THE OCCURRENCE OF BACILLUS BOTULINUS IN NATURE

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Van Ermengem, who was the first to isolate *B. botulinus* from poisonous food and who proved that it was the organism which produced the toxin causing the type of food poisoning known as botulism, made repeated efforts to isolate *B. botulinus* from nature. He made cultures of garden soil, dirt from the streets, mud from ponds and rivers, manure from stables, cow manure, horse manure, duck excreta and the intestinal contents of various species of fish. He was never able, however, to find the bacillus except in two specimens of ham which had caused outbreaks of poisoning (Van Ermengem, 1912).

The work of Kemper and Pollack (1897) who recovered a strain from the intestinal contents of a "normal" hog, is the only recorded case of the isolation of *B. botulinus* from nature. This finding, together with the fact that botulism was at that time chiefly connected with poisoning from sausages and ham, led to the belief that *B. botulinus* was a normal inhabitant of the hog intestine.

In 1917 Dr. E. C. Dickson[2] of Stanford Medical School, San Francisco, undertook to check up the work of Kemper and Pollack. Dr. Dickson examined the intestinal contents of 250 grain-fed hogs which were slaughtered for market in South San Francisco. The contents of the colon and ilium were the portions chosen for examination. No trace of *B. botulinus* was

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[2] Aided by a grant from the State Council of Defense of California.

found in any of the material, showing that *B. botulinus* is not a common inhabitant of the intestines of grain fed hogs.[3]

Dr. Dickson also collected 10 samples of manure from two groups of garbage fed hogs. Cultures were made and examined but no trace of *B. botulinus* was found.

Dr. Dickson collected samples of soil from two gardens where in the summer of 1917 beans were grown and canned, which caused two outbreaks of botulism in January and February, 1918. The specimens were collected in January and February, 1918, and represented a fair sample of the dirt in the yards, both surface and sub-surface, to a depth of 12 inches. *B. botulinus* was not found in any of the samples.

In the summer of 1918, when I made the survey described in the following pages, there were really no positive data as to the occurrence of *B. botulinus* in nature. There were four localities in central California where outbreaks of botulism from home canned fruits and vegetables had occurred during the previous winter, and it seemed that something should be learned by visiting these houses and gardens during the canning season. In collecting the samples, my object was to cover as wide a range of material as possible rather than to make a complete analysis of any one kind. For that reason this report must serve rather as an outline for further research than as a basis for any very definite conclusions.

METHOD OF COLLECTING AND TESTING THE MATERIAL

The samples were collected in sterile test tubes and petri dishes and reached the laboratory within four days. Sterile instruments were used in handling the material in the laboratory. Cultures were made in a double strength beef infusion broth (Van Ermengem's broth) with 2 per cent glucose and a reaction as near neutral as possible (Van Ermengem, 1912). The broth was oil stratified in the tubes. Witte's peptone was used in order to insure reliable toxin production. This broth medium is particularly favorable for the production of botulinus toxin. All the cultures were made in test tubes unless

[3] Dr. Dickson has a report of this work in process of publication.

otherwise stated in the tables. Where flasks were used a large quantity of material was cultured. Just before inoculation all the media were boiled for twenty minutes and immediately cooled by placing the tubes in cold water.

The broth cultures were incubated at 28°C. for from two to six months. They were then tested for toxin. About 10 cc. of the broth culture was passed through a Mandler filter and the filtrate at once oil stratified. One cubic centimeter of this filtrate was injected, subcutaneously, into a guinea-pig. Each filtrate which killed the guinea-pig in forty-eight hours or less, was tested against antitoxins of *B. botulinus* types A and B to determine whether it contained the specific toxin of *B. botulinus*.

For the toxin-antitoxin test three 250 gram guinea-pigs were injected subcutaneously, as follows:

GUINEA PIG NUMBER	FILTRATE	ANTITOXIN
1	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for *B. botulinus* type A*
2	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for *B. botulinus* type B
3	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	Control—no antitoxin

* The antitoxins used were of such strength that 1 cc. neutralized 200 minimum lethal doses of the homologous toxin. One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

A reliable toxin-antitoxin test may be obtained with a toxin, 1 cc. of which requires four days to kill. With a weaker toxin the antitoxin test is not reliable for in some cases the pigs develop typical symptoms of botulism but recover after about two weeks while in other cases they remain well for a month and then die with typical symptoms after a few days of illness. In

the following charts four cultures are marked as doubtful because the toxin in the filtrate was too weak to give a decisive toxin-antitoxin test although the symptoms of the guinea-pig that died were entirely typical of botulism.

Where a filtrate gave evidence of containing a weak toxin an effort was made to obtain a stronger toxin by making a transplant from the original culture into a fresh tube of "Van Ermengem's" broth. All the unfiltered portion of the original culture was transferred into the new tube, which was then incubated for about four weeks and tested for toxin. In one case (Palo Alto culture from a bird-pecked and moldy cherry) I was successful in getting from the transplanted culture a strong toxin which proved to be *B. botulinus*, type A.

The incubation period of from two to six months, was too long, as the average life of *B. botulinus* in broth cultures is about three months. My original plan was to incubate for from six to ten weeks. The longer period was necessitated by delays which were unavoidable. I believe it very probable that if the original plan had been adhered to the percentage of positive botulinus cultures might have been higher, for about one-fourth of the guinea-pigs receiving the initial dose of filtrate, died in from two to six weeks, some especially in the Oakdale series, showing marked symptoms of botulism. All efforts to obtain a strong toxin from sub-cultures failed, except in the one case in the Palo Alto series mentioned above.

SURVEY FOR B. BOTULINUS, JUNE, JULY AND AUGUST, 1918

The material was collected from five localities in the central section of California, two of these, Palo Alto and Hollister in the Santa Clara Valley, Oakdale and Madera in the San Joaquin Valley, and Berkeley on San Francisco Bay. The towns were all more than 50 miles distant from each other.

In the Palo Alto series the material had no connection with any previous outbreak of botulism. In the four other localities, however, outbreaks of botulism had occurred during the previous winter, which were caused by home canned fruits and vegetables put up in the summer of 1917. I visited the gardens and the orchard where the vegetables and fruit were grown, except in

Hollister where the peas had been purchased from a peddler. In Hollister and Berkeley an examination was made of the household conditions under which the canning was done. The time chosen for collecting the material corresponded as nearly as possible with the time at which the canning had been done the previous year.

The following is a detailed description of the places and conditions under which the various samples were collected, the character of the samples and the results of the toxin-antitoxin tests. The data are divided into groups according to the places from which the material was collected.

Palo Alto, California

There had been no recorded outbreak of botulism in or near Palo Alto for several years. The corn, beans, peas, peach and cherries which were cultured were obtained in a first class grocery store. All except the cherries were carried direct to the laboratory.

The corn had been set aside as unfit for sale and was badly worm eaten. Four test tube cultures were made of the caterpillars from the corn and five flask cultures of the corn itself. *B. botulinus* was not found in any of the cultures.

One test tube culture each was made of the beans, peas and peach. The peas and beans were dirty and spotted and the peach was partially decayed. The cultures were negative.

The cherries were bought for preserving and were all a little underripe. They were picked over on the unscreened porch of a house in a clean section of the town. There were no horses, cows, or pigs kept anywhere in the neighborhood and dogs were the only animals kept on the immediate place. There were no ants about the house and very few flies. The cherries from which the cultures were made were either slightly bruised or bird pecked. There was no decay or mold on them when they were separated from the other cherries. They were placed in a clean paper bag on the railing of the porch where they remained for two days before they were taken to the laboratory. There

were no spiders or insects in the bag when it was taken to the laboratory but the cherries had molded, wherever the skin was broken.

Nine test tube cultures were made of the cherries. In two of these cultures toxin of *B. botulinus* was produced. One of the positive cultures was made from a bird-pecked moldy cherry, the other from a bruised and moldy cherry. Both contained type A toxin.

Two cultures were made from a caterpillar and four from the crop and gizzard contents of birds killed in the yard of the house where the cherries were picked over. The cultures were all negative.

Hollister, California

The place from which this material was collected was a cross roads saloon about 7 miles from the town of Hollister. It was an exceedingly dirty place. Hogs, cows, and horses were kept in sheds clustering around the back of the house. There was an open privy about 100 feet from the kitchen door. Chickens, mud and manure were everywhere, even on the porches. A leaky water tank was built over a store room adjoining the kitchen. Gray slugs crawled over the walls of the kitchen and store room at night. There were swarms of flies in the house and out of doors.

In the summer of 1917 the housewife canned peas, which she had bought from a peddler. One jar of the peas had spoiled in February, 1918, and the contents was thrown into the garbage which was fed to two hogs. The hogs, one full grown and one small, developed typical symptoms of botulism and *B. botulinus*, type B, was recovered from the stools while the animals were sick. The animals recovered after a few weeks. Three months later, when these specimens were collected, the animals were perfectly healthy, and the small hog had grown to full size. They were later sold to a market.

Cultures were made of the tap water from the kitchen and also of flies collected in the kitchen. In the yard cultures were made from slugs, earth-worms and sowbugs, from chicken

manure, straw and dirt from the chicken yard, from mud under a leaky faucet and from fresh manure collected in the pen of the large hog referred to above.

Toxin of *B. botulinus* type B was produced in one of the five cultures of hog manure. The strain in this culture was of the same type as that isolated from a stool while the hog was sick. It was impossible to make a more complete investigation of the case as the hogs had been killed for market and the pens cleaned up by the time this culture had been tested out. Cultures of all the other material were negative.

Reference has already been made to the work of Kemper and Pollack who isolated a strain of *B. botulinus* from the intestinal contents of a "normal" hog and to the fact that Dr. E. C. Dickson of Stanford Medical School was unable to verify their work although he had examinations made of the intestinal contents of 250 grain-fed hogs and 10 samples of manure from two groups of garbage-fed hogs. The second finding of *B. botulinus*, type B in the manure of the Hollister hog, four months after the contaminated food was eaten suggests very strongly that Kemper and Pollack's "normal" hog may have been fed contaminated food a short time before their cultures were made. Hogs are fairly resistant to botulinus toxin so that there might easily have been no record of the animal's illness.

Oakdale, California

The garden from which material was collected at Oakdale was one in which pole string beans were grown in the summer of 1917. Some of these beans were canned. One of the jars spoiled and was the cause of an outbreak of botulism. In July, 1918, conditions in the garden were practically the same as in the previous year. The section of the town in which this garden was located was quite new. A garden had been made on the place only two years before and no manure had been used. There were no horses, cows, pigs or chickens kept on the place. Beside pole and bush beans, there were tomatoes, wax beans, and melons. The soil was clean sandy loam, well drained. There had been no rain for several months. Irrigating ditches

supplied the water. The samples were collected in the blazing sunlight with the thermometer at 100°F.

Twenty-three cultures were made from spotted leaves and pods of the pole and bush string beans, from ants, spiders, bugs and grasshoppers, collected about the plants and from dirt from the mouth of an ant hill. In one of the three cultures of pole bean leaves spotted by insects or some other small animal toxin of *B. botulinus* type A was produced.

Another culture, containing spiders and small bugs contained a weak toxin. In the toxin antitoxin test the control pig and the pig which received the filtrate botulinus antitoxin type A developed very evident symptoms of botulism about the fourth day but later recovered. As it was impossible to obtain a stronger toxin in transplants from the original, this culture was listed as "doubtful."

Madera, California

The samples were collected from a ranch about 2 miles out of Madera. It was an ordinary small country ranch, with an old house, tank-house, stables and chicken houses. A small orchard of fig and apricot trees adjoined the house on one side and walnut trees bordered the drive and the main road. Alfalfa fields surrounded the place and there were no houses very close by. In 1917 the owners lived on the ranch and kept chickens, turkeys, hogs and horses. There is no record as to whether they also kept cows or as to what sort of a garden they had. In July, 1917, a lot of apricots were canned in a screened summer kitchen. It was windfall fruit and unfit for market. One of the jars of apricots spoiled and caused an outbreak of botulism in which six persons died, February, 1918.

The surviving members of the family moved away and the place was rented to another family. They stayed only a short time. In July, 1918, when I visited the ranch, no one was living there and there were no domestic fowls or animals with the exception of two horses. There were noticeably few birds, bugs or insects other than ants.

One hundred and seventeen cultures were made from material

collected on the ranch. Five cultures were made of tap water from the house. The remaining cultures were made from windfall apricots, which were bird pecked, decayed, moldy or ant eaten; from bugs, flies, ants and spiders; from the beaks, claws and alimentary tract contents of English sparrows, linnets, dove and black birds; from dirt and manure collected in an old hog pen and in the chicken yard where all the chickens had died of botulism six months before; and from fresh horse manure.

No positive culture was found in any of the material. One culture of linnets' claws contained apparently a weak toxin of some sort but it was too weak to give a satisfactory toxin-antitoxin test. The control pig and the filtrate-antitoxin B pig developed typical symptoms of botulism on the third day, but gradually recovered while the filtrate-antitoxin A pig remained normal throughout. This was listed as "doubtful" since it was not possible to obtain a stronger toxin from the transplants.

The ranch at Madera differed from the other localities from which I collected material in that there were no human beings, dogs, cats, pigs, cows, or chickens, living on the place. There was no vegetable garden and very few birds, spiders, insects (other than ants), or other small animals. Neighboring ranch homes were a quarter of a mile distant.

Berkeley, California

In August, 1917, Mrs. M. of Berkeley, California, canned some string beans from her garden. She sterilized them by the fractional method. In January, 1918, it was found that four of the seven jars of beans contained *B. botulinus.* The contents of one jar were fed to some chickens and 24 of them died of botulism, "Limberneck." In August, 1918, Mrs. M. had a few beans growing in her garden in almost the same spot where they had grown the year before and she consented to can these for me, as nearly as she could, in the same manner as those which she had put up the year before. In addition, samples were collected from the house and garden.

The house in which Mrs. M. lived was an old one and was situated in a closely built up neighborhood. The yard was an

ordinary city lot, at the back of which were chicken houses and runs. Between the chicken runs and the house was a garden in which was a small patch of bush string beans and also a patch of mint. The yard was dry at the time my samples were collected. The kitchen and screen porch had been made as clean as possible and there were no flies in either place. Mrs. M. was apparently a woman of average cleanliness.

The beans were gathered from the garden about 1.30 p.m. by Mrs. M. and her daughter, and were picked over by Mrs. M. on the screened porch. She discarded all beans that were broken or decayed. Some of the beans which she passed as good had small hard scars or blemishes on the skin. The beans were washed in several changes of cold water under the faucet in the kitchen. The kettles used to hold the beans were clean. After washing, the beans were cut by Mrs. M. with a clean knife and packed in three Mason quart-jars. Mrs. M. said that she had washed the jars and tops the night before in scalding soap suds. In the morning she had rinsed them in about a dozen changes of cold water. They were again wiped out with a freshly laundered towel just before using. The rubbers were new and were washed in cold tap water from the laundry tub on the screen porch before they were put on the jars.

The jars, with tops loosely screwed, were placed in a deep pot of cold water. The water was brought to a boil. After thirty minutes in the boiling water the jars were removed and the tops screwed down. They were then taken to the laboratory and incubated for six months.

Broth cultures were made from the tap under which the beans were washed and from the tap under which the rubbers were washed. Portions of the beans discarded by Mrs. M. were cultured. Cultures were made of dirt from under the bean plants, of snails, slugs, ants, spiders and a caterpillar, and of beans direct from the garden.

In one of the three jars of beans, canned by Mrs. M. toxin of *B. botulinus* type A was produced, and toxin of *B. botulinus* type B was produced in a culture of a small spider from one of the bean plants. The spider was placed in a sterile tube at

once and did not come in contact with anything in Mrs. M's house. The other material was negative with the exception of two doubtful cultures, one of which was from a sowbug, while the other contained earth, web and droppings from a spider tube in the ground under the bean plants. There was apparently a trace of toxin present in the tubes but it was too weak to give a reliable toxin-antitoxin test.

Forage poisoning

I have recently obtained a culture of *B. botulinus* type A from one of four samples of discolored, moldy hay from a large stack on a ranch near Oakdale, California. This hay was suspected as the cause of one of a series of outbreaks of forage poisoning among horses and mules, occurring in the vicinity of Oakdale in January and February, 1919. The spoilage in the hay ran in veins down through the stack and the samples which were cultured were taken from the deeper layers.

The symptoms exhibited by the horses, as they were described to me by Dr. Eddy of Stockton, California, were very suggestive of botulism. The finding of *B. botulinus* in a sample of the spoiled hay is very suggestive. It does not, however, definitely prove that the animals died of botulism as I was unable to carry out feeding experiments to demonstrate the presence of toxin in the hay.

The successful therapeutic use of a polyvalent botulinus antitoxin in cases of forage poisoning will furnish the best proof of the identity of this poisoning with botulism. An antitoxin furnished by the Agricultural Experiment Station of the University of Illinois was used on two horses of the Oakdale series without success. I have recently tested a sample of this serum and find that it contains only type B antitoxin. In my experience *B. botulinus* type A is more common in California than type B.

Graham and Brueckner, who were the first to claim that forage poisoning was caused by the toxin of *B. botulinus*, have a report of some recent antitoxin work in the Journal of Bacteriology, January, 1919.

SUMMARY

A. Two hundred and thirty-five cultures were made from samples collected in five localities in central California, 50 or more miles distant from each other. The cultures covered a wide range of material, including tap water, hay, leaves, vegetables and fruits in various conditions, insects, spiders, sowbugs, snails, and caterpillars, garden soil, manure from horses, hogs, and chickens, and also samples from the claws and beaks, and crop, gizzard and intestinal contents of birds.

B. Seven cultures containing *B. botulinus* were found. The source of the material in these cultures and the type of toxin demonstrated was as follows:

1. Bruised and moldy cherries.....Palo Alto, Cal., Type A
2. Bird-pecked cherries.....Palo Alto, Cal., Type A
3. Pole bean leaf covered with spots or droppings of insects or small animals.....Oakdale, Cal., Type B
4. Spiders from bush bean plants.....Berkeley, Cal., Type B
5. Bush beans, some of which were slightly scarred, picked over, washed and packed in clean jars for canning.....Berkeley, Cal., Type A
6. Manure from large hog which had recovered from botulism 3 months before sample was taken.....Hollister, Cal., Type B
7. Discolored moldy hay from an outdoor stack..Oakdale, Cal., Type A

Four cultures were found in which there was evidence of toxin but it was so weak that the toxin-antitoxin tests were not considered reliable. The material from which these doubtful cultures were made is as follows:

1. Earth from spider tube, spider droppings and web.....Berkeley, Cal.
2. Sow bug from bush bean plant.....Berkeley, Cal.
3. Linnet claws.....Madera, Cal.
4. Spider and small bugs from bush beans.....Oakdale, Cal.

C. This survey is obviously brief and must serve chiefly as an outline for further research. Nevertheless, the following points seem to be quite clearly indicated.

1. *B. botulinus* is widely distributed in nature.

2. *B. botulinus* is present in the garden and may be on the fruit or vegetables when they are picked.

3. *B. botulinus* is not necessarily associated with active decay. It may be present in the blemishes or spots on the skin of apparently sound fruit and vegetables.

D. The strain of *B. botulinus* found in the hog manure of the Hollister series apparently indicates that *B. botulinus* may remain in the intestinal tract of an animal for at least four months after contaminated food has been eaten.

E. *B. botulinus* may not occur far from the habitation of man. Of the five localities visited, only one failed to give positive results as to the presence of *B. botulinus*. This locality, Madera, differed from the other four in being isolated and deserted. There were no human beings living on the place, no domestic animals other than horses, and there was no vegetable garden.

F. The evidence very strongly suggests that *B. botulinus* may be closely associated with or disseminated by spiders or insects common in gardens in California. Since *B. botulinus* grows readily at temperatures as low as 22°C., there is no reason for assuming that this organism must be associated with the stools of warm blooded animals.

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