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Incidence and Origin of *Clostridium botulinum* Spores in Honey

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ABSTRACT

Eighty honey samples, including some from foreign countries, were obtained from a local processor or from apiaries in Pennsylvania, Illinois and New Jersey. They were analyzed for *Clostridium botulinum* spores by a dilution-centrifugation (DC) procedure and by direct addition (DA) of honey to two different enrichment media. All were negative by the DC method; five were positive by DA in fluid thioglycollate media and six by DA in cooked meat media. Some samples positive in fluid thioglycollate media were negative in cooked meat media and vice versa.

Bees (*Apis mellifera*, 25,000 per hive) were experimentally inoculated with spores of *C. botulinum* by feeding a 50% sugar-water solution containing 1.6×10^5 spores of 20 strains (equal numbers of 11 type A and 9 type B). Honey collected from the hive 2 weeks later contained 1100 spores per g; that collected after 5 weeks contained 50 spores per g. Quantitative estimates of honey yield and spore contents indicated that all the spores originally ingested by the bees had been incorporated into the honey. No botulinal spores were found in the intestinal or rectal contents of the bees 2 weeks or more after spore ingestion.

Botulism is usually caused by ingesting foods in which the potent botulinum neurotoxin has been produced. A different type of toxico-infectious botulism has been recognized recently in infants under 1 year of age (*1,9*). In this type of botulism spores of *Clostridium botulinum* apparently germinate and produce toxin in the intestinal tracts of affected infants (*7*). Epidemiological investigation of food and environmental samples associated with cases of infant botulism (*1*) indicated that honey was a possible source of the disease, although 65% of the cases of infant botulism had no history of honey consumption. They found *C. botulinum* spores in several samples of honey obtained from affected households; the *C. botulinum* type in each case was the same as that isolated from the infant.

The incidence of *C. botulinum* spores in honey has been studied by several workers. Sugiyama et al. (*10*), using a dialysis method, reported that 18 of 241 honey samples contained the spores, while Midura et al. (*8*) using a dilution-centrifugation method reported that 9 of 90 honey samples were positive.

The work reported here was undertaken to determine the possible origin of *C. botulinum* in honey and to evaluate the efficacy of several methods for assaying the spores.

MATERIALS AND METHODS

Honey samples

Several hundred-gram samples of honey were obtained from bulk domestic and imported stocks which are blended for retail sale by a local processor. Honey samples from a number of small producers were also obtained through the courtesy of the Pennsylvania, Illinois and New Jersey Departments of Agriculture.

Experimental hives

Four hives were set up in a remote area of the USDA, Beltsville, MD facility. An *Apis mellifera* queen and workers (25,000) were placed in each hive and the bees were fed a 50% sucrose solution in a plastic gravity-feed bottle with several 1/16-in. holes in the cap. The bottle was placed over the frames and the hives were sealed by replacing the original tops with an inverted empty hive body. The original tops were replaced after the bees consumed the sugar solutions (24 h) thus allowing the bees to resume normal forage.

The sugar solutions (500 ml per hive) were of four kinds: (a) control, (b) inoculated with 1.6×10^5 spores of *C. botulinum* (20 strains consisting of 11 type A and 9 type B), (c) inoculated with 1×10^5 spores of *Bacillus larvae*, and (d) inoculated with spores of both organisms.

Two and 5 weeks after the bees had consumed all the sugar solutions, part of the honey that had been produced was removed from the hives and examined for spores. An estimate was made of the total honey in the hives.

Assay for C. botulinum *spores*

Media used for *C. botulinum* spore assays were the cooked meat medium (CM) of Dowell and Hawkins (*3*) and commercial (Difco) fluid thioglycollate (FT) medium. Two methods of assay were used. The first

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was a dilution-centrifugation (DC) method similar to that of Midura et al. (*8*) in which 10 g of honey were diluted with 30 ml of distilled water and centrifuged at 10,000 RCF at ambient temperature for 30 min. The pellet was transferred to 7 ml of either CM or FT medium in 16 × 125-mm tubes. The second was a direct addition method (DA) in which 10 g of honey were added directly to 90 ml CM or FT media and mixed by swirling. In both the DC and DA methods the inoculated media were held at 68 C for 30 min to kill non-sporeformers. The media were usually overlaid with vaspar before the heating step except when incubation was in an anaerobic incubator (National Appliance Company). When used this incubator was filled with N_2 after evacuation to 25 in. vacuum. Incubation was at 30 C for 1 week.

Presence of *C. botulinum* in the honey samples was determined by identifying the presence of toxin in the supernatant fluids of centrifuged enrichment cultures. Two 15-20 g mice were inoculated i.p. with 0.5 ml of supernatant fluid. Deaths of mice with typical symptoms of botulism within 3 days indicated positive samples. The toxin type was determined by monitoring the survival of mice after challenge with culture supernatant fluids that were treated with antisera (1I.U./mouse) and held for 1 h at room temperature before injection. Three-tube most probable number (MPN) determinations were made in CM medium. For this test three milk dilution bottles, containing 90 ml medium, were inoculated with 10 g portions of honey. After thorough mixing, two successive 10-fold dilutions were made in 16 × 125-mm culture tubes containing the same medium. The tubes and bottles were overlaid with vaspar, heated and incubated as previously described. The supernatant fluids from centrifuged cultures were tested for toxin.

Microbiological analysis of bees and other samples

Tests were made for spores in intact bees, bee intestinal and rectal contents, and environmental samples in or near the hives. The latter included frass (debris removed from the hives by the bees), bottom board debris and wax. These were assayed by the direct addition method.

Bees were examined by blending (30 sec in a Waring blender) 50 of them in 150 ml FT medium. The blender jar was flushed with N_2 for 10 min before and also during blending. After blending, the samples were analyzed by the direct addition method.

A loopful of the incubated culture was streaked onto Brewer anaerobic agar (Difco) plates. The agar was overlaid with 20 ml of the same medium and the plates were placed in anaerobic jars ("Torbal", Torsion Balance Co.). The jars were evacuated to 25 in. vacuum and the vacuum was replaced with a mixture of 4% H_2:10% CO_2:86% N_2. The procedure was repeated. Incubation was 2 days at 32 C. Isolated colonies were picked into Brewer anaerobic agar shakes (16 × 125-mm tubes with 10 ml agar) which were incubated at 32 C until growth was evident. Cultures showing anaerobiosis (deep growth with clear surface zone) were tested for biochemical attributes and for toxin production by standard techniques. Biochemical characterization of the cultures used media supplemented with 0.05% sodium thioglycollate and were incubated in the anaerobic incubator.

Intestinal or rectal contents were examined by dissecting the appropriate section of the bees and culturing it in tubes of FT medium. Incubation was in the anaerobic incubator. Isolation and culture identification methods were as described above.

RESULTS AND DISCUSSION

Table 1 shows the results of testing the bulk honey samples for *C. botulinum* spores. When assayed by the DC method, all 10 g samples were negative. The DA method in FT media gave positive results in 3 of twenty 10-g samples (17 of these were honey samples, 3 were comb or filter material). Of these three, one was type B (Australian eucalyptus honey); one was type A (the Albany, Canada sample). Sample number 7 cultured in

TABLE 1. *Comparison of two methods for determining* Clostridium botulinum *spores in honey.*

Sample No.	Source	*C. botulinum* spores recovered by direct addition to FT[a] medium	*C. botulinum* spores recovered by direct addition to CM[b] medium
1	North Dakota	-	ND[c]
2	Retail mixture (origin unknown)	-	ND
3	Mexico	-	0/3
4	Australia	+ (B)[d]	0/3
5	Mixture from Republic of China, Florida, Mexico, Brazil	-	ND
6	Colorado	-	ND
7	Mixture from South Dakota, Mexico, Guatemala	+ (negative on repeat)	2/3 (both A)
8	South Dakota	-	1/3 (A)
9	Montana	-	ND
10	Columbia, S. America	-	ND
11	Ontario, Canada	-	ND
12	Tennessee	-	ND
13	Albany, Canada	+ (A)	0/3
14	Guatemala	-	0/3
15	Filter residue	-	ND
16	Wax	-	ND
17	Republic of China	-	1/3 (B)
18	Canada	-	ND
19	Cappings	-	ND
20	Canada (half soybean)	-	ND

[a]FT=fluid thioglycollate (assayed single 10-g samples).
[b]CM=cooked meat (assayed triplicate 10-g portions).
[c]ND=not determined.
[d]Letter in parentheses indicates toxin type.

the FT medium gave a very weak toxin test when first assayed and was negative when the same extract was re-injected into mice a month later. When samples were reassayed in CM medium, three were positive; however, only one was positive in both the CM and FT media. Midura et al. (*8*) also found some samples positive by one method and negative by another; a reflection probably of the low incidence and/or uneven distribution of spores in the honey. The filter material used in clarifying the honey before bottling was negative.

Forty-two samples of honey from apiaries throughout the state of Pennsylvania were assayed; of these, only one was positive when assayed in the FT medium. The amount of toxin produced in this medium was very low; only 1 of 2 injected test mice died. Both mice survived when injected with the extract from another aliquot. All were negative by the DC method. Assay by the DA method using the CM medium yielded three positive samples; the one originally weakly positive was negative by this technique.

Fifteen samples from apiaries in Illinois were negative by the DC method in the FT medium; one was positive by the DA method in the same medium; a second sample obtained from this apiary was negative. Three samples from New Jersey were negative by both methods.

Of the methods used for assaying the spores, the DC method seemed to be the least reliable, although when irradiation-sterilized honey (obtained through the courtesy of Dr. E. Wierbicki, U.S. Army Natick Research and Development Command, Natick, Mass.) was inoculated with as few as one spore per g, it was positive by the DC as well as the DA methods. We found that some supernatant fluids from the DC method (using the inoculated irradiated honey) were also positive, indicating that some spores did not sediment during centrifugation.

Another part of our studies was to determine the possibility that bees were acting as carriers of the spores accidentally picked up from environmental sources. This was the Beltsville experiment consisting of the four hives described above. Two weeks after the bees had ingested the spores in the sugar-water solution and were released to forage again, honey samples from both hives inoculated with *C. botulinum* were positive. We did not assay for *B. larvae* spores; these were added to determine whether a foulbrood infested bee colony would be more susceptible to infection by *C. botulinum*. Both hives, however, originally had the same number of botulinum spores in the honey. At 2 weeks the MPN was 1100 spores/g and, at 5 weeks, it was 50/g.

An accurate determination of honey production was not possible but at 2 weeks we estimated that 0.5 to 2.5 kg of honey had been produced in each hive. Based on these figures, at 2 weeks the number of recovered spores was greater than the original number introduced (1.6×10^5), but was within the 95% confidence interval of the MPN method (the theoretical spore concentrations based on this confidence level could have ranged from 7.5×10^4 to 2.4×10^6). At 5 weeks, the estimate of honey produced was 12 kg, giving a total spore population, based on 50 spores/g, of 6.0×10^6. This was also within the MPN confidence limits. At 14 weeks, the honey samples were still positive; however, a quantitative estimate of spores was not made.

Clostridium botulinum spores were not found in frass (debris from the hives) or in the intact bees from the 4 hives. Intestinal and rectal contents were also negative. The possible presence of factors inhibiting *C. botulinum* spore germination or vegetative growth in the intestinal tract of the bees was investigated by preparing homogenates of the bees and inoculating them with spores. All cultures were toxic when assayed by the CM method indicating the absence of an inhibitor.

Cultures of intestinal and rectal contents of the bees recovered primarily facultative aerobic organisms but some strict anaerobes were also recovered. Some of these were purified and subjected to biochemical tests. The organisms were very similar to *C. sporogenes* in being motile and producing gas (including H_2S) but no toxin. Glucose, lactose, mannitol, arabinose, maltose, mannose, esculin, xylose and sorbitol were fermented, but dulcitol, melibiose, inulin, dextrin, starch and raffinose were not. Tests for indole, acetyl-methyl-carbinol, citrate, nitrate and urease were negative. Gram stains showed typical gram-positive rods, often in pairs with terminal spores swelling the sporangium.

Although aerobic species of bacteria have been reported in the bee intestinal tract (*4-6*), no reports of obligately anaerobic bactera in bees have appeared. However, Bengston (*2*) found *C. botulinum* type C in larvae of *Lucilia caesar*. The significance of these anaerobic bacteria in the bee is unclear; they may be merely transitory contaminants picked up during foraging. Nonetheless, their presence indicates that *C. botulinum* spores might gain entry into the intestinal tract in the same manner with subsequent deposition into honey. Our failure to find *C. botulinum* spores in the intestinal tract 2 weeks after the inoculated sugar-syrup feeding suggests that the bees do not retain the ingested organisms very long. Future work will help to determine whether *C. botulinum* spores enter honey as transitory contaminants carried by bees.

A comparison of the two procedures for assaying honey for *C. botulinum* spores suggests that neither of the methods is completely satisfactory, although the dilution-centrifugation method appears to be less satisfactory than the direct addition technique. More work is required to establish better assay methods for the spores in honey.

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availability of free histidine in the muscle to act both as an inducer and substrate makes scombroid fish muscle an ideal environment for histamine formation. As shown with tuna muscle, *P. morganii*, if given the opportunity to grow, can rapidly synthesize histidine decarboxylase and cause histamine accumulation to levels that can be toxic. Although the literature does not give a completely clear understanding of the frequency of occurrence of *P. morganii* on marine fish, its implication in several outbreaks of scombroid food poisoning suggests that it is a fairly common contaminant.

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