P – value	0.037				

-Total number of *C. botulinum* isolates in honey samples collected from apiaries was 10 however, was only 3 in retail jars

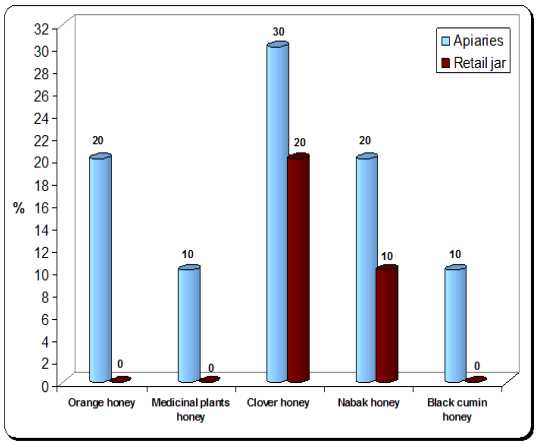


Figure (2): Prevalence of *C. botulinum* in honey samples collected from apiaries and retail jars

Table (4): Comparison of two methods used for detection of *C. botulinum* spores in honey

Types of honey	No. of samples analyzed	Methods							
		DC				SF			
		CMM (37 ℃)		TPGY (28 ℃)		CMM (37 ℃)		TPGY (28 ℃)	
		No.	%	No.	%	No.	%	No.	%
Orange honey	10	0	0.0	0	0.0	2	20.0	0	0.0
Medicinal plants honey	10	1	10.0	0	0.0	1	10.0	0	0.0
Clover honey	10	3	30.0	0	0.0	5	50.0	0	0.0
Nabak honey	10	1	10.0	0	0.0	3	30.0	0	0.0
Black cumin honey	10	2	20.0	0	0.0	2	20.0	0	0.0
P –value	0.134								

-SF method was more efficient than DC method where 13 isolates were obtained using SF method while, only 7 isolates were obtained using DC method.

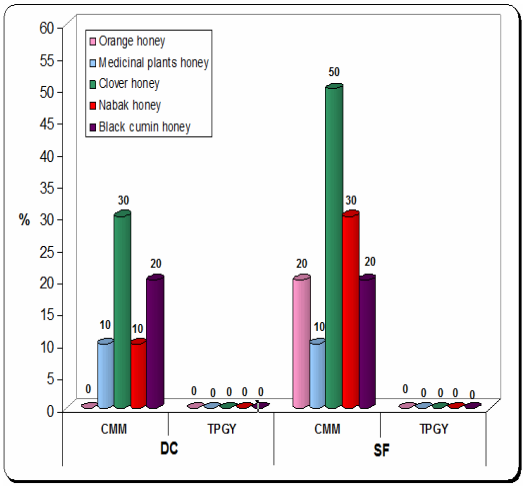


Figure (3): Comparison of two methods used for detection of *C. botulinum* spores in honey

Table (5): Prevalence of *C. botulinum* types (A, B, E and F) in fish products samples

Types of fish products	No. of samples Analyzed	Total isolates		*C. botulinum* type							
				A		B		E		F	
		No.	%	No.	%	No.	%	No.	%	No.	%
Canned Sardine	20	4	20.0	0	0.0	1	5.0	3	15.0	0	0.0
Canned Tuna	20	3	15.0	0	0.0	1	5.0	2	10.0	0	0.0
Faseikh	20	7	35.0	0	0.0	2	10.0	5	25.0	0	0.0
Moloha	20	1	5.0	0	0.0	0	0.0	1	5.0	0	0.0
Packaged smoked fish	20	4	20.0	0	0.0	1	5.0	3	15.0	0	0.0

-Total number of positive isolates in fish products samples = 19(19%)
-There is significant difference between faseikh vs. moloha (P= 0.044) Fisher Exact test

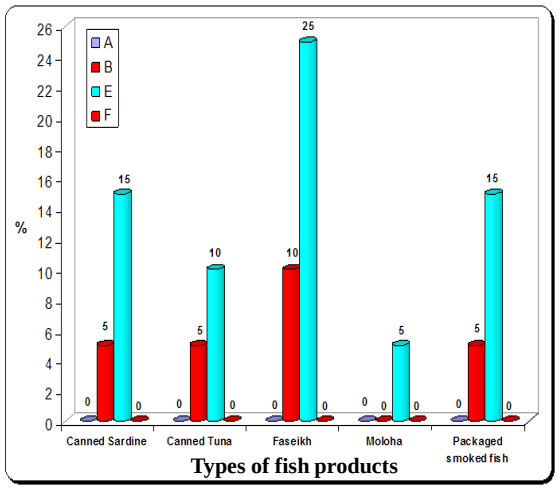


Figure (4): Prevalence of *C. botulinum* types (A, B, E and F) in fish products samples

Table (6): Prevalence of *C. botulinum* types (A, B, E and F) in meat products samples

Types of meat products	No. of samples Analyzed	Total isolates		*C. botulinum* type							
				A		B		E		F	
		No	%	No.	%	No.	%	No.	%	No.	%
Canned Sausage	20	3	15.0	1	5.0	2	10.0	0	0.0	0	0.0
Canned Luncheon	20	4	20.0	2	10.0	1	5.0	1	5.0	0	0.0
Egyptian fresh sausage (EFS)	20	8	40.0	1	5.0	5	25.0	2	10.0	0	0.0
Basturma	20	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Packaged smoked beef	20	6	30.0	2	10.0	3	15.0	1	5.0	0	0.0

-Total number of positive isolates in meat products samples =21(21%)
-There is significant difference between EFS vs. basturma (P=0.003)
-There is significant difference between packaged smoked beef vs. basturma (P=0.020)

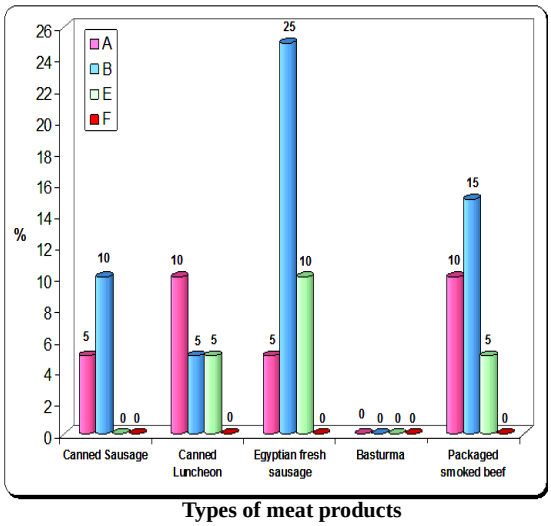


Figure (5): Prevalence of *C. botulinum* types (A, B, E and F) in meat products samples

Table (7): Frequency distribution of *C. botulinum* in canned meat and fish products samples

Types of canned products	No. of samples Analyzed	Total Isolates		Appearance			
				Normal		With signs of internal spoilage	
		No	%	No.	%	No.	%
Canned Sausage	20	3	15.0	0	0.0	3	15.0
Canned Luncheon	20	4	20.0	0	0.0	4	20.0
Canned Sardine	20	4	20.0	2	10.0	2	10.0
Canned Tuna	20	3	15.0	0	0.0	3	15.0
P- value	0.005						

-Total number of positive isolates for *C. botulinum* in canned meat and fish products with signs of internal spoilage was 12.
-However, only 2 samples were positive in canned meat and fish with normal appearance. This result is statistically significant.

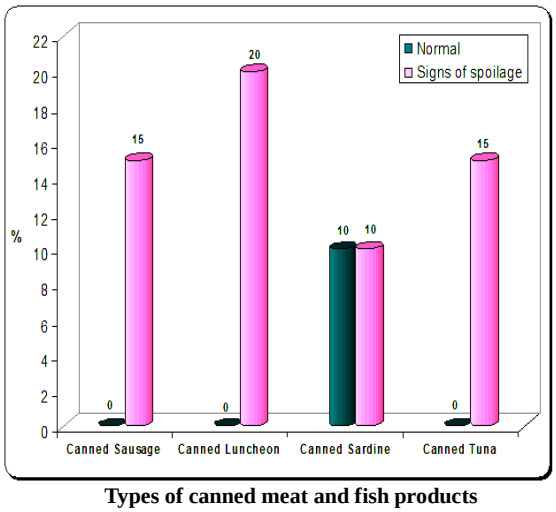
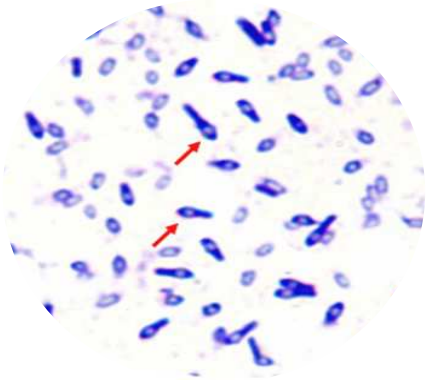


Figure (6): Frequency distribution of *C. botulinum* in canned meat and fish products samples

Photo (1): *C. botulinum* on Clostridial agar produced large, irregularly circular, grayish white colonies with spreading fibrillar edge.



Arrows point to *C. botulinum* endospores

Photo (2): Film from culture stained with Gram's stain showing Gram positive *C. botulinum* with oval subterminal spores.

Photo (3): Blood agar inoculated with *C. botulinum* showing narrow zone of β-haemolysis surrounding the colonies.

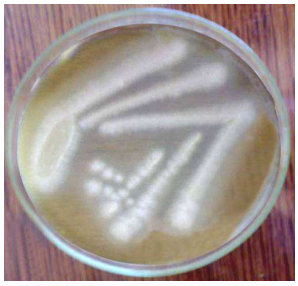


Photo (4): Anaerobic egg yolk medium inoculated with *C. botulinum* showing lipase positive colonies.

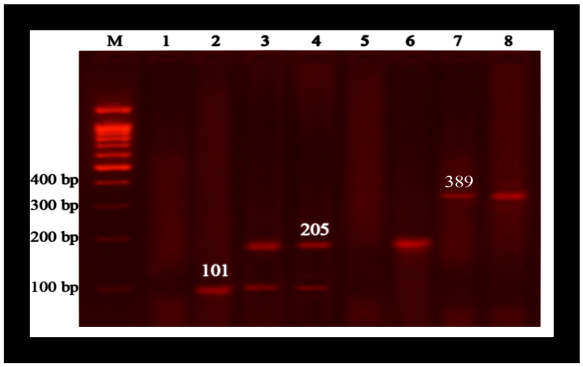


Photo (5): Agarose gel electrophoresis for detection of *C. botulinum* neurotoxin genes

M= 100 bp ladder
Lane (1): Negative control
Lane (2): Positive for *C. botulinum* type A
Lane (3) and (4): Positive for *C. botulinum* type A and type B
Lane (6): Positive for *C. botulinum* type B
Lane (7), (8): Positive for *C. botulinum* type E

4. Discussion:

Botulism is a fatal neuroparalytic illness resulting from the action of a highly potent neurotoxin formed during the growth of *C. botulinum*. Foodborne botulism, while rare, remains a public health emergency because of its severity and epidemic potential ***(Johnson et al., (2005)***. Honey has been known to contain *C. botulinum* spores and considered a potential source for infant botulism ***(CDC, 1998)***. About 60% of infant botulism cases in Europe had a history of honey consumption ***(Aureli et al., 2002)***.

In the present study the total number of positive samples for *C. botulinum* in honey samples was 13 in a percentage of (26 %) as shown in Table (2). The obtained results are adequately consistent with those reported by ***Özlem et al., (2006)*** who studied the incidence of *C. botulinum* spores in Turkish honey. They could isolate *C. botulinum* from 6 (12.5%) out of 48 honey samples collected from honey sold in retail markets in Ankara. The finding in Table (2) is also in general agreement with the results of ***Naves et al., (2002)*** who conducted a prevalence survey on *C. botulinum* spores in 190 honey samples. They detected *C. botulinum* spores in 8 (7%) of the 114 Finnish and in 12 (16%) of the 76 imported honey samples.

On the other hand, there are some studies reporting lower contamination levels with *C. botulinum* spores in honey. For example, 2 studies were done in Sao Paulo ***(Rall et al., 2003)*** and in Washington ***(Kautter et al., 1982)*** reported 3% and 2% contamination levels respectively.

Data presented in Table (2) revealed that the highest prevalence of *C. botulinum* spores was in Clover honey (50%). This may be attributed to the lower level of phytochemicals in Clover honey than in darker-colored honey varieties. The inhibitory activity of honey against different bacteria has been reported in several studies ***(Taormina et al., 2001, Mundo et al., 2004, Alnaqdy et al., 2005*** **and** ***Lusby et al., 2005)***. In addition to the osmolarity and slight acidity of honey, its antimicrobial effect is mainly due to the generation of hydrogen peroxide via glucose oxidase and due to a variety of phytochemicals as flavonoids and phenolic acids and lysozyme. Generally, darker honeys have higher phytochemicals content than lighter honeys ***(Perez et al., 2007)***.

A significantly higher prevalence of *C. botulinum* spores was noticed in honey samples collected from apiaries than in retail jars (P = 0.037), this is clear from the results in Table (3) as 10 isolates (20%) of *C. botulinum* were isolated from samples collected from apiaries however, only 3 isolates (6%) were detected from samples collected from retail stores and supermarkets in Assiut City. The finding may be due to ageing of honey in retail jars ***(Nakano et al., 1990)***. ***Nevas, (2006)*** suggested that the difference is more likely to be caused by dilution rather than ageing of the honey. When honey from different apiaries is extracted and mixed in large containers to produce retail packages, spores may be diluted to some extent. ***Nakano et al., (1990)*** studied the Incidence of *C. botulinum* in honey of various origins. *C. botulinum* spores were present in 6 of 58 Japanese honeys (10.3%), 9 of 76 honeys from China (12%), and 3 of 15 from Argentina (20%). Incidence was higher in samples taken from drums (18%) soon after import, and from apiaries in Osaka (23%), than in honeys from shops (5%).

Honey is a complex material for microbiological investigation ***(Nevas et al., 2002)***. High sugar content, low pH (3.9), high viscosity, low water activity (0.5- 0.6) ***(White, 1978)*** and existence of hydrogen peroxide via glucose oxidase enzyme reaction inhibits microorganisms from growth ***(Gross et al., 2004)***. Therefore, two methods for concentration of *C. botulinum* spores in honey were evaluated. SF method was more efficient than DC method. By using the (DC) method in preparing honey samples; some false negative results were obtained. This was clearly evident from the results obtained in Table (4) which shows the detection of 13 isolates using supernatant filtration method (SF) in contrast to 7 isolates only were obtained using dilution centrifugation method (DC). This may be attributed to the presence of *C. botulinum* spores in the supernatant which was collected to isolate the organism from it. However, the precipitate contains inhibitory substances that affect bacterial growth as hydrogen peroxide, flavonoids, lysozyme, phenolic acids and terpenes ***(Nevas, (2006)***.

Supporting this study, other investigators found that SF method was more efficient than DC method. ***Nevas, (2006)*** mentioned that SF method in processing honey samples provided sensitivity sufficiently high to detect 0.1 spore of *C. botulinum* in 1 gm of honey. This may be considered adequate in determining whether honey is a potential cause of infant botulism, as the number of spores in honey samples associated with infant botulism has been reported to vary from 5 to 80 spores/gm ***(Midura et al., 1979)***.

In contrary, ***Özlem et al., (2006)*** preferred DC method for isolation of *C. botulinum* spores in honey and failed to detect *C. botulinum* spores by using SF method.

The use of Multiplex PCR in screening for the presence of botulinal spores in honey samples was successful. It is clear from Table (2) that the number of isolated strains were 5 for *C. botulinum* type A (1

(10%) in Orange honey, 2 (20%) in Clover honey, 1 (10%) in Nabak honey and 1 (10%) in Black cumin honey) and 10 for *C. botulinum* type B (2 (20%) in Orange honey, 1 (10%) in Medicinal plants honey, 3 (30%) in Clover honey, 3 (30%) in Nabak honey and 1 (10%) in Black cumin honey). On the other hand, the number of isolated strains of *C. botulinum* type E and *C. botulinum* type F were 0. Moreover, 2 honey samples (1 orange honey and the other was from Nabak honey) were positive for both types of *C. botulinum* (type A and type B).

C. botulinum type B strains that were isolated from honey samples were all proteolytic but variable in the proteolytic activity. This result is in general agreement with ***Eric et al., (2005)*** who studied the culture phenotypic properties of *C. botulinum* type B strains isolated from infant botulism cases in the United Kingdom and found variation in the proteolytic activity of the isolated strains.

Foodborne botulism results from ingestion of food containing preformed botulinum neurotoxin. The toxin is heat labile and produced by *C. botulinum* in foods that have not been properly handled or canned as canned fish, meat and vegetables ***(Ahsan et al., 2005)***.

C. botulinum can grow and produce toxin in foods under the following conditions: 1) natural or nonprocessed food contaminated with spores or vegetative cells; 2) processing treatment is inadequate to inactivate *C. botulinum* spores, or the product is re-contaminated after processing; and 3) the food is particularly conductive to create anaerobic conditions (ex: addition of oil) that allow *C. botulinum* spores to germinate and outgrow or vegetative cells to grow and produce toxin ***(Bremer et al., 2003)***.

C. botulinum is ubiquitous in aquatic environments and has been isolated from water, ocean sediments, intestinal tract of fish and the gills and viscera of crabs and other shellfish ***(Huss, 1981)***.

C. botulinum type E which is most common in fish and fishery products is of particular concern because it grows at temperature as low as 3- 5 °C and produces little or no noticeable evidence of spoilage ***(Frerk, 2000)***.

Ingrid (2008) reported that even though a fish might be cleaned, gutted and packaged, risk of botulism still existed. This risk existed because *C. botulinum* spores could adhere to the surface of fishes and find their way into muscle tissue during processing. Muscle tissue below the surface of fish could provide an anaerobic environment, where outgrowth of vegetative cells and toxin production could occur if time and temperature permitted this.

In the present study the total number of positive samples for *C. botulinum* in fish products samples was 19 in a percentage of (19 %) as shown in Table (5).

Data presented in Table (5) and Figure (4) revealed that the number of isolated *C. botulinum* were 4 in canned sardine, 3 in canned tuna, 7 in faseikh, 1 in moloha and 4 in packaged smoked fish in a percentage of (20%), (15%), (35%), (5%) and (20%) respectively.

In the present study, faseikh showed the highest contamination level with *C. botulinum*. There is significant differences versus moloha (P= 0.044) Fisher Exact test. This result may be attributed to the traditional method that is used to process faseikh and to the high salt concentration in moloha that may exceed 7%.

Faseikh is a traditional product made by fermenting uneviscerated fresh mullet for up to 1 day. Then salt-curing is done in barrels which may be tightly sealed from 1 week to 1 year ***(Ingrid, 2008)***.

Researchers thought that, because the fish were uneviscerated, the gut could provide a relatively low-salt environment that protected the spores and allowed them to germinate and produce toxins. Despite adequate understanding of how foodborne botulism could be avoided, outbreaks still occur, particularly among people consuming certain high risk ethnic food ***(McLaughlin et al., 2004)***.

Lindström et al., (2005) reported that the inhibitory NaCl concentration in brine is 5% for Group II strains of *C. botulinum*. However, Group I strains can tolerate NaCl concentrations as high as 10% in brine.

In 1991 two botulism outbreaks occurred. "Faseikh" was implicated in at least 91 illnesses and 18 deaths in Egypt. An ethnic fish product called "moloha" caused a botulism outbreak involving four family members in New Jersey ***(Tara, 2004)***.

In 1997 two Germans got botulism after eating hot smoked Canadian whitefish produced in Finland ***(Korkeala et al., 1998)***.

In 2004, a woman in New Jersey, U.S was treated for type A botulism after eating whole, uneviscerated, salt-cured fish, called faseikh ***(Tara, 2004)***.

Sobel et al., (2007) reported that a variety of salted, fermented, smoked, and canned fish sources have been implicated in type E botulism outbreaks in the United States and worldwide. The bacterium has been found in these dishes in spite of salt levels far in excess of 7%. In patients exposed to low doses of botulinum toxin type E, gastrointestinal symptoms predominated.

The recorded results in Table (5) and Figure (4) pointed out the number and type of *C. botulinum* neurotoxin genes that were genotyped by Multiplex PCR in fish products samples. The number of

isolated strains were 5 for *C. botulinum* type B (1 (5%) in canned sardine, 1(5%) in canned tuna, 2 (10%) in faseikh, 1 (5%) in packaged smoked fish) and 14 for *C. botulinum* type E (3 (15%) in canned sardine, 2 (10%) in canned tuna, 5 (25%) in faseikh, 1 (5%) moloha and 3 (15%) in packaged smoked fish). On the other hand, the number of isolated strains of *C. botulinum* type A and *C. botulinum* type F were 0.

This finding is in general agreement with the results of ***Lalitha and Surendran (2002)*** who studied the occurrence of *Clostridium botulinum* in fresh (67) and cured fish (257) samples in retail trade in Cochin (India). An overall prevalence of 19% (13/67) was found in fresh retail fish and types A to D neurotoxin genes were detected in the positive samples.

The prevalence of *C. botulinum* type E in fish and fishery products of commercial importance in Finland was determined using PCR analysis. Five percent of 214 vacuum packaged fish products were positive for neurotoxin type E gene ***(Hyytiä et al., 1998)***.

In contrary, ***(Gibbs et al., 1994)*** reported that all 82 samples of vacuum packaged hot-smoked trout and mackerel purchased from retail outlets in the UK were negative for *C. botulinum.*

Isolated strains of *C. botulinum* type B in fish products samples were non proteolytic strains belong to Group II, as they grew on TPGY broth at 28 ºC while, no positive result was obtained on CMM at 42 ºC.

Group II strains of *C. botulinum* are psychrotrophic and thus they are able to grow at refrigeration temperatures. Anaerobic packages and extended shelf lives provide *C. botulinum* with favourable conditions for growth and toxin formation. Group II (non-proteolytic) *C. botulinum* poses a safety hazard in modern food processing technology, which consists of mild pasteurization treatments, anaerobic packaging, extended shelf lives and chilled storage ***(Lindström et al., 2005).***

The results in the present study indicated that current fish processing practices were inadequate to eliminate *C. botulinum* spores from fish products in Assiut City.

Control of growth and toxin production from *C. botulinum* in fishery products is based on spore destruction (e.g., retorting canned foods) or inhibition of vegetative cell growth (e.g., control of water activity below 0.93, or acidification to below pH 4.6, or use of approved chemical inhibitors as nitrates, nitrites and sufficiently high NaCl concentration). The control measures must be applied rapidly and uniformly throughout the product to protect consumers from this potentially life-threatening toxin ***(Korkeala et al., 1998)***.

The total number of positive samples for *C. botulinum* in meat products samples was 21 in a percentage of (21 %) as shown in Table (6).

The recorded result in Table (6) and Figure (5) revealed that the number of isolated *C. botulinum* were 3 in canned sausage, 4 in canned luncheon , 8 in Egyptian fresh sausage, 0 in basturma and 6 in packaged smoked beef in a percentage of (15%), (20%), (40%), (0%) and (30%) respectively.

The results reported in Table (6) pointed out a significant difference between basterma versus Egyptian fresh sausage (P= 0.003) and versus packaged smoked beef (P= 0.020).

Concerning the results in Table (6) the highest evidence of *C. botulinum* was in Egyptian fresh sausage. This may be due to contamination from unsanitary handling, materials and environment.

On the other hand, in the present study we failed to detect *C. botulinum* in basterma. This may be attributed to the ancient preservation method which ensures availability of basterma today in many countries. The preservation of meat is based on the use of dry salt in preparation of Egyptian basterma which maintain at least 8% NaCl concentration, whereas Turkish basterma is prepared primarily by pickling. Another important factor is the removal of water from meat by dryness and high salt concentration. This reduces the water activity to approximately 0.8 at which most undesirable microorganisms are unable to proliferate and grow. Moreover, the garlic paste covering the meat in basterma has bactericidal action ***(El-khateib, 1997)***.

In Poland, a total number of 85 cases of foodborne botulism were registered during 2002, including 5 deaths. Of the 85 cases, 58 were caused by contaminated meat, including 21 cases from contaminated, home-canned pork, and 17 cases caused by commercially produced sausages ***(Przybylska, 2004)***.

Multiplex PCR has the advantage of simultaneous detection of several clostridia possessing type A, B, E, and F botulinum neurotoxin genes. Therefore, it can be used to test food samples in outbreaks or in routine surveillance studies ***(Dario et al., 2009)***.

It is clearly evident from Table (6) and Figure (5) the number and type of *C. botulinum* neurotoxin genes that were genotyped by Multiplex PCR in meat products samples. They were 6 for *C. botulinum* type A (1 (5%) in canned sausage, 2 (10%) in canned luncheon, 1 (5%)in Egyptian fresh sausage and 2 (10%) in packaged smoked beef), 11 for *C. botulinum* type B (2 (10%) in canned sausage, 1 (5%) in canned luncheon, 5 (25%) in Egyptian fresh sausage and 3 (15%) in packaged smoked beef) and 4 for *C. botulinum* type E (1 (5%) in canned

luncheon, 2 (10%) in Egyptian fresh sausage and 1 (5%) in packaged smoked beef). While, the number of isolated strains of *C. botulinum* type F were 0.

This result is in general agreement with ***Raphael et al., (2010)*** who reported that botulism due to type F botulinum neurotoxin (BoNT/F) is rare (<1% of cases), and only a limited number of Clostridial strains producing this toxin type have been isolated.

Supporting this study, ***Dario et al., (2009)*** described a useful Multiplex PCR method for detection of *C. botulinum* type A, B, E, and F neurotoxin genes in clinical, food, and environmental samples. They detected 55 positive samples for *C. botulinum* in 234 examined honey samples. Of these 55 positive samples, 48 were positive for *C. botulinum* type A and 7 were positive for *C. botulinum* type B.

The prevalence of *C. botulinum* in semipreserved meat products was studied by ***Kerstin and Riemann (1971)***, they could detect *C. botulinum* type A in 5 of 100 smoked turkey samples and *C. botulinum* type B in one of 41 smoked chicken samples. On the other hand, they failed to detect *C. botulinum* in basterma, smoked beef and luncheon.

In 2007, four cases of botulism have been reported to CDC from Indiana (2 cases) and Texas (2 cases). All four persons were reported to have consumed Castleberry's brand Hot Dog Chili Sauce Original. Botulinum toxin was identified in leftover chili sauce from an unlabeled sealable bag collected from a patient's refrigerator ***(CDC, 2007)***.

Eight isolates of *C. botulinum* type B that were isolated from meat products samples were proteolytic belongs to Group I, as they grew on CMM at 42 ºC and caused blackening of the meat, decomposing it, reducing its volume with the formation of foul smelling. The degree of proteolysis in CMM was variable. However, 3 isolated *C. botulinum* type B strains from packaged smoked beef were non proteolytic belong to Group II.

Group II *C. botulinum* has been reported to be present in vacuum packaged frankfurters, cured luncheon meat and smoked turkey products ***(Lindström et al., 2005)***.

C. botulinum spores are widespread in soil, dust inside or outside houses, marine sediments, intestinal tracts of animals and fish, animal manure, vegetables, fruits, and honey. Therefore, they are potentially present in a wide range of raw materials used in the food industry, as well as in the environment of food processing factories ***(CDC, 2002)***.

Concerning the results in Table (7) it is evident that the total number of positive samples for *C. botulinum* in canned fish and meat products with normal appearance were 2 (2.5%). While, the total number of positive samples for *C. botulinum* in canned fish and meat products with signs of internal spoilage were 12 (15%). The recorded results pointed out higher prevalence of *C. botulinum* in canned food with signs of internal spoilage which is statistically significant (P= 0.005).

The number of the isolated strains was 2 (10%) in canned sardine with normal appearance. No positive samples were found in canned tuna, canned sausage and canned luncheon with normal appearance. The nonproteolytic strains of *C. botulinum* type E and some type B and F do not produce overt signs of food spoilage during growth and toxin production ***(Conner et al., 1989)***.

Commercially canned fish has been the source of type E botulism in a few outbreaks in the United States and Poland ***(Hauschild, 1992)***.

Also, it is apparent from Table (7) and Figure (6) that the number of the isolated strains was 3 (15%) in canned sausage, 4 (20%) in canned luncheon, 2 (10%) in canned sardine and 3 (15%) in canned Tuna that show signs of internal spoilage.

Canning is a method of preserving food in which the food contents are processed and sealed in an airtight container. During the canning process, foods undergo hot fill process and oxygen is removed leaving the food in an anaerobic environment. Certain foods, such as meat is able to bind oxygen to create an anaerobic environment. It contains hematin and glutathione that act as reducing agent. Home canning process for low acid food presents an extremely high risk because time and temperature are often inadequate. Spoiled acidic food should be discarded in a place where it will not be eaten by humans or pets ***(Sobel, 2005)***.

Conclusion and recommendations:

The present study revealed that some food products in Assiut City may present a potential hazard for foodborne and infant botulism. Therefore, strict hygienic measures should be done when canning foods occurred at home such as washing hands, utensils, and food contact surfaces with hot soapy water before food preparation and after they touch raw meat or seafood; home canned foods should be canned in pressure cookers to ensure the proper time, temperature and pressure requirements to avoid the growth of the bacteria and spores; Low acid home canned foods should be boiled for 10 minutes before consumption; commercially canned food should undergo a "botulinum cook" at 121 °C for 3 minutes to reduce the survival of *C. botulinum* spores; infants should not feed honey in the first year of life; food from dented, bulging or leaking home or commercially-canned food should be hygienically discarded; uneviscerated fishes that are salt-cured as

(faseikh and moloha) must not exceed 3-5 inches in length to ensure complete permeation of the flesh with the inhibitory levels of salt concentrations. Larger fishes should be avoided because they represent a potentially life-threatening health hazard.

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7. References

1. Ahsan C. R., Hajnoczky G., Maksymowych A. B. and Simpson L. L. (2005): Visualization of binding and transcytosis of botulinum toxin by human intestinal epithelial cells. J Pharmacol. Exp. Ther. 315:1028-35.
2. Alnaqdy A., Al-Jabri A., Al Mahrooqi Z., Nzeako B. and Nsanze H. (2005): Inhibition effect of honey on the adherence of Salmonella to intestinal epithelial cells in vitro. Int. J Food Microbiol. 103: 347-351.
3. Arnon S. S. (2004): Infant botulism, p. 1758-1766. In R. D. Feigin, J. D. Cherry, G. J. Demmler and S. L. Kaplan (ed.), Textbook of pediatric infectious diseases, 5th ed. W. B. Saunders, Philadelphia, Pa.
4. Aureli P., Franciosa G., and Fenicia L. (2002): Infant botulism and honey in Europe: A commentary. Pediatr. Infect. Dis. J 21: 866-868.
5. BAM, Bacteriological Analytical Manual Online (2001): Clostridium botulinum, Chapter 17. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM070879
6. Braconnier A., Broussolle V. Perelle S., Fach P., and Nguyen F. (2001): Screening for Clostridium botulinum type A, B, and E in cooked chilled foods containing vegetables and raw material using polymerase chain reaction and molecular probes. J Food Prot. Feb; 64 (2):201-7.
7. Bremer P., Fletcher G., and Osborne C. (2003): Clostridium botulinum in seafood. Crop and Food Research Limited, Christchurch, New Zealand. http://www.crop.cri.nz/home/research/marine/pathogens/clostridium.pdf
8. CDC, Centers for Disease Control and Prevention. (1998): Botulism in the United States, 1899-1996. Handbook for epidemiologists, clinicians and laboratory workers. Centers for Disease Control and Prevention, Atlanta, USA.
9. CDC, Centers for Disease Control and Prevention. (2002): Surveillance for botulism. Summary of 2001 data. Centers for Disease Control and Prevention, Foodborne and Diarrheal Diseases Branch, Atlanta, USA.
10. CDC, Centers for Disease Control and Prevention. (2004): Surveillance for botulism. Summary of 2002 data. Centers for Disease Control and Prevention, Foodborne and Diarrheal Diseases Branch, Atlanta, USA.
11. CDC, Centers for Disease Control and Prevention (2007): "Botulism associated with commercially canned chili sauce--Texas and Indiana," MMWR Morb Mortal Wkly Rep 56(30): 767-9.
12. Conner D., Scott V., and Bernard D. (1989). Potential Clostridium botulinum hazards associated with extended shelf-life refrigerated foods: A review. J. Food Safety 10: 131-153.
13. Dario De Medici, Fabrizio Anniballi, Gary M. Wyatt, Miia Lindström, Ute Messelha¨ußer, Clare F. Aldus, Elisabetta Delibato, Hannu Korkeala, Michael W. Peck, and Lucia Fenicia (2009): Multiplex PCR for Detection of Botulinum Neurotoxin-Producing Clostridia in Clinical, Food, and Environmental Samples. Applied and environmental microbiology, 75 (20): 6457–61.
14. El Khateib T. (1997): Microbiological status of Egyptian salted meat (basterma) and fresh sausage. J food safety 17 (3) 141-149.
15. Eric A. Johnson, William H. Tepp, Marite Bradshaw, Richard J. Gilbert, Paul E. Cook, and E. David G. McIntosh (2005): Characterization of Clostridium botulinum Strains Associated with an Infant Botulism Case in the United Kingdom. J Clin. Microbiol., 43 (6) p. 2602-2607.
16. Frerk F. (2000): The role of seafood in bacterial foodborne diseases. Microbes and Infection. : 2; (13): 1651-1660.
17. Gibbs P., Davies, A. and Fletcher, R. (1994): Incidence and growth of psychrotrophic Clostridium botulinum in foods. Food Control 5, 5-7.
18. Gross H., Polagruto J., Zhu Q., Kim S., Schramm D., and Keen C. (2004): Effect of honey consumption on plasma antioxidant status in human subjects. Paper presented at the

227th American Chemical Society Meeting, Anahein CA, March 28.
19. Hauschild, A.H.W. (1992): Epidemiology of human foodborne botulism. In: Hauschild A.H.W. and Dodds, K.L. (eds.) Clostridium botulinum - ecology and control in foods. Marcel Dekker, New York, USA, pp. 68-104. Available at http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhpb16-eng.php.
20. Huss H. (1981): Clostridium botulinum type E and botulism [DSci thesis]. Lyngby (DK): Technical University, Technological Laboratory of the Ministry of Fisheries. P.58.
21. Hyytiä E., Hielm S., and Korkeala H. (1998): Prevalence of Clostridium botulinum type E in Finnish fish and fishery products. Epidemiol Infect 120: 245-250.
22. Ingrid Artin, (2008): Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in Clostridium botulinum. Doctoral dissertation, Lund University, Faculty of Medicine,Department of Laboratory Medicine, Lund Division of Medical Microbiology, Sweden. Available at http://www.lunduniversity.lu.se/o.o.i.s?id=12683&postid=1022281
23. John A. and Greg S. (2009): Detection of Clostridium botulinum And Its Toxins in Suspected Foods and Clinical Specimens. Food & Nutrition; Compendium of Analytical Methods.Volume2
24. Johnson E., Tepp W., Bradshaw M., Gilbert R., Cook P., McIntosh E. (2005): Characterization of Clostridium botulinum strains associated with an infant botulism case in the United Kingdom. J Clin Microbiol.43: 2602-7.
25. Kautter D., Lilly T., Solomon H., and Lynt R. (1982): Clostridium botulinum spores in infant foods: a survey. J Food Protect 45: 1028-1029.
26. Kerstin A. and Riemann H. (1971): Prevalence of Clostridium botulinum in semipreserved meat products. J Applied Microbiology 21 (3): 543-544.
27. Korkeala H., Stengel G., Hyytiä E., Vogelsang B., Bohl A., Wihlman H., Pakkala P. and Hielm S. (1998): Type E botulism associated with vacuum-packaged hot-smoked whitefish. Int. J Food Microbiol. 43: 1-5.
28. Lalitha K. V. and Surendran P. K. (2002): Occurrence of Clostridium botulinum in fresh and cured fish in retail trade in Cochin (India) International Journal of Food Microbiology. 72: 169-174.
29. Lindström M., Kiviniemi K., Korkeala H., (2005): Hazard and control of group II (non proteolytic) Clostridium botulinum in modern food processing; Int J Food Microbiol 108: 92-104.
30. Lucia F. and Fabrizio A. (2009): Infant botulism, Ann Ist Super Sanità 45 (2): 134-146.
31. Lusby, P.E., Coombes, A.L., and Wilkinson, J.M. (2005): Bactericidal activity of different honeys against pathogenic bacteria. Arch Med Res 36: 464-467.
32. McLaughlin J., Sobel J., and Lynn T., (2004): Botulism type E outbreak associated with eating a beached whale, Alaska. Emerg Infect Dis. 10 (9):1685-7.
33. Midura T., Snowden S., Wood R. and Arnon S. (1979): Isolation of Clostridium botulinum from honey. J Clin Microbiol 9: 282-283.
34. Mundo M., Padilla-Zakour O., and Worobo R. (2004): Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. Int J Food Microbiol 97: 1-8.
35. Nakano H., Okabe T., Hashimoto H., and Sakaguchi G. (1990): Incidence of Clostridium botulinum in honey of various origins. Jpn J Med Sci Biol 43: 183-195.
36. Nevas M. (2006): Clostridium botulinum in honey production with respect to infant botulism. Doctoral dissertation, University of Helsinki, Faculty of Veterinary Medicine, Department of Food and Environmental Hygiene. http://ethesis.helsinki.fi/julkaisut/ela/elint/vk/nevas/abstract.html.
37. Nevas M., Hielm S., Lindström M., Horn H., Koivulehto K., and Korkeala H. (2002): High prevalence of Clostridium botulinum types A and B in honey samples detected by polymerase chain reaction. Int J Food Microbiol 72: 45-52.
38. Ornella R., Michela S., and Paola C. (2001): Tetanus and botulinum neurotoxins: turning bad guys into good by reseach. Toxicon 39, 27−41.
39. Özlem K., Muammer G., Haydar Ö. and Ahmet K. (2006): Incidence of Clostridium botulinum spores in honey in Turkey. Food Control. Volume 17, Issue 3, Pages 222-224.
40. Peck M. (2009): Biology and genomic analysis of Clostridium botulinum Adv Microb Physiol. 55:183-265.
41. Perez R., Iglesias M., Pueyo E., Gonzalez M. and de Lorenzo C. (2007): Amino acid composition and antioxidant capacity of Spanish honeys. J Agric Food Chem. Jan 24; 55 (2):360-5.
42. Przybylska A. (2004): Botulism in Poland in 2002. Przegl Epidemiol ; 58(1):103-10.
43. Rall V., Bombo A., Lopes T., Carvalho L., and Silva M. (2003): Honey consumption in the

state of São Paulo: a risk to human health? Anaerobe 9: 299-303.
44. Raphael B., Choudoir M., Lúquez C., Fernández R., Maslanka S. (2010): Sequence diversity of genes encoding botulinum neurotoxin type F. J Appl. Environ. Microbiol. 76(14):4805-12.
45. Sagua M. , Lúquez C., Barzola C., Bianco M., and Fernández R. (2009): Phenotypic characterization of Clostridium botulinum strains isolated from infant botulism cases in Argentina. Revista Argentina de Microbiología 41: 141-147.
46. Shunji Kozaki, Yoichi Kamata, Tei-Ichi Nishiki, Hiroaki Kakinuma, Hiromi Maruyama, Hiroaki Takahashi (1998): Characterization of Clostridium botulinum Type B Neurotoxin Associated with Infant Botulism in Japan, Infection and Immunity, 66, (10): 4811-4816.
47. Sobel J. (2005): Botulism. Clin Infect Dis; 41:1167-73.
48. Sobel J., Malavet M., and John S. (2007): Outbreak of clinically mild botulism type E illness from home-salted fish in patients presenting with predominantly gastrointestinal symptoms.Clin Infect Dis. 45:e14-6.
49. Solomon H., Johnson E., Bernard D., Arnon, S. and Ferreira S. (2001): Clostridium botulinum and its toxins. In F. P. Downes and K. Ito (Eds.), Compendium of methods for the microbiological examination of foods (pp. 357-380) Washington: APHA.
50. Taormina P., Niemira B., and Beuchat L. (2001): Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. Int J Food Microbiol 69: 217-225.
51. Tara, (2004): TKH Bacteriology Notes: Botulinus. http://www.tarakharper.com/b_botuln.htm
52. Underwood K., Rubin S., Deakers T., and Newth C. (2007): Infant botulism: a 30-year experience spanning the introduction of botulism immune globulin intravenous in the intensive care unit at Childrens Hospital Los Angeles. Pediatrics.120:1380-5.
53. Vu, Thi Lam (2006)**:** Incidence of Clostridium botulinum Spores in Honey and Infant Food Samples Collected from Vietnam and Germany, Dissertation zur Erlangung des Doktortitels, angenommen von: Georg-August-Universität Göttingen, Fakultät für Agrarwissenschaften. http://webdoc.sub.gwdg.de/diss/2006/vu/vu.pdf
54. White J.W. (1978): Honey. Adv Food Res 24: 288-374.