A COMPARISON OF PRACTICAL METHODS FOR DETERMINING THE BACTERIAL CONTENT OF MILK.*

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THE American Public Health Association appointed a committee in 1905 to formulate standard laboratory methods for the bacterial examination of milk, following the example set by a similar committee on water analysis. The methods of milk examination in vogue at present are so widely different in the different laboratories that results cannot be compared. These different results are due to at least four factors, viz., the medium employed, the temperature of incubation, the period of incubation, and the degree of shaking before plating.

The media usually employed are agar or gelatin. There are undoubtedly some arguments in favor of the use of gelatin, but the difficulty in handling gelatin plates, especially in warm weather, and the great loss occasioned by the presence of liquefying colonies probably more than counterbalance the advantages. The addition of litmus and dextrose or lactose is a common practice.

The temperature of incubation has been the subject of considerable discussion, inasmuch as some workers favor incubation at 37° C., others at 20°. Since certain bacteria multiply more rapidly at 37° than at 20°, the former temperature has been often employed, so that in many laboratories counts are made after 24 hours' incubation at 37°. It is true, however, that at 20° a higher count can be obtained than at 37°, and this fact seems to be of sufficient importance to advocate incubation at the lower temperature. It is generally conceded that at 37° multiplication is more rapid than at 20°, so that plates can be counted at an earlier date.

Finally the numbers may vary with the degree of violence used in shaking milk dilutions preparatory to plating, as has been shown recently by Rosenau and McCoy.¹

The points just mentioned explain to some extent the varying

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¹ *Jour. Med. Res.*, 1908, 18, p. 165.

results reported from different laboratories on the same milk. Within our own experience these results have varied enormously. The desirability of uniform methods is therefore emphasized, not only from a scientific point of view, but uniformity of methods should also be made a basis in the framing of ordinances for the regulation of milk supplies.

The ordinary bacteriological examination of milk consists chiefly of the colony count. Some significance has been attached to a study of bacterial species, a procedure which eventually may be of great value. The length of time required for a study of this nature and the uncertainty in interpreting results owing to our imperfect knowledge of microorganisms are the most serious objections to an extended study of species in milk. The total colony count therefore gives at present the most valuable control of milk and the efficiency of this method may be enhanced by establishing uniform methods. In water examinations we have different conditions from those obtaining in milk. The significance of fecal bacteria in water is well understood, total numbers being of secondary importance. Fecal bacteria in milk do not as a rule originate from human beings and therefore the significance of their presence is not the same as in water. The presence of fecal bacteria in milk signifies chiefly contamination with cow feces, in other words filthy methods in milking.

Bacteria as a rule are capable of enormous multiplication in milk. In water on the contrary they multiply but little. A high bacterial count in milk then has some decided significance. From this point of view it appears that numbers in milk are of more value than the differentiation of particular species.

To judge from the origin of bacterial contamination in milk we may distinguish several more or less distinct groups of bacteria, viz.:

1. Those gaining access to milk from the air.
2. Fecal bacteria.
3. Bacteria from contact with the hands and clothes of milkers.
4. Bacteria dropping into the milk from the cow during milking.
5. Bacteria in the utensils.
6. Pathogenic bacteria.

Fecal bacteria are chiefly acid formers and probably most bacteria originating from the cow and utensils are of this nature.

Many air bacteria do not form acid and we can thus by making a differential count of acid and non-acid forming colonies gain some insight into the conditions which have governed the production and handling of the milk. Pathogenic bacteria, if present, are usually in relatively small numbers, so that they can be detected with great difficulty.

The experiments reported in this paper were undertaken in order to put the considerations outlined above on a definite basis. The medium used was sugar-free¹ meat extract agar² with addition of 1 per cent dextrose in one set of plates and 1 per cent lactose in another set. A litmus solution prepared from Merck's pure extract of litmus was poured on a Petri dish before the milk dilution and the liquefied agar were added. Care was taken not to allow the milk dilution and the litmus to mix before the liquefied agar was added, on account of the alleged germicidal action of litmus. The samples were purchased from various dealers and dilutions of 1:1,000, 1:10,000, and 1:100,000 prepared. The dilution flasks used for this purpose were filled with 100 c.c. of distilled water and it was assumed as a result of frequent tests that they contained 99 c.c. after having been sterilized in the autoclave. Particular attention was paid to shaking all mixtures of milk and water in as uniform a manner as possible so as to break up the clumps of bacteria in the milk equally in all samples. Two sets of plates were prepared from each sample, two with lactose and two with dextrose. Then one from each set was incubated at 37° and one of each at 20°. The incubator for the low temperature, in which these plates were kept, is of very simple construction. An ordinary ice chest is kept cool by water from Lake Michigan circulating through a series of pipes and an incandescent electric light furnishes the heat. By a simple device the electric current is disconnected automatically as soon as 20° is reached and if the temperature falls below this the connection is re-established. The temperature was noted twice daily and the variations were less than one degree each way.

¹ Sugar-free meat extract agar was used in order to eliminate the error caused by the presence of muscle sugar in comparing dextrose and lactose agar. For routine work the small amount of muscle sugar in meat extract is of no consequence.

² Composition: 3 grams Liebig's extract of meat, 10 grams Witte's peptone, 15 grams agar in 1 liter of water. 8 c.c. of this medium were filled into each tube, so that after adding 1 c.c. milk dilution and 1 c.c. litmus solution the amount of agar was 14%. Reaction 1 per cent acid.

TABLE I.
SUMMARY OF RESULTS OBTAINED FROM EXAMINATION OF 40 SAMPLES OF MILK.

Days	ACID COLONIES			Non-ACID COLONIES			TOTAL COUNTS		
	Dextrose Agar		Lactose Agar	Dextrose Agar		Lactose Agar	Dextrose Agar		Lactose Agar
	20°	37°		20°	37°		20°	37°	
1.....	0	110.675	100	902.725	1,653.650	718,100	902.725	1,764.325	718,200
2.....	687.400	1,605.775	454.100	4,677.600	2,115.050	4,844.400	5,365.000	3,721.725	5,208.500
3.....	2,707.075	2,022.175	1,726.775	6,382.150	2,217.475	7,424.150	9,180.125	4,239.650	9,150.025
1.....	0%	6%	0%	100%	94%	100%		+ 861.600	
2.....	13%	43%	8.5%	84%	57%	91.5%	+ 1,643.275		+ 1,597.250
3.....	30%	48%	19%	70%	52%	81%	+ 4,045.475		+ 5,033.925

Spreaders: At 20° = 5%; at 37° = 15%.

In counting the colonies those plates were selected which showed the presence of 200 to 500 colonies; the acid forming colonies were counted separately. The total count was also recorded so that a definite ratio between acid and non-acid forming colonies could be established. The counts were repeated on each of three successive days. After this period the number of colonies still increases, but the increase is slow and the total is not increased very much although more pronounced at 20° than at 37°. In the Thirteenth Annual Report of the Board of Health of the Town of Montclair, N. J., it is stated that milk plates were incubated for seven days at room temperature, and in a private communication Dr. Baldwin stated that the count after three days at room temperature gave perfectly satisfactory results.

Table 1 here given is a summary of the results obtained from the examination of 40 samples of milk in the above-described manner. If we compare the total counts with reference to the temperature of incubation we find that in dextrose agar the number after one day is larger at 37°, after two days the number is higher at 20°, and after three days the number of colonies at 20° is about double that at 37°. In lactose agar conditions are practically the same.

As to the number of acid colonies, at 20° there are no acid colonies in either dextrose or lactose agar after 24 hours. After two days the number of acid colonies in both dextrose and lactose agar is considerably larger at 37° than at 20°, but this proportion is reversed after three days, the acid colonies developing at a more rapid rate after two days at 20° than at 37°. The proportionate number of acid colonies, as compared with the total number of colonies developed, is smaller at 20°, however, than at 37°, which would seem to indicate that acid formers which are chiefly of fecal origin are favored at 37° at the expense of non-acid formers. It is obvious then that at 20° we obtain a more complete representation of the groups of bacteria gaining access to milk than at 37°. And since the actual number of acid formers is really higher at 20° than at 37° after three days, it seems reasonable to assume that they are represented in fair proportion.

The relation of acid forming colonies to non-acid formers in

dextrose and lactose deserves special notice. There is in some samples a decided relative decrease of non-acid colonies after two and three days at both temperatures in lactose agar. This phenomenon is undoubtedly due to a fact which has been noted repeatedly, namely, that some milk bacteria of the *B. aërogenes* type form red colonies at first and then later these colonies assume the blue color again. This phenomenon was not observed in dextrose agar. We conclude from this observation that dextrose is to be preferred as an addition to the medium to lactose.

The number of plates lost by the overgrowth of spreading colonies is three times larger at 37° than at 20°. The loss might be prevented partially by using porous-cover petri dishes, but these covers render counting more difficult and inaccurate and the jelly is more liable to become dry. The insertion of a piece of filter paper under the cover of a petri dish before sterilization is perhaps a better method for the absorption of condensation water.

One of the advantages then of incubation at 20° over 37° is the larger count obtained. This may not be of prime importance since we cannot hope to demonstrate the presence of nearly all bacteria in milk by ordinary methods. The count after two days at 37° is, however, so much larger than after one day that, if 37° is to be the incubation temperature, the period of incubation should be at least two days. The incubation at 20° after two days gives a higher count than at 37° and a still higher one after three days. Thus the time gained is but one day by using the higher temperature and if the better differential count at 20° is taken into consideration this temperature is surely preferable. We favor the incubation at 20° for three days, therefore, chiefly because more groups of bacteria have a chance to develop than at 37° and this gives us a better insight into the production, handling, etc., of milk.

The results of our experiments are graphically illustrated in the following chart which is self-explanatory. It is shown here very clearly that the numbers of bacteria steadily increase at 37° until a high point is reached after two days, while at 20° the increase is slow for 24 hours and then the high point is reached after three days, although even after two days the numbers at 20° are higher than at 37°. It is also shown that the acid colonies develop more rapidly

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at 37° for two days after which time a higher number is reached at 20°. Finally we note that more acid colonies develop in dextrose agar than in lactose agar.

Several objections to the use of 20° as incubation temperature have been advanced, some of which will be briefly discussed.

1. Pathogenic bacteria do not grow so well at the lower temperature. This is undoubtedly true, but the finding of pathogenic bacteria in milk, as in water, is very difficult and in the majority of cases search for them is unsuccessful, and when successful is the result of long and laborious work. It may safely be stated that under present conditions the probability of finding pathogenic bacteria in milk is so remote that any attempt to find them is entirely out of the question in routine examinations of milk. Incubation at 37° therefore affords no benefit in this respect.

2. Incubation at 20° requires more time than at 37°. It has been shown in this paper that the difference is but one day and this is surely too short a period for which to sacrifice accuracy. A longer period of incubation also necessitates the use of more material and glassware, which, however, does not seem a sufficiently weighty objection to the use of 20° for three days if better results can be obtained.

3. Great stress is laid by some workers on the claim that it is difficult to procure incubators which can be kept uniformly at 20°, especially in summer weather. There is a description of a practical low-temperature incubator in the new edition of William's *Manual of Bacteriology*, which also refers to Rogers' paper describing an electrically controlled low-temperature incubator.¹ This type of low-temperature incubators has been used in several laboratories with entire satisfaction. It is possible to keep an incubator of this nature in any locality where electricity and ice are available.

The following conclusions seem warranted from the results of the experiments reported:

1. Official bacterial standards for milk should include a statement of the methods by which the bacteriological control is to be obtained.

2. For practical purposes incubation at 20° is superior to incuba-

¹ *Centralbl. f. Bakt.*, 1905, Abt. 2, 15, p. 236.

tion at 37°, because: (a) a higher count is obtained; (b) a better differential count is obtained.

3. Dextrose is to be preferred to lactose as an addition to the medium.

4. Since milk is usually consumed before the results of a bacteriological examination can possibly be reported, a gain of one or even two days is immaterial. The bacteriological and chemical examination of milk ought to be carried on chiefly with the object of improving the whole milk supply of a commonwealth rather than of punishing individual offenders. To this end the most accurate and scientific method of examination is the preferable one.