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THE DEVELOPMENT OF NEW TECHNICS IN THE MICROSCOPIC EXAMINATION OF MILK

Thesis for the Degree of M. S.

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Frank Robert Peabody

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THE DEVELOPMENT OF NEW TECHNICS IN THE MICROSCOPIC EXAMINATION OF MILK

by

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A THESIS

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OUTLINE

The Development of New Technics in the Microscopic Examination of Milk

INTRODUCTION

Of the many and varied tests for checking the sanitary quality of milk, both before and after pasteurization, the determination of the bacterial content is one of the most accurate. At the present time there are two accepted methods of bacterial examination which are given in the Standard Methods for the Examination of Dairy Products (1). The older method of the two is the standard plate count in which a suitable dilution of the sample is planted in an agar plate and incubated for a given time. A little over thirty years ago, another method of milk analysis was introduced; namely, the direct microscopic examination. During this period of time the latter method has been accepted gradually until at the present time it is in general use. The method is at present almost identical with the original procedure as outlined by Breed (2).

It is the purpose of this work to evaluate the method of direct microscopic examination especially from the standpoint of statistical methods to determine its accuracy and, also, to introduce some new modifications in the present procedures.

HISTORICAL

The use of the microscope for making counts of bacteria dates back almost to the origin of the microscope itself. Although counts had been made, it was not until shortly after the turn of the century that the idea was applied to milk. The first method was introduced by Slack (3), who in 1906 centrifuged a sample of 2 ml. at 2000-3000 revolutions per minute for 10 minutes. The entire sediment was then smeared on a slide and spread over an area of 4 sq. cm. The preparation was stained with methylene blue and examined with a 1/12 oil immersion lens. Slack suggested using it as a "presumptive" test for very high and low quality samples. Those samples coming between would be analyzed by the plate method. He especially noted that the accuracy was poor when dealing with clean milk.

The next method was introduced by Breed (2) who considered that centrifuging introduced too many unknown factors to be a reliable procedure. Breed found that in the microscopic examination for body cells in milk, the bacteria were also stained. A standard procedure for the examination of bacteria microscopically was developed which is almost identical with the pipette method in use today.

One of the greatest objections to this method was the belief that dead bacteria would influence the count to a large extent. Breed discounted this factor because he found that dead bacteria decompose rapidly and did not retain the stain.

In 1914, Brew (4) discovered that there was little relationship between the plate and microscopic methods. This was especially true in the low and high quality samples. The differences in the low-count milk were attributed to the fact that in the samples used many of the bacteria were of udder origin. These organisms fail to grow an agar plates at an incubation temperature of 21 degrees C. and thus would not show up on the plates while they would be counted by the microscopic method. This gave the advantage of more accuracy to the microscopic procedure.

Two years later, in 1916, Breed and Brew (5) in continuing this study, advised a few modifications in technic. When the films were being dried, it was found advisable to use a slight amount of heat, (warm table or similar device) because if the slides were dried slowly the increase in numbers would introduce a significant error. They also found that it was not necessary to use sterile pipettes - thorough rinsing was sufficient to remove all the organisms that would effect the count to a significant degree. They also made a study of the accuracy of the loop smear as compared to the pipette method. It was found that although a loop claibrated to deliver 0.01 ml. speeded up the procedure, it introduced a variation of 35 per cent while the pipette technic did not exceed 2 per cent variation. The

methods were checked by weighing the amount of milk discharged rather than by bacterial counts.

It was found by Brew and Dotterrer (6), in 1917, that more than 50 per cent of the plate counts of 643 samples gave results intermediate to the microscopic "group" and individual counts. This showed, as was expected, that the plate count represents fairly well the number of "groups" present after being broken apart by the dilution waters; the "group" count gave the number of groups originally present; and the individual count gave the number of bacteria actually present in the milk. They also found that the size of the "groups" increased with the number of bacteria up to a point where the lactic acid bacteria became predominant. These, of course, formed smaller "groups" and the size decreased again. Thus, they concluded that the accuracy of the plate method was affected by two highly variable factors - the size of the "groups" originally present and the extent to which they were broken up in making the dilutions. For this reason, the plate method was not considered accurate enough to grade milk into more than two or three grades.

In 1920, Breed and Stocking (7) published some data showing that the variations in results of both the plate count and the direct microscopic count were small enough to justify the use of either method for grading milk into two or three classes. Also, they agreed with the previous conclusions that the methods were not sufficiently accurate to allow any finer grading. Breed and Stocking also made some analytical studies to show the large standard deviation

found in the microscopic methods. These studies showed the variations among the results of different analysts, some of whom were inexperienced in the technic, and should not be used as a criterion for the reliability of the method. Their purpose was to show the need of experienced technicians in the microscopic as well as the plate method, if reliable results are to be expected, rather than to determine the accuracy of the procedures.

It was demonstrated by Robertson (8) that the ratios between plate counts and the individual microscopic counts became more uniform if the incubation periods were lengthened to five days at 21 degrees C. followed by two days at 37 degrees C. The use of lactose agar also reduced the number of widely discrepant counts.

Some statistical analysis was done in 1929 by Brew (9), who found that the standard deviation of a "group" count and the plate count were in approximate agreement.

It can be seen that in all these studies the aim has been a comparison with the plate method. At the same time, several workers have pointed out the highly variable factores involved in the plating technic. Thus, it would seem more reasonable to use only the microscopic method and obtain a sufficient volume of data so that statistical anlysis could by employed to determine the results. By recording the counts of each field, it is possible to determine the amount of error encountered as well as some data on distribution of organisms on the smear. This gives a much better and more accurate picture of the advantages as well as the limitations of a direct

microscopic method.

EXPERIMENTAL PROCEDURE

The milk used in all of these experiments was of good quality. When high counts were desired, the milk was incubated for a suitable period of time. By using one grade of milk and varying the bacterial content by incubation it was possible to have comparable types of bacteria giving comparable clumps so that any variation that might enter through change of organisms was largely eliminated. In all of the studies, comparisons between procedures were made so the writer believes that the selection of one type milk gave the results desired.

In the first series of studies, a comparison of methods of preparing the smear was made. Two procedures were examined; namely, the pipette and the loop methods.

All smears made by the pipette method were in accordance to Standard Methods for the Examination of Dairy Products (1).

The loop method was made according to the procedure recommended by Bryan (10) for the examination of producer milk. In this procedure, a standard platinum loop of 4 mm. outside diameter is used and a smear of 4 by 8 mm. is made. Using this method 20 smears were evenly spaced on one slide.

Smears were made by each method of three grades of milk so

the data could be obtained for smears showing less than 1 organism or clump per field, smears showing 10 to 15 organisms or clumps per field and smears showing numerous organisms or clumps per field.

All slides were stained in exactly the same manner using the same stains so that the only variable in the series was the method of smearing. Two hundred and fity fields were counted for each smear in the low and medium count milks and one hundred for the high count milk. In all instances the number of organisms per field was recorded for each smear.

Examinations were made with a 1.32 mm. oil immersion objective and a 5 X ocular. This yields a conversion factor of 240,000 - one organims per field represents 240,000 organisms per ml. of milk. The results represent a "clump count" in which the clumps of bacteria, as well as the individual organisms, are counted as units.

In the second series a study of two different methods of staining was made. One method is a modification of "standard methods" as it is used routinely in this laboratory. The slides are immersed in xylene to remove the fat, followed by a 95 per cent alcohol solution. About one minute is sufficient in each, and the slides are drained between solutions. The staining bath is prepared by adding 10 ml. of saturated alcoholic solution of methylene blue to 90 ml. of 30 per cent alcohol (12). The slides are dipped in the stain just long enough for proper staining. They are then rinsed in water and decolorized in alcohol if necessary followed by thorough drying.

The second is a method which is recommended by Mallmann and Churchill (11) for use in staining egg-meats. The slides are stained according to the following procedure:

The staining bath is made of

1 gm. methylene blue (Certified for bact. use)
500 ml. 95 per cent ethyl alcohol
5 ml. conc. hydrochloric acid.

The slide should remain in the staining bath from three to five minutes. It is then removed and dipped in a tap water bath only long enough to remove the excess stain. It is important to decolorize only partially in order to avoid decolorization of the background to such an extent that it is difficult to find the location of the various smears when making the examination. In addition, excessive washing will soften and loosen the film. The slide is air dried.

Because this stain contains 95 per cent alcohol, it was found more satisfactory to omit the alcohol bath before staining, using only the xylene to remove the fat.

In this series, all of the smears were made by the loop technic.

In the third series, a study was made of the effect of a colored light source in making bacterial counts. The work was done with the use of colored solutions - acid fuchsin for the red and potassium dichromate for the yellow. The concentrations of the dyes used are as follows:

Yellow	Solution	#1	12	parts	pot. dichromate	per 100,000
"	"	2	30	"	"	" 100,000
"	"	3	60	"	"	" 100,000
Red	"	1	0.4	"	fuchsin	" 100,000
"	"	2	1	"	"	" 100,000
"	"	3	2	"	"	" 100,000
Red & Yellow"		1	(24	"	pot. dichromate	" 100,000
			(0.4	"	fuchsin	" 100,000
" "	" "	2	(24	"	pot. dichromate	" 100,000
			(0.5	"	fuchsin	" 100,000

These solutions were made up in quantities of 200 ml. from stock solutions and were used in a special 250 ml. Florence flask with a ground glass side which fitted the "Stella" microscope light. In addition to the solutions, Wratten filters were employed which were fastened to the sub-stage of the microscope.

In much of the work described, the conclusions were reached by submitting the data to statistical treatment. All conclusions were made according to the following formulae:

$$\sigma_x = \sqrt{\frac{\Sigma(x^2)}{N} - \left(\frac{\Sigma(x)}{N}\right)^2}$$

$$\sigma_{mean} = \frac{\sigma_x}{\sqrt{N}}$$

$$t = \frac{\text{Dif. of means}}{\sigma_{\text{dif. of means}}} = \frac{\text{Dif. of means}}{\sqrt{\sigma_{m_1}^2 + \sigma_{m_2}^2}}$$

where

σ_x = standard deviation of the sample
x = organisms per field
N = number of fields
σ_{mean} = deviation of the mean
t = test for significance

The data used were obtained from at least 100 fields and in most

cases 250 or 300 fields were used. The deviations are calculated from the original counts of individual fields and not from a series of averages.

RESULTS

The results of the first series of studies will be found in Tables I to VII. Before starting a discussion of these data, it should be understood that the terms "low count", "medium count", and "high count" are used as relative terms in the statistical sense and do not in any way imply that the quality of milk is "high", "medium", and "low" since this is not the case as can be seen from the counts per ml.

A study of the data for the high count milk (Tables I and II) shows that these two methods give very comparable results. The pipette procedure yielded a slightly higher average. It can be seen from the sub-totals that there is some variation from one smear to another (652 to 1004) as well as from one field to another when dealing with the pipette method. In the case of the loop technic, the totals for the ten smears varied only from 667 to 922, or almost 100 less.

In order to determine the variations from one field to another, the standard deviation and mean deviation were calculated (Table VII). However if these values are submitted to the "students" t test, it

Table I - The bacteria per field of 10 smears made by the pipette technic on a high count milk. (Sample No. 22)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	85	101	90	122	76	67	83	53	91	84	
	110	76	97	88	74	73	82	63	102	100	
	75	83	108	81	73	89	90	73	80	108	
	72	81	90	105	50	128	96	92	79	92	
	105	85	71	115	52	61	99	74	100	52	
	78	87	96	120	91	105	74	95	88	110	
	89	99	97	97	60	80	81	108	92	90	
	116	86	66	107	51	72	83	94	81	72	
	90	93	73	86	70	84	100	92	67	61	
	94	86	119	83	55	76	78	75	70	84	
Total	914	877	907	1004	652	835	866	819	850	853	8,577
Average	91.4	87.7	90.7	100.4	65.2	83.5	86.6	81.9	85.0	85.3	85.77

Table II - The bacteria per field of 10 smears made by the loop technic on a high count milk. (Sample No. 22)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	92	61	103	85	100	114	83	78	65	116	
	98	105	56	55	79	104	88	65	70	92	
	73	67	95	79	83	100	96	58	63	106	
	106	94	96	57	69	68	93	96	74	90	
	95	59	51	100	81	98	69	72	77	116	
	83	62	69	86	78	70	96	66	61	115	
	117	100	77	76	89	61	70	80	67	78	
	90	110	80	85	66	73	64	77	69	72	
	75	83	87	82	86	59	85	83	64	66	
	98	50	90	68	107	72	70	99	57	78	
Total	927	791	804	773	838	819	814	774	667	929	8,136
Average	92.7	79.1	80.4	77.3	83.8	81.9	81.4	77.4	66.7	92.9	81.36

is found that the result is less than 2 and therefor the variations between methods is not significant.

In studying the data on a medium count milk, Tables III and IV, it is found that the results obtained were almost identical. This time the variations of the smears are very close - 207 for the pipette against 197 for the loop technic. However, the deviation of the fields show more striking results. Table VII shows that the standard deviation is 6.93 for the pipette and 6.50 for the loop; while the deviation of the mean was .438 and .411 respectively. In this case, the value of "t" exceeds the limit of 2 and therefor the variations have considerable significance.

In considering the results of Tables V and VI one should realize that in dealing with so few organisms on each smear, small variations will give relatively high percentage errors. In this respect it is wll to point out that the difference in the two totals would amount to only 15,000 organisms per ml. In practical use, this amount is not too important. For this reason as well as the low value of "students" t, the difference encountered here can be considered as insignificant even though they show slightly larger variations with the loop technic.

In making the series of studies on staining, counts were taken on two duplicate sets of smears of varying quality. In examining the smears made by the Breed procedure of staining, the usual difficulties were encountered; i. e., the background of milk and any debris which was on the slide retained the stain almost as well

Table III - The bacteria per field of 10 smears made by the pipette technic on a medium count milk. (Sample No. 23)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	15	20	9	7	27	13	13	15	18	10	
	9	11	7	15	44	9	8	17	8	6	
	9	30	6	7	14	5	4	8	6	12	
	9	16	7	33	18	14	6	6	16	26	
	12	15	9	24	10	8	7	13	13	6	
	5	11	6	22	12	15	4	7	6	14	
	9	11	15	25	33	10	5	12	14	14	
	12	10	6	25	36	16	4	7	6	10	
	11	11	10	26	37	17	6	6	7	15	
	6	12	10	14	22	6	3	11	7	4	
	18	21	11	12	10	15	6	11	14	3	
	8	25	8	14	13	13	6	10	5	10	
	8	6	10	26	5	10	5	9	7	20	
	5	8	13	13	2	10	12	6	14	16	
	10	23	3	6	11	14	14	6	17	21	
	9	17	15	7	7	11	10	9	10	18	
	19	10	16	12	6	14	4	6	9	15	
	9	9	6	9	12	9	6	4	9	18	
	6	7	5	1	5	3	11	7	10	10	
	8	12	7	4	10	10	9	19	9	9	
	16	17	15	8	30	12	13	15	8	16	
	8	27	14	2	25	10	5	8	5	6	
	10	7	13	8	5	6	10	6	18	32	
	13	17	5	10	17	10	25	21	16	16	
	10	15	11	20	8	6	13	7	9	34	
Total	254	368	237	350	419	266	209	246	261	361	2,971
Average	10.3	14.7	9.5	14.0	16.8	10.6	8.4	9.9	10.4	14.4	11.88

Table IV - The bacteria per field of 10 smears made by the loop technic on a medium count milk. (Sample No. 23)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	16	15	33	15	15	14	25	13	15	13	
	20	25	25	9	9	10	7	6	17	14	
	21	21	31	7	7	12	16	11	10	5	
	12	22	8	5	14	21	12	21	19	14	
	10	13	22	8	9	18	8	14	12	19	
	10	17	18	16	7	20	10	17	5	15	
	31	14	29	17	12	28	9	21	10	12	
	20	26	7	13	12	17	5	12	4	14	
	11	10	10	22	19	20	10	12	17	11	
	11	8	17	20	16	12	9	13	4	16	
	5	10	27	16	19	13	10	14	13	18	
	12	14	22	20	16	13	17	7	7	15	
	13	17	17	12	8	9	9	5	15	10	
	9	13	25	8	14	18	21	7	15	3	
	20	14	19	5	8	41	10	10	12	15	
	9	16	30	24	28	26	7	11	10	3	
	23	18	32	17	24	7	14	9	23	9	
	6	12	13	22	15	24	26	5	9	8	
	21	6	11	23	23	14	19	9	18	10	
	16	17	14	20	7	5	21	18	22	17	
	16	7	21	15	16	18	29	15	9	11	
	20	19	15	11	27	10	22	14	5	17	
	11	8	21	21	7	7	17	12	11	20	
	19	12	15	14	24	11	17	14	12	18	
	17	10	25	9	20	18	26	20	17	8	
Total	379	364	507	369	376	406	376	310	311	315	3,713
Average	15.1	14.5	20.3	14.8	15.0	16.2	15.0	12.4	12.4	12.6	14.85

Table V - The bacteria per field of 10 smears made by the pipette technic on a low count milk. (Sample No. 24)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	1	0	0	0	1	0	0	1	0	0	
	0	1	0	0	1	0	0	1	0	0	
	2	0	0	0	0	0	0	0	0	0	
	0	0	0	1	0	1	0	0	0	0	
	1	0	1	1	1	0	3	0	1	0	
	0	0	1	0	0	0	0	0	1	1	
	0	0	0	0	0	0	0	0	0	0	
	0	1	0	0	0	0	0	1	0	0	
	0	0	0	0	0	0	0	1	0	0	
	0	0	0	0	0	0	0	0	0	0	
	0	1	1	0	1	0	0	0	0	1	
	1	0	2	2	1	0	0	0	2	0	
	0	2	1	0	2	0	1	0	1	0	
	2	1	0	1	1	0	0	1	1	0	
	0	0	0	1	0	0	0	0	0	0	
	1	0	0	0	0	1	1	0	0	1	
	0	0	1	2	0	1	0	0	0	1	
	0	0	0	0	0	2	0	0	0	2	
	0	0	0	1	0	1	0	0	0	0	
	1	0	0	1	1	1	1	0	0	0	
	0	0	0	0	0	0	1	0	1	1	
	1	0	1	0	0	0	0	0	0	1	
	0	0	0	1	1	0	0	1	1	0	
	0	0	1	0	1	0	0	1	0	0	
	0	1	0	0	0	0	0	0	0	0	
Total	10	7	9	11	11	7	7	7	8	8	85
Average	0.40	0.28	0.36	0.44	0.44	0.28	0.28	0.28	0.32	0.32	0.34

Table VI - The bacteria per field of 10 smears made by the loop technic on a low count milk. (Sample No. 24)

Bacteria per field

	Smear No.										
	1	2	3	4	5	6	7	8	9	10	
	0	0	0	0	0	0	0	0	0	1	
	0	0	0	0	0	0	0	0	1	0	
	0	0	0	0	0	0	1	0	0	1	
	0	0	0	0	0	0	0	0	1	0	
	0	0	0	0	0	0	0	0	0	0	
	0	0	0	2	0	2	0	0	0	0	
	1	0	0	1	0	0	1	0	0	0	
	0	0	0	1	0	0	0	2	0	0	
	1	0	0	0	0	3	0	1	1	2	
	1	1	0	0	0	1	2	1	0	1	
	1	0	3	0	1	0	1	1	0	0	
	0	1	0	0	3	0	1	0	0	1	
	0	1	2	0	0	0	0	0	0	0	
	0	0	1	0	0	0	1	0	0	0	
	1	1	0	1	0	1	0	0	1	0	
	0	1	0	0	0	0	0	0	0	1	
	1	0	0	0	1	0	1	0	1	0	
	1	1	1	0	1	0	0	0	0	2	
	1	2	2	0	1	0	0	0	1	1	
	2	0	1	1	0	0	0	0	0	0	
	0	0	0	0	0	0	2	0	1	0	
	0	0	1	1	0	0	0	1	1	0	
	0	1	0	0	1	0	1	2	0	0	
	1	0	0	0	1	0	0	1	1	0	
	2	1	0	1	0	1	0	2	0	1	
Total	13	10	11	8	9	8	11	11	9	11	101
Average	0.52	0.40	0.44	0.32	0.36	0.32	0.44	0.44	0.36	0.44	0.404

Table VII - A Statistical Summary of Tables I through VI

Deviations Between the Two Methods

Method	Sample	Arithmetic mean	Standard deviation	Deviation of the mean
Pipette	22	85.77	16.66	1.67
"	23	11.88	6.93	.438
"	24	.340	.478	.032
Loop	22	81.36	16.48	1.65
"	23	14.85	6.50	.411
"	24	.404	.644	.047

Significance of Deviations Between the Two Methods

Sample No.	Value of "Students" t.	Conclusion
22	1.88	Not Significant
23	4.9	Significant
24	1.12	Not Significant

as the organisms themselves. This showed that many of the bacteria were obscured by the other stained material, and good results could be obtained only in the hands of a trained technician, if at all. On the other hand, in examining the smears made from duplicate samples which had been stained with the acid stain, the background was found clear of debris and stained very faintly. This slight amount of stain was retained during the washing procedure in order that there would be something to focus on while making the examination. The organisms retained the stain well and appeared very distinct from the rest of the smear. This afforded much more ease and speed in counting.

Another factor to consider is the ease of preparing the staining bath and the method of staining. It can be seen from the technics presented in the preceding section that the acid stain is even more simplified than the Breed technic. The preparation is about the same except that only two baths are required and there is no need for a separate decolorization in using the acid stain.

Of course in comparing the relative value of two staining technics, the important thing to consider is which will give the higher counts; i. e., which stains the larger number of bacteria. In this respect, the data in Table VIII and the first two columns of Table IX show that the acid stain gave consistently higher results. Thus, the acid stain was the better of the two in all respects.

The data on the studies on the effect of a colored light source are found in Tables IX to XI. Those presented in Table IX are of a preliminary nature. In several cases it was found upon

Table VIII - The Bacteria per ml. on Two Duplicate Sets of Smears using Different Methods of Staining.

Sample No.	Type of Staining	
	Breed technic	Acid stain
25	5,000	5,000
26	5,000	9,000
27	144,000	115,000
28	15,000	38,000
29	53,000	100,000
30	53,000	200,000
31	14,000	65,000
32	9,000	5,000
33	38,000	57,000
34	115,000	300,000
35	5,000	14,000
36	34,000	48,000
37	9,000	9,000
38	14,000	19,000
39	96,000	144,000
40	72,000	75,000
41	9,000	15,000
42	192,000	245,000
43	53,000	67,000
44	19,000	25,000

Table IX - The bacteria per ml. of milk (expressed in thousands) of various smears made by the loop technic using different stains and light sources.

Sample No.	Light Source												
	Blue		Red*	Yellow			Red			Red & Yellow		Wratten Filters	
				1	2	3	1	2	3	1	2	35 & 15	22
	Breed Stain	Acid Stain											
1	29	24	29	48	38	76	48	58	38	62	210		120
2	5	5	9	5	9	5	5	5	5				
3	245	576	384	620	864	912	740	653	480	614	614		672
4	10	15	43		53								
5	67	50	43		91								
6	14	15	5		9								
7	5	9	14		5								
8	100	173	173		168	240				153	168		230
9	9	15	9		5								
10	5	15	9		5								
11	9	5	5		5								
12	806	1,140	980		864	1,200				1,820			1,900
13	173	240	154		187	187				200	187		210
14	912	2,160	1,700		1,730	2,000				2,440	2,760		2,940
15	48	320	29		288	335				298	283		250
16	177	2,000	336		437	302				269	572		
17	2,340	3,040	2,380	2,600	2,880	2,680	1,800	2,480	2,160	2,640	2,790	1,180	2,880
18	2,520	2,080	1,800	2,300	2,450	2,400	1,600	2,300	2,400	2,200	2,120	1,870	1,560
19	50	68	92		144	128		62		120	82	72	182
20	3,840	4,512	2,400		2,800	1,900		1,630		3,460	4,500		4,360

* Filter used by Mallmann and Churchill for egg examination (11).

Table X - A Study of Five Different Filters of Sample No. 21 (Smear A)

Number of Bacteria per Field

Type of Filter

	Blue	Wratten 22	Pot. dichromate Sol. 2	Fuchsin Sol. 2	Fuchsin & dichromate Sol. 2
	2	2	4	3	2
	2	2	4	1	1
	3	2	2	2	3
	2	2	2	0	4
	2	1	1	0	6
	2	2	0	0	4
	1	4	1	0	2
	2	4	3	1	0
	4	2	1	0	1
	4	4	2	1	1
	3	3	1	3	2
	4	4	2	2	5
	3	1	1	1	2
	3	1	1	0	1
	2	1	1	0	3
	1	3	0	4	2
	3	2	0	1	2
	1	1	1	2	2
	2	2	1	3	2
	2	0	3	3	1
	2	4	1	1	2
	2	1	2	1	1
	4	5	3	2	1
	2	4	1	0	3
	4	1	1	1	2
Total	62	58	39	32	55
Average	2.5	2.3	1.6	1.3	2.2
Deviation of the mean	.19	.26	.22	.24	.27

Table XI - A Study of Five Different Filters of Sample No. 21 (Smear B)

Number of Bacteria per Field

	Type of filter				
	Blue	Wratten 22	Pot. dichromate Sol. 2	Fuchsin Sol. 2	Fuchsin & dichromate Sol. 2
	2	1	0	3	2
	4	3	0	2	0
	4	3	2	1	3
	1	3	4	1	2
	1	1	1	1	2
	0	2	1	1	3
	1	4	3	0	2
	3	1	2	0	2
	2	6	1	3	4
	1	0	1	4	2
	3	3	2	2	2
	3	2	1	2	1
	4	2	0	1	2
	6	2	2	1	1
	2	1	3	3	2
	2	1	1	2	3
	1	2	1	3	1
	4	4	1	2	3
	3	4	3	1	2
	2	2	2	2	3
	2	1	1	2	2
	1	1	1	3	0
	2	1	1	2	1
	1	1	2	2	3
	1	2	2	0	3
Total	56	53	38	44	51
Average	2.2	2.1	1.5	1.8	2.0
Deviation of the mean	.27	.27	.19	.21	.19

examining only a few slides that the filter used would be unsatisfactory and it was discarded. Some were too weak, some destroyed contrast by too great an intensity, and others were hard on the eyes. Those samples containing 15,000 organisms per ml. and less are not considered in these results because they represent less than four organisms per fifty fields and variations would be largely due to selectivity rather than variation of the light source.

Since it is rather hard to get a true picture from the averages as presented here, five of the lights which showed up the best were checked more carefully. The counts per field were recorded from 25 fields on two duplicate smears. The data are recorded in Tables X and XI. They show almost no difference between the light blue color and the yellow Wratten filter No. 22. In close agreement to these is the mixture of red and yellow (Solution 2) which is a comparable substitute for the yellow Wratten filter.

To complete the analysis of the procedures used in milk examination, it was thought advisable to make some study concerning the relative accuracy of the counts made by using a varying number offields. To do this satisfactorily, the counts were recorded for 300 fields on one smear (Table XII) and 200 fields on a second smear (Table XIII). It can reasonably be assumed that this amount of data would give results close to a "true mean". The data are given in Table XIV as Samples 3 and 17 respectively. In addition to these samples four more were selected to show the results for higher and lower figures. Although counts such as found in Samples 22 and 23

Table XII - The Bacteria per Field on One Smear of Sample No. 3

2	2	6	0	2	4
3	4	8	1	4	5
5	1	3	1	2	5
1	5	4	7	4	3
3	6	5	3	3	3
1	3	2	3	6	4
1	2	2	5	6	5
3	4	2	3	6	3
5	1	2	2	3	4
1	5	2	3	5	4
2	5	1	1	5	6
6	4	5	5	4	6
4	4	3	4	3	7
6	6	7	4	1	2
3	8	6	1	3	2
4	3	3	3	1	2
3	3	1	5	3	3
5	8	0	7	2	6
6	8	4	4	1	2
7	4	6	6	2	4
1	4	6	7	5	2
5	2	6	0	2	2
2	4	7	1	4	5
1	4	2	6	2	1
3	2	2	3	7	4
4	0	7	2	5	0
4	2	5	5	4	3
5	4	4	2	4	1
4	3	4	6	4	4
7	4	8	3	0	4
4	5	3	5	0	5
6	10	0	3	0	2
6	5	5	2	1	2
7	5	6	1	6	3
7	10	5	3	4	4
5	3	7	5	6	4
6	4	8	5	1	5
4	1	2	3	3	4
2	6	6	5	5	2
3	2	2	5	7	5
1	3	2	4	3	1
2	3	4	4	6	1
0	3	5	5	4	2
2	2	4	6	3	5
1	2	1	1	3	1
4	2	2	2	0	3
3	9	7	7	2	3
5	5	4	6	2	7
0	4	1	4	4	5
0	3	1	2	5	3

Total - 1098 Average - 3.66

Deviation of the mean - 0.117

Table XIII - The Bacteria per field on
One Smear of Sample No. 17.

14	15	11	14	10
10	19	20	15	15
17	14	11	12	17
10	7	12	21	7
11	15	14	13	18
12	16	6	13	7
23	15	11	12	18
14	17	15	13	17
10	14	15	8	13
12	11	13	12	17
13	9	15	10	6
10	10	17	18	16
9	13	12	13	15
14	20	19	17	8
11	13	23	6	14
13	10	15	12	12
10	6	14	20	8
9	7	15	10	10
14	14	18	13	22
11	11	11	15	12
10	10	15	13	7
15	12	13	11	16
16	16	14	13	20
9	10	13	14	15
20	5	17	11	7
12	11	11	11	16
11	14	15	19	10
15	19	17	9	19
8	8	14	16	6
19	5	14	5	13
14	19	12	15	11
15	14	13	11	10
13	16	21	6	14
10	21	20	14	16
20	16	19	15	10
11	12	9	9	10
11	9	12	11	10
15	10	12	12	17
14	15	14	8	14
13	16	19	22	15

Total - 2,643 Average - 13.22

Deviation of the mean - 0.28

Table XIV - To Show the Number of Fields Necessary to Obtain a Desired Accuracy

Sample	Arithmetic mean	Standard deviation	Allowable per cent error	Number of Fields
24	.404	.644	25	47
			15	113
			10	254
21	2.12	1.48	25	8
			15	22
			10	48
3	3.66	2.02	25	5
			15	14
			10	31
17	13.22	3.96	25	2
			15	4
			10	9
23	14.85	6.5	25	3
			15	9
			10	19
22	85.77	16.6	25	1
			15	2
			10	4

would not be encountered in actual practice, they were included here to show the trend of the results.

DISCUSSION

When the microscopic technic was proposed by Breed(2), it was standardized by using 0.01 ml. of milk spread over an area of 1 sq. cm. This procedure gave a sufficiently uniform thickness to the smear that consistent results were possible. However, the method is somewhat cumbersome and time-consuming because it is necessary to accurately measure the amount of milk in the pipette, and two operations are necessary to make the smear - depositing the milk and spreading it over the required area. In addition, it is advisable to have some sort of guide to indicate the proper area.

The solution appeared to be the use of a loop for depositing the 0.01 ml. of milk. However, Breed and Brew (5) found that this introduced an extremely large error, and the method was discarded in favor of the pipette procedure.

The purpose of the procedure is to obtain a smear of constant thickness. Since the area used is much larger than is necessary for the examination, it would be possible to reduce the area and the amount of milk used with a resultant smear of the same thickness as obtained by the original method. If this smaller amount of milk were used, it was thought that it might be possible to calibrate a loop that would have greater accuracy. This method was recently proposed

by Bryan (10) and was used in this study. The results showed the loop to be superior in every way. To further demonstrate its reliability, several distribution curves have been prepared from the foregoing data. Figure 1 was prepared from Tables III and IV in which 25 fields were counted for each of 10 smears. The pipette method shows two peaks indicating two different predominating sized fields. The wider distribution of the counts indicates more variability among the slides. The loop technic, on the other hand, shows a rather compact graph without too large a distribution.

Figure 2 was prepared from Tables XII and XIII in which 300 and 200 fields were examined on one smear for each sample respectively. These curves give a very good idea as to the small amount of variation found when using the loop technic.

In addition to the greater reliability of the results as previously shown, the loop method is much more rapid. By using three loops, it is possible to be flaming one and cooling the second while the smear is made with the third. In this way there is no waiting andclean, sterile equipment is always immediately available. The smears of 4 by 8 mm. are easily made by placing the 4 mm. loop on the slide and drawing the smear out to twice its width. This requires only one operation as the sample is deposited and spread over the required area in one motion.

Another consideration is the saving of equipment. The smaller size of the smear allows about four times as many samples to be placed on one slide, and the replacement and care of the pipettes are

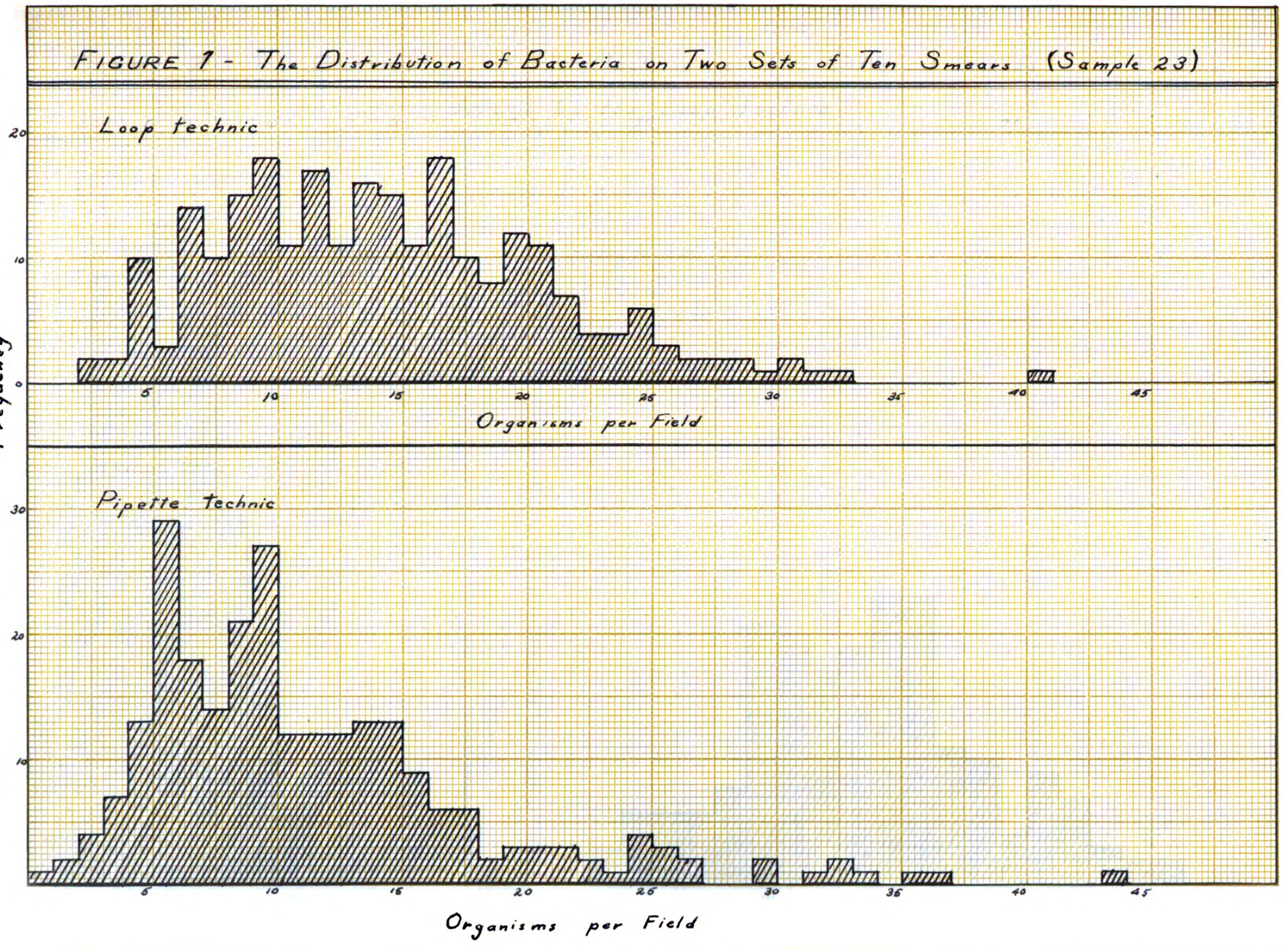
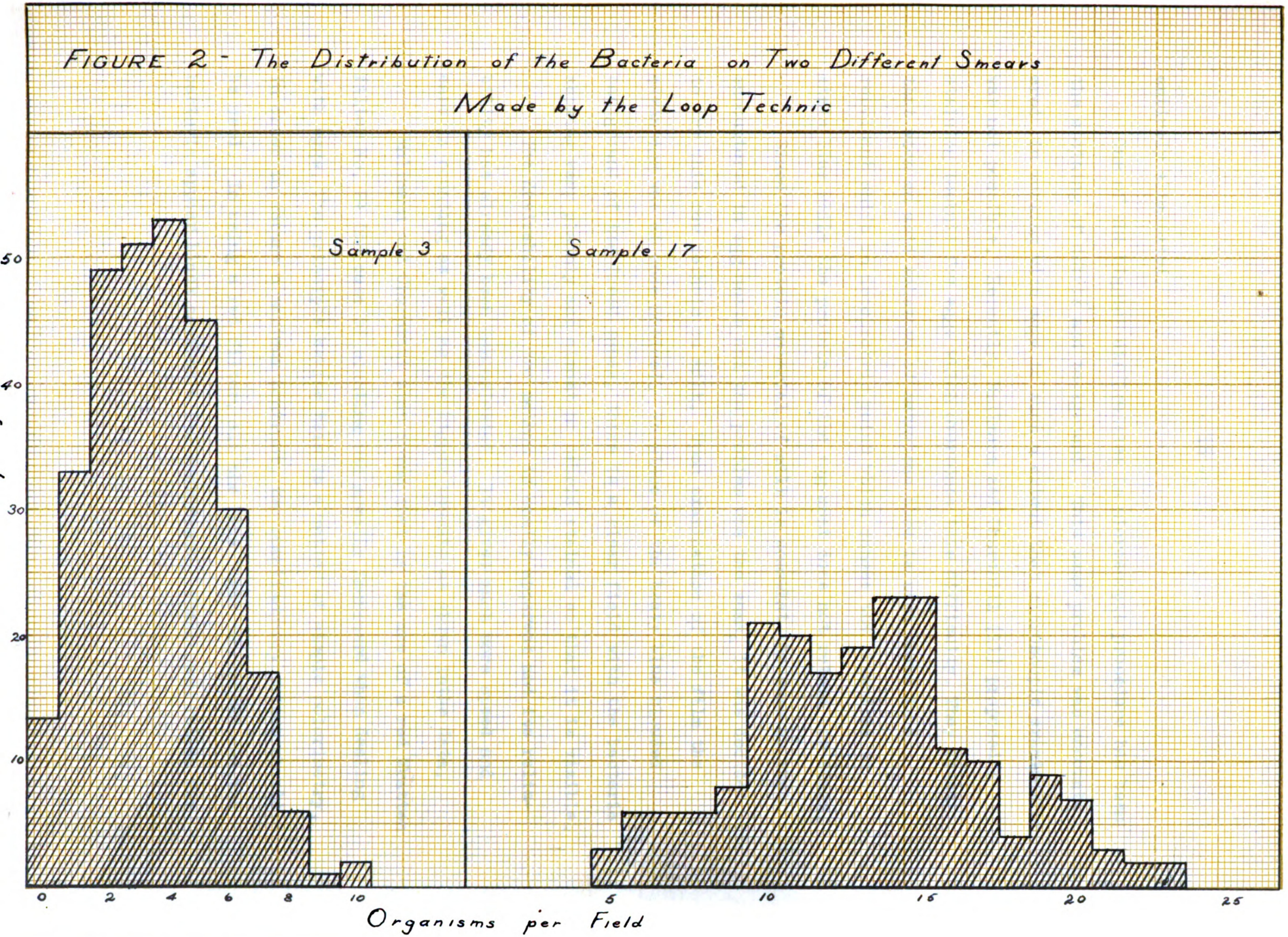


FIGURE 1 - The Distribution of Bacteria on Two Sets of Ten Smears (Sample 23)
Loop technic
20
10
0
5
10
15
20
25
30
35
40
45
Organisms per Field
Pipette technic
30
20
10
5
10
15
20
25
30
35
40
45
Organisms per Field

FIGURE 2 - The Distribution of the Bacteria on Two Different Smears Made by the Loop Technic



entirely eliminated.

One of the biggest difficulties in microscopic procedure has been the staining of debris and background. This same problem was encountered in working with eggs to such a extent that microscopic examination was not possible. Recently, Mallmann and Churchill (11) proposed a staining technic for eggs which overcomes this difficulty. They found that when an acid solution of methylene blue is used there is a resultant shift in isoelectric point which causes the organisms to stain blue while the protein of the egg-meat does not retain the stain.

Since the protein material of milk is somewhat the same as that encountered in egg-meats and the problem was the same, it was decided to try this new technic on milk. Upon examination of the film the advantage was at once noticed. In place of the heavy blue clouded background of the Breed smear, there is a clear field with the bacteria showing very plainly and almost no debris stained so that it is visible.

The desirability of the method is easily apparent and its reliability has already been demonstrated. It gives much more speed and ease in counting as well as the higher results as shown inthe data.

Mallmann and Churchill also found that by the use of a red Wratten filter No. 24 the ease of counting was greatly increased when dealing with eggs. The principle behing this is a matter of simple colorimetry. In order to secure a maximum contrast, two complimentary colors should be used. In using a red filter, all the blue light is removed and the bacteria appear black against a red background. Their main reason for

discarding the blue light was the fact that it would filter out many of the lightly stained organisms so that they were not counted. By the use of a red filter, these bacteria were easily seen. To avoid this error in making milk examinations, a study of light filters was made.

In studying the data obtained in this series, one might be led to think that the blue or daylight filter should be retained. However, there are several factors which will not show up in a tabulation of data.

First, is the fact that the results show that for these particular samples there was little difference between the blue and the Wratten No. 22 filters. As in working with eggs, one occasionally encounters some of those organisms which for some reason will not retain the stain as well as the others. In this case it is easy to see that the blue background would "hide" many of these bacteria so they would not be counted. Unfortunately, no organisms of this type were found while this study was being made so that it was not possible to tell exactly what difference it would make.

In addition, there must be considered factors such as ease and speed of counting which do not show up in the tables of data. It was found that where the yellow Wratten filter No. 22 is used, the bacteria could be seen distinctly and more easily than with a blue background. This filter has just enough red to make the organisms appear dark against the light background of yellow, thus providing

a very good contrast. When the red Wratten filter No. 24 was used, it was found to cause irritation of the eyes, while the yellow presented a restful, pleasing condition. If work was done continuously for an extended period, the eyes became fatigued much sooner with the red than with the yellow light source.

When these factors are considered it can be seen that the yellow filter has a distinct advantage over the blue, or daylight, and red filters.

In making an analytical study of the data obtained, one of the important factors which can be determined is the number of fields that must be counted to give any desired degree of accuracy. By taking the formula for determining the deviation of the mean and then calculating this value from the allowable per cent error, it is possible to find the number of fields which must be counted to obtain the desired accuracy. Referring again to Table XIV, it may be seen that for poor quality milk, samples 3 and 21, when only 10 fields are counted, the error might be as high as 25 per cent. At the same time if 50 fields were examined, there would occur an error of only 10 per cent.

In considering the high quality milk (sample 24), comparable accuracy was obtained by counting 47 fields for 50 per cent, and 254 to remain within 10 per cent. In routine work the examination of this number of fields is not possible for each sample, and so one might be inclined to think that the inaccuracies are so great that

the method is of little practical value. However, in turning to the practical side there is another consideration which must not be overlooked. This is the fact that an error as large as 50 per cent, when dealing with such low counts, is not too significant. By counting 50 fields, the amount of error can be kept within the limits of practical usage. In dealing with samples of higher counts, it is possible to reduce the number of fields counted to 25 and still maintain reasonable accuracy. Thus, if a sample had an average of three or four organisms per field one would be safe in counting only 25 fields. With lower counts the minimum of 50 fields should be counted.

Although this number is considered sufficiently accurate for routine work, in case a finer grading of milk is desired which requires greater accuracy, the number of fields will have to be varied accordingly.

SUMMARY

The results of these studies show that all three of the proposed modifications in the procedures used in examining milk gave very satisfactory results. The loop method of preparing the smear gives a preparation that can be interchanged with the pipette method and still obtain the same results. In addition, it is much easier and faster. The series on the acid stain showed that this method of staining the slides is much superior to the method now in use.

It has also been shown that the use of a yellow Wratten filter No. 22 greatly increased the ease and speed of counting.

On the basis of the results obtained, the following recommendations are proposed for the Standard Methods of Milk Analysis:

1. The method of preparation of the smear on the slide be changed to the loop method of Bryan in order to increase the speed of the examinations and also to obtain greater accuracy of results.

2. The use of the acid methylene blue stain be adopted in place of the Breed stain now in use. This stain gives a much cleaner field to work with and a resultant higher count is obtained.

3. When making the examinations of the smear, the light source containing a yellow Wratten filter No. 22 or its equivalent be adopted for standard use.

4. It is further recommended that the number of fields counted follow the data presented in the foregoing section. For routine work this would be 50 fields for those samples where one organism or less appears on each microscopic field. In those instances where there are more than two or three organisms per field, it would be necessary to examine only 25 fields.

These changes in procedure will give a greater accuracy in the results as well as speeding up the method of examination and making it possible for one technician to handle more samples in a shorter time with greater accuracies.

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