A STATISTICAL ANALYSIS OF REDUCTION TIMES IN RELATION TO PLATE COUNTS

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In judging the bacteriological quality of milk supplies, most laboratories use either of two general methods for estimating bacterial density: (1) counting of colonies on an agar plate or clumps in a stained microscopic film, or (2) indirect estimation from dye reduction times.

Grade limits have been recommended by the Public Health Service Milk Ordinance and Code for each type of test. The counting procedures are covered by a single set of limits, established in terms of colonies or clumps counted under conditions specified by Standard Methods for each test. In the dye reduction tests, grades are based on the hours required to reduce the dye completely to a given color standard.

PUBLIC HEALTH SERVICE MILK ORDINANCE STANDARDS

The 1953 edition of the Milk Ordinance and Code recommended by the Public Health Service (1) for adoption by states and communities contains the following grade standards for the alternative tests, based on an average of four samples taken during a period not to exceed six months.

The purpose of the present study was to examine the agreement in grades assigned to replicate samples of milk by plate count and reduction time tests carried out under the conditions specified in the Ninth Edition of Standard Methods for the Examination of Dairy Products (2). Various reduction time limits, including those recommended in the 1953 Milk Ordinance and Code (see Table 1) were examined to reveal discrepancies which might arise if the same milk were graded by total plate count and reduction time.

Statistical analysis was based on agar plate count rather than the direct microscopic count, or both, for several reasons: (a) recommended grade limits are the same for both counting procedures, (b) the direct count has been shown to be subject to considerable variation among individual readers — a source of error probably much less important in the plate count, and (c) it was desired to test agreement between reduction time and plate count when the latter was obtained after incubation at 32° and at 35° C.

EXPERIMENTAL WORK AND ANALYSIS

Data were obtained from 407 milk samples collected during fall, winter, spring, and summer seasons. A preliminary analysis of this material has been previously reported (3). Methylene blue tubes were read after thirty minutes and at hourly intervals thereafter. Resazurin tubes were examined every hour. All tubes were inverted after each reading until a completed test was obtained. The distribution of individual milk samples by reduction times and Grade as indicated by plate counts after 32°C. incubation, is summarized in Table 2 for methylene blue and Table 7 for resazurin.

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Results were analyzed by calculating per cent disagreements in grade classification under various reduction time limits. Consider, for example, a lower limit of three hours for the resazurin reduction time (to Munsell P 7/4) of Grade A raw milk. A sample which did not reduce resazurin within three hours but yielded a plate count greater that 200,000 per ml. would represent a disagreement in grade classification. For convenience, let us say that a milk placed in one grade by the plate count and in a higher grade by the reduction time has been "upgraded"; conversely, if placed in a lower grade by the reduction time, the milk has been "downgraded". Our purpose

TABLE 1 — BACTERIAL AND REDUCTION TEST STANDARDS FOR RAW MILK TO BE PASTEURIZED (AS DELIVERED FROM THE FARM)[a]

Grades	Log. average plate or direct microscopic clump count/ml not to exceed:	Arithmetic average reduction time in hours to be not less than: Methylene Blue	Resazurin (to Munsell P 7/4)
A	200,000	5½	2¾
B	1 million	3¾	2
C	No limit		No limit

[a]Source: 1953 Milk Ordinance and Code (1), footnotes 30 and 34, pp. 12 and 17, respectively.

is to select reduction time limits which lead to the smallest total error of upgrading and downgrading.

METHYLENE BLUE REDUCTION

The following example illustrates the procedure, utilizing data in the first section of Table 2, namely spring milk samples classified by methylene blue reduction time and plate count after incubation at 32°C. Five sets of limiting times were considered: (1) 6½ — 4½ — 0, implying that milk would be classified Grade A if its methylene blue reduction time were not less than 6½ hours, Grade B if between 4½ and 6½ hours, and Grade C if less than 4½ hours, (2) 5½ — 4½ — 0, (3) 5½ — 3½ — 0, (4) 4½ — 3½ — 0, and (5) 4½ — 2½ — 0, all with connotations analagous to those for the first set.

Under the first set, 6½ — 4½ — 0, Table 2 (Spring) shows that 20 of the 35 samples or 57.1 per cent, placed in Grade A by the plate count would be downgraded by the reduction time test. Similarly, 12 of the 29 samples, or 41.4 per cent, placed in Grade B by the plate count would be downgraded by the reduction test, while 4, or 12.8 per cent, would be upgraded. Finally, 8 of the 57 samples, or 14.0 per cent, placed in Grade C by the plate count would be upgraded by the reduction test. The overall average per cent change of grade would be 32 per cent, resulting from an average of 49 per cent downgrading change and 14 per cent upgrading change.

These results and similar calculations for the other sets are included in Table 3. A set of high reduction time limits would lead to considerable downgrading but little upgrading. The converse situation follows from a set of low reduction time limits.

Calculations such as these were carried out for each season and incubation temperature. The two temperatures yielded very similar results. The overall average per cent grade change under each set of methylene blue reduction time limits is given in Table 4 by season and temperature.

Even apart from the somewhat atypical results observed during winter, which were not confirmed on more precise analysis, Table 4 does not offer much basis for choosing any one among the middle three sets of standards. The reason for this, of course, is the great variation shown in reduction times and plate counts by milk samples of apparently equivalent quality.

This unreliability of individual samples has been recognized in the Milk Ordinance and Code (1) (p. 49) which recommends grading based on the average of four successive observations. As noted in Table 1, a geometric mean plate count and an arithmetic mean reduction times are used.

Of the 67 farms from which data was obtained during fall, winter, and spring, a large majority contributed at least four samples. Farms sampled during summer were rarely represented in the other seasons; consequently, this season was eliminated from further analysis. Table 4 shows that per cent disagreements observed in summer milk samples were similar to those of fall and spring samples. An analysis of winter averages, based on two samples yielded by each 40 farms, did not confirm the atypical winter findings observed during the other seasons, namely, a minimum per cent disagreement in the neighborhood of 5½ — 4½ — 0 and 5½ — 3½ — 0 grade limits for methylene blue reduction time.

Therefore, samples from each farm during fall, winter, and spring were averaged to produce a single mean plate count and reduction time. Altogether, 51 farms were represented, each contributing 4 to 6 samples, accounting for 87 percent of all samples collected during these seasons. The resulting frequency distribution at 32°C. incubation temperature is shown in Table 5. The intervals 2¾ — 3¾ and 3¾ — 4¾ hours were employed to permit an exact test of the current milk Ordinance and Code (1) Grade Limit recommendations.

Table 6 gives the average per cent disagreement under various methylene blue reduction time grade limits using means of 4 to 6 samples over several seasons. These findings support the presently recommended time limits, 5½ — 3¾ — 0, as the most suitable set for controlling discrepancies which may arise when grading by both plate count and methylene blue reduction time.

RESAURIN REDUCTION

The evaluation of resazurin followed the same pattern as that described in detail for methylene blue. Table 7 classifies individual milk samples according to resazurin reduction times and plate counts after 32°C. incubation, while Table 8 shows the distribution of means. Use of the interval 2¾ — 2 hours permits an

TABLE 2 — DISTRIBUTION OF INDIVIDUAL MILK SAMPLES ACCORDING TO METHYLENE BLUE REDUCTION TIME AND PLATE COUNT AT 32°C., BY SEASON

	Reduction time (hours)							
Spring: Plate Count (per ml)	<1½	1½-2½	2½-3½	3½-4½	4½ 5½	5½-6½	>6½	Total
<200,000 (Grade A)				5	5	10	15	35
200,000 - 1 million (Grade B)		1	2	9	10	3	4	29
>1 million (Grade C)	3	16	19	11	5	1	2	57
Total	3	17	21	25	20	14	21	121

Summer:	<1½	1½-2½	2½-3½	3½-4½	4½ 5½	5½-6½	>6½	Total
<200,000			3	7	5	10	16	41
200,000 - 1 million		2	6	4	6	6	5	29
>1 million	12	20	12	7	2			53
Total	12	22	21	18	13	16	21	123

Fall:	<1½	1½-2½	2½-3½	3½-4½	4½ 5½	5½-6½	>6½	Total
<200,000					6	4	5	15
200,000 - 1 million			1	5	6	1	1	14
>1 million	6	6	13	6	5	1	2	39
Total	6	6	14	11	17	6	8	68

Winter:	<1 1/2	1½-2½	2½-3½	3½-4½	4½ 5½	5½-6½	>6½	Total
<200,000				1	11	14	25	51
200,000 - 1 million			2	7	2	3	2	16
>1 million	5	8	7	5	1	1	1	28
Total	5	8	9	13	14	18	28	95

exact test of the Grade limits, 2¾ — 2 — 0, recommended by the current Milk Ordinance and Code.

Finally, Table 9 shows the average per cent disagreement under various resazurin reduction time grade limits. Again, the presently recommended limits appear to be most satisfactory.

DISCUSSION

It is interesting that the analysis of means revealed differences between the two incubation temperatures which were not apparent when individual samples were studied. Plates incubated at 32°C. were less likely to disagree with dye reduction time than plates incubated at 35°C., under the optimal reduction time limits. Nevertheless, even under these time limits, and despite the use of means, the probability of disagreement between resazurin or methylene blue reduction

TABLE 3 — EXAMPLE OF AVERAGE PER CENT CHANGES IN GRADE, BY TYPE OF CHANGE, WHEN VARIOUS SETS OF REDUCTION TIME LIMITS ARE COMPARED WITH PLATE COUNTS (DATA OF TABLE 2: SPRING, METHYLENE BLUE, PLATES INCUBATED AT 32°C.)

Reduction time limits	Overall average	Average per cent changes in grade: Downgraded	Upgraded
6½ — 4½ — 0	32	49	14
5½ — 4½ — 0	27	35	19
5½ — 3½ — 0	24	19	29
4½ — 3½ — 0	29	12	46
4½ — 2½ — 0	36	9	63

TABLE 4 — AVERAGE PER CENT CHANGES IN GRADE WHEN VARIOUS SETS OF METHYLENE BLUE REDUCTION TIME LIMITS ARE COMPARED WITH PLATE COUNTS, BY SEASON AND TEMPERATURE

Reduction time limits	Spring 32°C	35°C	Summer 32°C	35°C	Fall[a] 32°C	32°C	Winter 35°C
6½ — 4½ — 0	32	30	31	28	34	33	31
5½ — 4½ — 0	27	25	30	28	29	30	28
5½ — 3½ — 0	24	22	30	28	24	24	23
4½ — 3½ — 0	29	28	32	32	25	22	22
4½ — 2½ — 0	36	32	32	35	32	25	25

[a]Incubation at 35°C. omitted during fall

TABLE 5 — DISTRIBUTION OF ARITHMETIC MEAN METHYLENE BLUE REDUCTION TIMES AND GEOMETRIC MEAN PLATE COUNTS AT 32°C. INCUBATION FOR SAMPLES COLLECTED FROM 51 INDIVIDUAL FARMS DURING FALL, WINTER, AND SPRING

	Reduction time (hours)							
Plate count/ml.	0-1½-	1½-2½	2½-3½	3½-4½	4½ 5½	5½-6½	>6½	Total
<200,000					2	6	5	13
200,000 — 1 million			1	5	10	5		21
>1 million	1	4	9	3				17
Total	1	4	10	8	12	11	5	51

Table 6 — Average Per Cent Changes in Grade when Various Sets of Mean Methylene Blue Reduction Time Limits are Compared with Mean Plate Counts, By Temperature

Incubation Temperature	Reduction time (hours) 6½-4½-0	5½-4½-0	5½-3¾-0	4½-3¾-0	4½-2½-0
32°C.	23	17	15	23	36
35°C.	32	27	22	25	30

Table 7 — Distribution of Individual Milk Samples According to Resazurin Reduction Time and Plate Count At 32°C., by Season

Spring: Plate Count per ml.	Reduction time (hours) <1	1 - 2	2 - 3	3 - 4	>4	Total
<200,000		3	11	15	6	35
200,000 - 1 million		13	10	5	1	29
>1 million	19	30	5	3		57
Total	19	46	26	23	7	121
Summer: per ml.	**<1**	**1 - 2**	**2 - 3**	**3 - 4**	**>4**	**Total**
<200,000	3	8	16	9	5	41
200,000 - 1 million	1	11	14	3		29
>1 million	32	16	4	1		53
Total	36	35	34	13	5	123
Fall per ml.	**<1**	**1 - 2**	**2 - 3**	**3 - 4**	**>4**	**Total**
<200,000		1	4	5	5	15
200,000 - 1 million		1	8	4	1	14
>1 million	14	16	5	3	1	39
Total	14	18	17	12	7	68
Winter: per ml.	**<1**	**1 - 2**	**2 - 3**	**3 - 4**	**>4**	**Total**
<200,000		5	13	14	19	51
200,000 - 1 million	2	7	5	2		16
>1 million	15	11	2			28
Total	17	23	20	16	19	95

Table 8 — Distribution of Arithmetic Mean Resazurin Reduction Times and Geometric Mean Plate Counts at 32°C. Incubation for Milk Samples Collected from 51 Farms During Fall, Winter, and Spring

Plate Count/ml.	Reduction time (hours) 0-1	1-2	2-2¾	2¾-4	>4	Total
<200,000		1	1	11		13
200,000 - 1 million		3	15	3		21
>1 million	4	11	2			17
Total	4	15	18	14		51

Table 9 — Average Per Cent Changes in Grade When Various Sets of Mean Resazurin Reduction Time Limits Are Compared with Mean Plate Counts, by Temperature

Incubation Temperature	Reduction time (hours) 4-2¾-0	2¾-2-0	2-1-0
32°C.	46	14	42
35°C.	44	21	35

time and standard plate count remained substantial.

Other workers in this field, notably Barkworth *et al.* (4), in England, and, most recently, Johns (5), in Canada, have noted increased reduction times in cooler months, attributing this to an inhibiting effect of low temperature on the activity of individual organisms. The former authors point out, however, that an increase in average methylene blue reduction time was seen only in milk of relatively low bacterial count. A density greater than 200,000 organisms per ml., was sufficient to counteract effects of low temperature and show reduction times similar to those during summer.

The findings were confirmed in the present work, although more clearly in the case of resazurin than methylene blue. Average resazurin reduction time of individual Grade A milk samples was about 40 percent longer during winter than summer; methylene blue reduction time was only twelve per cent longer. In milks of higher bacterial count, average reduction times in winter and summer were almost indentical. Hence, no excessive upgrading error would be expected during winter, nor was one observed.

These results do not agree with those reported (in graphical form) by Johns. He observed average reduction times in high count milk between 50 and 100 percent higher during cooler months (March-April) than warmer months (May-June).

Summary

1. An analysis has been made of plate counts and

dye reduction times (methylene blue and resazurin) observed in 407 split milk samples as delivered from farms during all four seasons of the year.

2. A simple procedure is introduced for estimating disagreements in grading when accepted plate count and various dye reduction time grade limits are applied to these observations.

3. Observations in individual milk samples were found too variable to permit clear selection of the set of reduction time grade limits which minimizes disagreements. Therefore, an analysis was undertaken of logarithmic mean plate counts and arithmetic mean reduction times based on groups of 4 to 6 milk samples as delivered during fall, winter, and spring from each of 51 farms.

4. Results indicate that reduction time standards recommended in the 1953 Milk Ordinance and Code for methylene blue and resazurin are most suitable for minimizing disagreements between dye reduction time and plate count in grading milk supplies.

5. Average resazurin reduction time of individual Grade A milk samples was 40 per cent longer in winter than in summer; methylene blue reduction time was 12 per cent longer. However, in milks of higher bacterial count, average reduction times in winter and summer were almost identical.

References

1. *Milk Ordinance and Codes: 1953 Recommendations of the Public Health Service.* Public Health Service Publication No. 229, U. S. Government Printing Office, Washington, D.C., 1953. (See footnotes 30 and 34, pp. 12 and 17, respectively.)

2. Standard Methods for the Examination of Dairy Products: American Public Health Association. 9th Ed. 1948.

3. Thomas, R. C., Levine, B. S., and Black, L.A., Studies Showing the Effect of Changes in the New (9th) Edition of Standard Methods in Relation to the Bacteriological Analysis of Milk. Public Health, **38**:233-238. 1948.

4. Barkworth, H., Irwin, J. O. and Mattick, A.T.R. The Plate Count and Methylene-Blue Reduction Test Applied to Milk. J. Dairy Research, **11**:265-314. 1940.

5. Johns, C. K. Relation Between Reduction Times and Plate Counts of Milk Before and After Pasteurization. J. Milk and Food Technol. **17**:369-371. 1954.