CORRELATION BETWEEN STANDARD PLATE COUNT AND FOUR DIRECT MICROSCOPIC COUNT PROCEDURES FOR MILK

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SUMMARY

The standard plate count (SPC-32 C) and the direct microscopic count (DMC) of samples of commercially pasteurized milk inoculated with pure cultures of actively growing (18-24 hr growth) bacteria commonly found in milk were compared. Four staining procedures for DMC were used: *(a)* Levowitz-Weber's methylene blue stain; *(b)* a modified Levowitz-Weber stain incorporating basic fuchsin; *(c)* alcoholic-acetic acid fixation followed by periodic acid-bisulfite treatment and staining with pH_4 toluidine blue; and *(d)* alcoholic-acetic acid fixation and staining with pH_4 toluidine blue. Counting was standardized by the use of a geometrical pattern. Correlations between SPC and each DMC procedure, or among the DMC procedures were little influenced by the number of microscopic fields counted, their location on the smear or the definition of "clumping" used. Correlations were influenced by the type of bacterial culture inoculated in milk and by the staining procedure. Precision of DMC was shown to be independent from the staining procedure, but varied directly with the number of cells per field and inversely with the square root of the number of fields counted.

Standard plate counts (SPC), direct microscopic counts (DMC), and dye reduction methods are used interchangeably in the grading of raw milk. Frequently the disagreement between these methods is such that a substantial proportion of milk samples would be upgraded or downgraded depending on the grading method used *(2)*.

LaGrange and Nelson *(5)* reported that with pure cultures of various psychrophilic bacteria, SPC's invariably exceeded DMC's. They suggested that dispersal of clumps during dilution for SPC's was probably the main cause of the observed differences.

The distribution of bacteria in a microscopic smear has been shown to be non-uniform *(4)* and quite recently a definite ratio has been shown among leucocyte counts made from various positions on the microscopic smears *(7)*.

Although there is no report of a particular type of bacteria failing to stain by standard methods procedures, inadequate contrast with the background may, however, cause many cells to be overlooked or to be confused with artifacts in the smear. The type of bacteria in milk might conceivably be a factor in the discrepancy between SPC and DMC.

Since publication of the last edition of *Standard Methods for the Examination of Dairy Products (9)*, at least three new staining procedures have been proposed which show promise of being superior to procedures now in use. These are: *(a)* a modified Levowitz-Weber stain incorporating basic fuchsin in which bacteria are stained blue against a mottled pink background *(3)*; *(b)* a periodic acid-bisulfite-toluidine blue staining procedure which is outstanding for staining bacteria in heat-processed or dried milk *(6)*; and *(c)* a nucleic acid staining procedure using alcohol-acetic acid for fixation followed by staining with pH_4 toluidine blue. Stains described in *(b)* and *(c)* above stain bacteria purple against an absolutely featureless, colorless background *(6)*.

These new staining procedures were compared with SPC and with a procedure currently in use.

EXPERIMENTAL METHODS

SPC's in duplicate were done according to *Standard Methods (9)*. Plates were incubated at 32 C for 48 hours.

All microscopic smears were prepared according to *Standard Methods (9)*. Breed pipettes were used to transfer milk from sample to smear. The staining procedures were used in accordance with the specific instructions of their originators.

Pure cultures of *Pseudomonas fluorescens, Escherichia coli, Pseudomonas fragi, Alcaligenes viscolactis, Aerobacter aerogenes, Micrococcus varians,* and *Bacillus subtilus* were grown in nutrient broth for 18 to 24 hours. Portions of each actively growing bacterial culture were added to 50 ml of commercially pasteurized milk of good quality (in all cases less than 3,000/ml by SPC).

A procedure for counting microscopic fields was developed and standardized. Figure 1 shows the geometrical pattern used for the selection of microscopic fields. A graduated mechanical stage was used to determine the geometrical pattern on each smear. A total of 60 microscopic fields was selected as follows: 18 fields along AB (horizontal); 18 fields along CD (vertical) and 6 fields along each of the following lines, IJ, KL, EF, and GH. The distance between fields along each line was selected so as to be fairly representative and was determined with the graduated mechanical stage.

Counts were made of individual cells and of clumps, defining "clump" as *(a)* like cells visibly connected (touching); *(b)* like cells within one cell diameter, and *(c)* like cells separated by a distance less than twice the smallest diameter of the two cells nearest each other, the present standard definition according to "Standard Methods" *(9)*. The four

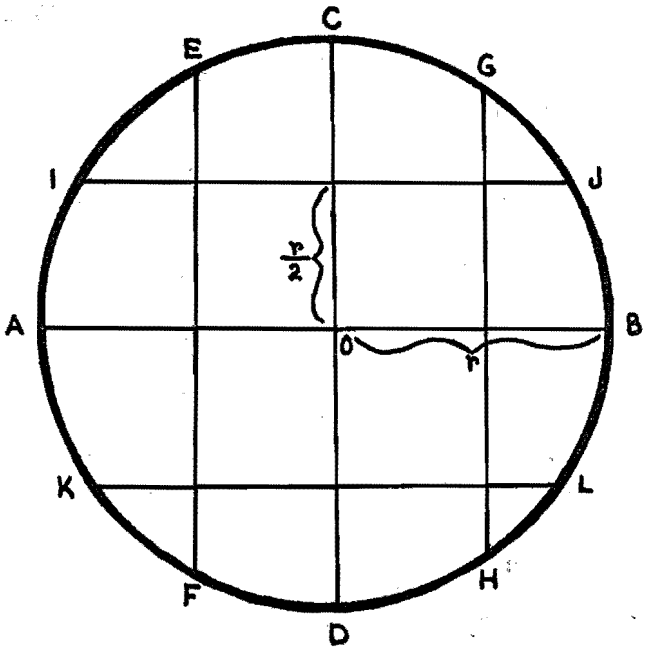

Figure 1. Geometrical pattern used for the selection of microscopic fields on smears.

counts were recorded for each microscopic field counted.

Counts per ml of milk were reported on the basis of 60 fields (total number of fields); on the basis of 36 fields (18 on AB and 18 on CD); on the basis of 18 horizontal fields (on AB); and on the basis of 18 vertical fields (on CD). In addition, two more counts were reported: one on the basis of 10 fields selected at random (5 on AB and 5 on CD), and the other on the basis of 5 fields selected at random along AB.

On the basis of SPC, bacteria populations investigated ranged from 180,000/ml to 60,000,000/ml.

A total of 132 microscopic smears was counted, that is, 33 sets of four smears: *Ps. fluorescens*, 7 replicate sets of four smears; *E. coli*, 5 sets; *M. varians*, 5 sets; *Ps. fragi*, 4 sets; *B. subtilus*, 4 sets; *A. aerogenes*, 4 sets; and *A. viscolactis*, 4 sets. Each smear was stained by one of the following procedures: Staining procedure A, Levowitz and Weber methylene blue stain *(9)*; staining procedure B, a modified Levowitz and Weber methylene blue stain with basic fuchsin *(3)*; staining procedure C, a periodic acid-bisulfite toluidine blue stain *(6)*; and by staining procedure D, an alcoholic-acetic acid fixation, toluidine blue stain *(6)*. A grand total of 7920 microscopic fields was counted (microscopic factor of 600,000); that is, 1980 microscopic fields for each staining procedure.

Results and discussion of the present study will be presented in three parts. Part one will deal with correlations between SPC and the four DMC procedures; part two will deal with correlation among the four DMC procedures; part three will deal with the precision of counting.

RESULTS AND DISCUSSION

Correlations between SPC and DMC procedures.

Table 1 shows the variations in correlation between SPC and DMC depending on the type of staining procedure used in DMC. Staining procedure D has the highest correlation with SPC. Inasmuch as SPC is a count of viable bacteria, staining procedure D appears to be more specific for viable bacteria than any of the other procedures.

Correlations between SPC and DMC's when the so-called "clump" definition was introduced are shown in Table 2. When staining procedures A, C, and D were used, the correlations between SPC and counts based on "touching" or "individual" were slightly higher than for counts based on "1 diameter" or "2 diameters". The number and the location of microscopic fields counted did not sensibly modify the correlations shown in Table 2, which were based on 60 fields per smear.

The correlations between SPC and DMC's for milk samples inoculated with various pure bacterial cultures are shown in Table 3. These correlations vary widely depending on the staining procedure and on the bacterial culture in the milk samples. Regardless of the staining procedure, SPC's and DMC's of milk inoculated with *Ps. fluorescens* or *A. aerogenes* were correlated significantly (all reference to statistical significance indicates significance at the 5% level of probability); SPC's and DMC's of milk inoculated with *B. subtilus*, however, were not correlated significantly. In milks inoculated with *E. coli* or *Ps. fragi*, SPC's and DMC's were correlated significantly except for staining procedure B. On the other hand, with procedure C, DMC's were not significantly correlated to their SPC for milks inoculated with *A. viscolactis* or *M. varians*.

TABLE 1. CORRELATION BETWEEN STANDARD PLATE COUNT (SPC) AND FOUR DIRECT MICROSCOPIC COUNT (DMC) PROCEDURES—ALL CULTURES BY NUMBER AND LOCATION OF MICROSCOPIC FIELDS COUNTED

Number and location of Field	Staining procedures[a]			
	A	B	C	D
60 H[b] & V[c]	0.906*	0.857*	0.864*	0.952*
36 H&V	0.913*	0.857*	0.859*	0.948*
18 H	0.912*	0.846*	0.870*	0.941*
18 V	0.903*	0.855*	0.841*	0.951*
10 random H&V	0.914*	0.846*	0.833*	0.955*
5 random H	0.926*	0.839*	0.827*	0.955*

[a]A—Levowitz-Weber's methylene blue stain.
B—Duitschaever-Leggat's fuchsin modification of stain A.
C—Moat's alcoholic-acetic acid, periodic acid-bisulfite treatment and staining with pH_4 toluidine blue.
D—Moats' alcoholic-acetic acid treatment and staining with pH_4 toluidine blue.
[b]H—Horizontal.
[c]V—Vertical.
*Significant correlation at the 5% level (31 df)

TABLE 2. EFFECT OF "CLUMP DEFINITION" ON THE CORRELATION BETWEEN SPC AND FOUR DMC PROCEDURES (ALL CULTURES, 60 FIELDS COUNTED)

"Clump" definition	Staining procedures[a]			
	A	B	C	D
Touching	0.919*	0.863*	0.866*	0.955*
1 diameter	0.900*	0.860*	0.863*	0.949*
2 diameters	0.893*	0.851*	0.857*	0.946*
Individual	0.911*	0.854*	0.868*	0.957*

*Significant correlation at the 5% level (31 df).
[a]See Table 1.

TABLE 3. CORRELATION BETWEEN SPC AND FOUR DMC PROCEDURES FOR MILK SAMPLES INOCULATED WITH PURE BACTERIAL CULTURES (COUNTING ON 60 FIELDS H&V)

Milk inoculated with:	df	Staining Procedures[a]			
		A	B	C	D
Ps. fluorescens	26	0.930*	0.713*	0.866*	0.908*
E. coli	18	0.858*	0.429	0.685*	0.917*
Ps. fragi	14	0.659*	−0.163	0.890*	0.752*
A. viscolactis	14	0.567*	0.676*	−0.138	0.606*
A. aerogenes	14	0.767*	0.833*	0.860*	0.942*
M. varians	18	0.651*	0.709*	0.337	0.862*
B. subtilus	14	0.146	−0.063	0.369	0.306

[a]See Table 1.
*Significant correlation at the 5% level.

Within each bacterial culture, the variation in correlation between SPC and DMC depended on the staining procedure used. In general, correlations between SPC and DMC were most consistently high for staining procedure D.

In a previous study (2) where staining procedure A was used, the correlation between SPC and DMC varied widely for milk supplies from different geographical locations in the United States. In the present study, correlation between SPC and DMC (staining procedure A) varied according to the type of bacterial population inoculated in milk. It is possible, then, that differences in the predominant bacterial flora from different geographic sources might account for variations in correlation between SPC and DMC noted in the previous study.

When correlations between SPC and DMC's were calculated for each type of bacterial population on the basis of numbers of fields counted or on their location on the smear, the pattern was the same as that shown in Table 3 which was based on 60 fields.

Data for *Ps. fluorescens* was further broken down on the basis of "clump" definition, number of fields counted, and location on the smear. Regardless of the "clump" definition used, DMC's and SPC's were significantly correlated, except for staining procedure B. In this case, DMC's based on "individual" or "touching" were not correlated significantly with their SPC regardless of the number or location of microscopic fields counted.

Correlations among the four DMC procedures.

Table 4 shows the correlations among the DMC procedures when all the cultures are lumped together and when counting is reported on the basis of 60 microscopic fields. Correlation was highest between staining procedures A and D. On the other hand, correlation was lowest between staining procedures B and C. When counts were reported on the basis of number of microscopic fields counted, correlations among the DMC procedures varied little from the correlations shown in Table 4.

When the data relative to all cultures were divided according to the "clump" definitions, correlations among the DMC procedures changed little and were not affected by the number of microscopic fields counted or by their location on the smear.

Correlations among DMC procedures for milk samples inoculated with various types of bacterial populations are shown in Table 5. Counting was based on 60 microscopic fields. Staining procedures B and C were not significantly correlated for milk inoculated with *E. coli, Ps. fragi, A. viscolactis,* or *B. subtilus.* On the other hand, procedures B and C were significantly correlated for milk inoculated with *Ps. fluorescens, A. aerogenes,* or *M. varians.* Again, as between SPC and the four DMC procedures, neither the number of microscopic fields counted nor their location on the smear affected the correlations among the four DMC procedures.

TABLE 4. CORRELATION AMONG FOUR *DMC* PROCEDURES-ALL CULTURES WITH COUNTING ON 60 FIELDS H&V

Staining[a] Procedures	B	C	D
A	0.939*	0.958*	0.968*
B		0.854*	0.904*
C			0.950*

*Significant at the 5% level (31 df).
[a]See Table 1.

These results show that correlations among various staining procedures depend on the type of organism inoculated in milk. This points to the danger of extrapolation of results based on a single type of bacterial culture to the correlation when mixed cultures are used. When data relative to *Ps. fluorescens* were analyzed, on the basis of "clump" definition,

number of microscopic fields counted, and their location on the smear, correlations varied little among the four DMC procedures.

All the correlations in this report were calculated on the basis of actual counts. Nevertheless, a statistical analysis was made of the same data after transformation into logarithms. When the data were analyzed without reference to the type of bacterial population inoculated in milk, the correlations between SPC and DMC's and within the DMC procedures were slightly higher than when actual counts were used. On the other hand, the correlations were always slightly lower when the data were analyzed on the basis of individual types of bacteria. In general, the use of logarithms in lieu of actual counts did not sensibly modify the results, *B. subtilus* was a notable exception. When actual counts were analyzed, correlations between SPC and DMC procedures were non-significant regardless of the staining procedure. On the other hand, analysis of logarithms for *B. subtilus* showed low, but significant, correlation between SPC and DMC for staining procedures C and D.

Precision of DMC

The accuracy of DMC is controlled by the systematic errors *(1)* which consistently produce results either too high or too low. On the other hand, the precision of DMC is controlled by the random errors *(1)* which introduce dispersion in the results.

In DMC, the term "accuracy" must be used with caution because there is no absolute standard for rating the accuracy of a procedure. On the other hand, the precision of DMC can be determined in terms of standard deviation, standard error, or confidence intervals. Although a confidence interval is usually expressed as percentage of the count per milliliter, we expressed it as percentage of the average bacterial count per field.

Bacteria are randomly distributed in a smear. So for the purpose of calculating precision, the counting of 60 fields in one smear is equivalent to the counting of one field in 60 smears of the same sample. Furthermore, the relative precision of DMC for counts based on 36, 18, 10, or 5 fields was calculated on the basis of the estimated standard error of the mean for counts of 60 microscopic fields.

Table 6 shows the influence of the level of bacterial population, on the precision of counting. Precision was shown to be significantly independent from the staining procedure, but was influenced significantly by the number of microscopic fields counted. Although only two staining procedures are shown in Table 6, the results apply to the other two staining procedures.

Over a wide range of counts the precision of counting is better when 60 microscopic fields are counted, but this is not practical. An adequate count, using a geometrical pattern to select microscopic fields, takes about 2 hours. The precision of counts based on 18 fields chosen horizontally and 18 fields chosen vertically is practically the same. On the other hand, the precision for counts based on 10 random field is low for low-count milk, but is acceptable for high-count milk. The precision of counts based on five fields selected at random is quite low even for high-count milk.

TABLE 5. CORRELATION AMONG FOUR DMC PROCEDURES FOR MILK SAMPLES INOCULATED WITH PURE BACTERIAL CULTURES (COUNTING ON 60 FIELDS H&V)

Staining[a] Procedure	*Ps. fluorescens*			*E. coli*			*Ps. fragi*		
	B	C	D	B	C	D	B	C	D
A	0.809*	0.944*	0.978*	0.784*	0.622*	0.910*	0.376	0.764*	0.982*
B		0.625*	0.736*		0.119	0.488*		−0.28	0.237
C			0.977*			0.721*			0.852*

Staining[a] Procedure	*A. viscolactis*			*A. aerogenes*			*M. varians*		
	B	C	D	B	C	D	B	C	D
A	0.938*	0.552*	0.942*	0.990*	0.982*	0.869*	0.907*	0.910*	0.839*
B		0.320	0.940*		0.986*	0.919*		0.742*	0.765*
C			0.487			0.921*			0.645*

Staining[a] Procedure	*B. subtilus*		
	B	C	D
A	0.345ns	0.849*	0.926*
B		0.400ns	0.176ns
C			0.944*

*Significant at the 5% level.

[a]See Table 1.

TABLE 6. PRECISION OF DMC EXPRESSED AS 95% CONFIDENCE INTERVAL IN TERMS OF PERCENTAGE OF AVERAGE NUMBER OF BACTERIA PER FIELD AND BASED ON THE ESTIMATED STANDARD ERROR OF THE MEAN COUNTS USING 60 FIELDS H & V — VARIATION IN PRECISION DUE TO LEVEL OF BACTERIAL POPULATION, STAINING PROCEDURE, NUMBER AND LOCATION OF MICROSCOPIC FIELDS COUNTED

	Average number of bacteria per field							
	0.72		4.83		11.3		40.0	
Staining procedure[a]	A	D	A	D	A	D	A	D
Number and location of field	Percent deviation from mean of the 95% confidence interval							
60 H & V[b]	23.7	27.6	10.2	10.7	8.4	8.0	7.1	7.3
36 H & V	27.9	32.0	16.3	15.7	12.3	11.7	10.3	9.9
18 H	38.9	46.1	24.8	24.1	16.1	18.2	14.9	15.6
18 V	41.7	50.0	23.0	22.8	21.3	16.2	15.3	13.8
10 random H & V	79.2	86.7	36.7	41.5	27.1	25.7	23.2	22.3
5 random H	117.1	89.0	68.7	81.6	46.1	47.4	42.1	47.0

[a]See Table 1.
[b]H = Horizontal
V = Vertical

Routine examination of milk samples by DMC, by counting five fields selected at random horizontally, does not appear to give adequate precision. For low-count milk 18 fields will give adequate precision, and for high-count milk 10 fields will give adequate precision.

While this report was being prepared, a paper by Schneider and Jasper *(8)* came to our attention. Their results on the precision of microscopic count of somatic cells(that it varies inversely with the working factor, i.e., the number of microscopic fields counted, and directly with the actual cell content) were similar to the variation of precision reported in the present study for direct microscopic counts of bacteria in milk.

Their results, as well as ours, are consistent with the Poisson distribution principles *(1)* that the precision of direct microscopic counts is a function of two variables, the number of fields counted and the number of cells per field (Table 6).

CONCLUSIONS

1. Correlation between SPC and DMC was influenced by the type of bacteria present in the milk. Type of bacteria present also influenced the correlations among the DMC procedures.

2. Correlations between SPC and DMC, and among four DMC procedures, were little influenced by the number of microscopic fields counted or by their location on the smear. Furthermore, the definition of "clumping" did not much affect the correlations between SPC and DMC procedures nor those among the four DMC procedures.

3. Correlation between SPC and the four DMC procedures varied according to the staining procedure used. Staining procedure D, an alcohol-acetic acid fixation followed by staining with pH_4 toluidine blue, appeared to be more specific for viable bacteria than any of the other staining procedures tested.

4. The precision of direct microscopic counting varied directly with the number of cells in the milk sample, but varied inversely with the square root of the number of fields counted. Because precision was low when counts were based on 5 fields selected at random, we recommend the use of 10 fields selected at random (5 horizontally and 5 vertically) for milk samples with high cell counts and of 18 fields horizontally or vertically for samples with low cell counts.

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REPRODUCIBLE VOLUME DELIVERED BY AN 0.01 ML "LOOP"

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SUMMARY

A platinum cylinder is described which may be used in place of a standard loop to give improved accuracy.

Jasper and Dellinger *(2)* recently made a thorough study of the variations in volume of milk delivered by a standard 0.01-ml loop. The volume was found to depend on the speed and angle of withdrawal of the loop and providing these two factors were kept under control the variation in volume delivered was found not to be too great for the purpose of making leukocyte counts in milk. However, it is clear that the variability must depend on the skill of the worker and it seems probable that if the variation between workers were included the total variation would be greater than that which was recorded. If something more accurate than an ordinary loop were available it would be *(a)* less dependent on a high level of skill in use or *(b)* available for more precise work.

As Jasper and Dellinger point out, the volume withdrawn by the loop is a function of the shape of the liquid-air interface. Thus the smaller this interface can be relative to the volume, other things being equal, the more reproducible will the volume be. Reducing the interface leads eventually to the capillary pipette, and the convenience of a loop has been sacrificed. A cylinder open both ends and of suitable dimensions forms an attractive compromise. A platinum cylinder 0.2 mm thick, 3 mm long and 2 mm in diameter (volume = .00943 ml) was tested by Berridge *(1)*. It was welded to a platinum wire 0.5 mm in diameter at right angles to the axis of the cylinder.

RESULTS

In a set of experiments to determine the reproducibility of volumes transferred by means of this cylinder, several workers were asked to make transfers in triplicate with no instructions or previous practice. The first group was seven in number and included inexperienced laboratory assistants and one engineer. The volumes transferred were measured by using 5.0 N hydrochloric acid, rinsing, and titrating with 0.25 N caustic alkali in a stream of nitrogen using an "Agla" micrometer syringe. In this experiment the individual values ranged from 86 to 118% of the general mean.

After this, five of the assistants were asked to repeat the experiment according to a set of instructions which was now provided. In this case the individual values ranged from 95 to 105% of the general mean, the spread for each worker being considerably smaller.

Contrary to expectation, the cylinder was found to be easy to keep clean. The only precaution beyond that used for an ordinary loop was to rinse once with distilled water before flaming to avoid the excessive accumulation of ash.

CONCLUSION

It is clear that a cylinder of this type is capable of higher reproducibility than is a normal loop and that it is less dependent on the skill of the worker.

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