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Clostridium botulinum in honey: prevalence and antibiotic susceptibility of isolated strains

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Abstract: *Clostridium botulinum* and rare strains of *C. butyricum* and *C. baratii* produce an extremely potent toxin, the botulinum neurotoxin (BoNT). Infant botulism is significant for both its high mortality rates and for the sophisticated treatment that it requires. Isolation and identification of *C. botulinum* according to standards depend on mouse bioassays to determine toxin-producing ability of strains. Polymerase chain reaction (PCR) is used to detect the types of toxins expressed by bacteria. Since honey is an important source of infant botulism, we determined the types of toxins secreted by *C. botulinum* strains and their antibiotic resistance. In this study, the first step was conducted to determine the prevalence of *C. botulinum* in different honey types; as the second step, antibiotic susceptibility of the strains was determined; and, finally, PCR typing of toxin genes was done. Nineteen strains were isolated from 250 honey samples. All *C. botulinum* strains were evaluated for BoNT types using PCR. BoNT type A was observed in 12 of the 19 (63.15%) strains, type B was observed in 3 (15.78%) strains, type F was recorded in 2 (10.52%) strains, and 2 of the 19 (10.52%) strains showed no amplification. All strains represented a resistance to amoxicillin and trimethoprim sulfamethoxazole (100%), followed with sulfamethoxazole and ampicillin (94.73%). Resistance to nalidixic acid was seen in 84.21%. The results show that different types of honey are contaminated with *C. botulinum* and toxin types also show different distribution. Additionally, antibiotic resistance patterns of the strains showed different distributions, which indicates obligatory application of antibiotic resistance testing for prevention of secondary infections.

Key words: *Clostridium botulinum*, honey, PCR

1. Introduction

Food-borne botulism is a severe type of food poisoning caused by ingesting foods containing the potent botulinum neurotoxin (BoNT), formed during microorganism growth. *Clostridium botulinum* and its spores are found in soil, in dust, and on surfaces (1). Infant botulism is a severe but transient neuroparalytic disease that affects infants under 1 year of age. Paralysis is caused by the action of BoNTs that block the release of neurotransmitter acetylcholine from motor endings of all peripheral cholinergic synapses. BoNTs are produced in vivo by *Clostridium botulinum* and other BoNT-producing clostridia that temporarily colonize the intestinal tract of infants (2).

Honey, syrup, and foods with high viscosity have been identified as potential sources of infection (3), while dust and honey have been shown to be sources of spores (2,4). There was also an exceptional case that occurred due to turkey consumption by 8-month-old baby girl in Italy (5). Honey has been identified both as a dietary risk factor for infant botulism and as a natural reservoir of *C. botulinum* type A and B spores (1). Immature infantile intestinal flora allows ingested spores to germinate, multiply, and produce botulinum neurotoxins in the intestinal lumen. Children aged between 2 weeks and 1 year are most susceptible (6). The main clinical features of infant botulism are constipation, hypotonia, listlessness, lethargy, difficulty in suckling and swallowing, weak crying, pooled oral secretions, general muscle weakness, and loss of head control. Infantile botulism management includes prevention of nosocomial complications, antitoxin administration, and supportive therapy. Antibiotics against *C. botulinum* are not used in infantile botulism. Antibiotics cause microorganisms to die and decompose, which leads to a leak of intracytoplasmic toxins into the intestinal lumen, thus causing severe results (7). Secondary infections should be treated with antibiotics that do not have bactericidal effects on C. *botulinum* (e.g., trimethoprim-sulfamethoxazole or amoxicillin clavulanate). It is known that aminoglycoside antibiotics have synergetic effects on toxin binding to the neuromuscular plate.

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The results of different studies, which have been conducted in different countries with different types of honey, indicate that honey can be contaminated with *C. botulinum* (8–13).

According to German Institute for Standardization (Deutsches Institut für Normung, DIN) standards, the isolation and identification of *C. botulinum* takes approximately 12 days (14). Toxinotyping can be performed with mouse bioassays. However, many researchers ignore this method for ethical reasons. In the second half of the 20th century, studies on the ethics of experimental animals gained interest and ethical codes relating to animal experimentation came into force. Several alternative methods that could be used instead of animal experiments were developed, with many organizations across the world working in this field. Both these "alternative methods" and ethical concerns, which brought about the assumption of responsibility for laboratory animals, constituted the basis of the principles adopted by national and international organizations operating in the area of animal experimentation, as well as the basis of relevant legislation enacted (15–18). The Turkish Food Codex Honey Communiqué, published in the Official Journal on 17 December 2005 with the number of 26026 (19), clearly stipulated zero tolerance for the presence of *C. botulinum* and other food-borne pathogenic bacteria, eggs of parasites, and adult parasites. To meet the legal requirements, good manufacturing practices are applied by honey producers.

The aim of this study was to determine the prevalence of *C. botulinum* in different types of honey. Since antibiotic administration is discouraged in infant botulism management and it is recommended only for treatment of secondary infections, we also investigated antibiotic resistance of the strains.

2. Materials and methods

A total of 250 honey samples were obtained from different markets in Ankara. The distribution of honeys was: 150 extracted honeys (117 were in original packaging and 33 were sold openly) and 100 comb honeys (39 were in original packaging and 61 were sold openly). All honey samples were collected from December 2007 to May 2008.

2.1. Methods

Isolation and identification was performed according to DIN 10102 (14), which is shown in the Figure.

2.1.1. Identification of toxin types of *C. botulinum* strains

The method indicated for toxin typing in the DIN 10102 procedure includes mouse bioassays. Due to animal welfare reasons, we preferred to apply the polymerase chain reaction (PCR) method reported by de Medici et al. with some modifications. The PCR primers are given in Table 1. In this study, no internal control primer was used (20).

For the PCR reaction, a 50-µL reaction mixture containing 2X multiplex PCR master mixture, 0.3 µM of each primer (Table 1), and 3 µL of purified DNA template was used. The reaction mixture was heated at 95 °C for 15 min to activate the Taq polymerase and then subjected to 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 7 min.

2.1.2. Antibiotic resistance of *C. botulinum* strains

C. botulinum isolates from honey were submitted to antibiotic susceptibility testing with the following molecules: sulfamethoxazole (25 µg), trimethoprim/sulfamethoxazole (1:19) (co-trimoxazole) (25 µg), nalidixic acid (30 µg), gentamicin (30 µg), penicillin G (5 units), tetracycline (30 µg), ampicillin (25 µg), amoxicillin (25 µg), and cefalotin (30 µg). All strains were spread on Mueller Hinton agar (Oxoid, UK) dishes, and disks were applied to the surface and incubated at 37 °C for 24 h. All dishes were evaluated for zones and the results were recorded. Dishes were incubated at 37 °C for an additional 24 h. The zones were checked and changes noted.

3. Results

In this study, 250 honey samples were analyzed to detect *C. botulinum*. *C. botulinum* was isolated and identified from 19 (7.6%) honey samples. All strains were typical in the microscopic evaluation. Biochemical identification showed a distribution of 19 *C. botulinum* strains, 12 strains of *C. butyricum*, and 1 strain of *C. noyvi*. Two strains' colony morphologies were atypical on anaerobic egg yolk (AEY) agar plates; one strain was isolated from comb honey and the other from extracted honey. Some strains gave weak or no turbidity at 7 days, but strong turbidity on day 10 of incubation. The 2 strains giving atypical colonies were taken from AEY and restreaked on AEY again when typical colonies were observed. All strains were gram-positive and spore-forming.

All *C. botulinum* strains were typed using PCR. BoNT type A was observed in 12 of the 19 (63.15%) strains, type B in 3 of the 19 (15.78%) strains, and type F in 2 (10.52%) strains, while 2 of the 19 (10.52%) strains showed no amplification. The 2 amplification-negative strains were extracted again and the test was repeated, but they again showed no BoNT amplification. The isolation and identification results are given in Table 2.

3.1. Antibiotic susceptibility

Table 3 shows the results of antibiotic susceptibility analysis. All strains were tested for resistance against several antibiotics and the results are given in Table 3.

4. Discussion

Several studies are reported in the world literature on the prevalence of *C. botulinum* in honey. Midura et al.

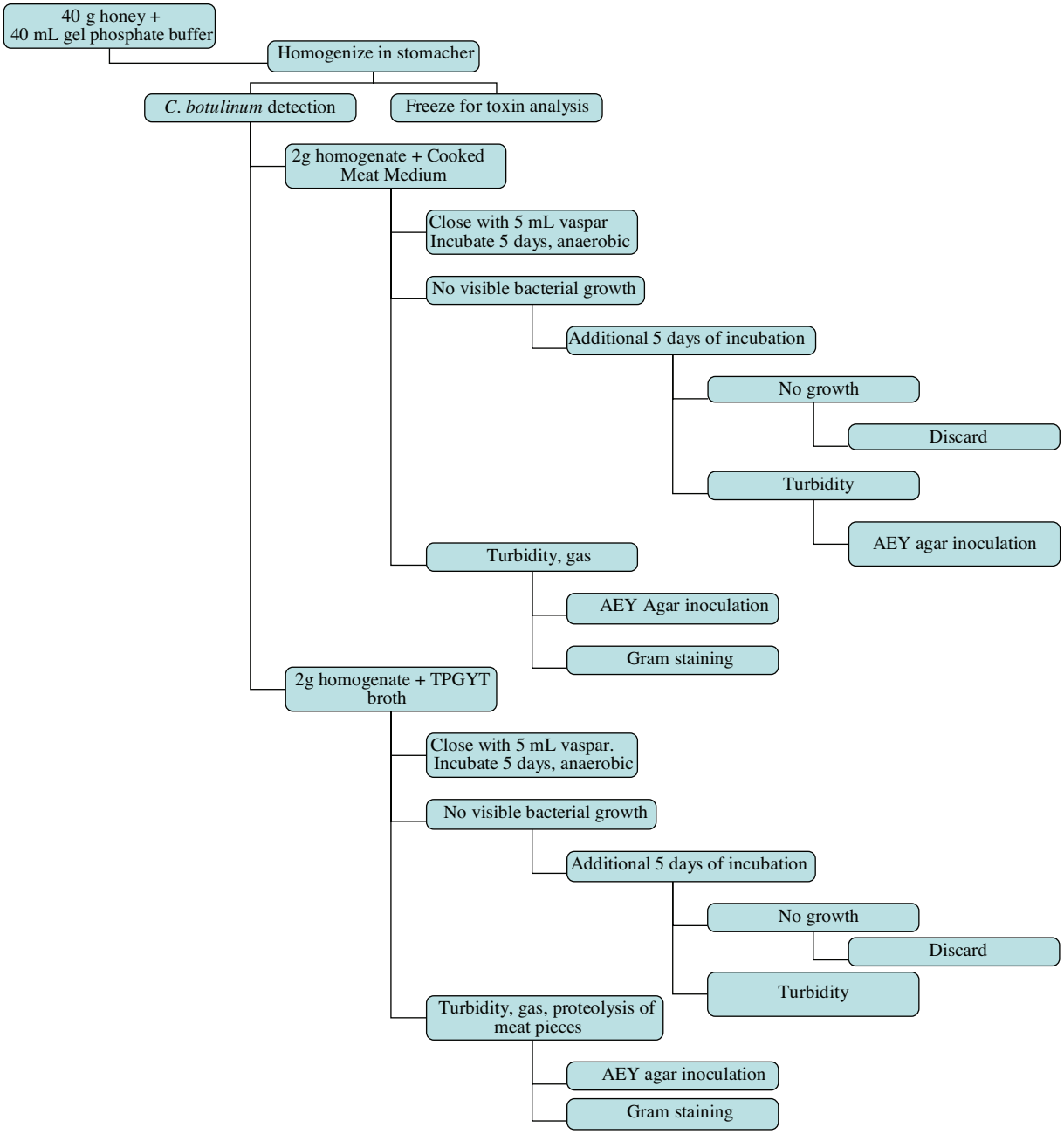

Figure. Isolation and identification scheme according to DIN 10102.

reported *C. botulinum* in 9 out of 90 honey samples, 6 of which were isolated from honeys given to infants who developed botulism (8). Nevas reported the prevalence of *C. botulinum* in the 448 extracted honey samples studied as 12%. Moreover, the overall prevalence of *C. botulinum* was 24% in comb honey samples (n = 256) in this study (12). In studies of 92 French (21,22), 282 German (23,24), and 134 Norwegian (25) honey samples, no *C. botulinum* spores were detected. In 2 different studies held in Argentina, Centorbi et al. isolated *C. botulinum* from 1 out of 42 honey samples (26) and Monetto et al. reported 2 positive samples out of 44 honey samples (27). Different studies held in the United States showed *C. botulinum* in 10 out of 80, 2 out of 100, and 18 out of 241 honey samples (28–30). In a study held in Italy, *C. botulinum* was isolated from 2 among 39 honey samples (31). In Turkey, *C. botulinum* contamination was reported in 6 out of 48 honey samples (11). The results of some of the authors above are consistent with those of our study. On the other hand, differences between results of our study

Table 1. Primers and product sizes for toxinotyping of *C. botulinum* strains.

Toxin type	Primer name		Product size (bp)
A	IOA (Forward)	GGG CCT AGA GGT AGC GTA CTG	101
	IOA (Reverse)	TCT TAA TTT CCA GAA GCA TAT TTT	
B	CBMLB (Forward)	CAG GAG AAG TGG AGC GAA AA	205
	CBMLB (Reverse)	CTT GCG CCT TTG TTT TCT TG	
E	CBMLE (Forward)	CCA AGA TTT TCA TCC GCC TA	389
	CBMLE (Reverse)	GCT ATT GAT CCA AAA CGG TGA	
F	CBMLF (Forward)	CGG CTT CAT TAG AGA ACG GA	543
	CBMLF (Reverse)	TAA CTC CCC TAG CCC CGT AT	

Table 2. The isolation and identification results obtained from this study.

No.	Type of honey	TPGY incubation		AEY	Gram	Toxin type				
		7 days	10 days			A	B	E	F	NAM
	EH (P)	+	+	+	+	+				
	EH (O)	+	+	+	+	+				
	CH (P)	+	+	+	+	+				
	CH (O)	+	+	+	+	+				
	CH (P)	+	+	+	+	+				
	EH (P)	+	+	+	+	+				
	CH (O)	+	+	+	+		+			
	EH (P)	+	+	AT	+	+				
	EH (O)	-	+	+	+					+
	EH (O)	+	+	+	+				+	
	EH (P)	+	+	+	+	+				
	EH (O)	+	+	+	+		+			
	CH (P)	+	+	AT	+	+				
	CH (O)	+	+	+	+	+				
	EH (P)	+	+	+	+	+				
	CH(O)	+	+	+	+				+	
	EH (P)	-	+	+	+	+				
	EH (O)	+	+	+	+		+			
	EH (O)	+	+	+	+					+

EH: Extracted honey, CH: comb honey, (O): sold openly, (P): in its original packaging, NAM: no amplification, AT: atypical, TPGY: trypticase peptone yeast extract broth, AEY: anaerobe egg yolk agar.

and those of others may be due to sampling, enrichment, and incubation. The study by Küplülü et al. showed that results obtained with different enrichment methods are statistically important (11).

In a study by Nevas, 61 of 256 comb honey samples were found to be contaminated with *C. botulinum*. These 61 strains showed toxin type distribution as follows: type A in 3 strains, type B in 54 strains, type E in 3 strains, and type F in 1. The authors also analyzed 448 extracted honey samples and reported contamination in 53 samples. These 53 strains showed toxin-type distribution as follows: 17 type-A, 40 type-B, and 5 type-E strains (12). The results are consistent with ours and the distribution of toxin types show parallelism with our results.

Table 3. Antibiotic susceptibility test results. R: Resistant, I: intermediate, S: susceptible.

Antibiotic	Concentration	R	I	S
Sulfamethoxazole	25 µg	18/19	1/19	-
Trimethoprim/sulfamethoxazole (1:19) (co-trimoxazole)	25 µg	19/19	-	-
Nalidixic Acid	30 µg	16/19	2/19	1/19
Gentamicin	30 µg	5/19	3/19	11/19
Penicillin G	5 units	4/19	9/19	6/19
Tetracycline	30 µg	6/19	4/19	9/19
Ampicillin	25 µg	18/19	1/19	-
Amoxycillin	25 µg	19/19	-	-
Cefalotin	30 µg	8/19	8/19	3/19

There are limited data about the antibiotic susceptibility of *C. botulinum* strains because the use of antibiotic drugs is discouraged in food-borne and infant botulism management. Antibiotics are administered only for treatment of secondary infections, considering that these drugs could not cause a worsening of neurological symptomatology (27–30).

There are limited data about the antibiotic resistance of *C. botulinum* strains. In a study by Swenson et al., susceptibility to tetracycline and penicillin was recorded. In the same study, *C. botulinum* strains were reported to be resistant to cefalotin, trimethoprim-sulfamethoxazole, nalidixic acid, and gentamicin (30). The data of our study are in accordance with this study. Resistance can be caused by application of antibiotics to prevent diseases during beekeeping. It was reported that 1714 honey samples were collected from beekeepers from 22 different regions in Turkey. The samples were analyzed for residues of sulfonamides, tetracyclines, and streptomycin-group antibiotics, which are not legally permitted to be used for beekeeping in Turkey. Enzyme-linked immunosorbent assay and high-performance liquid chromatographic techniques were used to analyze the compounds and the results showed that 10% to 15% of the beekeepers were still using these antibiotics against foulbrood diseases (28). In similar studies, antibiotic residues in honeys were found (28–30). All 19 strains isolated in our study represented resistance to sulfonamides. This may be caused by the illegal use of sulfonamides during beekeeping.

Nevas reported that the supernatant filtration method combined with PCR enables a sensitive, rapid, and ethically acceptable means of screening honey samples for the presence of *C. botulinum* spores (12). PCR is also used as an alternative method to animal models in scientific research. The fact that PCR results are accepted as more sensitive, specific, and rapid (12) complies with the ethical criteria required for toxin investigations in which animal models should be avoided. It was reported with different gene sequences for detecting *C. botulinum* (12). The results of that study indicated that the appropriate BoNT genes were detected in material from 7 out of the 8 cases of botulism and provided a supportive diagnosis faster than the conventional bioassay. These assays have already proven useful for public health microbiological investigations of suspected cases of human botulism by substantially improving the diagnostic process.

In conclusion, honey is known to be linked to infant botulism. There are various routes of honey contamination with *C. botulinum* spores. Our results show that beekeepers should stop the preventative application of antibiotics, which triggers antibiotic resistance in microorganisms found in honey. Using irradiation to eliminate *C. botulinum* contamination of honey is not acceptable due to significant changes in the sensory quality of honey. Good production practices should be applied in honey production. Public health and food safety concerns of *C. botulinum* should be taken into account, especially with regard to infant botulism. Turkish regulations are strict about *C. botulinum* in honey, underlining zero tolerance and obligatory labeling of honey. This kind of restriction is significant only for infant botulism because *C. botulinum* spores do not represent a risk for adults or young people. To this aim, in Turkey, like in the United States and in several European countries, honey labels report that the product is not recommended for an infant under 1 year of age. This may lead to an increased awareness, but more information to pediatricians and honey producers should be given to improve the overall quality of honey.

References

1. Solomon HM, Lilly T Jr. *Clostridium botulinum*. In: US Food and Drug Administration, editor. Bacteriological Analytical Manual. 8th ed. Silver Spring, MD, USA: FDA; 2001. Chapter 17. 2001. Available at http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070879.htm.
2. Arnon SS, Midura TF, Clay RM, Wood RM, Chin J. Infant botulism: epidemiological, clinical and laboratory aspects. J Am Med Assoc 1977; 237: 1946–1951.
3. Midura TF. Update: infant botulism. Clin Microbiol 1996; 9: 119–125.
4. Chin J, Arnon SS, Midura TF. Food and environmental aspects of infant botulism in California. Rev Inf Dis 1979; 1: 693–696.
5. Lonati D, Locatelli CA, Fenicia L, Anniballi F, Landri P, Giampreti A, Petrolini, VM, Vecchio S, Manzo L. Fatal course of foodborne botulism in an eight-month old infant. Pediatr Rep 2011; 3: e31.
6. Arnon SS, Damus K, Chin J. Infant botulism: epidemiology and sudden infant death syndrome. Epidemiol Rev 1981; 3: 45–66.
7. Long SS, Pickering LK, Prober CG. Principles and Practice of Pediatric Infectious Diseases, Revised Reprint, 3rd ed. Philadelphia: Churchill Livingstone, 2009.
8. Midura TF, Snowden S, Wood RM, Arnon SS. Isolation of *Clostridium botulinum* from honey. J Clin Microbiol 1979; 9: 282–283.
9. Nevas M, Lindstrom M, Hautamaki K, Puoskari S, Korkeala H. Prevalence and diversity of *Clostridium botulinum* types A, B, E and F in honey produced in the Nordic countries. Int J Food Microbiol 2005; 105: 145–151.
10. Gilbert S, Lake R, Hudson A, Cressey P. Risk Profile: *Clostridium botulinum* in honey. Christchurch, New Zealand: Institute of Environmental Science & Research, 2006.
11. Küplülü Ö, Göncüoğlu M, Özdemir H, Koluman A. Incidence of *Clostridium botulinum* spores in honey in Turkey. Food Cont 2006; 17: 222–224.
12. Nevas M. *Clostrıdium botulinum* in honey production with respect to infant botulism. Academic Dissertation, Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland, 2006. Available at https://helda.helsinki.fi/bitstream/handle/10138/18964/clostrid.pdf?sequence=2.
13. Nevas M, Lindström M, Hörman A, Keto-Timonen R, Korkeala H. Contamination routes of *Clostridium botulinum* in the honey production environment. Environ Microbiol 2006; 8: 1085–1094.
14. DIN. Detection of *Clostridium botulinum* and Botulinum Toxin. DIN-10102. Berlin: Deutsches Insitut für Normung, 1988.
15. Dolan K. Ethics Animals and Science. Oxford: Blackwell Science, 1999.
16. Nab J, Balls M, Hendriksen CFM. Alternatives to animal experimentation. In: Van Zutphen LFM, Baumans V, Beynen AC, editors. Principles of Laboratory Animal Science. Amsterdam: Elsevier Science Ltd.; 1993. pp. 319–333.
17. Singer P. Animal Liberation. London: Paladin Granada Publishing, 1978.
18. Zutphen LFM, Kruijt BC, Öbrink KJ. Introduction. In: Van Zutphen LFM, Baumans V, Beynen AC, editors. Principles of Laboratory Animal Science. Amsterdam: Elsevier Science Ltd.; 1993. pp. 1–8.
19. Ministry of Agriculture and Rural Affairs. Turkish Food Codex, Communique on Honey. Publication date: 17.12.2005. Publication no: 26026. Ankara: Ministry of Agriculture and Rural Affairs.
20. De Medici D, Anniballi F, Wyatt GM, Lindstrom M, Messelhaußer U, Aldus CF, Delibato E, Korkeala H, Peck MW, Fenicia L. Multiplex PCR for detection of botulinum neurotoxin-producing clostridia in clinical, food, and environmental samples. App Environ Microbiol 2009; 75: 6457–6461.
21. Nakano H, Okabe T, Hashimoto H, Sakaguchi G. Incidence of *Clostridium botulinum* in honey of various origins. Jpn J Med Sci Biol 1990; 43: 183–195.
22. Delmas C, Vidon DJM, Sebald M. Survey of honey for *Clostridium botulinum* spores in eastern France. Food Microbiol 1994; 11: 515–518.
23. Flemming R, Stojanowic V. Untersuchungen von Bienhonig auf *Clostridium botulinum*-Sporen. Arch Lebensmittelhyg 1980; 31: 179–180 (in German).
24. Hartgen H. Untersuchungen von Honigproben auf Botulinustoxin. Arch Lebensmittelhyg 1980; 31: 177–178 (in German).
25. Hetland A. *Clostridium botulinum* sporer I norskprodusert honning? Norsk Veterinærtidskrift 1986; 98: 725–727 (in Norwegian).
26. Centorbi OP, Alcaraz LE, and Centorbi HJ. Análisis bacteriológico e investigación de esporas de *Clostridium botulinum* en mieles. Rev Argen Microbiol 1994; 26: 96–100 (in Spanish).
27. Monetto AM, Francavilla A, Rondini A, Manca L, Siravegna M, Fernandez R. A study of botulinum spores in honey. Anaerobe 1999; 5: 185–186.
28. Huhtanen CN, Knox D, Shimanuki H. Incidence and origin of *Clostridium botulinum* spores in honey. J Food Protect 1981; 44: 812–814.
29. Kautter DA, Lilly T Jr, Solomon HM, Lynt RK. *Clostridium botulinum* spores in infant foods: a survey. J Food Protect 1982; 45: 1028–1029.
30. Sugiyama H, Mills DC, Kuo LJC. Number of *Clostridium botulinum* spores in honey. J Food Protect 1978; 41: 848–850.