INFLUENCE OF PRELIMINARY INCUBATION ON THE METHYLENE BLUE RESULTS OF FARM-REFRIGERATED MILKS

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SUMMARY

Samples of raw refrigerated milk were analysed bacteriologically for total bacterial, coliform, ps*chrophilic, casein digester, and thermoduric counts initially, and after storage overnight at 50°F, 60°F and 70°F, methylene blue and nitrate reduction tests were performed. The relationship of the results of analyses before and after incubation has been statistically examined.

The combination of methylene blue reduction and nitrate reduction tests after preliminary incubation of the sample for 24 hr at 60°F effectively eliminated milks of poor bacteriological quality. However, for practical purposes it is suggested that a methylene blue reduction test of 3 hr after such pre-incubation would give a good assessment of initial bacterial flora and would seem to be the most satisfactory standard for Queensland milk supplies.

I. INTRODUCTION

The main bacteriological test for raw milk in Queensland is the methylene blue test; a statutory standard of four hours is prescribed for the decolourization time. However, with the advent of refrigeration on most farms, 99 per cent. of the raw milks pass the test regardless of the quality of milk and hygiene of production. So it seemed necessary to re-evaluate the test in order to reveal the quality of the raw milk more effectively.

Workers in this field (e.g. Barkworth, Irwin, and Mattick 1940; Johns 1954) attributed increased reduction times in cooler months to an inhibiting effect of low temperature on the activity of individual organisms in the milk during storage, these organisms being so dormant that reduction of methylene blue is appreciably delayed. This position is similar to that of milks from

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farms with refrigeration. Johns (1958), Hadland (1962) and others have found that preliminary incubation is most useful in overcoming this dormancy. The initial poor agreement between standard plate counts and reduction times noted on fresh samples largely disappears following preliminary incubation.

Different temperatures and times of incubation have been used by various workers. The experiments reported here were made to determine the effect of different pre-incubation temperatures and to select the one most suitable for milk supplies in Queensland.

II. ANALYTICAL METHODS

Total bacterial count.—The fresh milks were plated prior to incubation using tryptone glucose-yeast extract (T.G.E.) agar and the plates incubated at 30°C for 72 hr.

Coliform count.—Desoxycholate agar was used, with an incubation time of 24 hr at 30°C.

Psychrophilic count.—Samples of milk were plated on T.G.E. agar and the plates incubated at 40°F for 14 days.

Casein digesters.—T.G.E. agar with the addition of 0·3 ml sterile milk was used, the plates being incubated at 30°C for 72 hr.

Thermoduric bacteria.—Samples were laboratory-pasteurized at 73°C for 15 sec, cooled, plated on T.G.E. agar and incubated at 30°C for 72 hr.

Methylene blue decolourization.—The standard method (American Public Health Association 1961) was used at 37°C.

Nitrate reduction.—A modification of the method of Kandler (1961) was used, with the addition of solutions of sulphonilic acid and alphanaphthyl-amine to the incubated milks after incubation with nitrate solution. Development of colour (pink-red) indicated reduction of nitrate to nitrite.

III. EXPERIMENTAL PROCEDURE

Fresh samples of individual suppliers' raw refrigerated milks were collected in sterile glass-stoppered bottles at the milk depot and immediately transported to the laboratory.

Prior to incubation, the samples were examined for total bacterial, coliform, casein digester, psychrophilic and thermoduric counts. A methylene blue test was also done on each of the fresh milks. Then each sample was divided aseptically into 6 x 10 ml aliquots and 3 subsamples added to 1 ml nitrate solution. One subsample with and one without nitrate solution were incubated at each of 50°, 60° and 70°F for 24 hr. After incubation, the tubes with added nitrate were stored at 37°C for 1 hr before the addition of the reagents, and methylene blue tests were performed on the incubated samples without added nitrate.

IV. RESULTS

The results of the bacterial analyses of fresh milks are set out in Table 1. A total of 288 samples was analysed for casein digesters and a total of 165 for thermoduric organisms.   No correlation was found between these counts initially and the results of testing subsequent to incubation, so these results were not considered further.

TABLE 1

BACTERIOLOGICAL QUALITY OF SAMPLES

Bacterial Counts	Total No. of Samples Tested	Standards Used (no./ml)	Percentage of Samples with Unsatisfactory Quality
Total bacteria ..	484	< 100,000	33
Coliform	654	< 100	14
Psychrophilic ..	425	< 10,000	16

(a) Methylene Blue Reduction

Of the 680 samples tested by the methylene blue test, 1 per cent. initially failed the 4-hr standard.   Both 50° and 70°F proved unsatisfactory for the 24-hr incubation, which is the most convenient time for subsequent analysis. At 50°F, the methylene blue results were similar to those of the fresh milks prior to incubation.   The methylene blue reduction times of the milks incubated at 70°F provided no correlation between initial bacterial flora and testing after incubation.

The numbers of samples and their reduction times after incubation at 60° F for 24 hr are shown in Table 2.

TABLE 2

INFLUENCE OF PRELIMINARY INCUBATION ON METHYLENE
BLUE REDUCTION TIME

Methylene Blue Reduction Times		Progressive Totals of Samples Failing Incubation Test		
Before Incubation	After Incubation at 60°F for 24 hr	Actual No.	Progressive Total	Progressive Percentage
Less than 4 hr	½ hr or less ..	7	7	1
More than 4 hr	1 hr	12	19	2·8
More than 4 hr	1½ hr	38	57	8·3
More than 4 hr	2 hr	37	94	14·8
More than 4 hr	2½ hr	47	141	20·7
More than 4 hr	3 hr	53	194	28·5
More than 4 hr	3½ hr	51	245	36
More than 4 hr	4 hr	52	297	43·6
More than 4 hr	More than 4 hr	383	680	100

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(b) Nitrate Reduction

The positive nitrate reduction results agree largely with the results of methylene blue testing in revealing milks with initial high counts of total bacteria, coliforms or psychrophiles. However, the nitrate test also eliminated milks with total counts of more than 100,000 per ml, but with methylene blue times greater than 3 hr. There was better correlation between the two reduction tests at the lower levels of methylene blue reduction times—86 per cent. of the milks with pre-incubated methylene blue reduction times of less than 3 hr gave positive nitrate results.

In 457 samples subjected to the nitrate reduction test, there were 109 positives, or 23 per cent. Of these, 16 per cent. were found to have high coliform and/or psychrophile counts and 5 per cent. high total bacterial counts but low psychrophilic and coliform counts.

(c) Relationship Between Initial Quality and Reduction Tests After Incubation

The chi-square test of hypothesis or application was used as a means of determining the most significant reduction time. Tables 3 and 4 summarize the chi-square determinations of analyses of 373 milks classified on the pre-incubated methylene blue and nitrate test results and on the basis of the arbitrary standards set out in Table 1. Tables 5 and 6 show the relationship of results of pre-incubated methylene blue tests and initial bacteriological quality but do not include the nitrate test results.

TABLE 3

COMPARISONS OF RESULTS OF TESTS OF METHYLENE BLUE AND NITRATE WITH COLIFORM
AND PSYCHROPHILIC COUNTS

Methylene Blue Reduction Times and Positive Nitrate Test	Unsatisfactory	Satisfactory	Totals	chi-square
< 2 hr & +ve nitrate	61	43	104	
2 hr & above	16	253	269	
Totals	77	296	373	126·69
< 2½ hr & +ve nitrate ..	63	45	108	
2½ hr & above	14	251	265	
Totals	77	296	373	131·35
< 3 hr & +ve nitrate	66	53	119	
3 hr & above	11	243	254	
Totals	77	296	373	128·54
< 3½ hr & +ve nitrate ..	69	70	139	
3½ hr & above	8	226	234	
Totals	77	296	373	113·36
Standards	Satisfactory—Less than 100 coli per ml			
	,, ,, 10,000 psychrophiles per ml			

Where the nitrate test results have been used with those of the methylene blue test, all positives regardless of their respective methylene blue times have been regarded as unsatisfactory.

As chi-square was most significant when the standard in methylene blue test was taken at $2\frac{1}{2}$ hr, this was the time at which the standard for the methylene blue reduction time was most efficient in failing milks indicative of unsatisfactory quality and passing the satisfactory ones.

Table 4 sets out the chi-square values for the milks when a total bacterial count of 100,000 per ml was also used as a standard for good quality milk. In this case, chi-square was most significant at the methylene blue reduction time of 3 hr.

TABLE 4

COMPARISON OF RESULTS OF TESTS OF METHYLENE BLUE AND NITRATE WITH COLIFORM, PSYCHROPHILIC AND TOTAL BACTERIAL COUNTS

Methylene Blue Reduction Times and Positive Nitrate Test	Unsatisfactory	Satisfactory	Totals	chi-square
< 2 hr & +ve nitrate	83	21	104	
2 hr & above	64	205	269	
Totals	147	226	373	98·72
< 2½ hr & +ve nitrate ..	86	22	108	
2½ hr & above	61	204	265	
Totals	147	226	373	102·93
< 3 hr	94	25	119	
3 hr & above	53	201	254	
Totals	147	226	373	114·02
< 3½ hr & +ve nitrate ..	102	37	139	
3½ hr & above	45	189	234	
Totals	147	226	373	106·49
Standards	Satisfactory—Less than 100 coli per ml ,, ,, 10,000 psychrophiles per ml ,, ,, 100,000 total bacteria per ml			

Omitting the results of the nitrate reduction tests, chi-square values for methylene blue results only were compared with satisfactory and unsatisfactory tests based on coliform and psychrophilic counts. These results are shown in Table 5. In this comparison, the most significant chi-square value was again that for the methylene blue reduction time of 3 hr.

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TABLE 5

COMPARISONS OF RESULTS OF METHYLENE BLUE TEST WITH COLIFORM AND PSYCHROPHILIC COUNTS

Methylene Blue Reduction Times	Unsatisfactory	Satisfactory	Totals	chi-square
< 2 hr	30	12	42	
2 hr & above	47	284	331	
Totals	77	296	373	74·08
< 2½ hr	40	25	65	
2½ hr & above	37	271	308	
Totals	77	296	373	76·13
< 3 hr	54	40	94	
3 hr & above	23	256	279	
Totals	77	296	373	103·79
< 3½ hr	61	68	129	
3½ hr & above	16	228	244	
Totals	77	296	373	83·48
Standards	Satisfactory—Less than 100 coli per ml ,, ,, 10,000 psychrophiles per ml			

The chi-square values for methylene blue results (omitting the nitrate results) were compared with satisfactory and unsatisfactory milks based on coliform, psychrophilic and total bacterial counts, and these figures are shown in Table 6. The chi-square value found to be most significant was again that for the methylene blue reduction time of 3 hr.

TABLE 6

COMPARISON OF RESULTS OF METHYLENE BLUE TESTS WITH COLIFORM, PSYCHROPHILIC AND TOTAL BACTERIAL COUNTS

Methylene Blue Reduction Times	Unsatisfactory	Satisfactory	Totals	chi-square
< 2 hr	39	4	43	
2 hr & above	108	222	330	
Totals	147	226	373	53·7
< 2½ hr	57	9	66	
2½ hr & above	90	217	307	
Totals	147	226	373	73·9
< 3 hr	77	17	94	
3 hr & above	70	209	279	
Totals	147	226	373	95·04
< 3½ hr	95	35	130	
3½ hr & above	52	191	243	
Totals	147	226	373	94·4
Standards	Satisfactory—Less than 100 coli per ml ,, ,, 10,000 psychrophiles per ml ,, ,, 100,000 total bacteria per ml			

V. DISCUSSION

It is always difficult to determine a standard for any test involving micro-biology, because the standard set must produce a balance between the number of unsatisfactory milks that pass and the number of satisfactory ones that fail, keeping these numbers to a minimum. The standard revolves around the criteria chosen for good quality milks.

On the basis of misclassification, it appears from the chi-square values in Tables 4, 5 and 6 that, in Queensland, 3 hr is the most significant methylene blue time after incubation at 60°F for 24 hr—when it is agreed that the acceptance of unsatisfactory milks and failure of satisfactory ones are equal errors.

Bockleman (1962) performed methylene blue and nitrate reduction tests on fresh milks, and also found good agreement between the tests, but stated that neither reflected the total bacterial or coliform counts in the milk. The results in this paper show that when the raw refrigerated farm milks were incubated at 60°F for 24 hr before testing, the methylene blue and nitrate reduction tests gave a very good reflection of initial bacterial flora.

The nitrate reduction test was used in conjunction with the methylene blue test to establish the suggested standard. When both the reduction tests were used (Table 4), 119 milks (31 per cent.) were down-graded; of these, 94 (25 per cent.) were unsatisfactory. When the nitrate test results were omitted (Table 6), the most significant time for the standard was again 3 hr, and 94 milks (25 per cent.) were down-graded; of these, 77 (21 per cent.) were unsatisfactory. In the light of these results, it is suggested that for daily routine testing the methylene blue test only be used after preliminary incubation of the milk samples.

The application of an advisory standard in Queensland of 3 hr methylene blue reduction time after preliminary incubation of the samples at 60°F for 24 hr would greatly assist the industry in improving the bacteriological quality of farm milks.

VI. ACKNOWLEDGEMENTS

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