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INCIDENCE OF *CLOSTRIDIUM BOTULINUM* IN HONEY OF VARIOUS ORIGINS

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SUMMARY: By the dilution-centrifugation method, 270 honey samples, both domestic and imported, were examined and *Clostridium botulinum* was detected in 23 samples (8.5%); type A in 11 samples, type B in two, type C in 10, and type F in one. Of 58 domestic honey samples, six (10%) were positive; three gave type A and the other two type C. Among imported honey samples, Chinese honey gave 12% positives (types A, B, and C) and Argentina honey 20% positives (types A and F). The incidence was higher with samples taken from drums (18%) and from apiaries (23%) than marketing honey (5%). It was estimated that most positive samples contained spores in one per gram or lower concentrations. One sample contained 4 type A spores per gram and another 36-60 type F spores per gram. No distinct biochemical properties were found with the honey isolates.

INTRODUCTION

Infant botulism was first reported in the United States in 1976 (1,2). Only infants aged between 3 weeks and 8 months develop the symptoms after ingestion of *Clostridium botulinum* spores which may germinate, proliferate and produce

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the toxin in the intestines (3,4). More than 650 cases were reported from 1976 to 1986 in the United States and also in other countries in North and South America, Europe, Australia and Asia (Arnon, S. S., personal communication). The causative organisms were principally of types A and B; *C. barati* producing type F botulinum toxin (5) was involved in a case and *C. butyricum* producing type E toxin (6) in two cases. The source of infection so far identified is honey. *C. botulinum* spores have been detected in honey at certain percentages (7-9), and a warning was issued in the United States not to feed honey to infants under 12 months of age.

In Japan, the first case of infant botulism associated with honey, was diagnosed in 1986 (10). The Ministry of Health and Welfare organized a study group to examine honey for contamination with *C. botulinum* (11). A total of 512 samples of domestic and imported honey were examined by the group and *C. botulinum* was detected in 27 samples (5.3%) (11).

The present communication describes the results of the surveys conducted during the period from 1986 to 1990 of 270 honey samples, including those collected by the Ministry of Health and Welfare in 1986 and examined by us. Attempts were made to correlate the incidence to the country of origin, the processing of honey or the storage period, and to characterize the isolates. The procedures we used for detection of *C. botulinum* in honey were evaluated by adding known numbers of *C. botulinum* spores to a honey sample.

MATERIALS AND METHODS

Honey samples: A total of 270 samples included 90 purchased at shops in Osaka in 1986 and 1987, 50 taken from drums soon after import, 13 taken directly from bee combs at apiaries in Osaka, and 117 purchased at shops in Hiroshima during the period from 1988 to 1990. These samples included 58 domestic, 177 imported, 10 mixtures of domestic and imported, and 25 of unknown origin. The imports were from China (76 samples), Argentina (15), Hungary (12), USA (10), Canada (5), USSR (4), Australia (3), New Zealand (3), France (2), Spain (2), Mexico (2), and UK (1). The nectar sources were acacia, clover, orange, Chinese milk vetch.

Procedures for detection of C. botulinum in honey: The dilution-centrifugation method of Midura et al. (8) was followed, with slight modifications. Honey samples were warmed to 37 C (solidified ones to 52 to 53 C), and a 20-g portion was taken from each and diluted with 100 to 150 ml of sterile distilled

water. The mixture was shaken and centrifuged at 8,000 to 10,000 $\times g$ for 30 min at 20 C. A small amount of distilled water was added to the precipitate to make a suspension of about 2 ml. Four tubes of 10 ml of cooked meat medium (Difco) supplemented with 0.1% glucose and 0.2% soluble starch were inoculated; two with 0.9 ml of the undiluted suspension and the other two with 1 ml of a 10-fold dilution. One set of tubes were heated at 80 C for 15 min, and the other untreated. All tubes were incubated in anaerobical jars at 30 C for 7 days. A 5-fold dilution of the centrifugal supernatant of each culture was injected intraperitoneally into a mouse, which was observed for 4 days. When the mouse developed symptoms and died, the toxin was typed with type-specific antitoxins (Chiba Serum Institute, Ichikawa, Chiba). For some samples, the suspension of the centrifugal precipitate of a 20-g sample was inoculated into only two tubes of cooked meat medium. For the 80 samples collected in 1988, distilled water with and without added 0.5% CaCO_3 was used to dilute the honey. Enrichment culture was repeated twice or three times with the 60 samples collected in 1986.

Viable counting of C. botulinum: When all four tubes gave positive results, viable counting of *C. botulinum* was attempted by three different procedures. As a direct plating method, 0.2 ml of a twofold dilution of the honey sample was plated on 5% egg yolk GAM agar (Nissui Pharmaceutical Co., Tokyo) supplemented with 0.1% cysteine and also on CBI agar plates when type A, B, or F toxin was detected. Colonies with pearly layers were counted. Besides, 10-fold serial dilutions of the twofold dilution were each inoculated in 1-ml amounts into three tubes of cooked meat medium, which were incubated and tested for the toxicity by mouse ip injection to calculate the most probable number (MPN) of *C. botulinum*. Furthermore, a diluted sample was filtered through HGMF (Hydrophobic grid membrane filter, QA Company., Toronto, Canada), which was cultured on the surface of a GAM agar plate with nitrocellulose membrane underneath the HGMF. Botulinum toxin blotted to the nitrocellulose membrane was detected by ELISA (13).

Isolation of C. botulinum: The toxic cultures in cooked meat medium were plated on 5% egg yolk GAM agar plates and, when proteolytic group I organisms (14) were suspected from the toxin type detected, on CBI agar plates. The plates were incubated anaerobically for 3 days at 30 C. Such colonies that were surrounded with pearly layers were picked up into tubes of cooked meat medium to test for the toxin production. When colony isolation from agar plates was difficult, the HGMF-ELISA method was applied prior to plating.

Biochemical tests of the isolates: The isolates were tested for some biochemical properties. Gram staining was performed by the modified method of Hucker; spore staining was made by the method of Schaeffer-Fulton on 7 day cultures in cooked meat medium. Aerobic culture, tests for lipase and lecithinase were made with 5% egg yolk GAM agar plates; hemolysis was tested on sheep blood agar (BBL) plates. Digestion of meat was tested in cooked meat medium by incubation at 30 C for 2 weeks. Carbohydrate fermentation was tested in GAM

semisolid base (Nissui Pharmaceutical Co.) with each carbohydrate added in 1%. In 2 and 5 days at 30 C, the BTB-MR indicator was added to compare the color with that of the carbohydrate-free culture. Motility was tested in the same medium. Growth was tested also on CBI agar plates containing cycloserine, sulfamethoxazole and trimethoprim. Nitrate reduction was tested in GAM semisolid medium containing 0.1% glucose and 0.3% KNO₃ in 2 days of incubation.

Evaluation of the procedure for detection of C. botulinum in honey: A spore suspension of *C. botulinum* type A strain 62 was so diluted that the dilutions would contain 100, 10, 5, 3, 1, and 0.5 spores/ml. Each dilution in 1.0- ml amounts was inoculated into a 10-g honey sample and three tubes of cooked meat medium. The inoculated honey was subjected to the above-described dilution-centrifugation method. In these tests, diluent with and without added 0.05% CaCO₃ was used. The experiment was repeated twice.

RESULTS

Detection of C. botulinum in Honey Samples

Of 270 honey samples, 23 (8.5%) were shown to contain *C. botulinum* (Table I). The toxin types found were type A in 11 samples, type B in two samples, type C in 10 samples, type F in one sample, and both types A and C in one sample. The incidences were 5% (11/ 207) in bottled samples from shops, 19% in 50 samples taken directly from drums, and 23% (3/13) in samples taken from apiaries. The incidence of *C. botulinum* in imported honey was 9.6% (17/177), while that in domestic honey 10.3% (6/58) (Table II). Among marketing honey samples, the incidence was 6.3% (8/127) in imported honey, while 6.7% (3/45) in domestic samples. As shown in Table II, incidences by country of origin were 9/76 (12%) of China origin, and 3/15 (20%) of Argentina origin. Samples from Hungary, Spain, and Mexico each gave one positive only. The toxin types found were types A, B and C in Chinese honey, types A and F in Argentina honey and type A in Japanese honey. With regard to bottled honey samples collected from shops, the incidences were 7/90 (7.8%) and 4/117 (3.4%) for samples collected in 1986-87 and 1988-90, respectively.

Table I. Honey samples tested for *C. botulinum* in 1986-1990

Sample source	Number of samples tested	Number of positive (%)	Toxin types ¹
Bottle	207	11(5.3)	A (5), C (5), F (1)
Drum	50	9(18.0)	A (3), B (2), C (3), A + C (1)
Apiary	13	3(23.1)	A (2), C (1)
Total	270	23(8.5)	A(10), B(2), C(9), F(1), A + C(1)

¹The figures in parentheses show the numbers of samples positive for *C. botulinum* of the type.

Table II. Country of origin of honey samples tested for *C. botulinum*

Country of origin	Number of samples	Number of positive (%)	Toxin type			
			A	B	C	D
China	76	9(11.8)	3	2	4	0
Japan	58	6(10.0)	3	0	3	0
Argentina	15	3(20.0)	2	0	0	1
Hungary	12	1(8.3)	1*	0	1*	0
Spain	2	1(50.0)	1	0	0	0
Mexico	2	1(50.0)	0	0	1	0
USA	10	0	0	0	0	0
Others	18	0	0	0	0	0
Unknown(foreign)	31	2(6.5)	1	0	1	0
Mixture(foreign)	11	0	0	0	0	0
Mixture(foreign + Japan)	10	0	0	0	0	0
Unknown	25	0	0	0	0	0
Total	270	23(8.5)	11	2	10	1

*One sample contained two types of *C. botulinum* spores.

Table III. Detection of *C. botulinum* in honey by method

No.	Country of origin	Nectar source	Sample source	Inoculum size/heat treatment ¹					Toxin type	Isolation
				20 g Yes	9 g Yes	9 g No	1 g Yes	1 g No		
1*	China	Acacia	Bottle	-	-	+			A	+
2	Foreign	NI	Bottle	+	-	-			C	+
3**	Foreign	NI	Bottle	-	+	+			A	+
4	Japan	Horse chestnut	Bottle	+	-	-			C	-
5	Japan	Chinese milk vetch	Bottle		-	+			C	-
6	China	NI	Bottle		-	+		-	A	+
7	Japan	Orange	Bottle		+	+	-	+	A	+
8	Japan	NI	Apiary		+	+	-	-	C	+
9	Japan	NI	Apiary		-	+	-	-	A	+
10	Japan	NI	Apiary		+	-	-	-	A	+
11	Hungary	Acacia	Drum		+(C)	+(A)	-	-	C+A	+, +
12	China	Acacia	Drum		+	-	-	-	B	+
13	Spain	Orange	Drum		+	-	-	-	A	+
14	China	White	Drum		-	+	-	-	C	-
15	Mexico	Orange	Drum		+	-	-	-	C	-
16	China	Acacia	Drum		+	+	+	-	C	-
17	China	NI	Drum		+	-	-	-	B	-
18	Argentina	Clover	Drum		-	-	-	+	A	+
19	China	Acacia	Drum		-	-	+	-	A	+
20	China	Acacia	Bottle#		-	+	-	-	C	-
21	China	Chinese milk vetch	Bottle#		-	+	-	-	C	-
22	Argentina	Clover	Bottle#		+	+	+	+	F2	+
23	Argentina	Clover	Bottle#		+	+	+	+	A3	+

¹Refer to the Materials and Methods. 260 spores/g by the direct method, 46/g by MPN and 36/g by HGMP-ELISA. 34 spores/g by the direct method, 4.6/g by MPN, and 3.5/g by HGMP-ELISA. NI: Not identified. *Negative in the 1st and 2nd but positive in the 3rd test. **Negative in the 1st but positive in the 2nd test. #Sampled in Hiroshima.

The Relation between the Positivity and the Procedure of Detection

Table III shows the results of detection of *C. botulinum* from honey samples with the country of origin, nectar source, sample source and the methods of detection. No correlation was seen between the toxin type and heat treatment or

Table IV. Biochemical properties of the *C. botulinum* isolates from honey

Toxin type	A	B	C	F
Positive samples	11	2	10	1
Number of isolates	11	1	3	1
Test item				
Gram	+	+	+	+
Aerobic growth	—	—	—	—
Meat digestion	+	+	—	+
Lipase	+	+	+	+
Lecithinase	—(9/11)*	—	+	+
Motility	+	+	+	+
Hemolysis	+(10/11)	+	+	+
Acid from:				
glucose	+	+	+	+
maltose	+(10/11)	+	+	+
lactose	—	—	—	—
sucrose	—	—	—	—
salicin	—	—	—	+
mannitol	—	—	—	—
glycerol	—(10/11)	—	+	—
sorbitol	—	+	—	—
trehalose	+	+	—	+
fructose	—(9/11)	—	—	+
Nitrate reduction	—	—	—	—
Growth on CBI agar	+	+	—	+
Spore	ST	ST	ST	ST

*Isolates showing the property/Number tested. ST: Subterminal.

the inoculum size. Only heat-treated tubes gave positive results with sample No. 16 and only diluted samples with No. 18. Samples Nos. 1 and 3 gave positive results only after twice or three-times repeated tests. Among the 80 samples collected in 1988, type C spores were detected from samples Nos. 20 and 21 only after centrifugation in the presence of CaCO_3 . All four tubes gave positive results with two samples, both of which were clover nectar and Argentina origin. Sample No. 22 contained 36 to 60 type F spores/g and No. 23 four type A spores/g. It was not difficult to isolate type A organisms from the 11 type A-positive samples, whereas type C organisms were isolated from only three of the 10 type-C-positive samples.

Some Properties of the Isolates

As Table IV shows, all type A, B, and F isolates belonged to group I (14). The type A isolate from sample No. 6 was atypical, possessing a high lecithinase and no hemolytic activity and fermenting glycerol. All the other isolates did not differ from the authentic strains of the respective types.

Table V. Detection of *C. botulinum* in honey to which spores were added

Number of spores added to 20 g of honey	Spore susp. (1 ml) Positives/ test tubes	9 g		1 g	
		Heated	Unheated	Heated	Unheated
100	3/3	2/2*	2/2	2/2	2/2
		2/2**	2/2	2/2	2/2
10	3/3	1/2	2/2	0/2	0/2
		2/2	1/2	0/2	0/2
5	3/3	1/2	0/2	0/2	0/2
		1/2	0/2	0/2	1/2
3	3/3	0/2	1/2	0/2	0/2
		0/2	0/2	0/2	0/2
1	2/3	0/2	0/2	0/2	0/2
		1/2	0/2	0/2	0/2
0.5	0/3	0/2	0/2	0/2	0/2
		0/2	0/2	0/2	0/2

*Upper row: by the standard method; **Lower row: CaCO_3 was added as a precipitation aid.

Evaluation of the Dilution-Centrifugation Method

When 100 spores were added to 20 g of honey, all four tubes gave positive results; when 10 or fewer spores were added, the results were variable (Table V). In some tests, the heat-treatment and/or addition of CaCO_3 as a precipitation aid enhanced the detection, but not in other tests.

DISCUSSION

In 1986, a case of infant botulism, after feeding honey, was first diagnosed in Japan. The survey conducted by the study group organized by Ministry of Health and Welfare detected *C. botulinum* in 5.3% of 512 honey samples (11). The consumption of honey is increasing and Japan imports honey from many countries. Although the fatality rate of infant botulism is fortunately low, the patients would amplify and keep excreting *C. botulinum* in their feces, which would create a serious public health problem.

To further scrutinize the contamination of honey with *C. botulinum* spores, a long-term survey was conducted. There was no difference in the incidence of *C. botulinum* between domestic and imported honey. Type C and E spores have often been detected in soil of this country (15), but type A and B spores have seldom been detected. It was, therefore, expected before conducting the survey that type A or B spores would seldom be detected, at least in domestic honey. However, type A spores were detected in domestic honey samples. This may indicate some other route of contamination of honey than from soil via nectar.

Most surveys of honey for *C. botulinum* have been conducted in USA. Chin et al. (16) detected *C. botulinum* in 9 of 90 honey samples. Most positive samples were those having been fed to the patients; only one sample was positive out of 63 samples not associated with infant botulism. Midura et al. (8) obtained an incidence of 10% among honey samples including those associated with cases. Sugiyama et al. (7) examined 241 samples of honey not associated with cases and detected *C. botulinum* in 18 samples (7.5%). The organisms detected in honey have exclusively been types A and B. Type C and F organisms were detected in honey in the present investigation.

Although only a limited number of samples were tested, the incidence was low among the samples from Europe. Hauschild et al. (17) stated that *C. botulinum* distributed in Europe is mainly of nonproteolytic type B, whose spores may have been killed by the heat-treatment for detection. In the present survey, honey samples from USA were all negative, while type A spores were detected in samples of Hungarian and Spanish honey.

As for Chinese honey, Mitamura et al. (18) have detected type E spores and Huhtanen et al. (19) type B spores. In the present survey, nine (11.8%) were positive among 76 Chinese honey samples and types A, B and C were detected in these positive samples. Although Yamakawa et al. (20) reported a dense distribution of type A and B spores in north-west China, Shinkiang district, Chinese honey is produced mostly in the south. It seems necessary to clarify whether contamination is from soil or some other sources. Japan imports a largest amount of honey from China and therefore attention should be paid to Chinese honey.

Type A and F spores were detected in Argentina honey. Distribution of type A and F spores in Argentina soil has been reported (21,22). Since the nectar source was clovers, contamination of honey from soil via nectar can not be ruled out.

The incidence of *C. botulinum* was higher in the samples taken from drums or apiary than marketing honey. Sugiyama et al. (7) also reported a higher incidence in samples taken from apiaries than from shops. We added type A and B spores to honey in 10^4 /ml and then kept at 25 C. After one year, the viable count of the spores decreased to less than 1% and the heat stability also decreased (23). From these results, it seems possible that some processes during purification and prolonged storage contributed to the lower incidences in marketing honey.

Several methods for testing honey for *C. botulinum* have been reported. Sugiyama et al. (7) reported the dilution-dialysis method, but it requires a large amount of medium and skillful techniques for use of dialysis tubing. Hauschild et al. (24) recommended the use of membrane filter, but our preliminary tests showed that it took fairly a long period for filtration of 20 g of honey. Amylase treatment accelerated filtration to a certain extent, but the technique is not so simple to treat many samples at a time. In contrast, the dilution-centrifugation method of Midura et al. (8) we used was simple and practicable. The recovery of the spores, however, should be improved, since Huhtanen et al. (19) reported that the samples giving positive results by the direct plating method gave negative

results by the dilution-centrifugation method. The recovery of spores by the method was increased from 20-40% to 40-50% when CaCO_3 was used as a precipitation aid (data not shown). Midura et al. (8) stated that the presence of seven to nine spores per g of honey was needed for detection. The present results show that spore concentrations higher than one per gram always gave positive results (Table V). It appeared that the heat treatment sometimes accelerated detection, but sometimes detection was positive only with unheated cultures. In case of soil samples that usually contain a large number of contaminants, dilution of the sample is often effective for detection of *C. botulinum*. Honey does not contain such many contaminants that dilution would not be so effective as for soil samples. With one sample (No. 18), a positive culture was obtained only when diluted (Table III). It was rare that both heated and unheated cultures gave positive results. It seems that such unstable results do not reflect the effect of heat treatment, but low population or uneven distribution of the spores in honey. It seemed justified to recommend the use of at least four tubes of enrichment medium for detection of *C. botulinum* in honey. As with samples Nos. 1 and 3, the first test gave negative results and the twice or three-times repeated tests gave positive results. From the foregoing facts, it is presumed that such honey containing only a few spores may have given negative results.

Quantitative determinations of spores were made for the two samples that gave four positive cultures; the other samples may have contained spores in one per gram or lower concentrations. Sugiyama et al. (7) reported that the highest concentration of spores in honey not associated with infant botulism cases was 7/25 g. Midura et al. (8) reported that honey fed to patients of infant botulism contained 5 to 15 spores/g and some contained as many as 70-80 /g. In the present investigation, two samples from Argentina contained spores compatible to these figures. It is unbelievable that such heavy contamination occurred from the environment. The question if *C. botulinum* would proliferate in honey before ripening has remained to be solved. As reported by Dezfulian et al. (25), the isolates from honey did not differ from the authentic strains of the respective types in the biochemical or physiological characters, but further characterization seems necessary in connection with the behavior of *C. botulinum* in honey.

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