STUDIES USING THE DIRECT MICROSCOPIC SOMATIC CELL COUNT

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ABSTRACT

Factors which might contribute to variance of cell estimates in milk, using the Direct Microscopic Somatic Cell Count (DMSCC) were investigated. Comparisons of cell estimates obtained from counting cells in nine areas of 20 circular milk films produced no evidence of uneven distribution of cells on these films. Estimates of cells by DMSCC were not affected by the temperature of the milk, or the method of spreading, when milk films were prepared. Standard deviations and means of counts indicated no significant difference in cell estimates obtained by three operators. Cell estimates from counts made in horizontal and vertical diameter strips of milk films showed no significant differences among means or variances relating to strip direction. Cells were counted in diameter strips (as defined by a wide and narrow ocular reticle) at two magnifications (450 x and 1,000 x). Cell estimates based on counts from the wide strips had lower standard deviations than those from the narrow strips.

Confidence limits were established for estimating 1 x 10^6 cells per ml of milk at p = 0.05.

A procedure for determining precision within laboratories and permitting a comparison between laboratories is presented.

The Direct Microscopic Somatic Cell Count (DMSCC) *(6)*, was developed by the Sub-Committee on Screening Tests of the National Mastitis Council to improve the accuracy and precision of the Direct Microscopic Leukocyte Count *(2)*. The area examined using the DMSCC is described as four diameter strips on two circular milk films. Expected confidence limits for the DMSCC have been determined *(6)*.

This study was initiated to determine factors that might contribute to variance of cell estimates using the DMSCC. Some factors which might influence cell estimates that were included in this study were: *(a)* distribution of cells over the surface of a film of milk, *(b)* effect of temperature of milk and method of spreading milk film, *(c)* variation between operators, *(d)* variation between direction of strips on milk films (horizontal and vertical), *(e)* effect of width of strip, and *(f)* effect of two magnifications (1,000 x and 450 x).

MATERIALS AND METHODS

Preparation of milk films

The milk films in this study were prepared as described for the DMSCC *(6)*, except that the slides contained 3 rows of 5 circular 1 cm^2 areas surrounded by etching[1].

Microscope

One ocular (wide field) of the binocular microscope[2] was equipped with a reticle with two sets of parallel lines[3] which permitted the examination of a portion of the milk film of known width as defined by the parallel boundaries in the microscope field. The band widths were 0.078 mm (wide band) and 0.026 mm (narrow band) using 1000 x magnification; and 0.179 mm and 0.058 mm using 450 x magnification. Horizontal and vertical diameter strips of the milk films were located as described elsewhere *(6)*.

Examination for distribution of cells on milk film

Eleven horizontal and 11 vertical strips were examined (1000 x magnification, wide reticle band) on each of 20 circular milk films. Following the location of the horizontal diameter strip, 5 additional strips on each side of and parrallel to the horizontal strip were examined. All strips were located with 1 mm distance between centers of adjacent strips. A similar pattern was followed for the location of 11 vertical strips. The relative location of 22 strips that were examined on each film is depicted in Fig. 1. The vernier scale on the mechanical stage permitted the location of all strips that were not diameter strips.

It was necessary to develop a mathematical equation for determining the area of each of the strips. In a circle whose area is 1 cm^2, the radius is approximately 5.6419 mm.

To determine the area of a parallel sided strip any place on the circle, the following equations were derived:

$$\text{(I)}\quad A(h) = h\sqrt{1 - h^2} + \arcsin h$$

$$\text{(II)}\quad A = 0.5 \sin 2\ominus + \ominus$$

These equations permit the determination of the area A between a diameter and a lesser chord, in this instance, one of the boundaries of a parallel sided strip (Fig. 2), where:

(a) The radius of circle is 1.
(b) h is expressed as a portion of the radius.
(c) $\sin \ominus = h$, and $\ominus$ ($\arcsin h$) is expressed in radian measure.

Let H_1 represent the distance in millimeters from the diameter to the nearer side of a strip, and H_2 represent the distance in millimeters from the diameter to the farther side of the strip (Fig. 2, B). Solve first for the area determined by using H_1, then that area determined by using H_2. It is necessary to convert the actual H values in millimeters into

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[2]American Optical Co., Eggert and Sugar Road, Buffalo, N. Y. 14215.

[3]Special reticle developed for SubCommittee of National Mastitis Council Research Committee.

units of h which fall between 0 and 1. To accomplish this:

(III) $h = \frac{H \text{ mm}}{5.6419 \text{ mm}}$

The steps necessary for solution of equation (II):

(a) Determine H_1 in mm from the vernier scale on the mechanical stage of the microscope (Note: The difference between H_1 and H_2 was 0.078 mm in each instance with the reticle-microscope combination used.)

(b) Convert H to h using equation (III).

(c) From a table of natural functions for angles expressed in radians, determine a value for

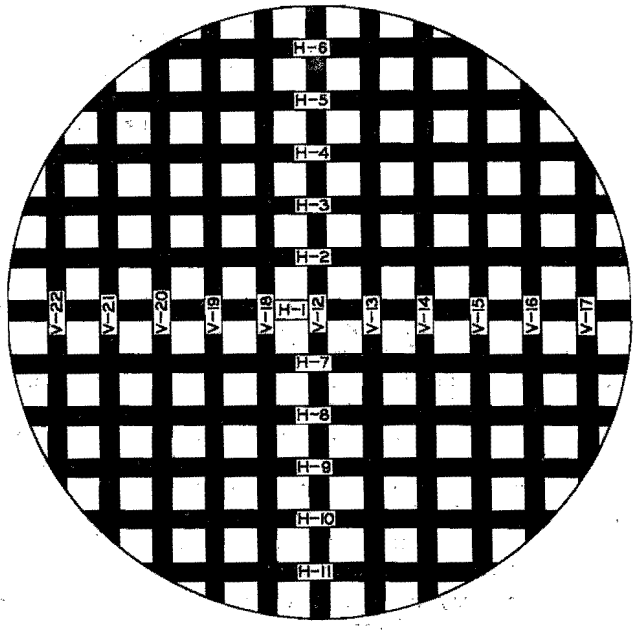
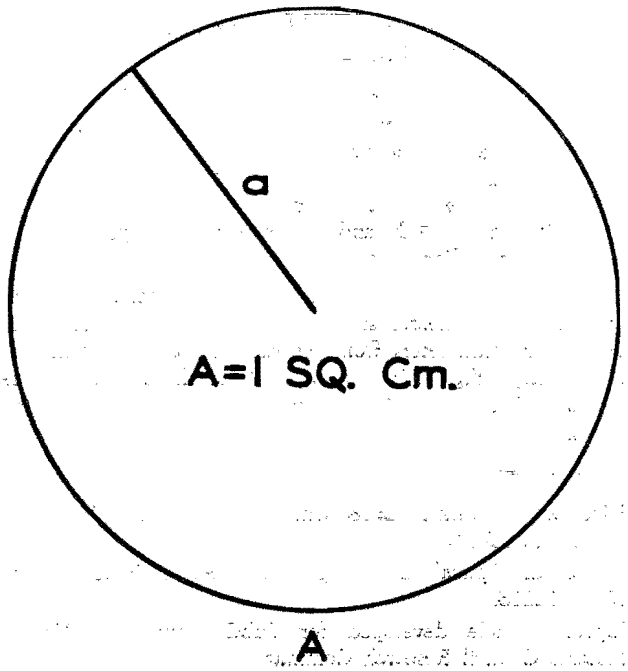


Figure 1. Diagramtic representation of relative location of 22 strips examined for distribution of cells on film of milk.



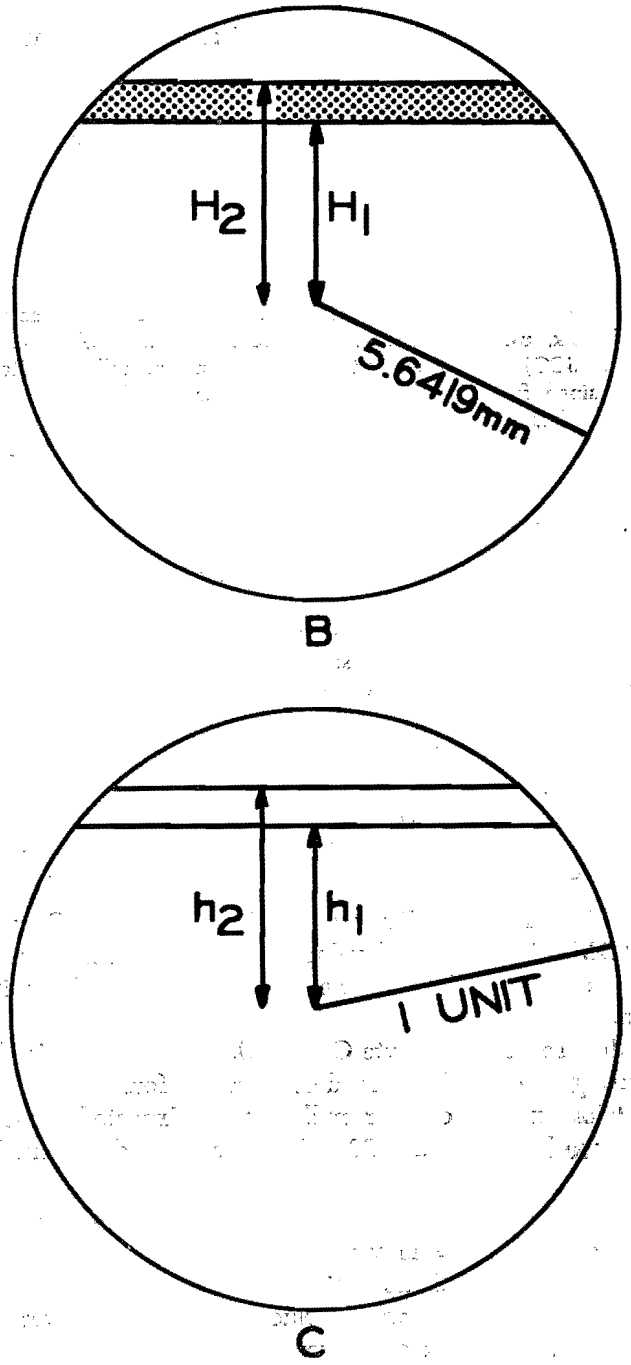


Figure 2. Figures used to explain the computation of the area of a strip of circular milk film.

$\ominus$, which is the arcsin h.

(d) Double $\ominus$.

(e) From the table (step 3), determine the sin of 2 $\ominus$.

(f) Divide this value (step 5), by 2.

(g) Add the value $\ominus$ and 0.5 sin 2 $\ominus$.

(h) Divide this value (step 7) by 3.14159 to determine the portion of the area of the circle between a diameter and the nearer side of the strip.

(i) Solve the equation again using H_2 value (steps 1 through 8).

(i) Subtract the value determined by using H_1 from the value determined by using H_2. This difference represents the portion of the area of the circle which falls within the strip under consideration.

It is of academic interest to note that a diameter strip, whose width is 0.078 mm with ends that are arcs of a circle with an area of 1 cm^2 has an area of 0.88012 mm^2, whereas, if the strip were considered to be a true rectangle 0.078 mm x 11.2838 mm, the area would be 0.880136 mm^2. Therefore, for practical purposes, the diameter strips described in this study could be considered rectangles.

Milk Samples

Fresh, bulk milk samples were obtained from Madison Milk Producers, Madison, Wisconsin. Films were prepared the same day that samples were obtained.

RESULTS

Distribution of cells on film of milk

Trial I. Ten milk samples with various concentrations of cells (150,000 to 3,000,000 cells/ml) were used. Strips were numbered from 1 to 22 (Fig. 1). Cell estimates were derived from 9 areas of each film using various combinations of strips, (Table 1). Analysis of variance indicated no significant difference among 9 estimates of cell counts within each film.

Trial II. Ten replicate films from one milk sample were used (approximately 400,000 cells/ml). Cell estimates were derived from 9 areas of each film using various combinations of strips as in trial I (Table 1). Analysis of variance indicated no significant differences within or between estimates from ten films.

Temperature and method of spreading milk

In order to determine if the temperature of milk or the method of spreading milk affected cell estimates, the following trial was conducted. Milk from five different bulk milk samples (range–600,000 to 1,500,000 cells/ml) were used to make films. One series was made with cold milk (4 C) using a metal rod to spread the milk. A second series was made from cold milk (4 C) using the pipette (0.01 ml) for spreading the milk. A third and fourth series were made from warm milk (35 C) and the milk was spread with a metal rod and the pipette as above. A 2 x 2 factorial analysis of variance indicated no significant difference between methods and no interaction between methods.

Operators

The analysis of variance comparing cell counts by three operators on 576 milk samples (Table 2) revealed that there was no significant difference between group means. The standard deviation of each of the three operators was similar.

Horizontal and vertical strips

The analysis of variance comparing cell estimates from counts in horizontal and vertical strips (Table 3) indicated that there was no significant difference in the means of either direction. The standard deviation from strips of each direction was similar.

Strip width and magnification

A summary of the results comparing strip width and magnification are presented (Table 4). A paired t test was used to compare the variance and means of each method. There was no significant difference between means or variance using either 450 x or 1000 x magnification. Also, there was no significant difference between the means obtained using wide or narrow strips. However, there was significantly more variation ($p = 0.001$) in cell estimates obtained using narrow strips than in cell estimates obtained using wide strips.

CONFIDENCE LIMITS

The components of variance of the remaining factors were computed using only data from wide strip, 1000 x magnification (Table 5). When the various components were separated, they were as follows:

Estimated residual variance	126
Estimated sub-sample variance	40
Estimated film variance	25

Using 1,000,000 cells/ml as the nominal rejection level for milk, 95% confidence limits were computed (Table 6). Confidence limits are expressed in cell numbers.

DISCUSSION

Precision within laboratories and standardization between laboratories

The ideal reticle as described for use in the DMSCC for the purpose of identifying milks that contain 1,000,000 or more cells per milliliter, will define a diameter strip on the milk film which contains approximately 100 cells. Microscopes equipped with different oculars (eg: wide field, huygenian, 10 x, 7.5 x, etc.) may require reticles with different dimensions in order to achieve a strip width that will include 100 cells *(6)*.

Comparison of cell counts between laboratories has little meaning unless something is known of the level of precision of the DMSCC in each laboratory. If confidence limits for DMSCC were developed for each laboratory and were of similar value, then meaningful comparison between laboratories could occur. Confidence limits would also give an indication of agreement between any laboratory and the guide lines suggested for the DMSCC *(6)*.

The following is a description of a procedure from which confidence limits for the DMSCC may be developed:

TABLE 1. DESCRIPTION AND IDENTIFICATION OF NINE AREAS OF MILK FILM WITH MICROSCOPE WORKING FACTORS AND MEANS OF CELL ESTIMATES

Area Examined	Strips	Working factor	Means x 10^{-6} Trial I	Trial II
Center horizontal strip	H-1	11,362	0.995	0.442
Center vertical strip	V-12	11,362	1.026	0.442
Center vertical and horizontal strips	H-1, V-12	5,681	1.011	0.442
All horizontal strips	H-1 through H-11	1,278	0.971	0.434
All vertical strips	V-12 through V-22	1,278	0.987	0.436
All strips	H-1 through V-22	639	0.979	0.435
Outer area strips	H-5, H-6, H-10, H-11, V-16, V-17, V-21, V-22	2,431	0.922	0.462
Intermediate area strips	H-3, H-4, H-8, H-9, V-14, V-15, V-19, V-20			
Center area strip		1,585	0.984	0.421
(3 horizontal and 3 vertical strips)	H-1, H-2, H-7, V-12, V-13, V-18	1,912	1.009	0.430

TABLE 2. COMPARISON OF CELL COUNTS (DMSCC) FOR THREE OPERATORS AND ANOVA TABLE

(Cell estimates = x 10^{-4})

Operator	Number of observations	Group mean	Group standard deviation
1	576	116.1	58.7
2	576	119.5	60.6
3	576	118.5	56.9

Analysis of Variance Table

Source	Degrees of freedom	Mean square
Between groups	2	1,814.2
Within groups	1,725	3,451.5
	F ratio = 0.526 n.s.	

TABLE 3. COMPARISON OF CELL ESTIMATES (DMSCC) FROM COUNTS MADE IN HORIZONTAL AND VERTICAL STRIPS

(Cell estimates = x 10^{-4})

Direction	Number of observations	Group mean	Group standard deviation
Horizontal	288	111.3	55.4
Vertical	288	111.5	53.7

Analysis of Variance Table

Source	Degrees of freedom	Mean square
Between groups	1	5.35
Within groups	574	2,977.72
	F ratio = .002	

A fresh milk sample is selected that contains approximately the cell concentration that is to be used as a rejection level for acceptable milks (example: 1 or 1.5 million). The cell count in the sample chosen may be approximated by a single microscope count. Use of indirect tests should be avoided for the selection of this sample.

This sample should be used to make 20 films. Each film should be examined across one diameter either vertical or horizontal according to the operator's preference. Variation (S^2) is determined using a standard method (5, p. 58). Example: *(a)* Determine mean (average of 20 counts). *(b)* Subtract each of the counts from the mean. *(c)* Square each of these values. *(d)* Add these values. *(e)* Divide this sum by 19. Confidence limits are obtained by using this equation (5, p. 58):

$$\frac{2S^2}{\sqrt{n}} = L$$

L = 95% confidence limits
n = number of films examined

Let us assume that $S^2 = 10$. Then, for n = 1, the confidence limits are ±20. Therefore, assuming the microscope working factor is 10,000 and the rejection level is 1,000,000 cells/ml, it would be necessary to obtain a count of 120 cells on a single strip in order to be confident that the original sample contained 1 million or more cells per milliliter. Similarly, a count of 80 or less per strip would be necessary to know with 95% confidence that the sample had less than 1 million cells per milliliter. The confidence limits would be reduced in the following manner: for two srtips, L = ± 14.1; for three strips, L = ± 11.5; for four strips, L = ± 10, etc.

The milk sample may be sent to another laboratory where four films should be prepared and counted across one diameter each. The average of these four counts should fall within the confidence limits of four strips as computed above. This is necessary to confirm the operator's ability to pipette 0.01 ml milk accurately and determine if cell identification was performed correctly.

TABLE 4. COMPARISON OF MEAN CELL ESTIMATES AND STANDARD DEVIATOINS OF TWO STRIP WIDTHS AND TWO MAGNIFICATIONS

Band	Width	Magnification	Strip working factor	Number of observations	Group mean	Group standard deviation
Wide	0.179 mm	450 x	4,950	80	66.4	9.20
	0.078 mm	1,000 x	11,362	80	65.8	9.67
Narrow	0.058 mm	450 x	15,384	80	69.7	12.15
	0.026 mm	1,000 x	33,333	80	62.4	13.17

TABLE 5. COMPONENTS OF VARIANCE FOR BULK, SUB-SAMPLE, AND FILMS

Sources	Degree of freedom	Mean square	Expected mean square
Bulk	3	541,889	$\frac{\sigma^2}{e} + 48\frac{\sigma^2}{B} + 4\frac{\sigma^2}{S} + 2\frac{\sigma^2}{F}$
Sub-sample	44	335	$\frac{\sigma^2}{e} + 4\frac{\sigma^2}{S} + 2\frac{\sigma^2}{F}$
Film	48	175	$\frac{\sigma^2}{e} + \frac{2\sigma^2}{F}$
Residual	480	125	$\frac{\sigma^2}{e}$

TABLE 6. 95% CONFIDENCE LIMITS FOR MEAN CELL COUNTS OF 100 CELLS PER STRIP ASSUMING THE MICROSCOPE WORKING FACTOR IS 10,000.

Area of film(s) examined to determine cell estimates	Cells per strip			
	12 sub-samples	4 sub-samples	2 sub-samples	1 sample
One strip on one film	±6.5	±11.3	±15.7	±22.6
Two strips on one film	±5.3	± 9.3	±13.1	±18.5
One strip on each of two films	±5.1	± 8.1	±12.4	±17.6
Two strips on each of two films	±4.3	± 7.5	±10.6	±15.0

By use of this method, laboratories could accept or reject milk with a 95% assurance that results of the DMSCC are comparable between different laboratories.

Results from this experiment reinforce the earlier report *(6)* of the possibility of repeatability of the DMSCC among different operators. Since no effects on cell counts were determined from temperature of milk or method of application of milk at the time films are prepared, there is no reason to vary from the standard procedure of the DMSCC. This investigation also reinforces the report *(6)* that no difference should be expected in cell numbers, related to direction of counting in strips (horizontal or vertical).

Unlike the distribution of cells that has been reported for films with a square format *(1, 3)* and circular format *(4)*, the distribution of cells on circular films appears to introduce no important bias. The method used in this study was not identical to the study of Smith *(4)*. This could explain the reason why this study revealed no important bias because of uneven distribution of cells.

It appears from results of this study that variance of counts will be lower using a wide strip than when a narrow strip is used. It would appear that a band width which would yield a working factor of approximately 10,000 would be satisfactory. Even though 450 x magnification yielded counts approximately equal to those at 1,000 x, a greater magnification than 450 x would reduce operator fatigue because of better resolution of detail. The higher magnification may permit more accurate identification of cells, especially in questionable situations.

The components of variance for bulk sub-samples and films were computed to give an indication of how these factors affect confidence limits (Table 6). It should be noted that if four films are examined using one strip on each film, the 95% confidence limits for a mean cell count of 100 are ± 11.3 cells. However, if two films are examined through two diameters

(horizontal and vertical) the confidence limits are raised to ± 15 cells. It would appear that in situations where narrower confidence limits are desired, as in research investigation, for an equivalent amount of microscope work, it may be adantageous to use four films and count one strip on each rather than two films with two strips each.

It would appear that the DMSCC has achieved the goal of offering a standardized method that permits determination of and control over precision of counting cells in milk films.

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THE CYCLAMATE STORY[1]

In spite of the effort made to clarify the events leading up to the ban on cyclamates—and the roles of the various organizations—confusion, misconception and misinformation still abound. For example, a CBS commentator, in observing the half-year anniversary of the ban, still referred to the misinformation widely disseminated earlier that the ban was based on the finding of bladder cancer in six of twelve rats fed cyclamate. Several renowned scientists, too, have made statements in the scientific and lay press based on a similar misunderstanding of the facts.

Perhaps, in once more setting the record straight, we should start by making unmistakably clear the role of FDRL, especially to point out that our involvement was solely with the sponsor of the research, Abbott Laboratories—and our reporting of findings was solely to the sponsor—and that FDRL was not in any way a party to the discussions which led to the decision by the Secretary of Health, Education and Welfare that cyclamates were no longer GRAS (generally recognized as safe under the conditions of use) and should be phased out of foods.

In a preliminary note in *Science* of February 20, 1970, the data leading up to the banning order were recapitulated. That article was signed by two scientists from Abbott Laboratories (J. M. Price, C. G. Biava), two from FDRL (B. L. Oser, E. E. Vogin), one from HEW (J. Steinfeld) and by former Commissioner H. S. Ley of the Food and Drug Administration. It recounted the earlier FDA findings that cyclamate fed for two years to rats at 1% or 5% concentration in the diet produced no effects at the lower dose and no distinct toxic effects at the higher dose. It mentioned results of research sponsored by Abbott Laboratories which showed an incidence of bladder tumors in Swiss mice which had received, by surgical implantation into their urinary bladders, pellets of 4 parts cholesterol and 1 part cyclamate. Scientists at the National Cancer Institute and the FDA agreed that this route of administration is too far removed from oral ingestion to be a suitable indicator of hazard from cylamates in foods.

Independently of such special tests, Abbott Laboratories in 1967 initiated two 2-year studies, one on cyclohexylamine sulfate at low dosage levels (conducted at another independent laboratory), and the FDRL study of a 10:1 mixture of sodium cyclamate and sodium saccharin (C/S) at daily dosage levels of 500, 1120, or 2500 mg/kg body weight, employing 80 rats on each dose. In the cyclohexylamine tests which were designed to study possible toxicity of the cyclohexylamine that may be present as an impurity in commercial cyclamates, one bladder tumor was found.

It must be emphasized that the FDRL study was designed to investigate the safety of a mixture of a

[1]Reprinted from Food and Drug Research Notes.

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