SOME FACTORS RESPONSIBLE FOR VARIATION IN COUNTING SOMATIC CELLS ON PRESCOTT-BREED SMEARS OF MILK[1]

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SUMMARY

By means of direct microscopic observation of cells in stained smears and projection of a series of color transparencies, differences in criteria used by individuals for identification of cells to be counted in Prescott-Breed smears were discovered and described. Extensive studies revealed a unique pattern of cell distribution within smears which can materially affect the outcome of a cell count depending upon the area selected for cell counting. Among other factors, variation in size of the smear was found to have a marked affect upon resultant sample count.

The current widespread trend toward screening of total herd milk (bulk tank samples) for market suitability by means of estimated leucocyte numbers has brought a new phase of application for the Prescott-Breed or direct microscopic cell count method *(6)* which emphasizes the importance of accuracy. In a critical examination of the Prescott-Breed method for counting somatic cells in milk *(9)* the highest counts were observed to be as much as three times the lowest count for some groups of counts, the average high count being 1.9 times the average low count. Others have also shown large differences in results when duplicate smears were counted *(4, 5)*.

Cell numbers have been observed to be distributed in a 3:4:3 ratio through the central 1/3 area of smears which had been divided into three equal parts *(3)*. The numbers of bacteria per field in milk smears have been shown to decrease in proportion to the distance of a given field from the center of the smear *(2)*.

Thus a likely source of error would involve the areas of the smear selected for counting as related to the distribution of cells within a smear. A second source of error could involve differences in criteria used for cell recognition by the counter. Some of the factors affecting cell distribution were therefore critically examined in this study and uniform criteria for cell recognition were explored.

MATERIALS AND METHODS

Criteria for determination of cells to be counted were studied subjectively utilizing 6 counters. Direct microscopic observation of cells in stained smears and projection of a series of color transparencies selected to illustrate the variety of visual objects seen in milk films served as a basis of discussion between participants. Differences in criteria used by individuals were thereby discovered and reconciled into substantial agreement.

Samples of milk at room temperature in 1-oz bottles were used for smear making. Mixing was assured by shaking through an arc of 180° and back again at least 25 times over a period of 30 to 45 seconds *(8)*. Smears were made on precleaned glass microscope slides placed over templates delineating round or square 1-cm^2 areas. A clean movable sheet of plate glass made level on a laboratory table provided a uniform surface for the slides during smear preparation and drying. Smears were dried at room temperature and were fixed and stained within a few hours using the Broadhurst-Paley triple step process *(7)*. Adequate observations on cell distribution required counting of representative microscopic fields from one edge of the smear to the other. Other than the above, procedures as outlined in Standard Methods for the Examination of Dairy Products *(1)* were followed.

In order to minimize any bias resulting from smear making in studying the distribution of cells in a smear, the main factors affecting smear making were varied in trials 1, 2 and 3A. Using both a 0.01-ml loop and a syringe[2], multiple round and square smears were made from various milk samples at separate times by different smear makers. Care was taken to keep the loop in a vertical position when withdrawing it from the milk.

In Trial 1 separate horizontal and vertical counts of 25 fields each through the center, were made on 6 smears. The number of cells in each of the parts of areas B and E (Figure 1) was calculated. The 3:4:3 ratio was thus tested by comparison of parts 4:5:6 and 2:5:8 (Figure 1).

Trial 2 tested whether the 3:4:3 relationship was constant across all areas of the slide. Thirty-two smears were utilized. To test whether cell numbers affected cell distribution, the smears were so selected that the cell counts were reasonably spaced within a range from 80,000 to 14,000,000 cells per ml of milk. By use of the substage calibrations the smears were divided into thirds, both horizontally (Figure 1, areas A, B, C) and vertically (areas D, E, F) and counts of 25 equally spaced fields across the entire smear were made through the center of each of the 6 resulting areas. The 3:4:3 ratio was tested by comparing counts of A:B:C and D:E:F.

The pattern of cell numbers throughout the smear was studied in trial 3A using 26 smears each of which was divided into 9 equal parts (Figure 1). The smears were first divided and the counts were made as in Trial 2, except that every field in sequence across the smear was counted

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[2]Available from Applied Research Institute, 2 East 23rd Street, New York 10, N.Y.

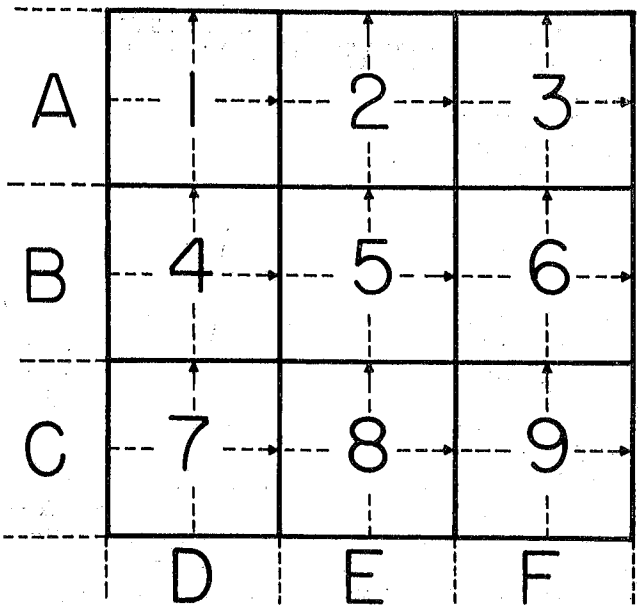

FIG. 1. SMEAR DIVIDED INTO 6 AREAS (A-F) AND 9 PARTS (1-9), SHOWING THE ORIGIN OF AREA ESTIMATES AND OF THE HORIZONTAL AND VERTICAL ESTIMATES FOR EACH PART.

and recorded. The fields in sequence for the 6 area counts on each smear were then divided into equal thirds and the number of cells in each part was totaled. There was thus one horizontal estimate and one vertical estimate from which the per cent of cells in each of the 9 parts was derived for each smear.

Since the tendency of the smear makers had been to start and stop in the upper portion of the smear, the possible effect of this practice was examined in Trial 3B utilizing 4 square smears in which the area of starting and stopping was in a different corner (Figure 1, Parts 1, 3, 7, 9). The smears were made by the same smear maker using the syringe.

In view of the errors in smear making *(8)* and variations in the distribution of cells found in smears 100 mm^2 in area, the possibility that another smear size could produce more accurate and uniform results was explored. Square smears were made covering areas of 49, 72, 100, 144 and 196 mm^2. Smears covering 289 and 400 mm^2 were also tried, but it was concluded that representative smears could not be made that large in size because portions of such large smears tended to dry before all of the spreading took place.

Two trials were conducted. Trial 4A included only smear sizes, 100, 144 and 196 mm^2. Trial 4B included all 5 smear sizes. In both trials, 5 separate milk samples ranging from high to low known cell counts were utilized. A syringe was used by the same smear maker to make 4 smears of each size in pairs from each sample in a set order.

Counts on the smear pairs were made in random order. In trial 4A, 25 field counts of both horizontal and vertical counts through the center were made on each smear regardless of size. The WF thus varied between 2,500 and approximately 5,000, dpending on the size of the smear. In trial 4B, 2 separate series of counts were made on all of the smears using a WF of 2,500 for each series, hence, when the 2 separate series were combined counts with a WF of 1,250 were produced.

To determine if the cell distribution was the same for all smear sizes, the 4 smears made for each size from the highest cell count sample of trial 4B, were counted as outlined for the Trial 3 study above.

RESULTS

From the observations and discussions, variation in criteria for cell recognition could be placed in 5 general categories. These involved the disposition to be made of ghost cells or cytoplasmic masses without a nucleus, cells in various stages of degeneration, nuclear and other particles or portions of cells, clumps of cells, and cells only partially within a field. The criteria for the recognition of cells to be counted was established as follows:

1. All stained material recognizable as containing more than 50% of a cell nucleus was counted as a cell. This applied also to cells only partially seen at the margin of a field.

2. Smaller cell fragments and cytoplasmic material were not counted as a cell.

3. All individual cells in cell clumps or masses were counted providing the total count of the field did not exceed the highest count obtained in any of the other fields without clumps. If the highest field count without clumps was exceeded, a count for that field equivalent to the highest count attained without clumps was recorded.

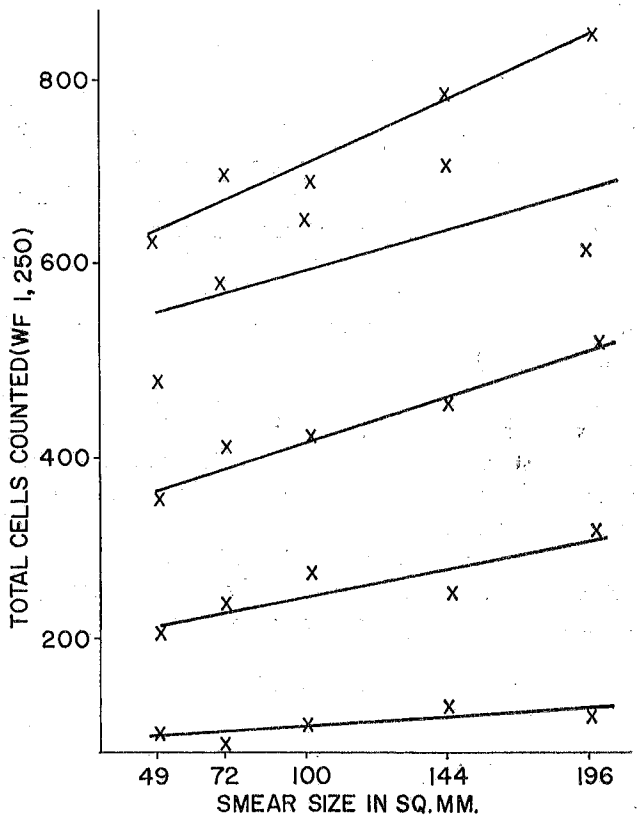

FIG. 2 TRIAL 4B, LINES OF "BEST FIT" VIA THE LEAST SQUARES METHOD FOR 5 SMEAR SIZES AND 5 MILK SAMPLES.

TABLE 1. THE PER CENT OF CELLS FOUND IN EACH AREA ON 32 SMEARS

	Horizontal counts			Vertical counts		
	A[a]	B	C	D	E	F
% of total cells counted[b]	33.4	38.5	28.1	31.8	37.7	30.5
Mean % when equal weight given each smear	31.9	37.8	30.3	31.0	34.8	34.2

[a]Letters refer to area designations in Figure 1.
[b]Chi Square on 3:4:3 ratio prior to conversion to % for each was P>.001.

TABLE 2. THE MEAN PER CENT OF CELLS AND STANDARD DEVIATIONS IN EACH OF NINE PARTS[a] FOR 26 SMEARS

10.4 ± 2.3	11.7 ± 3.3	10.9 ± 2.5
11.2 ± 1.7	15.1 ± 2.1	11.2 ± 3.6
8.6[b] ± 2.5	11.0 ± 1.5	10.0 ± 2.4

[a]Parts refer to those designated in Figure 1.
[b]Significantly different from other outside corner parts P>.01.

TABLE 3. THE EFFECT OF STARTING AND FINISHING THE SPREADING OF A SMEAR IN DIFFERENT CORNERS OF THE SMEAR

Smear	Part[a] started and stopped and the 2 adjacent parts[b]	Opposite 3 parts	% Cell parts Col'm 2	% Cell parts Col'm 3
1	6, 9, 8	2, 1, 4	36.8	28.5
3	2, 1, 4	6, 9, 8	35.4	31.8
2	2, 3, 6	4, 7, 8	33.0	32.8
4	4, 7, 8	2, 3, 6	33.8	32.6

[a]Parts refers to those designated in Figure 1.
[b]The part in which the smear making started and stopped is the center figure.

The pattern of cells found in Trial 1 by counting horizontally and vertically through the central areas of the smears (Figure 1 parts 4:5:6 and 2:5:8) confirmed the presence of a 3:4:3 ratio. No significant variations from this ratio was found in Chi Square tests. The 3:4:3 ratio for distribution of cells through the center was also verified in trial 3A (Table 2) and trial 4B (Table 5c).

The relative inconsistency of the 3:4:3 relationship when comparisons were made of counts across the entire smear (Trial 2) is shown in Table 1. A total of 16,410 cells were counted in the horizontal counts (Figure 1, areas A:B:C) and 16,546 cells on the vertical counts (Figure 1, areas D:E:F) on the 32 smears. It was found that the ratios of A:B:C and D:E:F varied significantly from the 3:4:3 ratio (P>.001). The effect of cell numbers on cell distribution is also illustrated in Table 1. Because smears with fewer cells tended to vary the most from a 3:4:3 ratio, such variability had a greater effect when equal weight was given to all smears.

The mean proportion of cells and standard deviations in each of the nine parts (Trial 3A) is shown in Table 2. Overall, approximately 15% of the cells were in the center ninth (Figure 1, part 5), and generally between 10 to 12% in each of the other parts. It was noticed that on the average there seemed to be more cells in the 4 middle outside parts (2, 4, 6 and 8) than in the 4 corner outside parts (1, 3, 7 and 9). On the 26 smears, the average proportion in each of parts 2, 4, 6 and 8 was 11.3%. The average in parts 1, 3, 7 and 9 was 10.0%. Part 7 varied significantly from the other corner outside parts (P>.01) and if it was excluded, the average for parts 1, 3 and 9 was 10.4%. Hence, on the average, each of the middle outside parts contained about 8% more cells than the corner outside parts, part 7 excepted.

Table 3 illustrates the effect of starting and finishing in different corners. There was always a higher proportion of cells in the total of the parts in which the smear was started and finished and those on either side, as compared to the total proportion of cells in the 3 opposite parts.

Trials 4A and 4B were comparisons of cell counts derived from smears of different sizes. Table 4 indicates the results of counts through the center for all smear sizes. The means of the ratios of the cell counts are also given for the different smear sizes

TABLE 4. TOTAL CELLS COUNTED[a] ON DIFFERENT SIZE SMEARS FOR 10 SEPARATE MILK SAMPLES AND THEIR MEAN RATIOS TO 100 SQ. MM COUNTS

	Smear size in mm²				
Sample No.	49	72	100	144	196
Trial 1					
1	-	-	851	984	1174
2	-	-	637	677	811
3	-	-	422	471	586
4	-	-	228	251	280
5	-	-	19	23	20
Mean of ratios	-	-	1.00	1.13	1.26
Trial 2					
1	314	349	345	392	429
2	236	292	326	354	308
3	178	202	210	230	264
4	104	121	138	127	163
5	50	44	56	66	62
Mean of ratios	.82	.91	1.00	1.09	1.15
Combined mean of ratios	.82	.91	1.00	1.11	1.21

[a]Cells counted adjusted to WF of 2,500 and rounded to the nearest whole cell.

studied using 100 mm² as the standard smear size. It is seen that the larger the smear, the higher will be the cell count.

Figure 2 shows the counts for each sample and

TABLE 5. THE MEAN PER CENT[a] OF CELLS IN EACH OF THE NINE PARTS[b] FOR DIFFERENT SIZE SMEARS

a. 49 Sq. Mm.			b. 72 Sq. Mm.		
10.2	11.1	9.0	9.1	11.7	10.3
14.2[c]	13.6	11.1	12.9	14.4	11.5
9.5	11.2	10.2	9.6	11.7	8.9

c. 100 Sq. Mm.		
7.7	12.6	8.8
13.0	16.0	12.1
8.9	12.5	8.5

d. 144 Sq. Mm.			e. 196 Sq. Mm.		
6.9	11.8	8.6	10.1[d]	11.1	8.3
11.7	20.2	12.2	11.2	22.8	10.9
7.8	12.3	8.6	7.8	10.6	7.1

[a]Mean of counts on 4 smears for each size.
[b]Parts refer to those designated in Figure 1.
[c]Significantly different from the other middle outside parts $P>.001$.
[d]Significantly different from the other outside corner parts $P>.01$.

TABLE 6. PER CENT VARIATIONS IN COUNTS MADE THROUGH DIFFERENT AREAS OF A SMEAR

Area[a]	6 Smear group	32 Smear group	26 Smear group	All smears
A	–	− 2.4	− 1.0	− 1.8
B	+9.5	+15.4	+12.2	+13.5
C	–	− 7.4	−11.2	− 9.1
D	–	− 8.9	− 9.5	− 9.2
E	+5.9	+ 2.6	+13.4	+ 7.3
F	–	+ .7	− 3.8	− 1.3

[a]Letters refer to areas designated in Figure 1.

smear size in trial 4B and the line of "best linear fit" for the counts on each sample computed via the least squares method. It is apparent that the higher the cell count, the steeper is the line of "best linear fit." This increased steepness appears to be due to a relatively constant per cent of change of cell count between the different smear sizes, regardless of sample cell content. Hence, the higher the cell count of a sample, the larger will be the difference in number of cells between smear sizes.

The pattern of cell distribution for different smear sizes is shown in Table 5. As also observed in Trial 3 there is a pattern of cell distribution with the outside corner parts, 1, 3, 7 and 9, comprising one group, the outside middle parts, 2, 4, 6 and 8, comprising a second group, and the center part, 5, comprising a third group. It is seen that with increased size of smears, there is an apparent decrease in cell concentration in the outside parts, with subsequent increase in concentration of cells in the center part.

DISCUSSION

The distribution of cells in smears indicates a tendency for cells to migrate from the outer parts, 1, 3, 7, 9 towards the inner parts, 2, 4, 5, 6 and 8, as the smear dries. The last part to dry is usually part 5, hence it has the most cells. This appears to be true regardless of the size of a smear. This pattern has also been observed for bacteria in milk smears *(2)*.

There is apparently a consistent variation in the distribution of cells in a smear. In general, a 3:4:3 distribution of cells can be expected if a smear is divided into equal thirds and counts are made either horizontally or vertically through the central third (Tables 2 and 5c). If a smear is divided into ninths (Figure 1), then approximately 15% of the cells are in the center ninth (part 5), and generally between 10 and 12% in each of the other outside parts (Table 2), with the outside middle parts containing an average of approximately 8% more cells than the outside corner parts. Since no differences in total count were obtained between smears dried at room temperature and at 37 C it would appear that differences in drying temperature within this range did not greatly alter migration patterns *(8)*.

Table 6 illustrates that counts made through different areas of a smear will not agree. Counts made through the center of the smear (Figure 1, B and E) will generally overestimate cell numbers by about 10.4%. This, of course, is due to the high proportion of cells in the middle ninth (part 5) and to a lesser extent the higher proportion of cells in the middle outside part, 2, 4, 6 and 8.

Theoretically, the area of the smear selected for counting should be based on the pattern of the cell distribution found on smears when the smear is divided into ninths (Figure 1 and Table 2), so representative fields are counted in each part. This, however, is not practical. In the interest of a good routine method that is repeatable and accurate, it is suggested that counts be made through the center as stated by Standard Methods for the Examination of Dairy Products *(1)*. Although counting in this fashion will overestimate by approximately 10%, a downward adjustment of 10% could be made whenever a more accurate estimate is needed. This adjustment could be built into the routine by adjusting proportionally either the WF or the number of fields to be counted. In most instances, this correction can be ignored but it is important that standard counting procedures be followed so that all counts in a group will be comparable.

Application of the Prescott-Breed method to any smear size other than 1 cm^2 (100 mm^2) would produce counts markedly different from those made on 1 cm^2 smears. Within the limits of the sizes examined, if larger smear sizes are counted through the center, the counts will be higher; if smaller smear sizes are used, the counts will be lower (Table 4). The change in cell count with change in smear size

is apparently due to the change in the pattern of cells found in smears (Table 5). With increased size of smears, there is a larger proportional concentration of cells in the center of smears, with a corresponding decrease of cells in the outside parts. The pattern of cell migration appears to be the same in all smear sizes, that of movement from parts 1, 3, 7 and 9 into parts 2, 4, 5, 6, and 8, with concentration of cells in the center part, part 5.

Results in Trial 3 had indicated that part 7 was unexplainably significantly lower than the other outside corner parts, 1, 3, and 9, (Table 2). It is noticed in trial 4B (Table 5) that part 7 did not vary significantly from the outside corners for any of the smear sizes, although the method of making and counting smears was not knowingly varied between the 2 trials. There is no reason to expect that part 7 should consistently differ from parts 1, 3, and 9.

CONCLUSIONS

1. Uniform criteria are necessary for recognition of somatic cells to be counted.

2. The number of cells counted in any given smear can vary considerably depending upon the area of the smear selected for counting.

3. Cells tend to migrate toward the center of the smear. This resulted in a 3:4:3 ratio for cell numbers in equal thirds of the smear when counted through the center, either horizontally or vertically on smears dried at room temperature.

4. Cell numbers also tend to increase in that portion of the smear where the making of the smear is started and stopped.

5. Higher cell counts are obtained for counts through the center if the smear area is increased. This results from a heightened tendency of cells to migrate toward the center. By the same token, smear areas of less than 1 cm^2 tend to give lower total cell counts by the same counting method.

6. Standardized procedures for counting cells are essential if uniform results are to be obtained. These include the following:

a. Uniform procedures for smear making.

b. Uniform smear size standardized at 1 cm^2.

c. Uniform criteria for cell recognition as described herein.

d. Uniform procedures for field selection. It is recommended that every other field be counted as the objective is moved through the center from one edge of the smear to the other.

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PROGRAM FOR CERTIFYING BAKERY EQUIPMENT UNDER WAY

The Baking Industry Sanitation Standards Committee, through its Office of Certification, began on January 1, 1966 its program to certify bakery equipment conforming to BISSC sanitation standards. Information supplied on registration and application forms furnished bakery equipment manufacturers and others interested forms the basis of certification.

A distinctive BISSC certification symbol has been approved and application for appropriate registration of the symbol has been filed. The BISSC program will require that the symbol be affixed to certified equipment in juxtaposition to the manufacturer's name plate or be a part thereof. Where this is impracticable it shall be stamped, etched or embossed on the equipment.

BISSC was established in 1949 and is comprised of representatives from six national trade organizations, four national sanitarian organizations, and FDA and USDA and USPHS. Twenty-three sanitation standards for bakery equipment have been developed, approved and published to date and a number of other standards are nearing completion. It is planned to continue the program until all bakery equipment and machinery has been covered effectively by BISSC sanitation standards.