Current Investigations of Laboratory Methods Used in Milk and Food Utensil Sanitation*

LUTHER A. BLACK, PH.D.

Senior Bacteriologist, Milk and Food Sanitation Laboratory, Water and Sanitation Investigations Station, U. S. Public Health Service, Cincinnati, Ohio

INTRODUCTION

DURING the recent war years it was possible for the U. S. Public Health Service to undertake only limited laboratory investigations pertaining to milk or food sanitation. In general this was dictated by the necessity of solving immediate problems of equipment or procedure posed by the mobile laboratories operating at that time in war areas in various parts of the United States. In this connection we were directly concerned with the application of Standard Methods in the bacteriological analysis of milk, water, and swabs from food utensils. Where modifications seemed warranted, recommendations have been made to the respective American Public Health Association committees on Standard Methods, and many such items have been incorporated in the current procedures. The background, as well as the supporting evidence, for some of the requirements retained and the modifications proposed, may not be generally known as is indicated by frequent inquiries received. Accordingly it seemed appropriate to review some of our recent and current investigations of laboratory methods that may be of interest to milk and food sanitarians.

AGAR PLATE COUNTS

Media. Compartive tests have been made of various ingredients for plating agar, in the interest of eliminating milk from the present standard medium, due to difficulties sometimes encountered with precipitates and turbidity. This is desirable, provided a suitable medium is selected which, without milk, will yield counts approximating those obtained with the present standard medium containing milk. While information on which to take action has not yet been secured, our investigations indicate the possibility of achieving a satisfactory medium, although this may be delayed pending investigation of nutritive requirements of certain milk bacteria. Ultimately this information may have a bearing on the materially higher counts obtained by a slight lowering of incubation temperature. Improvement in these factors concerned in the agar plate method is considered fundamental in correlation with other bacteriological and biochemical methods or with existent sanitary conditions.

Studies have been made in comparisons of various peptones, extracts, and other modifications of plating media, from the standpoint of obtaining more precise and uniform enumeration of the bacteria present in milk. Some 50 different modifications of media were used in comparing the better peptones generally available for bacteriological use, the effect of addition of certain other ingredients reported to influence counts, or variation in amounts of ingredients.

Time does not permit an analysis of the results obtained, but examination of the records indicates the following

* Presented at the Thirty-fourth Annual Meeting of INTERNATIONAL ASSOCIATION OF MILK SANITARIANS, INC., Milwaukee, Wis., October 16–18, 1947.

trends. While some media were considerably inferior to the present standard tryptone-glucose-beef-extract agar containing milk, the better peptones gave comparable results. With some milk samples counts were materially higher with media containing milk, in extreme instances up to 1000 percent or more.

A clearer media, permitting greater contrast with colonies, is desirable so that the smallest colonies may manifest themselves more readily, inasmuch as overlooking these colonies seemed to be the chief cause of the largest discrepancies observed. Better nutritive properties in plating media may likewise increase the size of such colonies so they may be visible readily.

Careful study, however, should be given before general acceptance of any modified medium that may be proposed. As an example, a comparison was undertaken of a sample of an experimental dehydrated medium submitted to us as a substitute for the standard tryptone-glucose-extract-milk agar. In some instances the proposed medium yielded equivalent counts, but with other milk samples only half as many colonies appeared as on the standard agar containing milk.

Incubation Temperature. We have made several comparisons of the effect of the alternative incubation temperature of 32° C. compared to 37° C., in many instances using media without milk as well as the standard agar containing milk. Differences due to media generally were slight compared to the larger numbers of colonies that developed following incubation at 32° C. compared to 37° C. Frequently samples of pasteurized milk showed 2, 3, or 4 times and in some instances 10, 20, or 30 times as many colonies as 32° C. This tendency was more pronounced for samples of pasteurized cream.

The present (8th) edition of *Standard Methods for the Examination of Dairy Products* permits incubation at either 37° C. or 32° C. For some years it has been recognized that incubation at the higher temperature is unsuitable in that it is above the optimum for the bacteria ordinarily present in milk, and that incubation at 32° C. is definitely advantageous. The current trend in public health laboratories is for incubation of pathogens at 35° C. instead of 37° C. The present edition of *Standard Methods of Water Analysis* accepts 35°–37° C. as the standard incubation temperature for agar plate counts and coliform tests. The A.P.H.A. Standard Methods Committee for Dairy Products determined by means of questionnaires that incubation of milk plates at 35° C. was acceptable to most directors of state and city laboratories, although there was a demand that 32° C. be used by those who understood the advantages of incubating milk plates at that temperature. Hence both 32° C. and 35° C. are the standard temperatures of incubation stipulated in the 9th edition of Standard Methods.[1]

Thomas, Levine, and Black[2] recently reported on several series of milk samples, subjected at different seasons of the year to each of the alternative bacteriological tests for sanitary quality, using the procedures to be required in the next (9th) edition of *Standard Methods for the Examination of Dairy Products.*

Table 1, taken from that report, presents a tabulation of plate counts and direct counts of raw milk to be pasteurized at one season of the year, grouped according to reduction times by the methylene blue test. This illustrates the general relationship of plate counts at the three incubation temperatures, both to each other, and in relation to microscopic clump counts, as well as reduction times. By classifying the counts according to reduction times, the table presents a wide range of comparative counts, from low to extremely high. It is apparent that there is a greater difference between

TABLE 1

AVERAGE PLATE COUNTS OF RAW-TO-PLANT MILK AND MICROSCOPIC CLUMP COUNTS, GROUPED BY METHYLENE BLUE REDUCTION TIME

Reduction hours	*Plate count* 37° C.	35° C.	32° C.	*Microscopic count*
1	28,000,000	36,000,000	25,000,000	7,400,000
2	4,700,000	5,200,000	5,600,000	4,300,000
3	1,800,000	2,100,000	2,300,000	2,500,000
4	940,000	1,700,000	1,500,000	1,500,000
5	480,000	670,000	690,000	1,100,000
6	130,000	220,000	230,000	400,000
7	99,000	190,000	190,000	270,000
8	160,000	280,000	290,000	510,000
9	24,000	47,000	53,000	300,000

counts obtained at 37° and 35° C., than between those at 35° and 32° C. Usually the plate count at 35° C. is only slightly lower than the 32° C. count.

REDUCTION TESTS

Methylene Blue. Several hundred samples of raw milk to be pasteurized were examined in 1944 using plate counts, clump counts, and three reductase tests, including the standard methods procedure, a modified test in which the tubes were inverted hourly, and a series in which the tubes were iced before examination. The modified reductase test in which the tubes were inverted hourly, as will be required in the next edition of Standard Methods, gave much better correlation with the bacterial plate counts and microscopic counts than did the reductase procedure then standard. Similar results were reported by Abele[3] in 1945, and Thomas, Levine, and Black[2] recently reported comparable results on milk samples subjected at different seasons of the year to the procedure to be required in the next edition of Standard Methods.

Table 2 summarizes the results by the methylene blue reduction test, reported by Thomas, Levine, and Black, showing the percentage of samples in each reduction group that fell in one of three grade groups, based on plate counts at 37°, 35°, or 32° C., and on the direct microscopic clump count. It

TABLE 2

PERCENTAGE OF RAW-TO-PLANT MILK FALLING INTO PLATE AND MICROSCOPIC CLUMP COUNT GROUPS, CLASSIFIED BY METHYLENE BLUE REDUCTION

Reduction hours	*Samples total*	*Count under 200,000* Plate count 37°	35°	32°	Clump count	*210,000–1,000,000* Plate count 37°	35°	32°	Clump count	*Over 1,000,000* Plate count 37°	35°	32°	Clump count
½	4	0	0	0	0	0	0	0	0	100	100	100	100
1	15	0	0	0	0	0	0	0	0	100	100	100	100
2	47	0	0	0	0	6	6	6	2	94	94	94	98
3	51	8	6	6	0	33	22	22	29	59	73	73	71
4	56	34	25	25	9	45	38	38	52	21	38	38	39
5	47	68	47	45	19	23	43	38	49	9	11	17	32
6	48	81	73	71	23	17	25	25	73	2	2	4	4
7	33	88	85	82	48	12	12	15	45	0	3	3	6
8	25	92	84	76	44	8	12	20	52	0	4	4	4
9	12	92	83	92	50	8	8	0	42	0	8	8	8

will be noted that when the direct clump count is considered, only 50 percent of the samples were in the low count group, even by the 9-hour reduction period.

Resazurin. An alternative reduction test using resazurin has been included in the 9th edition of Standard Methods, and equivalent values for this procedure are still to be established. Two methods of reading the test are included, the "one hour" and the "triple-reading" test. In work reported by Thomas, Levine, and Black,[2] both tests were followed but only the "triple-reading" test results were reported. The endpoint in this test is reduction beyond a certain color standard (P 7/4 according to the Munsell system). Samples were examined at hourly intervals and inverted after each reading. A record was made of the stage of reduction of all samples at each hour, but only the end point reduction beyond P 7/4 was used.

Table 3 summarizes the results by the resazurin reduction tests reported by Thomas, Levine, and Black, showing the percentage of samples in each reduction group that appear in one of three grade groups, based on plate counts at 37°, 35°, or 32° C., and on the direct microscopic clump count. All samples not reduced in 3 hours were recorded as reduced in 4 hours for the purpose of this paper, but complete records were kept up to 8 hours.

From table 3 it may be seen that most of the samples reducing in one hour fell into the higher plate count group (over 1,000,000). However, some samples that reduced in one hour appeared in the lower plate count group. A further microscopic study of these samples showed that they contained a large number of leucocytes. It has been reported [4] that leucocytes have a quick reducer effect on resazurin. The leucocyte content of these particular samples varied from 2 to 23 million per milliliter, and the methylene blue reduction time varied from 4 to 9 hours. On the basis of the direct microscopic clump count, only 41 percent of the samples were in the low count group, even after 4 hours.

MICROSCOPIC COUNTS

Studies on modifications of the direct microscopic examination of milk advocated by various individuals indicate wide variations in the results possible by this technique, and the desirability of remedying this situation. In addition to sampling error, there are various points in the preparation of the smear, in the nature and reaction of the stain, in the nature and amount of light source, as well as in the mechanism of examination, that influence results materially.

In preliminary microscopic examinations of raw and of pasteurized milk, under some circumstances use of a color filter resulted in observation of bacterial cells not discernible by

TABLE 3

PERCENTAGE OF RAW-TO-PLANT MILK FALLING INTO PLATE AND MICROSCOPIC CLUMP COUNT GROUPS, CLASSIFIED BY RESAZURIN REDUCTION

Reduction hours	Samples total	Count under 200,000				210,000–1,000,000				Over 1,000,000			
		Plate count			Clump count	Plate count			Clump count	Plate count			Clump count
		37°	35°	32°		37°	35°	32°		37°	35°	32°	
1	72	4	4	4	1	6	4	4	7	90	92	92	92
2	104	23	15	15	8	39	33	34	37	38	51	54	56
3	80	65	55	53	19	28	33	35	63	8	13	13	19
4	83	94	84	82	41	5	13	14	52	1	2	4	7

Standard Methods procedure. A series of several pasteurized milks and creams examined, using 4 staining procedures, resulted in higher microscopic counts in some samples compared to plate counts at 32° and 37° C., but in other samples microscopic counts were lower than plate counts on corresponding samples.

During the past year Dr. B. S. Levine of our laboratory has made a special study of the microscopic clump count, including stains, procedures, methods of examination, and comparative counts. He has developed a stain which in a recent series of samples, in many instances yielded twice the number of clumps observed by Standard Methods procedures. The detailed results will be made available in a separate paper.

COLIFORM MEDIA

During the past few years there has been an increased emphasis on examination for coliform organisms in pasteurized milk as an indicator of post-pasteurization contamination. In 1944 a sample was received of a revised formula for one of the direct plating media now listed in Standard Methods for presumptive tests for organisms of the comform group. Arrangements were made to obtain a seres of commercially pasteurized milk samples for use in a comparative study of the various media allowed by Standard Methods. In this investigation three tubes of each of three dilutions of alternative liquid media were used, together with single plates with 1 ml. portions of both alternative solid media allowed by Standard Methods, as well as standard plate counts at both 37° and 32° C.

Later three additional liquid media were compared, namely, desoxycholate lactose broth, EC media, and lauryl sulfate tryptose broth. Additional information obtained on the same samples included plate counts at 37° and at 32° C., together with preparations for direct microscopic examinations.

Inspection of the results showed good agreement among the various liquid and solid media, although fluctuations occurred. None of the newer liquid media appeared superior to those already in Standard Methods. Additional milk samples were examined and observation of the results obtained, together with those of former experiments, indicated that a solid plating media yielded the most quantitative information.

PHOSPHATASE TEST

In 1943 we received a copy of a new technique on the phosphatase test proposed by Scharer.[5] This test made use of the catalytic effect of copper on the formation of the indophenol blue color, and was reported to effect a more rapid and complete precipitation of protein, yielding about twice the color gain per unit of enzyme as obtained on the present laboratory technique, with the color formation being complete in about two minutes. It was also a simpler procedure inasmuch as it omitted the boiling and cooling steps now required and used a simplified buffer.

This technique will be included in the next edition of Standard Methods in the last chapter concerned with "Screening Tests," since the method has not been published and hence has not been considered by the Association of Official Agricultural Chemists. We have had considerable experience with this technique and can confirm the advantages reported. It was believed that inclusion of this procedure in the next edition of Standard Methods would allow a larger number of laboratories to utilize it, and determine if it appears generally satisfactory.

SWAB COUNTS

There has been widespread interest and activity in food sanitation during the past few years. In connection with

the bacteriological examination of eating utensils made by the various mobile laboratories operated during the recent war by the U. S. Public Health Service, it was found necessary to improve and standardize the equipment and procedure used.

An investigation was made of the types of containers used in connection with these food utensil examinations and their suitability, and steps were taken to determine the availability of satisfactory containers. Screw cap vials with well caps were ordered for use as swab containers, and these were distributed to each of the trailer laboratory units engaged in the bacteriological examination of food utensils.

A report was soon received that upon sterilization, some of plastic caps deteriorated, accompanied by the odor of formaldehyde. The stock of caps on hand was sorted out, and about 10 percent appeared to be of different composition, these seeming to be the caps that decomposed upon steam sterilization.

Arrangements were made with the Chemical Laboratory of the U. S. Public Health Service in Cincinnati for testing various lots of plastic caps for formaldehyde and phenol. Information concerning our experience with the caps was forwarded to the research laboratories of two of the large manufacturers whose caps were on hand, and their advice requested.

We also recorded the pH changes that occurred with various lots of plastic caps upon repeated sterilization. It appeared that the caps on hand surviving preliminary steam sterilization without appreciable change still gave off sufficient alkaline products to change the pH of the standard buffered phosphate diluent. With the stock of caps on hand this change was so slight that it seemed feasible to utilize these caps. It was found that increasing the concentration of the diluted phosphate buffer tendfold was a satisfactory way to compensate for the variations in plastic caps.

A comprehensive comparison of various types of plastic caps was made, including several types of experimental caps submitted by manufacturers, using both regular and 10 strength buffered phosphate, with and without applicators. It was soon ascertained that the wooden applicators gave off acid products upon steam sterilization while partially submerged in the dilution water. With plastic caps that gave off alkaline products, these acid and alkaline substances might neutralize each other and the final diluent be near the proper pH even with the regular phosphate buffer content. With the better mineral-filled plastic caps which did not yield alkaline products, it was found essential to use a more concentrated buffer (10 times that ordinarily recommended) in order to have the final pH of a sterilized swab vial outfit within the desired neutral range.

An order was placed for plastic well caps to be made of the mineral-filled plastic tested and found satisfactory for use in the bacteriological examination of food utensils. Upon receipt of these caps, tests were made. The results showed no similarity to the experimental caps of that material previously submitted by the two largest manufacturers. On the contrary, the caps reacted in a manner identical with the ordinary wood-filled plastic caps already available. This matter was taken up with the supply house, the supply house submitted representative caps to the manufacturers, and we were later advised that the manufacturer tested the sample caps submitted and admitted that the caps were made of the wrong material, and were making a replacement shipment.

Samples of the replacement shipment of caps were tested for suitability for use as closures in the bacteriological examination of food utensils, by deter-

minations of changes in pH of buffered phosphate diluent following steam sterilization on vials closed with these caps. Upon repeated daily testing of these, subsequent to autoclaving, they were found to be suitable closures.

These details have been supplied so that those affected by the unavailability of suitable plastics during the war may understand some of the factors responsible, and why we have recommended the use of a tenfold stronger buffered phosphate dilution water for restaurant utensil swab samples. This 10X buffered phosphate complies with the present A.P.H.A. "Technic for the Bacteriological Examination of Food Utensils" under the last statement on "Laboratory Procedures" that "Laboratories not conforming fully with this procedure should have comparative evidence to show that the variations used give comparable results."

One of the supply houses has developed longer vials designed to take a standard 3" cotton-tipped applicator without the necessity of cutting off about ⅜" as was heretofore necessary with the standard 21 x 70 mm. vials. The new vials now available are approximately 21 x 79 mm. and have been found convenient.

STORAGE OF SAMPLES

In 1944 studies were made of the effect of variations in storage of restaurant swab samples upon the plate counts per utensil surface examined. Observation of the data revealed a tendency to maintenance of approximately the same bacterial count durstorage of 6 to 12 and even 24 hours duration, using 10X buffered phosphate diluent with samples refrigerated. Only in some instances where the original count was near the border line of the standard of 100 organisms per utensil surface, would the interpretation of the results have been different if the swab samples had been analyzed at 12 or even 24 hours after collection.

It is still considered desirable to plate the dilution water samples as soon as possible, preferably within 4 hours of swabbing, but where this cannot be done, samples should be properly refrigerated and analyzed within 24 hours of swabbing.

INHIBITORS FOR QUATERNARY AMMONIUM COMPOUNDS

In connection with testing utensils in which quaternary ammonium and similar compounds have been used as bactericidal agents, it was considered desirable to study neutralizing agents for the purpose of inactivating the cationic germicides at the time swab samples are taken.

Experiments were undertaken to determine the neutralizing power of an inactivator recommended for this purpose. This was to be used where a residual amount of bactericide might be transferred to the sample vial by swabbing, using the swab-rinse plate count method for the bacteriological examination of eating utensils. This inactivator was checked by procedures comparable to field conditions of swabbing and subsequent handling of the sample. Eating utensil samples obtained from forty-seven restaurants or taverns were used as the source of the pooled or individual swabbings which were tested with various concentrations of bactericide, with and without inactivator. Bacteriological examinations were made immediately, and after 1, and in most instances, 4 and 24 hours storage under refrigeration. The majority of the plates were counted under a wide field binocular microscope to insure against overlooking any organisms surviving.

Although it was found that the inactivator itself was not bactericidal in the concentration recommended for use, it did not always neutralize the germicide under the conditions stipulated, or under the practical field conditions studied.

Further laboratory studies of inhibitors for this purpose were reported recently by Weber and Black.[6] In this work a gross screening procedure was first used to evaluate various compounds. Phosphate buffer, culture (*Escherichia coli*), and inhibitor were mixed in a Petri dish, allowed to act for 10 minutes, following which germicide was added. Ten minutes later the Petri dish was poured with concentrated agar known to arrest germicidal action. Each compound exhibiting satisfactory inhibitory action was further investigated by placing in a test tube appropriate amounts of phosphate buffer, culture, and germicide, followed by periodic plating. After a definite killing curve was established for each germicide, experiments were repeated introducing adequate amounts of inhibitors into the germicidal solution after 2½ and 5 minutes exposure.

TABLE 4

EFFECT OF INHIBITORS ADDED TO ALKENYL DIMETHYL ETHYL AMMONIUM BROMIDE (3 PPM)

Results expressed in percentages of *Escherichia coli* surviving

Exposure	*Experiments at room temperature (24–26° C.) pH 7.2*			
(Minutes)	*#242*	*#237*	*#238*	*#241*
0	100	100	100	100
1	72	74	73	74
2	33	50	68	53
2–½		Lecithin *	Naphuride Sodium *	Triton X500 *
3	2	60	67	24
10	0.0	60	63	22
60	0.0	62	60	17
300	0.0**	58	52	15
(Hours)	Lecithin			
24 (5° C.)		61	54	16
72 (24–26° C.)	0.0			

* Added 60 ppm. ** Added 500 ppm.

Table 4 shows such an interruption of killing curves upon the addition of certain inhibitors, as reported by Weber and Black. Generally speaking, a ratio of at least 10 : 1 and not greater than about 20 : 1 of inhibitor to germicide was required for effective immediate inhibition of germicidal action.

Certain compounds inhibited the germicidal action of quaternary ammonium compounds against *Escherichia coli* after this test organism had been exposed to the germicide for varying periods of time up to at least three hours at room temperature, which was the maximum period studied. Although lecithin was a satisfactory inhibitor in the crude edible grade used, it apparently had a stimulating action on growth of the test organism after a lag period of not less than about 4 hours at room temperature, but at refrigeration temperature (5° C.) there was no increase in the number of bacteria after 24 hours. It would appear that lecithin or certain other inhibitors may be employed satisfactorily in the buffered dilution swab-rinse water for food utensils which have been sanitized in quaternary ammonium germicides.

REFERENCES

1. Report of the Joint Editorial Committee for Standard Methods for the Examination of Dairy Products. *Am. J. Pub. Health* 37, 755 (1947).

2. 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. Presented at Annual Meeting of A.P.H.A., Atlantic City, N. J. (October 6, 1947).

(Continued on page 34)

feasible, unnecessary overhead structures shall be eliminated.

(f) *Hand-Washing Facilities.* Handwashing facilities shall be conveniently located and readily accessible, shall be provided with hot and cold running water, liquid soap or soap powder delivered from dispensers, individual cloth or paper towels, and suitable receptacles for disposal of towels, cleansing tissue, etc. Fixtures operated by foot, knee, or elbow are advised. The use of a common towel is prohibited.

Clearly legible signs shall be posted conspicuously in each toilet room instructing employees to wash their hands before returning to work. The common drinking glass and insanitary drinking facilities shall be eliminated.

(g) *Toilet Facilities.* Toilet facilities shall comply with local or state ordinances; shall be kept clean, well-ventilated, and in good repair.

(1) Toilet rooms shall be free from flies and shall not open directly into rooms where paper or paperboard products are fabricated, handled, or stored.

(2) Toilet room doors shall be self-closing; a room or enclosure open at the top is not satisfactory.

(3) Toilet rooms shall not be used for storage of clothing, shoes, or lunches.

(4) Toilet rooms shall be used only by operators for whom facilities are intended.

(h) *Lunching Facilities and Disposal of Food Residues.* Lunching at machines, in washrooms, and wherever materials and *these products* are held, fabricated, and stored shall not be permitted. Proper facilities shall be maintained for disposal of food scraps and bottles containing residues of milk and other drinks. Distribution and accumulation about plant of food residues, bottles, and other containers of food and drink shall be prohibited.

Item IX. *Personnel, Cleanliness and Health.* All persons coming in contact with materials used and *these products* shall handle them in a clean and sanitary manner; all such persons shall, (a) wear clean outer garments, (b) wash hands thoroughly with soap and water and dry them on a clean towel before commencing work, (c) wash hands thoroughly with soap and water and dry them on a clean towel before resuming work after visiting the toilet, and (d) keep the hands clean at all times while engaged in this work. Female employees shall confine the hair by means of a cap or net. Any person with acute contagious or infectious disease, receiving medication and/or requiring bandaging of fingers shall be promptly relieved of any connection with the manufacture of *these products* until, under advice of physician, all public health and sanitation hazards have been removed.

July 16, 1947

CURRENT INVESTIGATIONS OF LABORATORY METHODS USED IN MILK AND FOOD UTENSIL SANITATION

(Continued from page 12)

3. Abele, C. A. The Methylene-Blue Reduction Test as a Means of Estimating the Bacterial Content of Milk, to Determine Its Suitability for Pasteurization or as a Basis for Grading. *J. Milk Tech.* 8, 67–79 (1945).

4. Davis, J. C. and Jones, V. E. The Differentiation of Cells from Bacteria in the Resazurin Test. (Abstract) *Proc. Soc. Agric. Bact.* (1944.) N.I.R.D. Paper No. 820 from National Institute for Research in Dairying. University of Reading (England).

5. Scharer, H. Personal communication (July 28, 1943).

6. Weber, G. R. and Black, L. A. Inhibitors for Neutralizing the Germicidal Action of Quaternary Ammonium Compounds. *J. Bact.* 54, 44 (1947).