Number of *Clostridium botulinum* Spores in Honey

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### ABSTRACT

A dialysis-enrichment culture method for detecting *Clostridium botulinum* spores in honey is described. The method was used to survey 55 honey samples representative of 53 lots being sold at retail and 186 honey collections from 154 individual producers. Based on finding the organism in at least one of three 25-g test portions of a sample, one type A and one type B positive were found among the retail samples. Type A spores were found in collections of five different producers and type B in those of five others. Five of 13 different lots from one producer were positive for type A spores. One producer sample had both types A and B spores. Maximum most probable number by the five-tube method was seven botulinum spores (upper 95% confidence limit of 17) per 25 g of sample.

At least 58 (33 type A and 25 type B) cases of human infant botulism have been identified in the United States since the disease was recognized in 1976 as a distinct entity (4). One type A case has been found in England (11). The illness apparently starts when *Clostridium botulinum*, most likely the spore form, is swallowed and initiates an intraintestinal infection with accompanying toxin production (2,8). This etiology differs from that of the classical botulism food poisoning in which the toxin is already formed in the peccant food. Some of the infant crib deaths may be a form of infant botulism (3) having rapid onset and sudden death.

Botulinum spores are naturally present in soils (9) so that they may be in foods that are exposed to the environment. Infants might acquire the spores with any such foods but of those known to have been fed to the victims, the spores have been found only in honey (4,5). Although not proven, the limited data suggest a possible correlation between type B infant botulism cases and the feeding of honey (5). An association of honey producers has considered the possible health implications and with the public interest in mind has issued a press release about the presently known facts (6).

We report here on a study done to determine the incidence of *C. botulinum* types A and B spores in honey samples and to gain some idea of their concentrations in positive samples.

### MATERIALS AND METHODS

Honey samples from a leading cooperative of honey producers were representative of crude honey lots collected in 32 states of the U.S. and of honey being sold by the cooperative in retail stores. Included were 55 retail samples of 53 different lots and 186 samples representing honey collections from 154 individual producers. Specimens were in jars containing 0.5 to 2.0 lb (227 to 908 g).

Testing was based on the principle that botulinum toxin is produced in enrichment cultures that are made of samples containing *C. botulinum* (7). Because of indications that low concentrations of the organism might be encountered, 25-g test portions were tried. Growth from an experimental botulinum spore inoculum was significantly suppressed when this amount of honey was cultured in 300 ml of culture medium. Since the inhibition was probably due to the high sugar concentration resulting from the sample, honey was dialyzed before being cultured.

Honey in the shipping jar was warmed to 45 C and then mixed to distribute organisms through the sample. Full jars were inverted about 50 times; vigorous shaking was used when there was sufficient headspace. A 25-g portion was then weighed into a sterile beaker and diluted with 20 ml of sterile distilled water to permit easier handling. The thinned honey was transferred aseptically, along with a 5-ml water rinse of the beaker, into a 1.75-inch (44 mm) flat wide dialysis sac that had been tied off at one end before being sterilized (120 C, 20 min) in water contained in a beaker. All manipulations were done with precautions to avoid contaminating the honey; e.g., beakers were sterilized with covers of aluminum foil and were uncovered only when necessary.

The open end of the filled sac was closed by ties to leave a working length of about 45 cm. Up to 15 such filled sacs were suspended in a 15-liter capacity container with the sample columns submerged in unsterilized distilled water. Dialysis was done in a 4-C cold room with water cooled to 10 C or lower. During the approximate 24-h dialysis, water changes were made at 2, 2, and 15-h intervals. Sample volumes after dialysis were approximately 140 ml.

Enrichment cultures were made in 300-ml capacity, screw-capped prescription bottles in which about 5 g of cooked meat particles (Difco Lab., Detroit, Mich.) had been moistened with a small amount of water and sterilized. The area around one end of the sac was sanitized by swabbing with 5% hypochlorite solution and the sac cut so that the content could be poured into such a bottle. Fluid level was brought to the 150-ml mark with sterile water. The inside of the sac was rinsed twice with 60-ml volumes of double strength TPGY (trypsin omitted from TPGYT of ref. 7) and the rinsings added to the bottle. Final volume was made to 300 ml with the medium. TPGY was adjusted to pH 7.2 before sterilization and was at 80 C when used.

Bottles were immediately placed in an 80-C water bath and held for
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25 min to select for heat resistant spores such as those of *C. botulinum*. After incubating for 4 days at 37 C, portions of cultures were clarified by centrifugation and tested by injecting 0.5 ml intraperitoneally into a mouse. Those that killed mice within 4 days were retested in mice unprotected, protected with type A and protected with type B antitoxin (7). Deaths from nonbotulinal agents were not a problem.

Routine examination of a honey specimen consisted of three separate tests done at different times with a 25-g portion. A positive sample was one in which *C. botulinum* was identified in at least one of these tests. When a positive result was obtained, other 25-g units were tested so that a total of five such amounts would be examined. Additionally, enrichment cultures were prepared with five portions of 2.5 g and five of 0.25 g by adding honey, without dialysis, to separate tubes containing 35 and 20 ml, respectively, of cooked meat medium (7). The botulinum toxicity results were used as a 5-tube most probable number (MPN) test (1) to determine the number of *C. botulinum* in 25 g of honey.

RESULTS

Sensitivity of the dialysis-enrichment procedure for detecting samples containing botulinum spores was tested with a type A spore suspension whose viable count was known (10). Ten honey samples, in which the botulinum organism had not been found, were chosen at random and an estimated one spore was added to each of a 25-g honey portion being readied for dialysis. Six of the 10 resulting enrichment cultures contained the expected type of botulinum toxin. Of 10 tubes of cooked meat medium, each inoculated with the same spore suspension volume added to the honey samples, seven developed toxin. The number of positive tests in the two culture sets indicated the culturing method would identify honey containing small numbers of *C. botulinum* spores.

Possibility of contamination was examined. Periodically during the survey, a 25-ml volume of sterilized water was dialyzed and cultured as if it was a honey sample. None of 25 such controls showed growth.

The results are not reported specifically, but all samples were tested at least once, and most two or more times, with modified procedures. The common procedural variations were (a) dialyzing at room temperature, (b) heating at 60 C for 15 min, or omitting heating, preparatory to incubation for toxin production, (c) incubating culture at 30 C, and (d) collecting the precipitate formed during dialysis on a membrane filter (Millipore) with pores of 0.45-μm diameter and cultivating membrane and precipitate together in cooked meat medium. None of these methods seemed superior to that used as the routine test procedure; when a positive result was obtained with a modification, the sample was positive by the routine method.

Of the 241 samples tested, 18 had *C. botulinum* spores (Table 1). The preponderance of type A over B was due to five and two type A positives among the 13 samples from each of two different producers. When these two positive groups are considered as representing only two producing areas, types A and B were found with similar frequencies. Both types were present in a producer sample. Positive samples originated in the states of California, Florida, Iowa, Michigan, Minnesota, Nebraska, Tennessee, Texas and Washington.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. tested</th>
<th>No. specimens</th>
<th>Type</th>
<th>MPN/25 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retail</td>
<td>55</td>
<td>1</td>
<td>A</td>
<td>7 (1-17)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>B</td>
<td>2</td>
<td>(0.5-7)</td>
</tr>
<tr>
<td>Producer</td>
<td>186</td>
<td>2</td>
<td>A</td>
<td>7 (1-17)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>A</td>
<td>5</td>
<td>(0.5-13)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>A</td>
<td>4</td>
<td>(&lt;0.5-11)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>A</td>
<td>2</td>
<td>(0.5-7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>B</td>
<td>5</td>
<td>(0.5-13)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>B</td>
<td>4</td>
<td>(&lt;0.5-11)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>B</td>
<td>2</td>
<td>(0.5-7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>A and B</td>
<td>Each</td>
<td>2 (0.5-7)</td>
</tr>
</tbody>
</table>

Each tested with three 25-g portions.
Most probable number by 5-tube method; ( ) = 95% confidence limits.
Includes three of same lot.
Includes three others. Nevertheless, two other jars of the same retail lot did not have botulinum spores when five 25-g portions of each were examined. Similarly, the botulinum organism was not detected in two other sampling units (each tested with five 25-g portions) of a producer lot whose first sample unit had four type B spores per 25 g.

DISCUSSION

This study confirms the report that *C. botulinum* is present in some retail honey samples (2,5) and suggests that positive samples are likely to be found among honey produced in most, if not all, states of the U.S. when sufficient numbers of different lots are examined. Because of the toxico-infection basis of infant botulism, a theoretical hazard exists in feeding honey to infants (5) of up to 26 weeks old when the illness is known to occur (4). However, actual danger has not been established. The assessment depends on two important factors: the number of botulinum spores in honey and, as yet totally unknown, the number of spores required to infect an infant.

An upper limit of 17 botulinum spores per 25 g of honey was enumerated by the MPN tests. The number of botulinum spores necessary to intraintestinally infect 50% of an infant mouse group was, per animal, 700 (95% confidence range of 170 to 3,000) spores of one type A strain (10) and 170 (80 to 360 range) spores of a different type A culture (unpublished data). The dose response curves were such that in the infant mouse population with normal distribution of susceptibilities to infection, a few
individuals were infected with a dose of 10 to 20 spores. The validity of extrapolating the mouse data to humans can be questioned but the available information does not as yet rule out the possibility of human infants having comparable susceptibility. By this assumption, some human infants could be infected by the number of botulinum spores present in one, or several closely spaced, feeding(s) of some of the honey samples encountered in the present survey. A similar potential problem would exist with other substances containing the spore load of honey.

The opposite viewpoint can be argued if the observed maximum botulinum spore concentrations holds for all lots. Honey would not be of danger provided that human infants have some natural resistance against intraintestinal colonization by *C. botulinum*. Older people probably swallow these spores occasionally without suffering ill effects (9,10).

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REFERENCES