QUANTITATIVE DEMONSTRATION OF THE PRESENCE OF SPORES OF BACILLUS LARVAE IN HONEY CONTAMINATED BY CONTACT WITH AMERICAN FOULBROOD [1]

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INTRODUCTION

In a previous paper [3] the writer showed that it is possible to demonstrate the presence of spores of *Bacillus larvae*, the cause of American foulbrood, in samples of commercial honey that have had contact with American foulbrood in the course of their production or preparation for the market. Since this work was reported, 25 additional samples, making a total of 212 samples of commercial honey, obtained on the open market from 28 States and 2 Territories have been examined by the same method, and spores of *B. larvae* have been found in 17, or 8 percent, of these samples.[4] In most cases the spores were present in relatively small numbers.

The method of examination used in the work thus far reported gave only a qualitative indication of the number of spores present, the observations being recorded as showing "the presence of a sufficient number of spores resembling spores of *B. larvae* to be designated as positive." [5] This amounted to from one or two definite spores to a very few spores seen in numerous microscopic fields of each stained sediment examined. The primary object was to demonstrate only their presence or absence. It was assumed that in most cases the number of spores found was considerably smaller than would be found in honey containing numbers comparable with the observed minimum infective dose of 50,000,000 per liter.

The only way of demonstrating the accuracy of this assumption has been to feed such "positive" samples of commercial honey to healthy colonies of bees. This was done with 15 of the 16 samples in which spores were demonstrated, and only 1 sample, or 6.7 percent, was found to contain sufficient infection to produce the disease in a healthy colony. These investigations indicate that the requirement of certification of honey, as has been proposed and even placed in operation in certain States, is not a justifiable measure in the control of American foulbrood under the present conditions of inspection and control of disease in this country.

To permit a more accurate, quantitative study of the infectivity of honey that has been in contact with American foulbrood, on the

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[3] STURTEVANT, A. P. RELATION OF COMMERCIAL HONEY TO THE SPREAD OF AMERICAN FOULBROOD. Jour. Agr. Research 45: 257–285, illus. 1932.

[4] STURTEVANT, A. P. HONEY OF THE INTERMOUNTAIN REGION. Gleanings Bee Cult. 63: 463–468, illus. 1935.

[5] STURTEVANT, A. P. See footnote 3.

basis of its spore content—that is, a detailed study of the distribution of spores of *B. larvae* in the honey from infected hives or apiaries, or in commercial honey obtained on the open market, or of the effect of mixing infected honey with disease-free honey in the course of production or blending and preparation for the market—a more detailed investigation has been made of the spore content of honey containing approximately known numbers of spores. This has been accomplished by an improved and more accurate method of determining the number of spores in such honey, and the accuracy of the results and method has been demonstrated by means of a statistical analysis of the data obtained.

METHOD OF OBTAINING THE DATA

PREPARATION OF SAMPLES OF HONEY

A series of samples of honey containing approximately known numbers of spores per cubic centimeter were prepared in the manner described previously,[6] by adding to 100-cc quantities of spore-free honey the necessary quantities of various dilutions of a stock suspension of spores of *Bacillus larvae* containing approximately 5,000,000,000 spores per cubic centimeter. Five samples of honey were prepared in this way containing approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter, respectively. These samples, each considered as a unit and not as a dilution of the 1,000,000-spore sample, were heated in a water bath to 120°–130° F., and then thoroughly mixed with a mechanical stirrer for 5 minutes. Duplicate 5-cc quantities of each sample were then placed in 50-cc conical centrifuge tubes, and 45 cc of distilled water of approximately the same temperature was added. When the honey and water were completely mixed, the samples were centrifuged at 2,000 revolutions per minute for 45 minutes. All but about 1 cc of the supernatant honey-water solution of each sample was then removed by means of a pipette and suction. Again approximately 45 cc of distilled water was added, and after thorough mixing the suspensions were centrifuged for 30 minutes longer. The removal of the supernatant solution was repeated until all but approximately 0.1 cc[7] of the water had been removed from each centrifuge tube, and each sample of sediment was completely suspended in this remaining quantity of water by blowing gently through a capillary pipette dipped into the water. Duplicate 0.01-cc quantities of each suspension were then transferred with the capillary pipette (calibrated to deliver 0.01 cc) to microscope cover glasses. Circular cover glasses, size 12, no. 1 thickness, having an area of 1.13 cm^2, proved satisfactory for this purpose. A small (2 to 3 mm) loopful of carbolfuchsin stain was added to the drop of suspension on the cover glass and thoroughly mixed with it. This stained liquid was then spread uniformly over a 1-cm^2 area of the cover glass, a narrow ring at the outside edge being left uncovered. The smears were allowed to dry in the air and were then mounted on microscope slides either with water or, preferably, with Canada balsam, for examination under the microscope. These stained smears were not washed in water, as this might have caused some spores to be lost.

[6] STURTEVANT, A. P. See footnote 3.
[7] A mark was placed on the outside of the conical centrifuge tubes to indicate the 0.1-cc volume.

The foregoing process gives a concentration of spores in the sediment from the 5-cc samples of honey suspended in 0.1 cc of water, or one-fiftieth the original volume.

METHOD OF COUNTING SPORES

A method similar to that of Breed and Brew [8] for counting bacteria in milk was used for counting the spores of *Bacillus larvae* in these stained smears. This method is similar to that described in a previous paper [9] and is represented by the formula

$$\text{Number of spores per cubic centimeter} = \frac{KNX \times 100 \times D}{N}$$

where K is the factor for the number of circular fields per 1-cm^2 area, N is the number of circular fields counted, X is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and D is the dilution.

TABLE 1.—*Spore counts in stained smears of the sediments resulting from the centrifuging of duplicate 5-cc portions of five samples of honey containing known numbers of spores of Bacillus larvae*

Field no.	Spore counts in samples [1] containing the indicated number of spores per cubic centimeter									
	50,000		300,000		500,000		800,000		1,000,000	
	A	B	A	B	A	B	A	B	A	B
1	2	1	7	8	14	15	19	21	24	21
2	2	2	8	9	12	12	18	20	24	29
3	1	1	8	10	12	13	24	18	26	38
4	0	2	7	5	10	15	22	18	23	24
5	2	0	9	6	10	11	20	20	30	34
6	1	3	9	7	12	13	18	17	23	29
7	0	1	8	7	15	14	21	18	26	24
8	2	1	7	8	12	13	27	17	29	21
9	1	0	8	6	16	16	19	21	19	26
10	0	2	9	12	17	13	21	20	30	31
11	1	1	7	11	14	12	16	19	25	36
12	1	3	10	6	16	12	21	20	28	26
13	1	3	9	5	13	10	22	22	27	33
14	2	1	7	9	18	14	25	24	25	22
15	1	0	8	10	12	13	21	25	29	26
16	2	1	8	5	10	11	20	25	24	26
17	1	0	6	9	11	16	18	28	35	23
18	2	0	5	6	13	14	26	23	24	28
19	0	2	7	10	17	16	24	23	25	34
20	0	1	9	5	13	18	26	28	27	30
21	2	1	10	15	13	15	16	23	25	32
22	2	3	11	10	8	16	19	18	26	25
23	1	1	7	8	18	10	20	16	27	25
24	3	1	6	5	15	12	18	22	28	34
25	1	1	7	9	12	15	21	21	29	28
26	0	1	6	7	15	10	21	20	27	22
27	1	1	5	10	10	14	26	18	34	23
28	2	1	7	8	12	15	22	26	22	27
29	2	2	8	10	11	11	22	28	29	28
30	2	2	10	8	12	11	25	22	21	30
Total	38	39	233	244	393	400	638	641	791	835
Total for 60 fields	77		477		793		1,279		1,626	
Mean number of spores per field	1.2833		7.9500		13.2167		21.3167		27.1000	

[1] A and B represent duplicate portions of the samples.

[8] BREED, R. S., and BREW, J. D. COUNTING BACTERIA BY MEANS OF THE MICROSCOPE. N. Y. State Agr. Expt. Sta. Tech. Bull. 49, 31 pp., illus. 1916.

[9] STURTEVANT, A. P. See footnote 3.

An ocular micrometer disk, such as is used for counting bacteria in milk, was used in counting spores in the fields of the stained smears. The area of the circle etched on this disk was found to be 0.00006082 cm^2 when used in a binocular microscope with 15 × paired eyepieces and a 1.8-mm oil-immersion objective. Therefore, the factor K became 16,441.96.

The spores in 30 fields from each of the duplicate smears were counted, making a total of 60 fields (N) for each honey-spore sample. The fields were counted at random from various parts of the smear. From these counts the actual mean number of spores per field recovered in 60 fields for each honey-spore sample was determined (table 1).

Substituting the values for K and N and 0.02 (1/50) for D, the spore dilution in the foregoing formula gives

Number of spores per cubic centimeter

$$=\frac{16{,}442\times 60X\times 100\times 0.02}{60}=32{,}884X$$

COMPUTATION OF THEORETICAL MEAN NUMBER OF SPORES PER FIELD

The theoretical mean numbers of spores per field that should be recovered from each of five honey-spore samples used, under ideal conditions where there is no loss of spores during the process, were calculated by the foregoing formula, which for this purpose may be stated as follows:

$$X=\frac{\text{Number of spores per cubic centimeter}}{32{,}884}$$

X now designates the theoretical mean number of spores per field. In table 2 these values are given in comparison with the corresponding actual mean number of spores per field for each honey-spore sample.

TABLE 2.—*Relation between the actual and the theoretical mean numbers of spores of Bacillus larvae per field recovered from five samples of honey containing known numbers of spores per cubic centimeter*

Spores per cubic centimeter in sample (number)	Mean spores per field			Ratio of actual mean to theoretical mean
	Theoretical	Actual	Standard deviation	
	Number	*Number*	*Number*	*Percent*
1,000,000	30.4100	27.1000±0.3554	4.0812	89.12
800,000	24.3279	21.3167± .2751	3.1596	87.62
500,000	15.2050	13.2167± .2011	2.3100	86.92
300,000	9.1230	7.9500± .1708	1.9615	87.14
50,000	1.5205	1.2833± .0747	.8582	84.40

RESULTS OBTAINED BY USE OF THE METHOD

By the method used, the actual mean number of spores per field obtained by counting 60 fields from each honey-spore sample differed from the calculated theoretical mean number of spores per field by 10.88 percent for the honey containing 1,000,000 spores per cubic centimeter to 15.60 percent for the honey containing 50,000 spores per

cubic centimeter (table 2). This difference, which is relatively constant for each sample, may be due to the fact that some spores are lost during the centrifuging, but more probably to the fact that a certain proportion of the spores in each smear are covered up and not seen in the masses of stained debris always present even in honey of the highest quality.

DETERMINATION OF ACCURACY OF THE METHOD

STATISTICAL ANALYSIS OF THE DATA

Since the data obtained for the actual mean number of spores per field (table 1) for each honey-spore sample, if plotted against the data calculated for the theoretical mean number of spores per field (table 2), give practically a straight line having a trend similar to that of a line plotted for the theoretical data alone, the relation between the theoretical means and the actual means, for the five honey-spore samples used, was determined by the customary statistical methods.

The standard deviation and the probable error for the actual mean number of spores per field were determined from frequency tables prepared from the original data (table 1) for each honey-spore sample used [10] (table 2). The actual means were derived from large samples (60 fields each), and the calculated probable errors and standard deviations were shown statistically to be small.

The coefficient of correlation [11] between the values for the actual mean number and those for the theoretical mean number of spores per field for each sample as given in table 2 was found to be 0.9999 ± 0.0001.

The relation between the actual mean number of spores per field recovered from each honey-spore sample and the corresponding most probable values estimated from the theoretical mean number of spores per field for each sample was determined by use of the regression equation for the actual mean number of spores. This was found to be $\overline{Y}=0.8905X-0.1791$. Substituting the various values of the theoretical mean number of spores per field (table 2) for X in this equation gave the most probable estimated values for the actual mean number of spores per field ($\overline{Y}$) that should have been recovered from each sample (table 3). These most probable estimated values were found to be in excellent agreement with the actual values obtained.

TABLE 3.—*Theoretical and actual mean numbers of spores per field and the most probable estimated theoretical and actual mean numbers of spores per field*

Number of spores per cubic centimeter in sample	Mean number of spores per field			
	Theoretical	Estimated theoretical	Actual	Estimated actual
1,000,000	30.4100	30.6313	27.1000	26.9010
800,000	24.3280	24.1378	21.3167	21.4850
500,000	15.2050	15.0431	13.2167	13.3610
300,000	9.1230	9.1297	7.9500	7.9449
50,000	1.5205	1.6443	1.2833	1.1749

[10] CHADDOCK, R. E. PRINCIPLES AND METHODS OF STATISTICS. pp. 160–164, 240–241. Boston, New York [etc.]. 1925.

[11] CROXTON, F. E., and COWDEN, D. J. PRACTICAL BUSINESS STATISTICS. p. 416. New York. 1934.

The purpose of this investigation, however, was to develop an equation with which, if the actual mean number of spores per field is obtained with sufficient accuracy, the theoretical number of spores per field may be estimated, thereby giving the data necessary for estimating the number of spores per cubic centimeter in an unknown sample of honey. The regression equation or the theoretical mean number of spores per field can be used for this purpose, and was found to be $\overline{X}=1.1228Y+0.2034$. Substituting for Y in this equation, the various values of the actual mean number of spores per field, as obtained in table 1, gave the most probable estimated values for the theoretical mean number of spores per field that should be obtained from the actual counts for each honey-spore sample (table 3). By this method of estimation these values were found to agree closely with the original calculated values for the theoretical mean number of spores per field for each honey-spore sample (table 2).

DETERMINATION OF PERMISSIBLE LIMITS OF ERROR

The analysis of the data so far indicates the accuracy of the method outlined above for determining the most probable actual mean spore count per field from the mean of 60 fields counted. Variations in the counts may occur in individual samples, however, owing to the failure to recover all the spores, as stated previously.

The permissible limits of error in the statistical analysis of such cases are customarily determined by use of the standard error of estimate. This, for the most probable estimated actual means derived from the theoretical means, was found to be small, ± 0.1298 spore, and indicates the closeness with which new estimated values may be expected to approximate the true but unknown values. Since two of the five actual means fall within ± 0.1298 spore of the estimated actual means while the other three are only from 0.11 to 0.26 percent outside this zone, within which approximately two-thirds of the observations may be expected to fall in relation to the most probable values, a sufficient accuracy for the method is indicated.

The standard error of estimate for the most probable theoretical means derived from the actual means (which were found to agree closely with the estimated actual means) was found to be ± 0.1458 spore. As is to be expected in this case, again two of the original theoretical means fall within the zone of ± 0.1458 spore while the other three are only from 0.11 to 0.25 percent outside this zone. However, since ± 3 times the standard error of estimate, which should include 99.7 percent of all observations, is used customarily in delineating the largest error to which statistical analyses of this type are subject, it is found that all the theoretical means fall well within this zone, or within ± 0.4374 spore. This indicates the probable accuracy of estimating the number of spores per cubic centimeter in an unknown sample by calculating the most probable theoretical number of spores per field from the actual mean number counted.

PRACTICAL APPLICATION OF THE METHOD

In a previous paper [12] it was shown that during observations covering 5 years no cases of American foulbrood developed in 19 colonies of bees fed less than approximately 50,000,000 spores of *Bacillus*

[12] STURTEVANT, A. P. See table 1 of reference in footnote 3.

larvae in 1 liter of sugar sirup, or less than 50,000 spores per cubic centimeter. Of 11 colonies fed 50,000 spores per cubic centimeter, 2 developed disease and 9 remained healthy; of 6 colonies fed 75,000 per cubic centimeter, 3 developed positive disease and 1 probable disease, and 2 remained healthy; of 6 colonies fed 100,000 per cubic centimeter, 2 were positive, 1 probable, and 3 remained healthy; of 4 colonies fed 200,000 spores per cubic centimeter, 3 were positive and 1 probable. Thus it was assumed that 50,000 spores per cubic centimeter of sirup could be considered the critical number or minimum infectious dose of spores that will produce disease, when 1 liter is used as the unit volume to be fed.

Since the foregoing analysis of the data indicates, by the method of estimating used, that the actual mean number of spores per field falls well within the limits of permissible error for the estimated actual means (± 3 times the standard error of estimate), the most probable value for such a mean for use in determining the number of spores per cubic centimeter of an unknown sample is the actual mean number of spores per field determined by counting 30 fields each from stained smears from two centrifuged sediments of this sample. If the formula $\overline{X}=1.1228Y+0.2034$ is used to estimate $\overline{X}$, the most probable theoretical number of spores that should have been recovered, when Y represents the actual mean number of spores per field, and if this value is then multiplied by 32,884, the most probable number of spores per cubic centimeter in the unknown sample can be calculated. Applying the limits of error for $\overline{X}$, ± 3 times the standard error of estimate, or ± 0.4374 spore, and carrying it through into the second formula will give the possible range in which the number of spores per cubic centimeter might fall within the precision of the method.

Further work is in progress to determine whether the same accuracy will be obtained by counting a smaller number of fields to obtain the mean number of spores per field from a larger number of smears from sediments.

Since in the experimental work the samples of known spore content contained approximately round numbers of spores—multiples of 50,000—it probably would be sufficiently accurate to designate the number of spores as the nearest multiple of 50,000 to the actual figures derived from the formulas. When using the limits of error 0 ± 0.4374 spore per field, for the estimated mean number of spores per field, it will be found that for numbers below 100,000 there will be some overlapping between 10,000-spore increments, and the value will have to be expressed approximately (for example, the honey contains between 40,000 and 60,000 spores per cubic centimeter); neverthless the honey can still be designated either as dangerous or as not dangerous.

SUMMARY

Previous work on the qualitative demonstration of the presence or absence of spores of *Bacillus larvae* in honey that has been in contact with American foulbrood has been followed by the development of a quantitative method for determining the approximate number of spores per cubic centimeter in such honey. The method is represented by the formula

$$\text{Number of spores per cubic centimeter}=\frac{KNX\times 100\times D}{N}$$

where K is the factor for the number of circular fields per 1-cm^2 area, N is the number of circular fields counted, X is the actual mean number of spores per field, 100 is the factor that gives the number of spores per cubic centimeter from 0.01 cc of the suspension, and D is the dilution. The mean number of spores of *Bacillus larvae* per field counted in 60 fields of stained smears made from the sediments obtained by centrifuging 5-cc quantities of honey containing approximately known numbers of spores have been determined by this method.

The mean actual spore count per field was determined for a series of samples of honey prepared to contain approximately 1,000,000, 800,000, 500,000, 300,000, and 50,000 spores per cubic centimeter. The mean theoretical spore count per field that should have been recovered was determined by use of the formula

$$X=\frac{\text{Number of spores per cubic centimeter}}{32{,}884}$$

The actual mean numbers of spores per field were similar in trend to the calculated theoretical means but were from 10.88 to 15.60 percent smaller. A statistical analysis of the data to determine the accuracy of the method showed that the calculated probable errors and standard deviations were small. The coefficient of correlation between the actual and the theoretical mean number of spores per field for each sample was found to be 0.9999 ± 0.0001.

The relation between the actual mean number of spores per field ($\overline{Y}$) and the corresponding most probable values that should have been recovered, estimated from the theoretical mean number of spores per field (X), was determined by means of the regression equation $\overline{Y}=0.8905X-0.1791$. These most probable estimated values were found to be in excellent agreement with the actual values obtained, well within the customary limits of ± 3 times the standard error of estimate, which was found to be ± 0.1298 spore.

The most probable theoretical mean number of spores per field ($\overline{X}$) was estimated by means of the regression equation $\overline{X}=1.1228Y+0.2034$. These values were found to be in excellent agreement with the original calculated values for the theoretical mean, well within ± 3 times the standard error of estimate, ± 0.1458 spore.

The statistical analysis of the data therefore indicates that the method used is sufficiently accurate for determining the spore content of unknown samples of honey. For this purpose the following formulas are used:

$$\overline{X}=1.1228Y+0.2034 \pm 0.4374$$

where Y=the actual mean number of spores per field counted from 60 fields, and

$$\text{Number of spores per cubic centimeter}=32{,}884\overline{X}.$$