THE NUMBER OF COLONIES ALLOWABLE ON SATISFACTORY AGAR PLATES

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INTRODUCTION

A point which is of much importance in making bacteriological counts is the limit in the number of colonies that may be allowed to grow on a plate without introducing serious errors. Probably every bacteriological worker has this point in mind in making counts and has his own opinion based on experience; but there are few published data on the subject. The matter has been specially under discussion in connection with the proposed revision of standard methods of milk analysis. This investigation was made in order to increase the amount of information available for the use of the Committees who have undertaken the work of revision.

HISTORICAL

It is interesting to note the published opinions of different workers on this point. In 1895 Neisser (1895) published an article in which he reached the conclusion that plates should be so made that they will have about 10,000 colonies per plate, which numbers should then be estimated by means of the low power lenses of a compound microscope. He undoubtedly believed that each bacterial cell put into an agar plate would produce a colony regardless of overcrowding. Three years later Hesse and Niedner, (1898) realizing, to some extent at

[1] The senior author of this paper is responsible for the original suggestion of this problem, for direction in carrying it out, and aid in preparing the esults for publication. The junior author has carried out the laboratory work and has helped in preparing the results for publication.

least, the true state of affairs published an article in which they claim that plates having more than 100 colonies should be disregarded and that under these conditions the microscope should not be used for counting. In 1897, Hill (Hill and Ellms, 1897) contended that overcrowded plates would not give reliable results in water analysis. In 1899, Jordan and Irons (1899) independently urged the same thing. Again Hill (1908) called attention to the point in a paper read before the laboratory section of the American Public Health Association in 1907, in which he pointed out that wide discrepancies in counts might be caused by different methods of computation and concluded that only those plates having numbers of colonies falling between 40 and 200 per plate should be considered in reporting results. These figures were adopted in the report presented by the Committee on Standard Methods for the Bacterial Examination of Milk at the Richmond meeting of the American Public Health Association (1910). In the Report presented at the Rochester meeting in September 1915 (Comm. Stand. Meth. 1915) the lower limit in the number of colonies allowable on agar plates was changed from 40 to 30, and the limits of 30 and 200 were also accepted by the Committee on Standard Methods of Bacterial Water Analysis in their Report presented at the same meeting.

STATEMENT OF PROBLEM

It is generally recognized that the kind of bacteria present in the material under examination will have an influence on the size of the colonies, and, consequently, on the number that can develop on a plate. Some of the commonest and most important bacteria in milk do not produce colonies larger than pin points on ordinary agars even when only a few are present. Other colonies grow large and in the case of spreaders may cover the entire plate.

Just what prevents the development of all the bacteria into colonies on crowded plates is not thoroughly understood. In some cases it may be because the food material is all used up;

in others it is clearly due to the fact that by-products of bacterial growth inhibit the growth of other colonies; and occasionally colonies fuse or overgrow each other and so reduce the count. On the other hand colonies growing side by side sometimes stimulate each other, a phenomenon which has been noted in this work on plates containing large numbers of *B. bulgaricus* with an occasional mold or bacterial colony of a different type. The molds and many bacteria so stimulate the *B. bulgaricus* that these organisms form visible colonies in the region of the larger colonies, failing to develop in all other parts of the plate. The same condition has been noted in plating material containing large numbers of long chained streptococci. This phenomenon naturally produces marked irregularities in count when it occurs.

Because of these and other difficulties certain plates in any series made from a given sample are more satisfactory for use in computing a total count than are others. The matter of selecting plates to be used in computing a count becomes therefore a matter requiring considerable judgment.

EXPERIMENTAL DATA

a. Analyses made in the Station Laboratory

The object of this study has been to determine the limits in the number of colonies on plates which are satisfactory for making bacterial counts. The data used have been obtained by plating market milk samples on standard agar in triplicate and in three different dilutions, incubating for five days at 21°C., following with an incubation for two days at 37°C. The plates were counted at the end of five days and again after the two days incubation at 37°C. The five day and seven day counts are tabulated separately and show the conditions for each period of incubation.

In deciding which plate counts to select as probably nearest correct it became necessary to discard all of the counts on a few samples where no satisfactory average could be made because of spreaders or because the milk contained more bacteria

than was anticipated and the dilutions were not carried far enough to give assurance that the count was not affected by overcrowding. In selecting individual plate counts which were to be tabulated as satisfactory, those counts were chosen which could be used in making an average without any individual figure varying more than 20 per cent from the average. All others were listed as discrepancies. For example, one sample gave the following counts per plate, 1: 100 dilution 1944, 1472 and 1928 colonies; 1: 1000 dilution 484, 515 and 610 colonies; 1: 10000 dilution 43, 45, and 46 colonies. The counts of 484 and 515 from the 1: 1000 dilution were averaged with the 1: 10000 counts of 43, 45 and 46; and this average was taken as the final count on the sample. The counts made on the 1: 100 plates were all listed as discrepancies because they are more than 20 per cent lower than the average, and the count of 610 from one of the 1: 1000 plates was also listed as a discrepancy because it was more than 20 per cent higher than the average. Occasionally all of the nine plates made from a sample could be included in the final average.

Table I gives the number of plate counts made after five days of incubation at 21°C., arranged in groups according to the number of colonies which appeared on the plates. Four hundred and thirty-nine of the 1435 plates had less than 10 colonies per plate. Only 22.3 per cent of these checked within the 20 per cent limit. One hundred and eighty plates fell in the group having more than 10 and less than 20 colonies per plate. Of these 53.9 per cent checked within the 20 per cent limit. Percentages calculated for the groups of plates having 20 to 30, 30 to 50, 50 to 100, 100 to 200 and 200 to 400 colonies per plate were more or less variable, showing that from 66.3 per cent to 93.2 per cent of the total number of plates agreed within the 20 per cent limit. The best percentage of agreement is shown by the group having more than 100 and less than 200 colonies per plate, and the next highest by the group having between 50 and 100 colonies per plate. There were decidedly fewer plates giving satisfactory results among those which had more than 400 colonies per plate, the percentage of plates which checked within 20 per cent being 44.4.

The results given in the lower part of table 1 were calculated from the same counts, the groups of plates having been arranged differently. From this part of the table it will be seen that the percentage of discrepant plates is practically the same for the groups of plates having 20 to 400, 30 to 400, 20 to 200, 30 to 200, or 40 to 200 colonies per plate, the best showing being made by the group of plates having more than 40 and less than

TABLE 1

Plate counts after incubation at 21° C. arranged to show the number and percentage of counts in groups according to the number of colonies per plate

GROUP	CHECKED WITHIN 20 PER CENT OF AVERAGE		DISCREPANT PLATES, DID NOT CHECK WITHIN 20 PER CENT OF AVERAGE				TOTAL NUMBER OF PLATES IN GROUP
	Number	Per cent	Too low	Too high	Total number	Per cent	
0 to 10	98	22.3	172	169	341	77.7	439
10 to 20	97	53.9	29	54	83	46.1	180
20 to 30	54	72.9	6	14	20	27.1	74
30 to 50	67	66.3	11	23	34	33.7	101
50 to 100	162	84.8	17	12	29	15.2	191
100 to 200	179	93.2	8	5	13	6.8	192
200 to 400	105	78.9	25	3	28	21.1	133
Over 400	100	44.4	114	11	125	55.6	225
0 to 30	249	35.9	207	237	444	64.1	693
20 to 400	567	82.0	67	57	124	18.0	691
30 to 400	513	83.1	61	43	104	16.9	617
20 to 200	470	82.9	43	54	97	17.1	567
30 to 200	416	84.3	37	40	77	15.7	493
40 to 200	376	86.0	23	28	61	14.0	437
Over 400	100	44.4	114	11	125	55.6	225

Total number of counts summarized in this table 1435.

200 colonies per plate. Plates having less than 30 colonies or more than 400 colonies show very large percentages of discrepancies.

Table 2 gives the results obtained by counting 1056 of the same plates as those whose counts are summarized in table 1 after two days of additional incubation at 37°C. In general the results obtained from these counts are similar to those given in table 1. However the best showings are made in this case by groups of plates having more than 200 and less than 400 colo-

nies per plate (87 per cent of satisfactory plates), the group of plates having 100 to 200 colonies (82.4 per cent) and the group having 30 to 400 colonies per plate (81.4 per cent). As in table 1 there is a marked increase in the number of discrepant counts from plates having less than 30 or more than 400 colonies per plate While the results in table 1 favor the 40 to 200 group rather than the 30 to 400 group by 2.9 per cent., the same

TABLE 2

Plate counts after two additional days of incubation at 37° C. arranged to show the number and percentage of counts in groups according to the number of colonies per plate

GROUP	CHECKED WITHIN 20 PER CENT OF AVERAGE		DISCREPANT PLATES, DID NOT CHECK WITHIN 20 PER CENT OF AVERAGE				TOTAL NUMBER OF PLATES IN GROUP
	Number	Per cent	Too low	Too high	Total number	Per cent	
0 to 10	60	28.4	60	91	151	71.6	211
10 to 20	76	60.0	23	28	51	40.0	127
20 to 30	46	63.0	8	19	27	37.0	73
30 to 50	55	72.3	5	16	21	27.7	76
50 to 100	117	81.0	14	12	26	19.0	143
100 to 200	127	82.4	16	11	27	17.6	154
200 to 400	101	87	14	1	15	13	116
Over 400	78	50	74	4	78	50	156
0 to 30	182	44.2	91	138	229	55.8	411
20 to 400	445	79.2	57	61	117	20.8	562
30 to 400	399	81.4	49	42	91	18.6	490
20 to 200	353	77	45	60	105	23	458
30 to 200	307	79.7	37	41	78	20.3	385
40 to 200	277	79.8	36	34	70	20.2	347
Over 400	78	50	74	4	78	50	156

Total number of counts summarized in this table 1056.

comparison in table 2 shows an advantage of 1.6 per cent for the 30 to 400 group This indicates that there is little advantage in selecting one group of plates in preference to the other.

In the fourth and fifth columns of these two tables, the number of cases is shown in which the discrepancy was caused by having too few or too many colonies on the plate Arranging the plates in the groups 0 to 10, 10 to 20, 20 to 30, 30 to 50, 50 to 100, 100 to 200, 200 to 400 and more than 400 colonies per

plate, it is seen that there is a tendency for discrepancies caused by having too many colonies on a plate to occur in all groups having less than 50 colonies per plate (one exception to this statement is seen in the group 0 to 10 in table 1). In all cases where more than 50 colonies occurred on the plates, the greater number of discrepancies was caused by having too few colonies on the plates. The tendency toward discrepancies caused by having too few colonies on the plates becomes very marked as soon as the limit of 200 colonies per plate is passed.

These findings indicate that while the greater proportion of the discrepancies on plates having less than 50 colonies per plate are caused by the operations of the laws of choice and chance, yet there is some factor present which tends to cause more colonies to develop than should do so. In all probability this factor is chance contamination from the air which occurs during planting. As is well known, it is common for supposedly sterile check plates to develop one, two or more colonies on prolonged incubation. The presence of these colonies on inoculated plates having fewer than 50 colonies per plate causes a relatively large error in the counts which in some cases would cause the individual plate count to exceed the 20 per cent limit specified here as necessary before the plates were classed as satisfactory.

The tendency for irregularities, due to having too few colonies on plates, to occur in counts having 50 or more colonies per plate is too well known to all bacteriologists to require extended discussion. These are undoubtedly caused by the effect of overcrowding. The fact that not all of the discrepancies on plates having more than 400 colonies per plate were of this sort is more significant, for it shows that not all of the discrepancies on plates having numerous colonies are due to overcrowding. Irregularities in the number of bacteria used in inoculating or chance contaminations are two things which might produce plates having too many colonies even on crowded plates.

When all of these things are taken into consideration, it becomes a difficult matter to decide upon the limits in number of colonies which should be allowed on plates. It is at once

clear that plates having less than 20 and more than 400 colonies are so apt to be widely discrepant that counts from plates of this sort should be disregarded. There are likewise clear indications that plates having between 40 and 200 colonies per plate are as satisfactory as any that can be selected. However the results secured in this investigation do not indicate that serious errors would be introduced in routine work by extending these limits to 30 and 400, or even to 20 and 400, thereby lessening the amount of work necessary to secure acceptable counts.

b. New York City analyses

Another set of data which is more satisfactory in one way because of the fact that a very large number of plates were made from a single sample of milk but which is also less satisfactory in another way because of the fact that it is more limited in its application, has been secured from a set of analyses made on November 19, 1915, by five New York State laboratories,[2] under the supervision of Prof. H. W. Conn. In this series 20 samples of the same milk were sent to each laboratory for analysis. Four laboratories made plate counts, one making them in duplicate, so that five sets of plate counts are available. These were made from two dilutions of 1: 100 and 1: 1000 each. Two plates were made for each dilution. Three laboratories made microscopic counts, one making them in duplicate so that four sets of these counts are available.

The average of the accepted plate counts was 4250. The average of the microscopic counts of clumps, or sources, was 5590. The close correspondence in results obtained by these two very different methods of counting makes it very probable that the total number of groups of bacteria in this milk was close to 5000 per cubic centimeter. The 1: 100 dilution plates gave counts in which the average number of colonies on the two plates varied between 24 and 125. The 1: 1000 plates gave counts in which the average number of colonies from the two plates varied be-

[2] Lederle Laboratories, North's Sanitary Laboratories, N. Y. City Board of Health Laboratory, Borden's Laboratory, N. Y. Agric. Exp. Sta. Laboratory.

tween 0.5 and 16.5 with a single case where the average of the two plates was 44.

If we arbitrarily assume that plates giving a count more than 2500 above or below the average fail to check with the accepted count, we find that the averages of all but three of the 100 pairs of 1: 100 plates check with the accepted count while there are 27 cases out of the 100 where the count from the 1: 1000 dilution fails to check within these limits. It is important to note also that 23 of these 27 cases are instances where the discrepancy was such as to give a higher count that the accepted count, indicating that chance contaminations were probably the chief cause of trouble.

SUMMARY

1. The work here reported includes a study of the counts made from 1435 agar plates inoculated from samples of market milk and incubated five days at 21°C.; and also a study of the counts made from 1056 of the same plates after two days additional incubation at 37°C. The results obtained indicate that, for milk analyses, the counts made from plates having more than 30 and less than 400 colonies on the plates are very nearly as satisfactory as those obtained from plates having more than 40 and less than 200 colonies, the latter being the limits in numbers originally recommended by the Committee on Standard Methods for the Bacterial Examination of Milk.

2. Plates having less than 20 or more than 400 colonies on them are shown to be so frequently discrepant that counts obtained from them should never be trusted unless checked by comparison with plates from different dilutions having more than 30 or less than 400 colonies. The acceptance of counts from plates having 20 to 30 colonies per plate would not greatly increase the percentage of discrepancies.

3. All groups of plates, regardless of the number of colonies showed a certain percentage of plates which gave counts which varied more than 20 per cent from the accepted count. The percentage of discrepant counts of this sort varied between 37

and 7 for all groups of plates having more than 20 and less than 400 colonies per plate, the worst showing being made by the plates having 20 to 30 colonies per plate and the best by the plates having 100 to 200 colonies per plate.

4. The discrepancies which occurred in counts made from plates having less than 50 colonies per plate were more frequently caused by too many colonies on the plates than by too few colonies. This excess is undoubtedly due to the influence of chance air contaminations which took place during the plating. Where the plates have a small number of colonies on them a few extra colonies of this sort produce relatively wide discrepancies.

5. The discrepancies in counts made from plates having more than 50 colonies per plate were more frequently caused by having too few rather than too many colonies on the plates. The frequency of this type of discrepancy became very marked where the number of colonies exceeded 200 per plate. The probable explanation of the excess of this type of irregularity is that of overcrowding. Since however there was always a certain percentage of discrepancies caused by having too many colonies on the plate even where there were more than 400 colonies per plate, it is evident that not all of the irregularities are caused in this way.

6. Counts made from 20 duplicate samples of the same milk in five series of analyses showed 27 out of a possible 100 wide discrepancies in the counts obtained from an average of two plates made from a 1: 1000 dilution. The number of colonies of these plates averaged more than 0.5 and less than 16.5 for the two plates, with one exception where the average was 44. Counts made from the 100 pairs of 1: 100 plates which had more than 24 and less than 125 colonies as the average of the two plates, showed only 3 out of a possible 100 wide discrepancies.

REFERENCES

HESSE, W. UND NIEDNER. (1898) Die Methodik der bakteriologischen Wasseruntersuchung. Zeitschr. f. Hyg. u. Infektionskrankh. **29**: 454–462,

HILL, H. W. AND ELLMS, J. W. (1897) Report on Brooklyn Water Supply. pp. 164–169.

HILL, H. W. (1908) The Mathematics of the Bacteria' Count. Amer. Jour. Pub. Hyg. **18** (N. S. 4): 300–310.

JORDAN E. O. AND IRONS, E. E. (1899) Notes on Bacterial Water Analysis, Public Health Papers and Reports Amer. Pub. Health Ass'n. **25**: 564–569.

NEISSER, MAX. (1895) Die mikroskopische Plattenzählung und ihre specielle Anwendung auf die Zählung von Wasserplatten. Zeitschr. f. Hyg. u. Infektionskrankh. **20** 119–146.

Report of the Committee on Standard Methods of Bacterial Milk Analysis. 1910. Amer. Jour. Pub. Hyg. **20** (N. S. 6): 315–345.

Report of the Committee on Standard Methods of Bacterial Examination of Milk. 1915. Amer. Jour. Pub. Health, **5**: 1261–1262.