IS THE STATISTICAL METHOD APPLICABLE TO THE BACTERIAL PLATE COUNT?

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Statistical analyses of biological data are quite common today. The method is applied to various types of data, oftentimes distinctly to the detriment of well established practices which are supported by opinions of capable men. The recent criticisms of the Koch plate method for the enumeration of bacteria in milk is a case in point. Much good has come from the use of the agar plate in the past. Through its agency as a control measure, our milk supplies as a whole are immeasurably better now than ever before. That there are faults in the method cannot be denied; duplicate plates often vary to a large degree. Variations in plate counts are obtained by different workers with the same milk. The explanation is not far to seek when one studies the report of the Referee in which the needless variation of a supposed "Standard Procedure" is shown to exist (Breed, 1926).

The criticisms that the statistician makes are of a much more serious nature. Wright and Thornton (1927), using the same medium, the same methods, by the same operator, report that a "variation of less than 100 per cent within a series cannot be depended upon . . . the extreme variation was 1500 per cent. The average coefficient of variability for all counts was 25 per cent. High and low extremes were 57 and 15 per cent." If all of this is generally true then the final statement in their brief report is likewise true when they say that "this method is no more accurate for grading milk than other means available." It must be borne in mind however that with the agar plate as a foundation there has been built up a very stable system of milk control. Destroy confidence in the agar plate and milk control will suffer.

The statistical method in itself as a mathematical procedure is free from criticism but there are many pitfalls for the unwary in the unrestricted use of the method. E. B. Wilson (1923) has pointed out that "a method is a dangerous thing unless its underlying philosophy is understood and none more dangerous than the statistical. Our aim should be with care to avoid, in the main, erroneous conclusions. In the long run it is only clear thinking . . . that wins the strong hold of science."

The results reported by Wright and Thornton were so pointed that we decided to determine for our own working conditions the probable error of the plate method. We plated nine samples of milk in a single series using 75 plates to a series. In this work the procedure in every detail followed the accepted standard methods. The dilutions were chosen after a direct count had been made upon the milk, and were so made that the dilution used was one of 100 cc. From this dilution bottle 75 plates were made which were previously marked so that the first cubic centimeter of the dilution was placed in the plate marked one, the second in the plate marked two, and so on to the end. One pipette was used for 10 plates and then discarded. In this work only calibrated pipettes were used to avoid error from this source. Twelve-cubic-centimeter amounts of agar were kept in tubes at a constant temperature of 40°C. and this was poured into the plates, twenty plates being poured at a time as the experiment progressed. The plates were stacked in the incubator in piles of six, with spaces of one or two inches between the stacks. They were incubated for forty-eight hours at 37°C. after which time they were counted. Throughout the experiment the same person performed all the operations. The incubator is a room 10 feet on a side in which a fan was kept in motion.

The data so obtained are summarized in table 1 in which are given the low, high and mean counts for each of the nine trials. The calculated standard deviation and coefficient of variability are also given.

It is quite apparent that in the first five trials the results indicate that from a statistical point of view the method is of little value. The spread from low to high in each case is very large.

It was apparent however in studying the tabulated data from which the summaries of table 1 were taken that the high counts usually came from the plates made at the end of the experiment. This was so apparent that a graph was made in which the bacterial counts were expressed as ordinates and the number of plates as abscissae. It will be recalled that when the plates were made the precaution was taken to number the plates in the order in which they were used, thus giving a measure of time. When results of each of the experiments were plotted there were disclosed some interesting facts. Since trial no. 1, however, is quite typical

TABLE 1
High and low bacteria counts with statistical constants for nine milk samples

TRIAL	LOW	HIGH	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY
					<i>per cent</i>
1	38,000	1,600,000	292,506 $\pm$ 30,531	392,000 $\pm$ 21,607	134.0
2	58,000	490,000	183,693 $\pm$ 7,406	95,100 $\pm$ 5,240	51.4
3	3,000	60,000	13,773 $\pm$ 661	8,490 $\pm$ 467	61.6
4	5,000	110,000	26,653 $\pm$ 2,000	25,700 $\pm$ 1,416	86.4
5	48,000	620,000	138,333 $\pm$ 8,956	115,600 $\pm$ 6,370	83.7
6	53,000	148,000	108,640 $\pm$ 1,456	18,700 $\pm$ 1,030	17.0
7	37,000	63,000	46,346 $\pm$ 487	6,260 $\pm$ 344	13.5
8	6,000	66,000	21,013 $\pm$ 804	10,330 $\pm$ 569	49.0
9	6,000	118,000	34,013 $\pm$ 1,853	23,800 $\pm$ 1,311	69.9

it may be discussed at length since the other four of the first five are all similar and differ from it but slightly. In making the graphs the scale of the ordinates has been changed in each case to fit the spread of the counts for the experiment. It will be seen from figure 1 that while the counts vary but little for the first 40 plates poured there is a sharp rise in the count at this point. The mean of the 75 is 292,000 and is indicated in the figure, but it lies well above the range of the count of the first 40 plates whose mean is shown as 62,000. There are a few counts near the end of the trial which are also low but the majority after the first 40 steadily rise to a high point of 1,600,000. This increase in number of bacteria in the plates made toward the end of the experiment

cannot reasonably be considered as due to the growth of the organism since the temperature of the milk was about 50°C. and that of the dilution water about 20°C., while the average time necessary to make the 75 plates was less than 90 minutes. It is also unlikely that the increase in the number of organisms in the plates could be due to carelessness since it would have taken from

FIG. 1. DISTRIBUTION OF BACTERIA COUNTS THROUGH 75 PLATES OF TRIAL I, SHOWING MEAN OF THE 75 PLATES AND MEAN OF THE FIRST 40

20 to 40 cc. of the dilution to obtain the high counts recorded. Obviously a coefficient of variability of 134 per cent indicates that the mean value of all the counts in experiment 1 is meaningless. The mean of the first 40 plates for all of the trials was next calculated.

TABLE 2
Statistical constants for 75 plates compared with those of the first 40 plates

TRIAL	MEAN		STANDARD DEVIATION		COEFFICIENT OF VARIABILITY	
	75	First 40	75	First 40	75	First 40
					per cent	per cent
1	292,506 ± 30,531	62,970 ± 1,739	392,000 ± 21,607	16,300 ± 1,229	134.0	25.8
2	183,693 ± 7,406	134,400 ± 4,909	95,100 ± 5,240	46,000 ± 3,470	51.4	34.0
3	13,773 ± 661	11,625 ± 368	8,490 ± 467	3,450 ± 260	61.6	29.6
4	26,653 ± 2,000	15,150 ± 673	25,700 ± 1,416	6,310 ± 481	86.4	41.6
5	138,333 ± 8,956	83,100 ± 1,430	115,600 ± 6,370	13,400 ± 1,010	83.7	16.1
6	108,640 ± 1,456	103,850 ± 2,113	18,700 ± 1,030	19,800 ± 1,493	17.0	19.0
7	46,346 ± 487	44,255 ± 515	6,260 ± 344	4,830 ± 364	13.5	10.0
8	21,013 ± 804	19,050 ± 1,000	10,330 ± 569	9,380 ± 707	49.0	49.2
9	34,013 ± 1,853	17,375 ± 1,163	23,800 ± 1,311	10,900 ± 822	69.9	62.7

In table 2 the means for both the 75 plates and the first 40 plates are given together with the statistical constants. It will be seen that the coefficient of variability for trial no. 1 decreases from 134 per cent for the 75 plates to 25 per cent for the first 40 plates. The plates used in a routine analysis would be the first two and very little variation is found.

FIG. 2

Wright and Thornton report a coefficient of variations of 57 per cent. It is our suggestion that these authors failed to take into consideration the time factor which is evidently of importance. Had we not considered this in our work and had we counted plate number one first, plate 75 second, plate 2 third, and so on, we would have found the same coefficient of variability we did

find, 134 per cent, but there would have been no explanation for it with a resulting misinterpretation of our findings. The reason for the increase in plate counts in the plates numbered above 40 is not known. It was thought that the clumps broke up on standing, but a microscopical study of the dilutions did not substantiate this opinion, although it seems to be the only

FIG. 3

rational explanation. It is also suggested that clumping is found at the normal pH of the milk which is offset by the addition of the milk to the water and, although the microscope does not disclose the fact, the clumps may be less firmly held together with the change in pH. We are at present working upon an explanation which will be reported in the future.

Figures 2 to 5, which are obtained from trials 2 to 5, show much the same increase in counts in the higher number plates. The decreases in the statistical constants are shown in table 2.

Figures 6 to 8 show what might be considered normal distribution of counts. Figure 6 is interesting since it shows a slight but progressive increase in counts, although the coefficient of varia-

FIG. 4

bility is low. Further mention of this trial will be made later in this paper.

Trial 9 is of more than passing interest. A low count of 6000 and a high count of 118,000 are recorded. Plotted as a scatter diagram in figure 9 it will be seen that there are two distinct groups of counts; one from the plates numbered below 40 and the

other from the plates numbered above 40. The counts are higher after the first 40 plates but the increase is not a gradual one as in the first five trials. The mean for the 75 is 34,000, that for the first 40, 17,000, but the coefficients of variability are the same; indicating that they both have the same relative magnitude of error. This is doubtless true mathematically, but from the

FIG. 5

standpoint of the milk control a range of 6000 to 37,000, high though it may be, is more to be desired than the far greater one of 6000 to 118,000.

The foregoing would resolve itself into the question: Can we use the coefficient of variability or attempt a measure of relative variability as seems to be the increasing practice? Pearl (1927)

says that particular constant has never been an entirely satisfactory one for biologists to use. We think that Pearl is right. In support of this statement let us assume two hypothetical cases which incidentally are not far removed from a possibility in milk

FIG. 6

control. Assume that we have two sets of data; as in table 3 (a) with nine scores of 10 and one of 100, and (b) nine scores of 100 and one of 10.

The mean for (a) is 19, for (b) 91. The standard deviation would be the same in each case. The coefficient of variability of (a) is 144 per cent, while that of (b) is but 29 per cent, but the

FIG. 7

TABLE 3

	(a)	(b)
	100	10
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
Mean.....	19	91
Standard deviation.....	27	27
Coefficient of variability.....	144.4%	29.7%

standard deviation is the same. The reason for this is that in the formula

$$V = \frac{\sigma \times 100}{m}$$

the m is in the demoninator. A large m would give a small V and conversely. It is our opinion that this is a partial explana-

FIG. 8

tion of the low coefficient of variability for trial 6 as compared to the high value of 49 per cent for the same constant for trial 8.

This paper is frankly critical; its object is to point out constructively consequences of too close adherence to a mathematical formula. It would seem well then to mention two recent publica-

tions in which the coefficient of variability has been determined in studies upon the agar plate method. Norton and Seymour (1926) studied the variations resulting from plating a number of samples upon five different media. A statistical analysis of the results was attempted by tabulating the average total counts, the average deviation from the average counts, standard deviation and the

FIG. 9

coefficient of variability. Their average, however, was an average of the variations in the milk samples and has nothing to do with the method. There is no statistical reason for such average being taken and certainly no justification for calculating standard deviations from an average so taken, yet they cite coefficients of variations from 63 to 79 per cent which are abnormally large.

Ellenberger et al. (1927) also have apparently missed the point in a paper comparing the methylene blue reductase test to the agar plate count. They made trials on 79 milk samples. Each sample was divided into six smaller samples and two plates and two tubes were used which were supposed to furnish an opportunity to determine the coefficient of variation for both the agar plate count and the reductase test. It is a question whether they can assume that the six samples taken from the larger sample are all similar. If they are similar then the 12 plates and 12 tubes are hardly enough to constitute a fair sample. If the six samples are not to be considered as similar, and we doubt that they are, then the two plates and the two tubes are certainly not representative; yet they conclude that the "reduction test shows much less variability between check or duplicate tests than does the agar plate method" and they *apparently* have the statistical evidence to prove their statement.

It has not been our intention to discredit the statistical method *per se*; such an attempt would be foolhardy since its value to science is beyond cavil. It is open to question however whether increasing the "scores" obtained from a milk-water mixture is a correct statistical procedure. It is suggested that the key to the difficulty is the statement found in "Standard Methods of Milk Analysis" (1923) to the effect that "in no case should the interval (after the dilution of the milk and before the agar is poured) be allowed to exceed twenty minutes." It would seem from the work reported here that the milk-water mixture is not stable and that dispatch in completing the operation is of considerable importance. When it is fully understood that the bacterial counts cannot be used to split fine differences in grades of milk, but that counts can be used as a splendid means to the end of better milk control, the so-called "errors" will be considered less and less, for after all "probably the best safeguard against error is the certain knowledge on the part of the investigator that error is always possible and seldom absent. Dull the appreciation of this fact by insistence on infallibility of any formula and the way to error is thrown open" (Salmon, 1923).

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