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Study of the presence of the spores of *Clostridium botulinum* in honey in Brazil

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Abstract

The isolation of *Clostridium botulinum* from honey samples is described. Botulism is characterized as an intoxication provoked by ingestion of contaminated foods with this toxin. Infant botulism happens by the ingestion of spores of *C. botulinum* together with food that in special conditions of the intestinal tract, such as those present in babies of less than 1 year old, will allow the germination and colonization of the intestine with production and absorption of botulinic toxin. The samples were subjected to dilution and to a thermal shock and cultivated in modified CMM (Difco). Cultures were subjected to Gram smears and toxicity tests in mice. The toxic cultures were purified in RFCA (Oxoid) plates and incubated in anaerobic jars. Positive samples were typed using the mouse assay neutralization test. From the 85 honey samples analyzed, six were positive for *C. botulinum* (7.06%), and identified as producers of type A, B, and D toxins.

Keywords: *Clostridium botulinum*; Infant botulism; Honey; Toxin

1. Introduction

Infant botulism has been under investigation in the USA since it was identified in late 1976, as a distinct clinical entity that results from intestinal colonization and toxin production by *Clostridium botulinum* spores in babies under 1 year old [1,2]. Later it was also identified in others countries such as England, France, Canada, Japan and Argentina [3–8].

The origin of these spores is largely unknown in infant botulism, but honey has been identified as a possible source of contamination [9]. Honey, which was the only food item associated with cases of infant botulism found to contain *C. botulinum* spores, has been examined extensively in the USA and spores were found in the product [10,11]. More than 600 cases of infant botulism have been notified in the USA since the identification of the disease in 1976 [12]. Ten categories of infant food, including dry cereals, non-fat milk, pasteurized whole cow's milk, canned fruits and fruit juices, granulated cane sugar, fresh carrots, honey, and corn syrup, in a total of 910 samples were analyzed for *C. botulinum* spores and all products were negative, except two of 100

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samples of honey and eight of 40 corn syrup samples containing viable *C. botulinum* spores [13].

The purpose of this report was to describe the presence of *C. botulinum* in honey from Brazil and to alert pediatricians.

2. Materials and methods

Honey samples were randomly obtained from different sources: grocery stores, small apiaries, and commercial honey processing plants, in different states (Mato Grosso do Sul, MS; Rio Grande do Norte, RN; Rio Grande do Sul, RS; e São Paulo, SP) of Brazil.

All samples of honey were warmed in a 37°C water bath for homogenization, before the removal of an aliquot of 10 g which was diluted in 25 ml of sterile saline solution and sown in bottles with 150 ml of sterile Roberts medium (CMM, Difco, supplemented with 1% ammonium sulfate, yeast extract and three ground white hard-boiled eggs for each 500 ml of medium) and subjected to a thermal shock, of 80–82°C during 10 min and rapidly cooled at 30°C in water with ice [5] and then incubated at 37°C for 5–10 days. On the 5th and 10th days of incubation they were checked for evidence of growth, gas and toxin production. Bottles showing turbidity were subjected to Gram smears and tested for the presence of toxin in mice. Samples were centrifuged at $12\,000 \times g$ for 0.5 h in a 5°C refrigerated centrifuge [14]. The supernatant was removed carefully and divided into two, one portion was trypsinized (0.1% trypsin (Difco) 1:250 adjusted to pH 6.1 and incubated at 35°C for 1 h), the other untrypsinized and used to assay for botulinal toxin with the mouse neutralization test following the techniques described in [15,16].

Isolation of the organism was also attempted. A loopful from the positive bottles was streaked onto reinforced Clostridium agar (Oxoid) plates which were incubated at 37°C for 48 h in a Brewer GasPak jar with the BBL system.

Isolated colonies were picked, subjected to smears, stained by the Gram method and inoculated into (16×125 mm) screw-capped tubes with 10 ml of cooked meat enrichment broth, and incubated at 37°C for 24–48 h for the execution of the biochemical characterization using the API 20 system (bioMérieux) and to confirm toxin production.

3. Results and discussion

Eighty-five samples of honey commercially available in Brazil were analyzed for the presence of *C. botulinum* spores. From the analyzed samples, 23 (27.06%) presented growth with turbidity and gas production. Cultures were confirmed through smears as Gram-positive sporulate rods, the other 62 (72.94%) samples presented other types of bacteria, principally cocci.

The supernatant of the samples suspected to be *C. botulinum*, when tested for the presence of toxins, using the mice assay, demonstrated six positives, causing paralysis and death of mice in approximately 3 days (Table 1).

The fact that samples were positive only after 10

Table 1
C. botulinum spores found in samples of honey with presence of Gram-positive spore-forming rods, commercially available in Brazil

Samples analyzed	Incubation at 37°C		Toxin type
	5 days	10 days	
1	−	−	
2	−	−	
3	−	+	A
4	−	−	
5	−	−	
6	−	−	
7	−	−	
8	−	−	
9	−	+	A
10	−	−	
11	−	+	B
12	−	−	
13	−	+	D
14	−	−	
15	−	−	
16	−	−	
17	−	−	
18	−	+	D
19	−	−	
20	−	−	
21	+	+	D
22	−	−	
23	−	−	

+: mice died (3 days after inoculation); −: mice survived.

Table 2
Typing of toxins produced by cultures isolated from honey samples, using the neutralization test with specific *C. botulinum* antitoxin

Sample	Inoculation in mice						
	Boiled 100°C	'In natura'	Serum control	Neutraliz. c ant. A	Neutraliz. c ant. B	Neutraliz. c ant. C	Neutraliz. c ant. D
3	−	+	−	−	+	+	+
9	−	+	−	−	+	+	+
11	−	+	−	+	−	+	+
13	−	+	−	+	+	+	−
18	−	+	−	+	+	+	−
21	−	+	−	+	+	+	−

+: caused death of mice; −: mice survived.

days of incubation may be explained by the presence of a small number of spores in the samples, producing a low concentration of toxin that only affected the mice after this period of incubation. Similar results were cited by Sugiyama et al. [17], in an experiment with 4 days of incubation.

The typing of the toxins was done using specific *C. botulinum* antitoxins from the Institut Pasteur, Paris. Two samples produced type A toxin, considered one of the most dangerous to man. Analyzing 100 honey samples in the USA, Kautter et al. [13] also isolated this type of toxin. The other positive samples were one type B and three type D toxins (Table 2).

3.1. Isolation of the organism

From the six bottles that presented *C. botulinum* toxin, only in four was it possible to isolate pure colonies with characteristics of *C. botulinum* as described by Smith [18]. They were two type A, one type B and one type D samples, in which toxin production was confirmed.

This study confirms that *C. botulinum* is present in some retail honey samples in Brazil and that the situation is similar to other countries as reported before [5,10,13].

More studies are needed to determine not only the prevalence of *C. botulinum* in this kind of food but also how *C. botulinum* becomes incorporated into honey.

Because infant botulism is considered an infectious disease that results from in vivo toxin production by *C. botulinum*, it is a risk to feed babies under 1 year old with honey and pediatricians need to be alert to this.

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References

[1] Arnon, S.S., Midura, T.F., Clay, S.A., Wood, R.M. and Chin, J. (1977) Infant botulism: epidemiological, clinical and laboratory aspects. J. Am. Med. Assoc. 237, 1946–1951.
[2] Pickett, J., Berg, B., Chaplin, E. and Brunstetter-Shafer, M. (1976) Syndrome of botulism in infancy: clinical and electrophysiologic study. New Engl. J. Med. 295, 770–772.
[3] Turner, H.D., Brett, E.M., Gilbert, R.J., Gosh, A.C. and Liebeschuetz, H.J. (1978) Infant botulism in England. Lancet i, 1277–1278.
[4] Paty, E., Valdes, L., Harpey, J.P., Dore, F., Hubert, P., Roy, C. and Caille, B. (1987) Un cas de botulisme chez un nourrisson de 11 mois. Arch. Fr. Pediatr. 44, 129–130.
[5] Delmas, C., Vidon, D.J.M. and Sebald, M. (1994) Survey of honey for *Clostridium botulinum* spores in eastern France. Food Microbiol. 11, 515–518.
[6] Hauschild, A.H.W., Hilsheimer, R., Weiss, K.F. and Burke, R.B. (1988) *Clostridium botulinum* in honey, syrups and dry infants cereals. J. Food Prot. 51, 892–894.
[7] Sakaguchi, G., Sakaguchi, S., Kamata, Y., Tabita, K., Asao, T. and Kozaki, S. (1990) Distinct characters of *Clostridium botulinum* type A strain and their toxin associated with infant botulism in Japan. Int. J. Food Microbiol. 11, 231–242.
[8] Centorbi, deO.P., Acaraz, L.E. and Centorbi, H.J. (1994) Análisis bacteriológico e investigación de esporas de *Clostridium botulinum* en mieles. Rev. Argent. Microbiol. 26, 96–100.
[9] Arnon, S.S., Damus, K. and Chin, J. (1981) Infant botulism: epidemiology and relation to sudden infant death syndrome. Epidemiol. Rev. 3, 45–66.
[10] Huhtanen, C.N., Knox, D. and Shimanuki, H. (1981) Incidence and origin of *Clostridium botulinum* spores in honey. J. Food Prot. 44, 812–814.

[11] Sugiyama, H. (1979) Animal models for the study of infant botulism. Rev. Infect. Dis. 4, 683–687.
[12] Spika, J., Shaffer, N., Hargrett-Bean, N., Collins, S., MacDonald, K. and Blake, P. (1989) Risk factors for infant botulism in the United States. Am. J. Dis. Child. 143, 828–832.
[13] Kautter, D.A., Lilly, T.J., Solomon, H.M. and Lynt, R.K. (1982) *Clostridium botulinum* spores in infant foods: A survey. J. Food Prot. 45, 1028–1029.
[14] Midura, T.F., Snowden, S., Wood, R.M. and Arnon, S.S. (1979) Isolation of *Clostridium botulinum* from honey. J. Clin. Microbiol. 9, 282–283.
[15] Dowell, V.R. and Hawkins, T.M. (1974) Laboratory Methods in Anaerobic Bacteriology, Publication No. 748272. Center for Disease Control, Atlanta, GA.
[16] Midura, T.F. and Arnon, S.S. (1976) Infant botulism: identification of *Clostridium botulinum* and its toxins in faeces. Lancet ii, 934–936.
[17] Sugiyama, H., Mills, D.C. and Kuo, L.J.C. (1978) Number of *Clostridium botulinum* spores in honey. J. Food Prot. 41, 848–850.
[18] Smith, L.S. (1977) Botulism, the Organism, its Toxins, the Disease. Charles C. Thomas, Springfield, IL.