COMPARISON OF THE OVAL TUBE WITH THE STANDARD PLATE COUNT METHOD FOR DETERMINING VIABLE COUNTS OF RAW MILK

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SUMMARY

Standard plate counts (SPC) and oval tube counts (OTC) were compared and analyzed by the statistical method on 547 samples of raw milk from the Wichita milk shed. 389 of these milks were paired as routine two-dilution standard plates vs. single tube estimates of viable bacteria; 127 were set in duplicate by each method; and 31 samples were set in replicates of five for the purpose of two-factor variance analysis. Using the criterion of the IAMFES Committee on Applied Laboratory Methods, the experimental results indicated that the comparison odds were approximately even for equivalent counts between methods (48.9% of samples); two out of five random samples showed that the oval tube counts were higher (39.6% of samples) and that one out of ten of the standard plate counts was superior (11.5% of samples). Also, revised data of differences between two-dilution SPC's and single OTC's that passed the chi-square test for goodness of fit for a normal distribution gave a significant difference of means at the 1% level (89 samples). However, with another trial run, two-factor analysis did not yield a significant difference between methods (33 samples x 5 replicates), and the F ratio of the means of the pooled variance data showed no difference in precision between methods; the interaction between samples and methods was highly significant. A comparison of the results from these experiments with data obtained in five other laboratories over a span of 25 years is shown in Table form. It was concluded that there is no significant difference between the two methods where paired estimates are based on replication, but the oval tube is preferred where time and economy are important.

Among the several miscellaneous microbiological methods that may be substituted for the standard plate count is the oval tube technique *(1)*. This rapid method which evolved from the Burri quantitative smear culture of the 1930's has the well known advantages, first discussed by Myers and Pence *(10)*, of taking one-half the time of standard plating, of using one third the amount of agar, of eliminating dilution blanks and pipettes and requiring less incubator space. And, although the general consensus of opinion by authorities *(5, 8)* of this field that the oval tube is essentially equivalent or compares favorably with the standard plate count, Chapter 9 of "Standard Methods" *(1)* suggests that there is still wide opportunity for systematic and critical studies of any of the rapid methods in comparison with the conventional standard.

A loop method using an agar slope for counting bacteria was originally described as a quantitative smear culture by Burri in 1928 *(9)*. Later, Dorner found that the agar slope method gave higher counts than the standard Petri plate procedure *(7)*. In 1937, G. S. Rydzewski modified the Burri technique by using multiple plates *(10)*. Hucker and Haynes *(9)* found that 73% of the milks examined gave higher counts with the Burri slants as compared to 27% equivalent counts. Myers and Pence *(10)* felt that both the Burri and Rydzewski methods had certain disadvantages due to spreaders, coalescing colonies, the surface dry-slant requirement, etc., and introduced the standard 0.001 ml loop filled with pasteurized milk directly into melted agar at 45 C. With this oval tube method, they obtained geometric means of 1879 (SPC) and 1816 (OTC) for 76 comparisons involving thermoduric bacteria. Donnelly, Black, and Lewis *(5)*, found no significant difference between the oval tube and the standard plate count except that the former showed statistically better agreement between duplicates. With a technique using a 0.001 ml calibrated loop attached to a continuous volume syringe for rinsing a sample into a standard Petri dish, Thompson et al. *(14)* found this method equivalent to the standard plate count in a study of 85 samples used in four experiments.

PROCEDURES

The milk samples used in the following experiments were picked up by the Wichita City-County Health Department sanitarians at farms from 250 gallon bulk tanks. These samples were drawn with 18-inch straw pipettes, transferred to sterile bottles or plastic bags, placed in an ice chest, and brought to the laboratory where they were held at 2 C for not more than 24 hr before initial plating.

Only 10 samples per day were picked at random to plate and loop until it was desired to increase the number of samples in certain ranges. The procedure was thus modified to include an immediate oval tube count in order to find samples of a certain category, and these milks were held at 2 C until ready for use. After shaking the bottles or bags vigorously 25 times, the standard plate method was first performed while the milks were held at 2-5 C in the refrigerator. The small elapse of time that did not exceed 20 min in the refrigerator allowed the foam to break before the loop was inserted 2-3 mm below the surface.

TABLE 1. ANALYSIS OF OVAL TUBE AND STANDARD PLATE COMPARISONS OF 547 PRODUCERS SAMPLES ACCORDING TO CRITERION OF THE IAMFES COMMITTEE ON APPLIED LABORATORY METHODS (2)

Experiment	Category or range (Thousands/ml)		Equivalent counts		Significantly higher OTC		Significantly higher SPC		Totals	
			No.	%	No.	%	No.	%	No.	%
1	I	(1-9)	40	43.5	38	41.3	14	15.2	92	100
	II	(10-29)	57	49.0	49	42.4	10	8.6	116	100
	III	(30-99)	47	44.5	38	35.7	21	19.8	106	100
	IV	(100-300)	24	51.0	17	36.2	6	12.8	47	100
	V	(> 300)	16	57.0	11	39.3	1	3.7	28	100
Total or Weighted Average			184	47.3	153	39.3	52	13.4	389	100
2	I-V		66	52.0	54	42.4	7	5.6	127	100
3	I-V		17	54.8	10	32.4	4	12.8	31	100
Grand Total and weighted Means			267	48.9	217	39.6	63	11.5	547	100

Standard methods for the Examination of Dairy Products, 11th ed. *(1)*, were followed for both the standard plate count and the oval tube. Special attention was given to preliminary tests for growth inhibition or stimulation and tests for toxicity of the distilled water. Freshly prepared Difco Plate Count Agar, whose pH was verified electrometrically, and never held beyond 48 hr in the oval tubes or kept in screw cap bottles for the standard plate count, was used throughout the experiments. All counts recorded were averages of at least two count-readings whose difference was never greater than 5%.

The 0.001-ml welded platinum-rhodium loops, Lot no. 24105 2, were purchased from the Central Scientific Company. Four of the loops were calibrated gravimetrically by rapid weighings of milk-loaded loops and an average volume of 9.69×10^{-4} (SD 6×10^{-6} ml) was recorded. This work was checked by measuring the diameters at $1.46 \pm$ SD 0.02 mm and the volume calculated as 7.9×10^{-4} ml. The discrepancy of 1.8×10^{-4} ml between the gravimetrical and metrical method for determining the volume of the loop could be accounted for as the difference in viscosity between milk and water and was observed as the greater adhesion of the former to the "doughnut-rim" of the metal loop. This observation will be referred to later to help interpret results.

The rules of Section 3.33, Standard Methods of Dairy Products, 11th ed. *(1)*, for estimating colonies on crowded plates were followed exactly for the standard plate count. This provides for a lower limit of estimated counts at 5 colonies/cm^2 which would be equivalent to a total count of only 85 colonies with the oval tube due to the OTC/SPC area-ratio of 1:3.8. By using a 4X hand lens in conjunction with a dark-field Quebec counter for both methods, counts of the oval tube were not estimated unless the density exceeded 18 colonies/cm^2; or, in other words, no quantity less than 300 was a factor-count by either method.

RESULTS AND ANALYSIS

The analysis of three experiments in which the oval tube method was compared to the standard plate is summarized in Table 1. Experiment 1 involved a comparison of single tube and standard two-dilution plate counts from 389 producers samples. Experiment 2 consisted of 127 samples done in duplicate by each method while the counts of Experiment 3 were averages of replicates of five by each method. Table 1 shows that the largest percentage (54.8%) of equivalent counts occurred in Experiment 3. Results of Experiment 3 differ, also, from the other two by producing the least percentage (32.4% as compared with 39.3%, and 42.4%) of significantly higher OTC according to the arbitrary 80% rule set by the IAMFES Sub-committee *(2)*. Results of experiment 1 and 3 agree on the percent of significantly higher SPC's. The weighted averages of the grand totals of the three experiments indicate that only about half of the samples gave equivalent counts while there were approximately four times as many significantly higher OTC's than SPC's. Reasons for this interaction between samples and milks will be discussed in a later paragraph.

The breakdown of the results of Experiment 1 into five categories according to range is given in Table 1 and shown graphically in Figure 1 where degree of agreement is recorded as a mean concordance ratio with a 95% confidence interval. By inspection the chart indicates, with the exception of the very low counts of Category 1 which actually has only one significant figure, that there is no significant difference among the mean arithmetic ratios of the five categories. Also, if the true mean log ratio is no higher than 1.01, we can conclude that the average of the comparisons shows equality of methods. Also, such a low ratio is derived from logs whose variance would be less than the critical 0.012 set by Donnelly, et al. *(6)* for acceptability of observed results reported between a pair of SPC's by a single analysis, or between SPC's reported by two different analysts. But if the true value of the ratio is as high as 1.02, the results cannot be accepted as equivalent by the same critical standard.

The results of Experiment 1 were also analyzed by selecting at random twenty paired counts from each

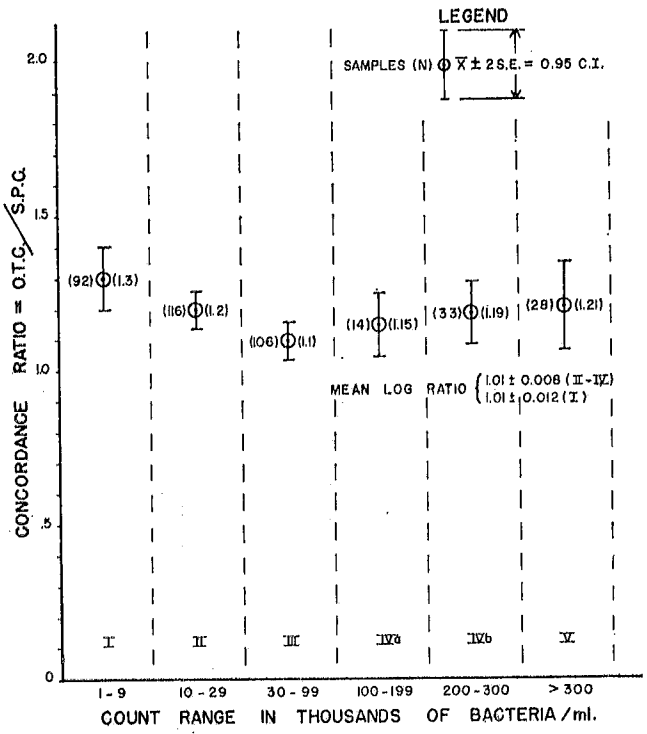

Figure 1. Mean Arithmetic Ratio's and Mean Log Ratio's with 95% Confidence Intervals of Viable Count Densities of 389 Raw Milk Samples Comparing Oval Tube with the Standard Plate Method.

of the five categories. Revised data of the square roots of counts passed a goodness of fit test for a normal distribution only after rejecting eleven paired counts from the original hundred. With these 89 paired counts, the "Student's" t statistic was 3.92 and highly significant in rejecting the null hypothesis that the means were equal. However, the result was not taken too seriously since the data were both revised and the counts based on a single dilution plate and oval tube.

In Experiment 2, 127 samples of raw milk were set in duplicate; the 52.0% equivalent counts were slightly higher than the 47.3% of Experiment 1. There was also a slight increase of the significantly higher OTC's with a relatively large decrease of the significantly higher SPC's. The geometric means for the OTC and SPC of this series was, respectively, 120 X and 100 X 10^3/ml where the mean of the SPC's was 83.4% of the mean OTC, indicating that the two methods could be considered equivalent. Just for curiosity each OTC was multiplied by 0.83, the reciprocal of the $\frac{OTC}{SPC}$ mean found in Experiment 1. When these revised data were averaged, the two geometric means differed now only by 10 X 10^3/ml, and the lower counts of the standard plate averaged 90.9% of the higher OTC's.

Tables 2 and 3 are summaries of the same data of Experiment 3. Each sample was subjected to the same replication of five by each method. Again, the square roots of the counts were used and in pooling sums of square to arrive at generalized pooled variances, it was necessary to M test *(12, 4)* for homogenity and to prepare a contingency Table. When tested, the series of results by each method showed that their variances were homogeneous; in other words, the variability was the same for the 31 milks and, also, independent of the means in magnitude. Experiment 3 differed from the other two in that none of the results exceeded 300 colonies where factor-counts can contribute to an excessive range of concordance ratios.

Table 2 shows the mean of the counts, the average of the pooled variances, and the average Coefficient of variation for each method when the reported count is an average of five plates or tubes and transformed into square roots to obtain normality. The F ratio of 1.09 for variance indicates there is no difference in precision between the two methods. The Coefficients of variation are also similar, and the t statistic value of 0.72 for difference of means is not significant.

TABLE 2. SUMMARY DATA OF SQUARE ROOT TRANSFORMATIONS OF SPC AND OTC SET IN REPLICATES OF 5 WITH 31 PRODUCERS SAMPLES

	(n-1)		Mean ($\bar{x}$)		$\sum (\bar{x} - x)^2$		Variance (s^2)		Standard deviation		Coef. of variation	
	SPC	OTC	SPC	OTC	SPC	OTC	SPC	OTC	SPC	OTC	SPC	OTC
Total or Mean	119	122	9.67	10.13	39.7841	36.5164	0.321	0.294	0.546	0.503	6.1	5.6
$\bar{x}$ SPC/$\bar{x}$ OTC			1/1.05		1.09		1.09		1.09		1.09	

s (SPC) = 3.54
t = 0.72
t 0.25, 30 = 2.042

F.025, 30/30 = 2.07

TABLE 3. ANALYSIS OF VARIANCE OF OVAL TUBE AND STANDARD PLATE METHOD COMPARISON DATA (SQUARE ROOTS OF COUNTS WITH REPLICATION OF 5) OF THIRTY SAMPLES OF RAW MILK

Source of variability	Sum of Squares	Degrees of freedom	Mean square
Between Methods	23.4	1	23.4
Among Milks	2377	29	82**
Interaction	255.6	29	8.81**
Subtotal	2656	59	
Within Subsclasses	229.7	235	0.9816
Total	2885.7	294	

**Highly Significant

The data of Experiment 3 was again analyzed and presented in Table 3 by partitioning total sum of squares of deviations from mean into a methods factor, a milk factor, interaction of milk and methods factor, and a factor associated with experimental error (within subclasses), along with a parallel partitioning of total number of degrees of freedom. Due to accidental omission of one set of results, only 30 samples are included in the Table. Since this two-factor analysis of variance is a mixed model (methods factor is fixed), the F tests for methods and milks are against interaction while the mean square of the error is used in the denominator for testing interaction. The F value for both milks and interaction is highly significant, while the difference between methods is less than significant and agrees with the acceptance of the null hypothesis of equality of means shown by the t statistic of Table 2.

DISCUSSION AND CONCLUSION

These results show clearly that there is a differential response among milks to the two methods. There are several factors that could be responsible for the 4 to 1 ratio of significantly higher OTC's according to the 80% rule. Among factors that could cause an increase in viscosity which would tend to produce significantly higher than normal variation in OTC/SPC ratios are the leucocyte contents of the milks *(12)*, certain proteolytic bacteria which produce changes in the hydrophilic properties of casein and, possibly, the effect of fat content at the temperatures (6 -10 C) at which the milks were looped. An extreme difference of this property of "stickiness" or just simple adhesion was observed in preliminary standardization of the loops with water and milk.

Of lesser importance is the probability of more surface colonies of strict aerobic bacteria developing in the oval tube since the surface to volume of media is about 1.35 times as great with the latter when compared to the standard plate. It is well known that surface streaking of agar slants give higher counts than the standard plate method. Punch and Olson *(13)* has shown that five-day surface counts of raw milk were significantly higher than seven-day SPC's when estimating aerobic psychrophilic bacteria in milk. The antithesis of this surface growth is, of course, the possibility of more microaerophilic streptococci and lactobacilli developing in the deeper agar of the plate and producing a large discrepancy in favor of the dilution method.

Table 4 is a summery of the work done in comparing four different loop methods with the standard plate count by six laboratories within an interval of 25 years. The data of Table 4 was prepared by converting actual counts reported in these papers into mean arithmetic ratios, mean log ratios, and percent differences in order to have a common denominator of comparison among laboratories. Half of the laboratories (the Sealtest, Inc., the Robert A. Taft Engineering Center, and the Wisconsin State Laboratory of Hygiene) reported nearly perfect agreement between methods when they used a grand total of 203 samples and obtained a range of counts not quite as broad as the second group of laboratories. The other three laboratories (The New York Agricultural Experiment Station, the Producers Creamery of Springfield, Missouri, and the Wichita City-County Health Department) reported slightly higher counts for a loop method with a grand total of 1,342 samples. To these reported comparisons could be added the early work (1930) of Dorner *(7)* at the New York Agricultural Experiment Station where he found that the agar slope method gave higher counts than the commonly used standard Petri plate procedure. In contrast with these results, Donnelly, Black, and Lewis *(5)* reported from four trials and a total of 43 samples at the Chicago Board of Health that there was no difference between methods, but the oval tube showed statistically better agreement between duplicity. Surface streaking in one instance and placing the loop directly in the melted agar in another instance is, no doubt, an important factor in explaining such differences of results.

All of the counts included in Table 4 were done with raw milks except the original tube work of Myers and Pence *(2)* who recommended this method for counting the surviving thermoduric bacteria of pasteurization as a better criterion for correcting unsanitary practices of milking machines, open seam utensils, and conditions of cows' udders. With the exception of the preliminary 71 samples where the mean lower count was 69% of the mean higher one reported by the Robert A. Taft Engineering Center, every one of the paired geometric means shown in

TABLE 4. COMPARISON OF RESULTS REPORTED BY SIX LABORATORIES IN EVALUATING RAPID LOOP METHODS WITH THE STANDARD PLATE COUNT

Laboratory	Workers	Year	Rapid method used	No. of paired samples	Range of counts (Thousands/ml)	Geometric means (Thousands/ml)		Geometric mean ratios	Geometric mean log ratios	% Difference $\frac{SPC-OTC}{SPC} \times 100$
N. Y. State Agr. Exp. Station (Geneva)	Hucker & Haynes	1939	Buri slant	299	1-10	Rapid Loop	SPC	>1.2 (69.5%) <1.2 (30.5%)		
				74	10-100	Count		>1.2 (79.7%) <1.2 (20.3%)		
				14	>100			>1.2 (100%)		
Sealtest, Inc. Baltimore, Md.	Myers & Pence	1941	Oval tube	77	0-59	1.816	1.879	0.967	0.996	3.4
Robt. A. Taft Sanitary Engineering Center	Donnelly Black Lewis	1960	Oval tube	71 (Prelim)	19-139[a]	47	68	0.690	0.968	30.9
				41	27-59	43	43	1.00	1.000	0.0
Wisconsin Lab. of Hygiene Madison, Wis.	Thompson Donnelly Black	1960	Loop plate	85	<3->290	27	27	1.00	1.00	0.0
Producers Creamery Co. Springfield, Mo.	Heinemann & Rohn	1953	Loop bottle	104	<1->100	61	53	1.15	1.01	15.2
				100	<1->100	84	84	1.00	1.00	0.0
			Total	204	<1->100	72	68	1.06	1.01	5.9
Wichita-Sedwick County Health Department	Wilson	1963	Oval tube	132	3->300	40	34	1.18	1.015	17.7
				132	3->300	32	28	1.14	1.01	14.3
				125	3->300	26	24	1.08	1.01	8.3
				389	3->300	32	28	1.14	1.01	14.3
				127	3->300	120	100	1.20	1.016	20.0
				31	3->300	93	84	1.11	1.01	10.8
								*1.09[b] ± SE 0.022	*1.01 ± SE 0.001	* 9.2 ± SE 2.0

[a]Result not included in averaging.
[b]Average result of five research laboratories.

Table 4 could be considered an equivalent result. The mean log ratio with its approximate 95% confidence limits for five laboratories, 1.01 ± 0.002, would also indicate that these specific microbiological loop methods are equivalent to the standard plate count method.

From the results of the t statistic, two-factor variance analysis with replication, and the F test for variance ratio for normal distribution, it is concluded that there is no significant difference in either accuracy or precision between the oval tube and standard plate method. Although the results discussed in this paper are not new and do not differ significantly from what has been found by others, the data confirms that the oval tube can be used with confidence by analysts preferring a simplified method for the purpose of screening and sanitary control of producer's samples.

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PET FOODS AND SALMONELLA

An interesting editorial on the role of pet foods in the increase of salmonellosis appears in the February, 1966, issue of the *Public Health Inspector*, the journal of the Association of Public Health Inspectors, London, England. The editorial reads as follows:

"Salmonellosis still remains the major problem in food poisoning and in 1964 there was an increase of 17 per cent in the number of persons affected with salmonellae compared with the figure for 1963.

"A recent report by the Public Health Laboratory Service shows that the total number of cases of food poisoning in 1964 was 9,975 of which 5,115 were caused by salmonella organisms. It was not possible to trace the food responsible for these in more than half the general outbreaks and only in very few of the family outbreaks, but where a particular food was incriminated it was generally found to be one of the meat products.

"It is known that many of the salmonellae causing disease in man are also found in domestic animals and on occasions a relationship is established between organisms isolated at abattoirs and the prevalence of the same serotype in human infections. Contaminated feeding stuffs, infection among farm animals and contamination in abattoirs all contribute to the introduction of salmonellae into the food factory, shop and home. If the increase in salmonella infections is to be stopped every possible step must be taken to break the chain of infection.

"In the October 1965 issue of *Public Health Inspector* a paper by Dr. Betty Hobbs was published in which the author referred to a survey that had been carried out to find the amount of raw meat contaminated with salmonellae that was being sold for pets. It was found that 21.9 per cent of the 214 samples were contaminated by salmonellae and for comparison purposes the examination of 195 samples of butchers' meat revealed a salmonella contamination in 1.5 per cent.

"When investigating family outbreaks of food poisoning, are questions always put to the housewife about the type and sources of raw pet meat? It has been demonstrated that this meat may be heavily contaminated by organisms of the salmonella group and cross infection can occur very easily in the domestic kitchen. Storage of the pet meat is invariably close to other foods, it may not be kept in a closed container, and frequently the same utensils and knives are used in the preparation of the family's meal.

"Several years ago a very small boy, whose family had been patiently providing faecal samples for the 'three negatives' asked the public health inspector why he did not have a sample from his cat. Perhaps the unconscious wisdom of a five-year old gave the clue. Perhaps the public health inspector would have asked about the pet foods in the house in any case. It was in any event a line of enquiry that should now be one of the standard questions on family outbreaks."