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A STUDY ON PREVALENCE OF HUMAN TOXICOGENIC TYPES (A, B, E) OF CLOSTRIDIUM BOTULINUM BY NESTED PCR METHOD IN DOMESTIC AND COMMERCIAL HONEYSIN ARDEBIL (NORTHWEST OF IRAN)

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ABSTRACT

To determine the prevalence of human toxicogenic types (A, B and E) of Clostridium botulinum spores in honey, which is one of the most important risk factors for food-borne botulism, especially in child, we collect 229 honey samples in Ardebil. Iran. For this purpose, 59 commercial and 167 domestic honey samples from all retailers and stories that sell honey were obtained. Enrichment media as TPGY broth for germination of spores was used. In addition, three pair primers were designed based the Nested PCR protocol and provided for distinguishes different types of *C.botulinum*. Result showed, in the enrichment stage 83 of 226 samples were positive (germinated by turbidity and growth) and next based on Nested PCR, number of *C. botulinum* types A, B and B were 0.38 and 4 respectively. Total prevalence of *C. botulinum* in honey samples was 18% and proportion of type B was about 90%. However the prevalence of *C.botulinum* in domestics groups samples were higher than commercials but this difference was not significantly ($p>0.05$). While the prevalence of the type A was shown to be lowest. No significant difference was present in the prevalence of botulinal spores in extracted honey compared with honeycomb samples. This is the first report of type A, B and E spores of *C. botulinum* in Iran. It is suggested that this Nested PCR method will be useful for epidemiological studies of food source botulism. This study

demonstrates the utility of molecular genotyping of *C. botulinum* and how it contributes to our understanding the epidemiology and variation of boNT gene.

Keywords: Clostridium Botulinum, Nested PCR, Honey, Ardebil

INTRODUCTION

*C. botulinum*is an anaerobic spore-forming organism which producing an extremely potent neurotoxin in foods **[1]**. There are seven types of *C. botulinum* (types A to G), which produce serologically distinct neurotoxins that involve flaccid paralysis and death in humans and other animals **[2]**. Types A, B, and E have been reported as the most common causes in human botulism, while type F is involved in rare cases. Botulism is caused by the ingestion of preformed botulinal toxin produced by *Clostridium botulinum* (*C. botulinum*), which can grow in improperly preserved foods. It remains the most frequent form in humans worldwide. Epidemiologic and laboratory studies have shown spores of Clostridium when ingestedininfant less than 1 year of age, they may germinatein the intestinal lumen and produce botulinum neurotoxin,one of the most potent toxins known **[3]**.The infection occurs in early infancy because of the immaturity of the micro flora of the intestine on the other hand, Only some infants are infected, and several other factorsInfluence the susceptibility of an infant than the lackof competing flora **[4-5]**. Since the first case recognized in 1976, there have been over 1,500 infant botulism cases reported in more than 15 countries worldwide. The youngest baby was just 56 hours of age. The first case of infant botulism due to *C. botulinum* type C was reported in Japan **[6]**. Most reported cases of infant botulism were caused by type A or B **[7]**. A few infant botulism cases were associated with *C. butyricum* and *C. baratii*, which produce type E and F neurotoxins respectively **[8-10]**. *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 **[11]**. 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. To date, honey has been regarded as the only foodstuff being a significant risk factor for infant botulism **[7]**, although scientific studies have recognized the approximately 30% of the infant botulismcases in California, where the highest number of cases in the United States are reported **[12]**, were associated with the ingestion of honey containing botulinal spores **[13]**. In Europe, a history of honey consumption has been reported for more than half of the reported

infant botulism cases **[9]**. Dust has be enimplicated as another important vehicle for infection **[14]**, but in many cases the source of infection remains unclear **[4]**. The contamination levels were estimated at between 5 and 80 *C.botulinum* spores per gram. About 60% of infant botulism cases in Europe had a history of honey consumption **[9]**. The contamination routes of *C. botulinum* in honey have not been identified, but it's possible contamination sources are likely to include pollen, dust and air **[15]**.Also demonstrated that botulinalspores are able to germinate and multiply under aerobicconditions in dead bees and pupae, which couldact as a source of contamination inside the beehive **[16]**. Due to the high sugar content, low pH, low proteinconcentration, and the existence of different oxidasesand other antimicrobial substances, thespores are unable to germinate and grow in honey **[17]**. For detection and identification of botulinal neurotoxins, the only universally recognized method is the mouse toxicity and toxin neutralization bioassay **[18]**. The use of Polymerase Chain Reaction (PCR) in screening for the presence of botulinal spores in honey samples may decrease detection time, costs, and avoiding ethical concerns associated with animal testing. Detection of the presence and the number of *C. botulinum* spores in honey is necessary, because, it give information about the *C. botulinum* spore contamination load which caused decision whether honey is suitable for human consumption or not. Moreover, the presence of *C. botulinum* spores in honey reflects the *C. botulinum* contamination of the environment because honey is considered an environmental indicator of the *C. botulinum* load. Ardebil province is located in North West of Iran and it is top three provinces in the production of natural honey in Iran. Nevertheless, there is a lack of information about the prevalence of pathogenic bacteria and their toxins such as *C.botulinum* in that region honey. With respect to infant botulism, there have been no surveys carried out to investigation the presence of *C. botulinum* and its spores in foodstuff. Therefore, findings related to infant botulism are insufficient in Iran. On the other hand, current methods used for detection of this microorganism are outdate and inefficient. So; in this study we collected two groups of samples from retails supermarkets randomly from different areas of Ardebil city, domestic & commercial, until have a comparison of prevalence of *C.botolinum* between then and we used Nested PCR with highest sensitivity and specificity for fist time.

MATERIALS AND METHOD

Honey Samples

A total 226 honey samples including 167 commercial and 59 domestic samples were collected randomly. These were provided from beekeepers, supermarkets, free markets, sales clerks and retail stories of Ardebil. Materials consisted of extracted honey and honeycomb samples. Commercial honey samples were analyzed from original containers while the others were collected aseptically into sterile containers; all samples were stored in the dark and cold places based of Codex and ISIRI no.12-1981 and 92.

Sample Preparation

The samples were prepared according to a Nevas et al described method (14). Briefly, 2×25 g of each honey sample were separately diluted in 225 ml of 1% Tween 80 (Merck-Schuchardt, Hohebrunn, Germany) solutions in a water bath at 65 C, after which they were centrifuged for 30 min at 8700–9000×g. The supernatants were filtered through 0.45-Am membrane filters with a diameter of 47 mm (Millipore, Bedford, MA, USA). The filters were then transferred to duplicate tubes containing 10 ml of trypticase–peptone–glucose–yeast extract TPGY broth(19). The tubes were incubated under anaerobic conditions for 5 days, one tube at 30 C and the other at 37 C, to enable germination and growth of non-proteolytic and proteolytic strains of *C. botulinum*, respectively. After the incubation, 1 ml of each broth was transferred to a fresh 10-ml TPGY medium for further 16h incubation under the same conditions. Cells from 1 ml of this overnight culture were then washed with TE buffer and made into a DNA extraction process as PCR template according to Hielm *et al*. **[20]**.

Statistic Analysis

On sample T test were carried out to assess association between categorical variable by SPSS v.16. Result considered statically significant when $p<0.05$.

DNA Extraction and Standard Cutter Preparation

We used the reference strains such as type A (69 proteotylic), type B (okra J2 proteolytic), type E (bulga non proteotylic) and *C. perfrengens* from microbial lab of food hygiene Department of Islamic Azad University by Dr. Razavilar efforts and *C. perfringens* ATTCas control negative for analytical sensitivity and specify testing and optimization nested PCR. DNA of reference bacteria was purified to DNEasy Kit (Qiagen), following the manufactures instruction. The quality and concentration of the DNA samples were assessed using spectrophotometery (NanodropND100).Themo electrophoress and UV by 2% agarose gel SYBR. In the pretest PCR, we used DNA extraction of standard strains and C. perfringens for

negative control but in main test, no DNA used for negative control. The DNA extraction was done according to the methodology used by Hielm *et al*. 1996.(20) Briefly, reference bacteria cells were cultured in pre-reduced cooked meat medium overnight. Cells were harvested by centrifugation at 6000g for 10 min and resuspended in 567 µL of TE buffer (0.01 M Tris HCL, 0.001 M EDTA). This was added with 30 µL of 10% sodium dodecyl sulfate and 3 µL proteinase K (20 mg/mL in distilled water) and incubated for 1hr at 37ºC. After this, 100 µL 5 M NaCl and 80 µL CTAB-NaCl solution (10% cetyltrimethyl ammonium bromide in 0.7M NaCl) were added and incubated for 10 min at 65ºC. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution and DNA was precipitated with absolute ethanol by incubation at -20ºC overnight. DNA was harvested by centrifugation at 12000 g for 20 min, washed with 70% ethanol and resuspended in 100 µL distilled water, after being dried at 37ºC incubation. DNA was stored at -20ºC.

Primers and PCR Procedure

Base on the DNA sequence of *C.botulinum* available in Genebank-NCBI and Basic Local Alignment Search Tool programs, specific targets were selected for DNA amplification. The primers were designed using primer express V.2.0 software. The target selected for amplification was BONT gene that encodes Neurotoxin botulinum in different types of *C.botulinum* such as A, B and E. 2 set of primers were designed for each of types of C. botulinum: outer and internal primers **(Table 1)**. PCRs (50-ml reaction mixtures) were performed with a DNA thermal cycler (model Peqstar96-Advanced Gradiant). The amplification reaction mixtures contained PCR buffer (50 mMKCl, 10 mMTris-HCl [pH 8.3]), 3.75 mM$MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (Boehringer Mannheim Biochemicals, Indianapols, Ind.), 1.0 mM each primer, 1.25 U of Taq DNA polymerase (Promega, Madison,Wis.), and 5.0 ml of DNA template in a 0.65-ml thin walled, polypropylene PCR tube under a layer of light mineral oil (Sigma). Samples were heated to 80°C for 5 min prior to addition of the deoxynucleoside triphosphates, second primer, and Taq DNA polymerase. An amplification profile of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min was performed for 30 cycles, followed by one cycle of 72°C for 10 min. Nested amplification reactions (15cycles) were performed with 1 ml of the initial amplification reaction mixture as the template. A no-template control (NTC) was included in every experiment two combinations of primers located within the

light-chain region of the toxin gene were used to detect the BoNT gene. In the initial amplification step, Af1, Bf1, Ef1 and Ar1, Br1, Er1 primer combinations were used. In the nested amplification reactions, the Af2, Bf2, Ef2 and Ar2, Br2, Er2 primer were used to give the primer combinations. At this phase before the Nested PCR, the sensitivity and specificity of each of the primers designed using standard strains and negative controls were evaluated Amplification reaction mixtures were size fractionated through 2% agarose gels in 13 TAE buffer (40 mM Tris acetate, 1 mM EDTA) containing 0.5 mg of ethidium bromide per ml.

RESULTS

83 of the 226 honey samples obtained from retailers and supermarkets and sales clerks gave a positive result (36.72%) and presence growth and turbidity when cultured into TPGY broth under anaerobic conditions. These cultures were examined macroscopically to also purity investigated, the typical cultures had rod morphology, width varied in length a width depend on the types. Production of organic acids during fermentation in grown cultures caused PH values ranged from 5.9-6.6. Only 42 (18%) of the 83 samples that presented turbidity and growth in the previous stage categorized and illustrated special bands in molecular assay. Prevalence of types A, B and E was 0, 38 and 4 respectively. In 4 samples that were positive in terms of type E, type B spores were collected too. Thus, both *C. botulinum* types E and B were isolated from four of the samples. The fragments obtained for each strain correlated with the size reported for the toxin gene with the primers used, corresponding to310 bp for A strain, 162 bp for B and 220 bp for E. Nevertheless, DNA extracted from strain A represented a very big quantity of DNA. However the prevalence of *C. botulinum* in domestics groups samples were higher than Commercials but this difference was not significantly ($p>0.05$). While the prevalence of the type A was shown to be lowest. **(Chart1)** No significant difference was present in the prevalence of botulinal spores in extracted honey compared with honeycomb samples. However, a trend towards higher prevalence in honeycomb samples was noted ($x2=3.0$, $p >0.05$).

DISCUSSION & CONCLUSION

Since the discovery of infant butolism in 1979, there have not been any reliable studies in Iran. This survey is first investigation about food stuff related by botulism because epidemiological risk factor of this disease include residence where agricultural practice area , urban area where building or earth moving for reach construction are frequent and honey and corn syrup consumption. Nested PCR assay

for the simultaneous detection and identification of *C. botulinum* types A, B, and E in honey was developed. The method is based on a primer cocktail consisting of 3 new pairs of oligonucleotide primers, each being specific for the botulinum neurotoxin type A, B, E gene. The number of *C.botulinum* 83 (36.72%) growth and turbidity amount 226 samples is represented several issues: First, samples may be taken from the part of honeys that was free of spores and despite the presence of sporesno growth in enrichment stage was observed. there for, false negative error be reported. Secondly, the final 42 molecular positive from 83 cultural shows that due to the high sensitivity and specificity of molecular Nested PCR, it may include other Clostridium types(C, F) or some anaerobic, sulfide reduction aspect bacteria. In this study the standard strains of type C, F were not provided. Hence, presence of spores of this type in honey samples is not clear.Finally, with regarding to prevalence type B (90%) can be said that the results are consistent with other investigations. However, the prevalence of (18%) *C. botulinum* in Ardebil honey samples are slightly higher than results that obtained in similar studies. Whereas the dust, surrounding environment, water resources carrying spores, we should focus our attention to these Areas. Because studies have shown a direct correlation between them and presence of *C.botolinum* in honey **[14]**.The prevalence of *C. botulinum* spores in honeymay be considered to reflect the overall existence ofbotulinal spores in the environment. In Denmark, a survey of 47 soil samples from cultivatedareas resulted in a 30% prevalence rate for *C. botulinum*; most of these were type B spores(21) In Sweden, of 69 terrestrial soil samples, 20 (29%) were shown to be positive for *C. botulinum* type E and 1 positive for both types E and B (22). No data is available on the prevalence of C. botulinum Ardebil soils, but the occurrence of type B spores in similar studies it is congruent with the existence of these serotypes in soils of Ardebil. Because of proteolytic and non proteolytic characteristics, specific optimal temperature and incubation duration of different types of *C.botulinum*, we take them, several studies have been conducted since 1978 and prevalence of *C.botulinum* spores in honey Approximately 2 to 24% have been reported **[9]**.While current study is the first study in this regard at Iran, that was achieved around 18%. The spores have not disappeared during the food processing over the years and survive steadily by Maintenance. If you want to destroyed spores by high thermal method, inevitably taste, structure and physicochemical aspects of honey change to worse. During the study

by Nevas et al 2005, the number of spores in honey samples were between 60-1200 and by the average it was about 180 spores per kg. In this study because of the enrichment and proliferation of spores, the number of spores was not estimate correctly, however, at least 180 sp/kg needs to Despite of sampling, spores are existed and grown signs appear. According to CDC, annually 2 per100.00 birth children under 14 months, in United States affected by botulism and more than 20% of them consumed honey before the appearance of botulism symptoms(10). While there aren't any reliable and comparable studies and data about this events in Iranian child. In 2004 Uzlem *et al* in Turkey used 3 different spore isolation methods include DA (Direct addition), DC (Dilution centrifugation) and SF (supernatant filtration) with TPGY broth or CMM (cooked meat medium) for detection of *C. botulinum* in honey samples and they illustrated the combination of TPGY broth with SP method is more useful for their approach. They estimated Ankara's retail honeys contamination to *C. botulinum* around 12.5% , but current study we apply TPGY broth with nested PCR and our results are higher than their findings **[23]**. Midura *et al.* found nine positives of 90 honey samples (10%) while Nakano, Okabe, Hashimoto, and Sakaguchi reported 23 *C.botulinum* positive of 270 honey samples (8.5%)(15, 24).Huhtanen et al. &Schocken-Iturino et al. reported 7.5% and 7.1% of *C. botulinum* spores positive of honey samples respectively(16). In a French study (Delmas, Vidon, & Sebald) 6.7% of honey samples were found to be contaminated with *C.botulinum* while in Cordoba (Monetto *et al*., 1999) found 3 of 45 samples (7%) were positive(25). In another study Nevas *et al*., 190 honey samples were collected from different countries, 20 samples (11%) were found to be positive. On the other hand there aresome studies reporting lower contamination levels in honey **[26]**. In two studies conducted in Washington DC (Kautter, Lilly, Solomon, & Lynt,) and Argentina (De Centorbi, Alcaraz, &Centorbi,) 2% and 2.3% of contamination levels were reported respectively **[27-28]**. De Centorbi, Satorres, Alcaraz, Centorbi, and Fernandez and Hauschild, Hilsheimer, Weiss, and Burke reported 1.1% and 0.93% of *C.botulinum* contamination from the honey samples respectively(29-30). In a study by Ralletal.in Sao Paulo, *C.botulinum* isolation level was found to be 3%. Botulism continues to be a public health problem all over the world and clearly needs further investigation **[31]**. Schocken-Iturino *et al*. stated that more studies are necessary to determine not only to state the prevalence of *C.botulinum* in honey but also know how this microorganism is incorporated to into it

[16]. In terms of difference between domestic and commercial honey should be pointed the number of positive samples of honey, a little more than the commercial samples, but the difference was not significantly ($p>0.05$). Our results show that honey sold in retail markets in Ardebil are significantly (18%) contaminated with *C.botulinum*. Being commonly consumed by all age groups of consumers and being potential infantile botulism agents for the children younger than 1 year the pediatricians should consider old, this food. Further, this may be used to overcome the cumbersome and time-consuming animal tests used to identify toxigenic Clostridium. Its application for the direct detection of pathogenic Clostridium in other food samples needs to be evaluated further.

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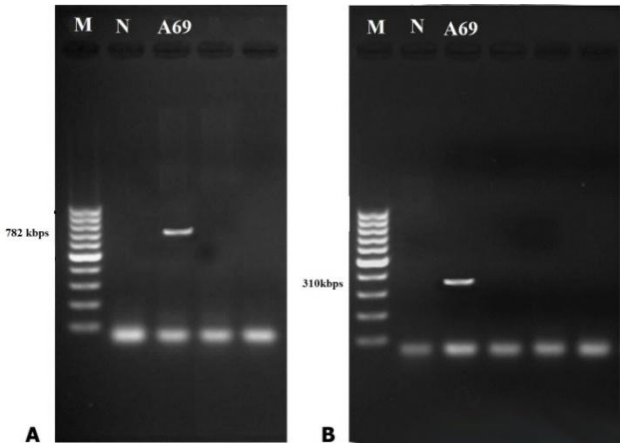

Figure 1: Detection of BONT A gene at standard culture A69 by A1, A2 primers

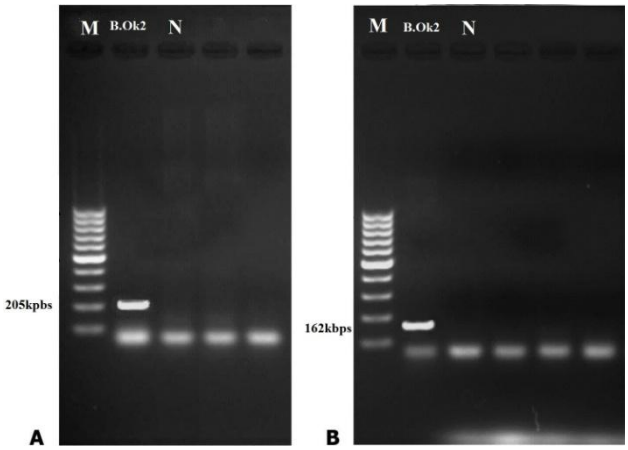

Figure 2: Detection of BONT gene at standard culture B Okraby B1, B2 primers

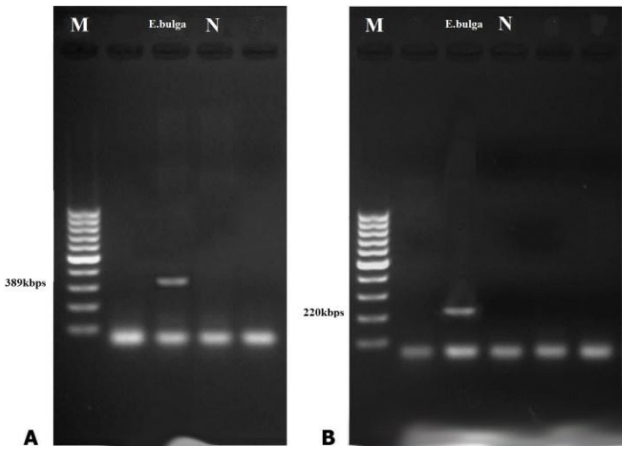

Figure 3: Detection of BONT A gene at standard culture E bulgaby E1, E2 primers

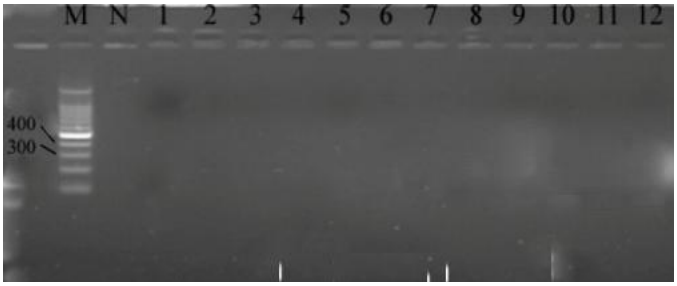

Figure 4: No detected of the A toxin gene amplification products in DNA isolated from honey samples. Images were generated with Photoshop version 5.02 and Freehand version 7.02 software. Letters and numbers above the lanes are sample designations. NTC, no-template control.

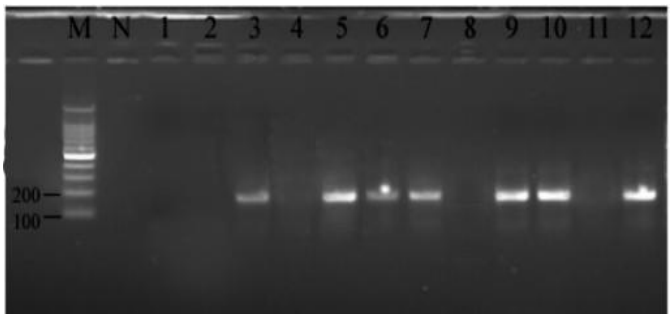

Figure 5: Detection of the B toxin gene amplification products in DNA isolated from honey samples. Images were generated with Photoshop version 5.02 and Freehand version 7.02 software. Letters and numbers above the lanes are sample designations. NTC, no-template control.

Table 1: Primers used for the Nested PCR detection of C. botulinum types A, B and E

Type	Primer	Sequence 5`→3`	Length of PCR product	Location on gene (coding region)
A	A_{f1}	AGS TAC GGA GGC AGC TAT GTT	782	1788-1808
	A_{r1}	CGT ATT TGG AAA GCT GAA AGG G		2569-2548
	A_{f2}	GAG TTT TCT AA	310	434-453
	A_{r2}	AAA GCT CGG GTT		638-619
B	B_{f1}	CAG GAG AAG TGG AGC GAA AA	205	98-117
	B_{r1}	CTT GCG CCT TTG TTT TCT TG		530-549
	B_{f2}	GAT TCT GCC ATT	162	32-52
	B_{r2}	TAC GGC GCT TGA		508-528
E	E_{f1}	CCA AGA TTT TCA TCC GCC TA	389	156-175
	E_{r1}	GCT ATT GAT CCA AAA CGG TGA		544-525
	E_{f2}	AGG TAC GAA CTT	220	185-194
	E_{r2}	GGA ATT GAG GAA		727-708

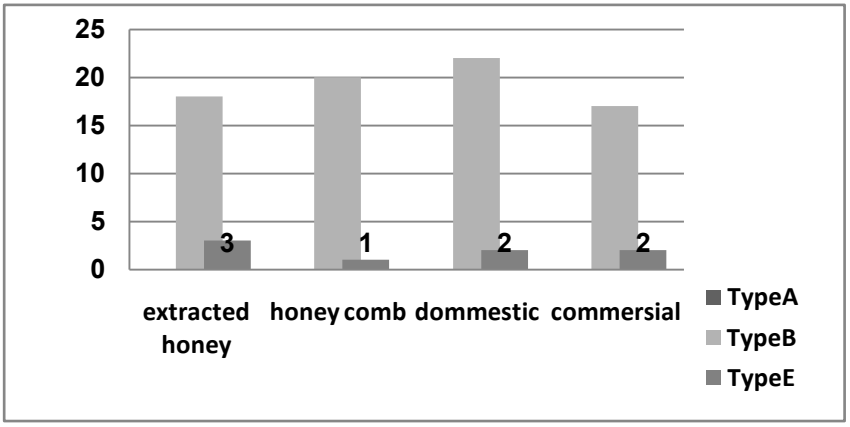

Chart 1: The number of *C. botulinum* in different categories