Practical Applications of Several Coliform Tests to Pasteurized Milk

WALTER D. TIEDEMAN AND S. EMERSON SMITH

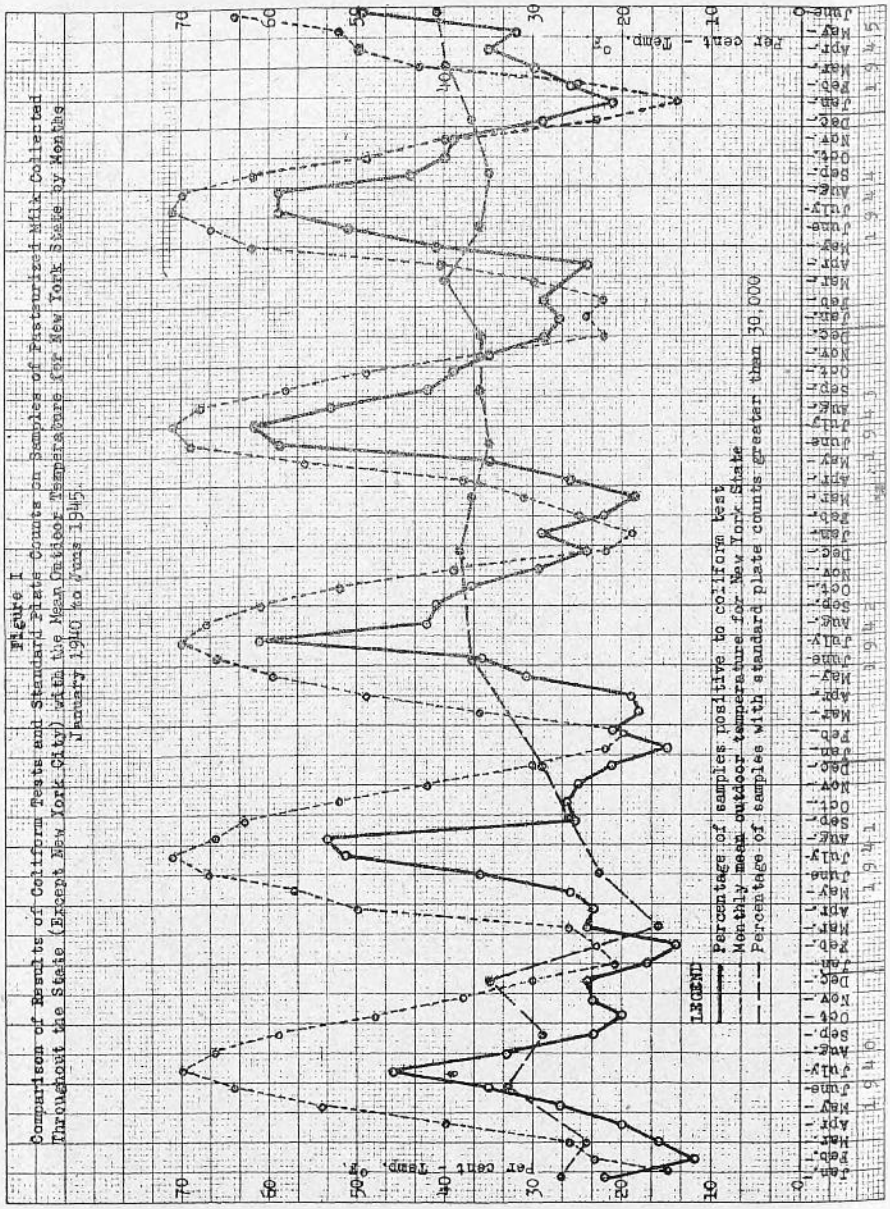
New York State Department of Health, Albany, N. Y.

THE frequency with which coliform organisms are found in bottled pasteurized milk by the standard fermentation tube procedure has led to the suggestion by some authorities that freedom from coliform organisms in three 1 ml. portions is too much to expect. Others in trying to explain the marked increase in positive findings during the summer months have theorized that the growth of coliform bacteria in samples of pasteurized milk held at 50° F. or lower for as much as 24 hours while enroute to the laboratory was responsible for positive findings that otherwise would be negative. The investigations here reported were instigated with a view to finding an answer to some of the questions and, if possible, to suggest improved testing and enforcement procedures.

Health officials are interested in coliform organisms in pasteurized milk not because they are harmful in themselves but because the presence of these organisms offers a useful index of recontamination after pasteurization. Different kinds of bacteria ferment lactose and therefore are included in the coliform group. Outstanding among these are *Escherichia coli,* a common inhabitant of the intestinal tracts of animals including men, cows, rodents and insects, and *Aerobacter aerogenes* frequently present on dust particles in the air in cow barns and milk plants.

It often is assumed that because of the prevalence of these bacteria in manure and in dust, they always must be present in raw milk. However, in a survey made by mobile laboratory crews of the New York State Department of Health in 1931, about 6 percent of more than a thousand samples of raw milk for pasteurization showed no coliform organisms in the single 1 ml. portions tested. Of course this cannot be interpreted as meaning freedom from coliforms in this 6 percent but simply a very low concentration. A standard of fewer than 10 coliform organisms per ml. is set for certified raw milk.

The seasonal variation in coliform positive findings on market pasteurized milk has been mentioned. We know this to be true at least in the northern states. A summary of the results of coliform tests on samples of bottled pasteurized milk in New York State shows a marked seasonal trend in the coliform positive findings. The curve (Figure 1) showing the percentage of positive samples by months almost parallels the mean temperature curve. However, pasteurized milk in most of our plants is cooled to and held at the same temperature in summer as in winter. Also the fact that the percentages by months of standard plate counts in excess of 30,000 on milk from the same bottles from which the coliform samples were taken do not show this fluctuation (Figure 1) indicates that temperature is not a direct factor. It is of interest to note that the period of maximum positive coliform tests comes during the fly season. Dr. G. S. Graham-Smith in an English investigation in 1909 reported finding coliform organisms in the feces of 35 out of 148 flies examined. There is some evidence that flies in city plants which do not have access to privies may carry coliform bacteria. Gilcreas reports finding coliform organisms on the legs of



a fly caught in a city milk pasteurizing plant. Colvin reports finding flies and roaches in the spreader pipe of a surface cooler. His explanation is that these insects crawled under the loosely fitting covers into the warm pasteurizer after it was sterilized and were flushed by the milk through the outlet and into the perforated spreader tube where they were caught. When bottles are sterilized well in advance of filling and are allowed to stand inverted in cases to cool they also constitute preferred resting places for flies. However, if flies

were the main factor the percentage of coliform positive samples of pasteurized milk would continue to be high in September and probably October instead of dropping with the mean outdoor temperature. In advocating the use of in-the-bottle pasteurization in England on a large scale Enock points to improperly sterilized bottles as a major factor and reports coliform organisms generally absent in 1 ml. portions of such milk. In pasteurizing milk in the bottle on a small scale in New York State we have not found a sample that was coliform positive in 1 ml. No doubt other factors such as dust blown through open windows and doors in the warm months, condensation dripping from pipes and perspiration from workers may contribute to the positive coliform findings. We do not as yet have sufficient evidence positively to state the cause of the increase in positive findings in the warm months.

In looking for possible sources of coliform organisms in bottled pasteurized milk it is well to keep in mind that the improper sterilization or recontamination of any of the equipment such as the piping, cooler, bottler and capper or of the bottle or cap may result in introducing coliform organisms. It is interesting to note, however, that we have examined samples of pasteurized milk from some well operated plants at intervals throughout the year without finding any positive coliform results on the 1 ml. portions examined.

A study of the results of coliform tests made in routine work indicates that the fermentation tube method is too sensitive a test to be applied to pasteurized milk with plant sanitation at its present level. If all three 1 ml. tubes are positive this gives a most probable number of 230 or more per 100 ml. which means that the number of coliform organisms per ml. may be as low as 2 or 3. However, the count frequently is higher than this and the enforcement official should know whether it is 2 or 3 per ml. or 200 or 2,000. When results by the fermentation tube method frequently are positive in one or more tubes the use of the plate method is indicated.

This is demonstrated by the summary in Table 1 of the results of 1,630 tests by the formate ricinoleate fermentation tube, and by the desoxycholate agar plate method on samples of pasteurized milk, cream and milk products. These are combined because there is not enough difference in the trend of results to justify individual study. All the tests herein reported were made in accordance with Standard Methods for the Examination of Dairy Products, 8th Edition, 1941. It is interesting to note that of the 896 samples showing no fermentation, i.e., gas production, 821 or 91.6 percent gave no count and

TABLE 1

COMPARATIVE COLIFORM TESTS BY FERMENTATION TUBE AND DESOXYCHOLATE PLATE METHODS ON SAMPLES OF PASTEURIZED MILK (1,227 SAMPLES), CREAM (315) AND MILK PRODUCTS (88) ALL BOTTLED AT PLANTS

AUGUST 18, 1944, THROUGH AUGUST 10, 1945

Fermentation Tube Results on 3—1 ml. Portions + = *Gas* — = *No Gas*	*Desoxycholate Plate Counts on 1 ml. Portions from Same Samples* 0	1 to 3	4 to 10	11 to 100	*More than 100*	*No. of Samples*
— — —	821	56	6	9	4	896
+ — —	89	36	2	3	1	131
+ + —	41	40	17	7	1	106
Sub-total for all having less than 3 tubes +	951	132	25	19	6	1133
+ + +	12	46	89	152	198	497
Totals	963	176	114	171	204	1630

an additional 56 or 6.2 percent gave counts of between 1 and 3 colonies per ml. which is good correlation for bacteriological tests. Due to the possibilities of chance contamination too much significance should not be attached to the presence of from 1 to 3 colonies per ml. A total of 877 of the 896 negative samples or 97.8 percent gave counts of 3 colonies or less per ml.

The groups of samples developing gas in one, two and three 1 ml. portions respectively show progressively higher counts as would be expected. However, 95.4 percent of the samples with one of the 1 ml. portions positive for gas and 76.4 percent of those having two 1 ml. portions positive gave counts of 3 colonies or less on desoxycholate agar.

Examining the results in Table 1 of the desoxycholate plate counts on the 497 samples which showed gas in all of three 1 ml. portions, we find only 12 or 2.4 percent of these produced no growth on desoxycholate plates and only 58 or 11.7 percent produced 3 colonies or less per ml. Of this group 350 samples or 70.4 percent gave counts of more than 10 colonies per ml.

It is apparent that the results showing gas in only one or two of three 1 ml. portions are generally speaking of little importance. Only 12 of 237 samples or about 5 percent gave counts of more than 10 per ml, and 206 or about 87 percent gave counts of 3 colonies or less per ml. Grouping these with the negative results we find that of 1,133 samples showing no gas in at least one of the three 1 ml. portions 1,083 or 95.5 percent gave counts of 3 colonies or less and only 25 or 2.2 percent gave counts in excess of 10.

Although the production of gas in all three 1 ml. portions examined is good evidence of real coliform contamination it is apparent that the desoxycholate plate count gives more information on samples of this kind than does the examination of three 1 ml. portions. With desoxycholate plate counts the control official has the opportunity to concentrate his attention first on the plants showing the highest counts rather than dispersing it among plants furnishing samples showing gas in all three 1 ml. portions the counts on which may vary from 0 to too numerous to count. In Table 1 the 375 counts in excess of 10 per ml. represents 23 percent of all the samples included in the study extending over a year. It appears that a temporary standard of not more than 10 coliform organisms per ml. would be a good starting point for a clean up program. These samples were from all sorts of plants, large and small, city and rural. In dealing only with large city plants compliance with a more rigid standard could be expected. After working with rural plants a more severe standard could be met. After plant methods generally are materially improved a return to the use of the fermentation tube may be in order.

A series of tests summarized in Table 2 were made to determine whether the violet red bile agar would give results comparable with those obtained with desoxycholate agar. It will be seen that the correlation is very good between the results obtained in plating 836 samples of milk, cream and milk products on each of these media. Differences of the order of those shown in this table are likely to occur between duplicate plate poured with the same media. It is evident from this that these media are equally satisfactory for this use.

To investigate the possible increases in coliform counts on iced samples enroute to the laboratory a set of 4 samples was examined immediately by the fermentation tube method using three 1 ml. portions in formate ricinoleate broth and by the desoxycholate and violet red bile plate methods and then held at refrigeration temperature varying from 6° C. (48.8° F.) to 12.5° C. (54.5° F.) and portions were examined by the same tests after 1, 2, 3 and 8 days storage respectively. A simi-

TABLE 2

COMPARATIVE COLIFORM TESTS BY FERMENTATION TUBE, DESOXYCHOLATE PLATE AND VIOLET RED BILE PLATE METHODS ON SAMPLES OF PASTEURIZED MILK (598 SAMPLES), CREAM (192) AND MILK PRODUCTS (46) ALL BOTTLED AT PLANTS

AUGUST 18, 1944, THROUGH AUGUST 10, 1945

Fermentation Tube Results on 3 — 1 ml. Portions + = *Gas* — = *No Gas*	*Plate Counts on 1 ml. Portions from Same Samples*											
	0		*1 to 3*		*4 to 10*		*11 to 100*		*More than 100*		*No. of Samples*	
	Des.[1]	*VRB*[2]	*Des.*[1]	*VRB*[2]	*Des.*[1]	*VRB*[2]	*Des.*[1]	*VRB*[2]	*Des.*[1]	*VRB*[2]	*Des.*[1]	*VRB*[2]
— — —	421	414	24	28	5	3	4	4	0	0	454	454
+ — —	58	50	17	21	0	3	2	2	0	1	77	77
+ + —	19	11	18	23	8	9	0	2	0	0	45	45
Sub-total for all having less than 3 tubes +	498	475	59	72	13	20	6	8	0	1	576	576
+ + +	5	5	22	20	31	37	79	80	123	118	260	260
Totals	503	480	81	92	44	57	85	88	123	119	836	836

[1] Desoxycholate agar. [2] Violet red bile agar.

lar set of samples was held in ice water in the refrigerator and examined at the same intervals. However, the ice melted on the 6th day (Sunday, July 15) and the temperature of the water in which they were being held in the refrigerator rose to 48° F. They were reiced during the morning of the seventh day. The results of this test are shown in Table 3.

In another series 3 samples of pasteurized milk and 2 of pasteurized cream were tested for coliform organisms by all three methods and then stored in ice water in the electric refrigerator and portions examined again after one, two and three days, respectively. All results were negative by all three tests except one of the samples of cream which on initial test showed gas in two out of three 1 ml. portions with the desoxycholate plate count 0 and the violet red bile count 1. After one day these were + — —, 0 and 1 respectively, after 2 days + + —, 0 and 1, and after 3 days — — —, 2 and 1.

Carrying this further Table 3 also shows the results of coliform tests by all three methods on split samples of pasteurized milk and cream at intervals with part held at refrigerator and the other part at incubator temperature. The results on the refrigerated samples confirms the results given in Table 3 for other samples. The results on the incubated samples seem to show that in samples A and B all coliform organisms were destroyed by pasteurization. This has been a disputed point, the argument being that a few organisms must be there although they were not discovered in the 1 ml. portions examined. Although samples C and D had a very low initial coliform content as indicated by the presence of gas in only one of the three 1 ml. portions, these organisms multiplied rapidly at incubation temperature. This shows that coliform bacteria will multiply rapidly in milk at 37° C. if a few are present at the start.

These results make it apparent that samples of pasteurized milk or cream showing none or few coliform organisms per ml. may be held in ice water or in an electric refrigerator without affecting the results of coliform tests. The results do not support the theory that coliform organisms grow in iced samples enroute to the laboratory and that such samples cannot be shipped. Since some members of the coliform groups may react differently than others the examination of larger numbers of samples by this method is necessary before concluding that holding

TABLE 3

EFFECT OF COLD STORAGE AND INCUBATION ON COLIFORM CONTENT OF PASTEURIZED MILK AND CREAM

Source of Sample—Date	Initial Test *F	Initial Test *D	Initial Test *V	Av. Storage Temp. °F.	1 Day *F	1 Day *D	1 Day *V	2 Days *F	2 Days *D	2 Days *V	3 Days *F	3 Days *D	3 Days *V	8 Days *F	8 Days *D	8 Days *V	9 Days *F	9 Days *D	9 Days *V	10 Days *F	10 Days *D	10 Days *V
6/18/45																						
A Past. Milk—Bottle	−−−	1	0	32+	+−−	1	0	++−	1	0	−−−	0	0									
A Past. Lt. Cream—Bot.	+++	37	50	32+	+++	34	43	+++	45	52	+++	42	43									
A Past. Milk—Bottle	−−−	0	0	32+	−−−	0	0	−−−	0	0	−−−	0	0									
A Past. Lt. Cream—Bot.	+++	6	9	32+	+++	7	6	+++	7	7	+++	5	7									
7/9/45																						
A Past. Milk—Vat	−−−	0	0	32+	+−−	0	0	−−−	0	0	−−−	1	0	+++	14	37						
A Past. Lt. Cr.—Vat	−−−	0	0	32+	−−−	0	0	−−−	0	0	−−−	0	0	+−−	19	42						
A Past. Milk—Bottle	−−−	0	0	32+	+−−	0	0	−−−	0	0	−−−	0	0	+++	11	32						
A Past. Lt. Cr.—Bottle	++−	1	1	32+	++−	1	1	+−−	1	1	++−	1	0	+++	110	81						
Following 4 samples split from 4 above																						
A Past. Milk—Vat	−−−	0	0	48±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0						
A Past. Lt. Cr.—Vat	−−−	0	0	48±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0						
A Past. Milk—Bottle	−−−	0	0	48±	−−−	0	0	−−−	0	0	−−−	0	0	+−−	0	0						
A Past. Lt. Cr.—Bottle	+++	1	1	48±	+++	1	1	+++	23	15	+++	44	54	+++	5000+	5000+						
7/30/45																						
A Past. Milk—Vat	−−−	0	0	46±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0
A Past. Lt.Cr.—Vat	−−−	0	0	46±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0
A Past. Homo. Milk—Bot.	+−−	0	0	46±	+−−	0	0	+++	1	0	+++	2	2	+++	26	31	+++	28	88	−−−	130	160
A Past. H. Vit. D Milk—B.	−−−	0	0	46±	+−−	0	0	+−−	0	0	++−	0	2	+−−	1	0	−−−	1	0	++−	0	4
Following 4 samples split from 4 above																						
A Past. Milk—Vat	−−−	0	0	97±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0
A Past. Lt. Cr.—Vat	−−−	0	0	97±	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0	−−−	0	0
A Past. Homo. Milk—Bot.	+−−	0	0	97±	+++	5000+	5000+	Discontinued														
A Past. H. Vit. D Milk—B.	+−−	0	0	97±	+++	5000+	5000+	Discontinued														

*F = Formate ricinoleate broth, 3 - 1 ml. portions, + = gas, − = no gas. D = colony count per ml. on desoxycholate agar.
V = colony count per ml. on violet red bile agar.

samples cold for from 24 to 48 hours before examining them does not change the result materially.

In striving for perfection in preventing the recontamination of pasteurized milk it is desirable to examine more than one or even three 1 ml. portions as is common practice. Tests were run to determine whether this could be readily accomplished by adding more than 1 ml. of milk to each desoxycholate or violet red bile agar plate. In the first series all the milk used was from a pint bottle of Grade A pasteurized milk showing on initial examination gas in two out of three 1 ml. portions. Twenty plates were poured with 1 ml. each, then 10 with 2 ml. and so on with 3, 4 and 5 ml. aggregating 20 ml. of milk for each. The agar in all these plates hardened well although those containing 4 and 5 ml. of milk were rather cloudy and therefore more difficult to count. These results are summarized in Table 4. The counts were low but the results show that if a single plate were poured the chance of getting the true picture were greater using 4 or 5 ml. of milk. A second series also is shown in Table 4 made on milk having a higher coliform count. The results show the highest count on both desoxycholate and violet red bile agar using 4 ml. per plate. A single test was made plating 6, 7, 8, 9, and 10 ml. per plate on desoxycholate agar. The agar hardened satisfactorily but more slowly than when less milk was used. The plates were so cloudy it was difficult to see the guide lines in counting. This work indicates that 4 or 5 ml. of milk per plate may be used satisfactorily.

CONCLUSIONS

It is evident from this discussion that much of the bottled pasteurized milk on the market is not completely free

TABLE 4

COMPARATIVE COUNTS PLATING 20 ML. PORTIONS OF PASTEURIZED MILK ON DESOXYCHOLATE AND VIOLET RED BILE AGAR USING FROM 1 TO 5 ML. OF MILK PER PLATE

Sample	No. and Size of Portions Plated	Colonies per Plate		Total Count Per 20 ml.	
		Desoxycholate	Violet Red Bile	Desoxycholate	Violet Red Bile
A	20 — 1 ml.	3 with 1 each 17 with 0 each	3 with 1 each 17 with none	3	3
A	10 — 2 ml.	3 with 1 each 17 with none	3 with 1 each 17 with none	3	3
A	6 — 3 ml. and 1 — 2 ml.	1 with 1 6 with none	7 with none	1	0
A	5 — 4 ml.	2 with 1 each 3 with none	3 with 1 each 1 with 2 1 with none	2	5
A	4 — 5 ml.	2 with 1 each 2 with 2 each	1 with 2 3 with none	6	2
B	20 — 1 ml.	9, 5, 9, 13, 8, 9, 15, 10, 2, 1, 6, 8, 8, 7, 5, 7, 8, 6, 3, 18	8, 8, 11, 6, 8, 9, 13; 8, 5, 7, 16, 8, 9, 9, 17, 7, 7, 7, 8, 8	159	179
B	10 — 2 ml.	25, 30, 45, 25, 18 26, 25, 30, 22, 21	29, 21; 19, 22, 27 31, 21, 27, 18, 20	267	235
B	6 — 3 ml. and 1 — 2 ml.	47, 61, 55, 46, 42, 31, 24	41, 38, 36, 56, 44, 38, 24	306	277
B	5 — 4 ml,	80, 75, 56, 64, 68	65, 55, 46, 54, 65	343	285
B	4 — 5 ml.	68, 67, 48, 72	61, 60, 80, 72	255	273

Sample A — 1 pint bottle Grade A Pasteurized milk with 2 out of 3 — 1 ml. portions positive for gas on initial examination.

Sample B — ½ pint bottle Grade A Pasteurized milk with 3 — 1 ml. portions positive for gas on initial examination.

from coliform organisms particularly during the summer months. The fermentation tube method of examination for coliform bacteria is a very critical test which does not differentiate between milk containing 2 or 3 coliform bacteria per ml. and that containing several hundreds or thousands. The plate methods, either desoxycholate or violet red bile give such differentiation and provide for the examination of 4 or 5 ml. of milk on a single plate. With plants at the level of sanitation of those furnishing the samples herein summarized the standard for raw certified milk is suggested as a temporary year around standard namely less than 10 coliform bacteria per ml. Although better results will be obtained during the winter months it is not considered necessary to set a higher standard for the winter months to attain that end. After considerable work has been done on sanitation in the plants failing to meet this suggested standard it should be possible to set a higher standard which need not necessarily be the ultimate one. The evidence indicates that freedom from coliform organisms in 1 ml. portions of pasteurized milk in well operated plants is an attainable goal.