PERFORMANCE IN MICROSCOPIC COUNTING OF SOMATIC CELLS IN MILK

I. EFFECTS OF PROCEDURAL VARIATIONS ON ACCURACY AND PRECISION[1]

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ABSTRACT

The effects of three changes in the optical equipment used for Direct Microscopic Somatic Cell Count in milk (National Mastitis Council method) on performance of the method have been measured and subjected to statistical analysis of probable significance. Replicate slides of six milk samples were counted by eight technicians among the authors' laboratories. Three levels of cell concentration were represented among the samples. No consistent differences in estimated cell concentration were demonstrated in the comparisons of narrow versus wide reticle band, wide field versus Huyghenian eyepieces, or low (high dry) versus high (oil immersion) magnification. In a few instances within each comparison, individual counters produced markedly different results when the optical equipment was modified. These individual biases were large and consistent with regard to change in objective magnification. No evidence was found to preclude use of wide field eyepieces in performing the DMSCC.

In developing the Direct Microscopic Somatic Cell Count in milk (DMSCC) *(1)*, the need to specify appropriate optical equipment forced us to make certain restrictive decisions. If, for example, reticles of the same band width were to be used with both Huyghenian and uncorrected wide field eyepieces, counts made with the latter equipment would be derived from a considerably smaller area of milk film and thus in theory would be expected to exhibit a greater variance. We decided to specify only Huyghenian eyepieces rather than introduce the confusion of a multiplicity of reticles *(2)* in describing a procedure with defined precision.

Since cells are difficult to recognize, there was no question that the high magnification achieved with oil immersion objectives should be required for general use in the DMSCC. Several participating researchers subsequently had occasion to make counts in parallel at lower magnifications and found a marked discrepancy in estimated cell concentration which seemd to be a function of objective magnification. Similarly, counts made using both wide and narrow bands of the two-band reticle seemed for some operators to show a consistent discrepancy. Optical physics offered no theoretical explanation. The latter difference was of immediate concern, for it would constitute a bias in our recommended procedure to minimize differences in expected coefficient of variation by varying the number and width of strips to be counted *(3)*. Consequently, a collaborative study was designed to test the effects of these three procedural variations through counting replicate sets of milk films.

MATERIALS AND METHODS

Milks containing approximately 0.5, 0.75, and 1.0 million somatic cells/ml were prepared by admixing bucket milks from individual cows. Each sample was subdivided into two portions. These were labeled I and IV, II and VI, and III and V to correspond in duplicate with the three desired levels of cell concentration. Sets of DMSCC slides containing two milk films of each of the six samples were prepared by a single technician. The order of film preparation among sets was randomized. Film preparation and staining were performed as specified for the DMSCC *(1)*.

One set of slides was mailed to each of the participating research laboratories with instructions for counting according to eight procedural variations, as shown in Table 1. The two-band reticle originally designed by the Subcommittee on Screening Tests, National Mastitis Council, and described by Schultze *(2)* was used throughout. Counts were recorded separately for each of the four strips examined for each cell count as specified in the DMSCC.

Because there was good reason not to assume normality of distribution among the cell count data in this study we used nonparametric methods of statistical analysis, in which distributions rather than means are compared. To compare counts produced on a given milk sample by any two procedures we

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TABLE 1. VARIANT COUNTING PROCEDURES USED IN COLLABORATIVE STUDY.

Count type code	Objective	Eyepieces	Reticle band
1	Oil-immersion	Huyghenian	Wide
2	Oil-immersion	Huyghenian	Narrow
3	High-dry	Huyghenian	Wide
4	High-dry	Huyghenian	Narrow
5	Oil-immersion	Wide field	Wide
6	Oil-immersion	Wide field	Narrow
7	High-dry	Wide field	Wide
8	High-dry	Wide field	Narrow

computed count differences as percentage, as follows:

Difference (count b from count a) = b/a − 1

This corrects for inequality of cell concentration and permits pooling of results across samples.

Wilcoxon's Signed Rank Test for Paired Replicates *(4, 5)* is a nonparametric method which considers medians rather than means of distributions and makes some use of the magnitudes of difference. N rank numbers are assigned to the differences between pairs and each rank number is given a sign according to the sign of the difference. The positive and negative ranks are summed separately, and the smaller sum in absolute value is designated "T". The expected total of one sign, $\overline{T}$, is equal to N [(N + 1)/4] and the variance of the total is equal to 2 N$\overline{T}$/6. The totals corresponding to the 0.05% and 0.01% levels of significance are computed as follows:

$T_{.05} = \overline{T} - 1.96\ (2N\overline{T}/6)^{1/2}$ $T_{.01} = \overline{T} - 2.576\ (2N\overline{T}/6)^{1/2}$

In each comparison, the null hypothesis to be tested is that the difference has a median value of zero.

Three variations were included in the experimental design, each a function of the other two. Thus, analysis of any one procedural variation required the comparison of four pairs of counts, selected from among the count types shown in Table 1. For the comparison of narrow versus wide reticle band we used pairs 2:1, 4:3, 6:5, and 8:7; for wide field versus Huyghenian eyepieces we used 5:1, 6:2, 7:3, and 8:4; for high dry versus oil immersion magnification we used 3:1, 4:2, 7:5, and 8:6.

RESULTS AND DISCUSSION

All estimated cell concentrations (factored counts) obtained in the study are shown in Table 2. Counts were not made with wide field eyepieces by Counter 2 of Laboratory C nor by Counter 1 of Laboratory E. Because preliminary work led us to suspect that individual counters might not respond similarly to changes in the DMSCC procedure and equipment all such comparisons were made in parallel within counter.

As an example of the computations involved in the Signed Rank Test, all desired differences which were determined for Counter 1 of Laboratory A are shown in Table 3. Table 4 illustrates the assignment of signed rank numbers to the computed signed differences, using the comparison of narrow versus wide reticle band for counts made with oil-immersion objectives and Huyghenian eyepieces. Only the cumulative ranking used to quantitate the comparison across all counters is shown. Ranking was also done within each counter's data. The computed values of "T" obtained for all three procedural comparisons are tabulated in Table 5.

The overall analysis indicated high probability that counts made using a narrow reticle band were higher than counts made on the same milk film using a wide reticle band (984 - and 1010 -, as shown in Table 5a). It is instructive, however, to examine individual counter performance. Only three of eight counters produced deviations from count equivalence significant at even the 5% level, and in several instances the direction of trend was reversed. These observations suggest that we are dealing with a problem of psychological bias rather than of physical optics. The magnitude of mean differences in factored cell count was not great, averaging 5.9%.

No evidence was found that counts made using wide field eyepieces might be expected to differ consistently either above or below counts on the same material using Huyghenian eyepieces (Table 5b). Some counter individuality was apparent in the results, however. In both Laboratories A and B, two technicians trained by the same researcher tended toward opposite biases. Subject to investigation of the respective count variances, which will be dealt with in a companion publication, we see no reason to discourage the use of wide field eyepieces for the DMSCC.

The overall analysis of pooled cell counts made with high dry and oil-immersion objectives appeared to establish their nonequivalence (1333 + and 671 +, as shown in Table 5c). Counts made with the high-dry objective might be expected to be lower with greater than 99% probability. But again it was necessary to consider the counters as individuals. The overall bias was contributed largely by two technicians, C-1 and E-1. Since for N = 12, the expected total for one sign (T) is 39 and the critical point for P = 0.05 is 14, it is apparent that among the other technicians only B-1 shared this strong counting bias.

Originally, only one technician from Laboratory C took part in the investigation. At a meeting of the Subcommittee on Screening Tests held at Laboratory C a preliminary analysis of these data was offered for comment. It was suggested that the uniqueness of count relationships produced by C-1 might relate to the microscope used, an unusually fine instrument of European manufacture. To test this, two of the authors recounted sample I by procedures 1 and 3 at this same microscope. The factored cell counts in thousands were, respectively, 1,146 versus 1,310 and 1,156 versus 1,263. Thus, these two checks produced

TABLE 2. FACTORED CELL COUNTS ($\times 10^{-3}$ PER ML.) OBTAINED BY ALL PARTICIPATING COUNTERS.

Milk sample	Count type	Lab A Ctr 1	Lab A Ctr 2	Lab B Ctr 1	Lab B Ctr 2	Lab C Ctr 1	Lab C Ctr 2	Lab D Ctr 1	Lab E Ctr 1	Mean
I	1	1,128	1,022	1,312	1,247	1,398	1,304	1,308	1,511	1,279
	2	1,243	1.089	1,463	1,257	1,470	1,262	1,199	1,390	1,297
	3	1,174	1,125	1,270	1,423	1,137	1,162	1,263	1,025	1,197
	4	1,160	1,162	1,248	1,266	1,291	1,038	1,196	1,326	1,211
	5	1,203	1,123	1,264	1,364	1,430	—	1,335	—	1,287
	6	1,156	1,062	1,568	1,245	1,693	—	1,372	—	1,349
	7	1,141	1,100	1,161	1,232	1,181	—	1,247	—	1,177
	8	1,299	1,184	1,270	1,375	1,210	—	1,452	—	1,298
II	1	432	480	506	488	590	475	491	592	507
	2	484	470	516	449	706	386	490	635	517
	3	472	455	470	461	412	385	449	296	425
	4	481	440	525	493	470	390	515	493	476
	5	474	440	457	429	483	—	510	—	466
	6	457	423	511	486	656	—	494	—	505
	7	450	469	443	396	413	—	447	—	436
	8	438	462	432	473	490	—	432	—	455
III	1	724	700	802	729	953	731	793	1,042	809
	2	759	847	923	710	1,009	909	752	839	844
	3	733	694	818	838	712	772	759	584	739
	4	741	654	852	863	752	908	840	831	805
	5	696	625	739	784	1,002	—	1,065	—	819
	6	732	517	818	733	1,410	—	1,202	—	902
	7	708	670	743	730	694	—	855	—	733
	8	714	673	691	684	738	—	879	—	730
IV	1	1,089	1,280	1,299	1,218	1,384	1,209	1,209	1,334	1,253
	2	1,236	1,290	1,324	1,245	1,820	1,347	1,374	1,563	1,400
	3	1,189	1,155	1,369	1,425	1,005	1,177	1,292	834	1,181
	4	1,242	1,149	1,369	1,309	1,267	1,101	1,268	1,169	1,234
	5	1,232	1,154	1,270	1,395	1,457	—	1,254	—	1,294
	6	1,304	1,277	1,227	1,287	1,640	—	1,355	—	1,348
	7	1,093	1,179	1,248	1,248	1,056	—	1,304	—	1,188
	8	1,201	1,151	1,360	1,352	1,068	—	1,357	—	1,248
V	1	730	704	858	783	991	760	796	987	826
	2	719	941	880	704	1,056	619	821	922	833
	3	723	722	750	887	652	740	753	860	761
	4	706	730	781	937	788	777	727	352	725
	5	712	631	779	796	938	—	842	—	783
	6	780	726	716	852	1,197	—	869	—	857
	7	719	667	716	835	730	—	819	—	748
	8	692	640	744	849	807	—	757	—	748
VI	1	391	442	482	443	677	427	479	588	491
	2	430	585	540	492	589	421	443	581	510
	3	434	483	469	511	426	437	425	326	439
	4	440	459	454	451	494	438	466	507	464
	5	483	385	469	480	668	—	429	—	486
	6	578	376	520	486	798	—	443	—	534
	7	446	424	465	516	432	—	478	—	460
	8	490	416	500	500	545	—	439	—	482

somewhat higher counts at high dry magnification. Following this, a second technician at Laboratory C was assigned to the study and also failed to confirm the strong bias of Counter C-1. We conclude that there is no evidence of a necessary difference in cell counts referable to the level of objective magnification. Other difficulties, in particular the problem of cell recognition, are stronger arguments against departure from oil-immersion objectives for the DMSCC. Idiosyncracies among individual technicians can produce such strong biases in cell counting, however, that we are cautioned against accepting radical innovations as free options in counting procedure. Procedural variations should be minimized, and first evaluated in a collaborative study before their acceptance.

Through this study it became apparent that individual counter biases can be an important source of error in quantitative microscopic procedures. Even though minor changes in the optical equipment or

TABLE 3. COMPUTED SIGNED DIFFERENCES FOR COUNTER 1, LABORATORY A

Count pair (b:a)	Milk sample I	II	III	IV	V	VI
2:1	+10.20	+12.04	+4.83	+13.50	− 1.51	+ 9.97
4:3	− 1.19	+ 1.91	+1.09	+ 4.46	− 2.35	+ 1.38
6:5	− 3.91	− 3.59	+5.17	+ 5.84	+ 9.55	+19.67
8:7	+13.85	− 2.67	+0.85	+ 9.88	− 3.76	+ 9.87
5:1	+ 6.65	+ 9.72	−3.87	+13.13	− 2.47	+23.53
6:2	− 7.00	− 5.58	−2.56	+ 5.50	+ 8.48	+34.42
7:3	− 2.81	− 4.66	−3.41	− 8.07	− 0.55	+ 2.76
8:4	+11.98	− 8.94	−3.64	− 3.30	− 1.98	+11.36
3:1	+ 4.08	+ 9.26	+1.24	+ 9.18	− 0.96	+11.00
4:2	− 6.68	− 0.62	−2.37	+ 0.49	− 1.81	+ 2.33
7:5	− 5.15	− 5.06	+1.72	−11.28	+ 0.98	− 7.66
8:6	+12.37	− 4.16	−2.46	− 7.90	−11.28	−15.22

apparent magnification can in theory be compensated for by calculation of a revised microscope factor, they can have a large and unpredictable effect on the counting performance of individual technicians. This observation argues strongly for the precise definition of detail of any quantitative microscopic procedure, and also for the selection of only a single reference method. The DMSCC (National Mastitis

TABLE 5. VALUES OF "T" COMPUTED FOR THE SIGNED RANK TEST FOR PAIRED DIFFERENCES[1]

a. Comparison of NARROW vs. WIDE RETICLE BAND

Counter	Used in combination with: Oil immersion N	"T"	High dry N	"T"
A-1	12	6 − **	12	24 −
A-2	12	23 −	12	23 +
B-1	12	9 − *	11	13 −
B-2	12	35 +	12	35 −
C-1	12	5 − **	12	0 − **
C-2	6	9 +	6	9 −
D-1	12	30 −	12	29 −
E-1	6	8 +	6	5 −
All	84	984 − **	83	1010 − **

b. Comparison of WIDE FIELD vs. HUYGHENIAN EYEPIECES

Counter	Used in combination with: Oil immersion N	"T"	High dry N	"T"
A-1	12	18 −	12	26 +
A-2	12	5 + **	12	25 +
B-1	12	6 + **	12	13 + *
B-2	12	13 − *	12	15 +
C-1	12	29 −	12	29 −
D-1	11	10 −	12	21 −
All	71	1229 −	72	1029 +

TABLE 4. ASSIGNMENT OF RANK NUMBERS TO COMPUTED SIGNED DIFFERENCES: COMPARISON OF NARROW VERSUS WIDE RETICLE BANDS USED WITH OIL-IMMERSION OBJECTIVES

Counter	Count pair		Milk sample I	II	III	IV	V	VI
A-1	2:1	CSD[1]	+10.20	+12.04	+ 4.83	+13.50	− 1.51	+ 9.97
		Rank[2]	+49	+58	+25	+63	−7	+47
A-1	6:5	CSD	− 3.91	− 3.59	+ 5.17	+ 5.84	+ 9.55	+19.67
		Rank	−24	−22	+27.5	+30	+46	+75
A-2	2:1	CSD	+ 6.56	− 2.08	+21.00	+ 0.78	+33.66	+32.35
		Rank	+33	−10	+76	+2	+82	+81
A-2	6:5	CSD	− 5.43	− 3.86	−17.28	+10.66	+15.06	− 2.34
		Rank	−29	−23	−68	+50	+65	−12
B-1	2:1	CSD	+11.51	+ 1.98	+15.09	+ 1.92	+ 2.56	+12.03
		Rank	+55	+9	+66	+8	+13	+57
B-1	6:5	CSD	+24.05	+11.82	+10.69	− 3.39	− 8.09	+10.87
		Rank	+77	+56	+51	−21	−43	+52
B-2	2:1	CSD	+ 0.80	− 7.99	− 2.61	+ 2.22	−10.09	+11.06
		Rank	+3	−40	−14	+11	−48	+53
B-2	6:5	CSD	− 8.72	+13.29	− 6.51	− 7.74	+ 7.04	+ 1.25
		Rank	−45	+62	−32	−39	+36	+5
C-1	2:1	CSD	+ 5.15	+19.66	+ 5.88	+31.50	+ 6.56	−13.00
		Rank	+26	+74	+31	+80	+34	−61
C-1	6:5	CSD	+18.39	+35.82	+40.72	+12.56	+27.61	+19.46
		Rank	+69	+82	+84	+59	+79	+72
C-2	2:1	CSD	− 3.22	−18.74	+24.35	+11.41	−18.55	− 1.41
		Rank	−19	−71	+78	+54	−70	−6
D-1	2:1	CSD	− 8.33	− 0.20	− 5.17	+13.65	+ 3.14	− 7.52
		Rank	−44	−1	−27.5	+64	+16.5	−38
D-1	6:5	CSD	+ 2.77	− 3.14	+12.86	+ 8.05	+ 3.21	+ 3.26
		Rank	+15	−16.5	+60	+42	+18	+20
E-1	2:1	CSD	− 8.01	+ 7.26	−19.48	+17.17	− 6.59	− 1.19
		Rank	−41	+37	−73	+67	−35	−4

[1]Computed signed difference
[2]Rank among counts from all counters

c. Comparison of HIGH DRY vs. OIL IMMERSION OBJECTIVES

	Used in combination with:			
	Huyghenian		Wide field	
Counter	N	"T"	N	"T"
A-1	12	26 −	12	14 + *
A-2	12	21 +	12	19 −
B-1	12	15 +	12	16 +
B-2	12	7 − *	12	31 +
C-1	12	0 + **	12	0 + **
C-2	12	30 +	−	
D-1	12	28 +	12	17 +
E-1	12	0 + **	−	
All	96	1333 + **	72	671 + **

[1]Significance at P = .05 designated *
Significance at P = .01 designated **

Council method) is uniquely susceptible to detailed performance analysis, and counter idiosyncracies can be identified and corrected through retraining.

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AMENDMENT TO THE 3-A ACCEPTED PRACTICES FOR THE SANITARY CONSTRUCTION, INSTALLATION, TESTING AND OPERATION OF HIGH-TEMPERATURE SHORT-TIME PASTEURIZERS, REVISED

Serial #60304

Formulated by
International Association of Milk, Food and Environmental Sanitarians
United States Public Health Service
The Dairy Industry Committee

The "3-A Accepted Practices for the Sanitary Construction, Installation, Testing and Operation of High-Temperature Short-Time Pasteurizers, Revised, (Effective January 22, 1967)", as amended, Serial #60303, are hereby further amended by substituting the following for definition B.1:

B.1

HTST Pasteurization: Heating every particle of (1) milk or milk product to a temperature of at least 161° F, and holding it continuously at or above this temperature for at least 15 seconds in a holding tube, provided that milk products which have a higher milkfat content than milk and/or contain added sweeteners, and concentrated milk products to be repasteurized before drying, shall be heated to at least 166° F, and held continuously at or above this temperature for at least 15 seconds in a holding tube, or (2) frozen dessert mix to at least 175° F, and holding at or above this temperature continuously for at least 25 seconds in a holding tube.

This Amendment is effective October 23, 1971.