STAINING OF BACTERIA IN MILK FOR DIRECT MICROSCOPIC EXAMINATION — A REVIEW

WILLIAM A. MOATS

United States Department of Agriculture
Agricultural Research Service
Market Quality Research Division
Beltsville, Maryland

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In the direct microscopic method for examining milk for bacteria, it is important that an adequate staining method be used. The method should stain clearly all bacteria present so that they can be identified readily and counted under the microscope. The problem of adequate staining has become more acute with the introduction of the direct microscopic count into Federal Standards for nonfat dry milk, (33) since the processing kills most of the bacteria and decreases their ability to take up stains. The direct microscopic count, despite its shortcomings (12), gives a general picture of the quality of raw milk used and the hygiene of manufacture of nonfat dry milk (7, 12), and is probably the best method for this purpose.

Staining of biological materials is essentially the binding of dyes to various chemical constituents of the object under study. In the case of milk, the objective is, of course, to stain the bacteria as heavily as possible without staining the milk film unduly. Biological staining methods are necessarily somewhat empirical because of the complex stereochemistry of both the dyes and the biological compounds to which they are bound. It is nevertheless, possible in many cases to apply sound physical and chemical principles to the development of staining methods. Until quite recently, the approach to the staining of bacteria in milk has been entirely empirical, and has consisted mostly of formulating a single dye, methylene blue, in various solvents. Results were never entirely satisfactory, although over the years procedures have improved considerably.

In the development of suitably selective staining procedures for bacteria in milk, it is helpful to consider the chemical composition of the bacteria and the milk. Both contain protein; bacteria, in addition, contain considerable amounts of nucleic acids and perhaps some other polyphosphate materials; gram-positive bacteria, at least, contain a great deal of polysaccharide material in the cell wall (30). By using stains specific for nucleic acids or polysaccharides, then, it should be possible to selectively stain bacteria with little staining of the milk background.

Selective staining of nucleic acids with basic dyes is based on the fact that phosphoric acid is a stronger acid than the carboxylic acids commonly found in biological materials such as proteins (28, 32). Nucleic acids are polyphosphates in the structure,

\[ \text{QH} \rightarrow \text{Q}^- \]

\[ R-P-R' \rightarrow R-P-R'+H^+, \]

\[ 0 \rightarrow 0 \]

where R and R' represent the remainder of the nucleic acid molecule. Proteins contain free carboxylic acid groups in the form,

\[ R-COO \rightarrow H^+ + RCOO^- , \]

where R represents the remainder of the protein molecule. In slightly acid solution, basic dyes pick up a hydrogen ion and carry a positive charge. The positively charged dye is then bound to the negatively charged groups on the proteins or nucleic acids by the attraction of the oppositely charged ions. The above equations show that, as the hydrogen ion concentration of the medium is increased, the equilibrium will shift toward the unionized forms of the acids and they will lose their ability to take up basic dyes. The phosphates remain dissociated into ions at a lower pH than the carboxyls, therefore, nucleic acids can be selectively stained by suitably adjusting the pH of the dye solution. In practice, dissociation of carboxyls is almost completely suppressed at pH 4 while phosphates of the nucleic acids remain in ionic form to about pH 2 (28, 32). Another means of suppressing the dissociation of molecules into ions is to lower the dielectric constant of the medium (11) by using organic solvents instead of water. Dissociation of the weaker carboxylic acids is affected more than is dissociation of the phosphates. Lowering the dielectric constant will also, however, tend to reduce the percentage of dye which is in ionic form, whereas lowering the pH will not.

The standard methylene blue stains used for staining bacteria in milk are, in fact, nucleic acid stains which use one or a combination of the above methods to selectively stain bacterial nucleic acids. The stains were developed empirically. The original Breed stain (4) used a water-ethanol solvent and tended to overstain the milk proteins. Levine (15, 16, 17) found that 95% ethanol was more a satisfactory
solvent and attempted to explain his results solely on the basis of changes in surface tension, without considering the reduced dielectric constant of the medium. The carbolated aqueous methylene blue stain (6) used phenol to acidify the medium which also tended to overstain the background (17, 18). Phenol is a very weak acid and evidently did not lower the pH sufficiently to suppress staining of proteins. Levine and Black (17) observed that strong mineral acids or even too much acetic acid in the medium gave very light staining, as would be expected in too acid a medium. North (24) used an aniline-HCl buffer in ethanol-water and Levowitz and Weber (19) used organic solvents slightly acidified with acetic acid in their modification of the Newman-Lampert formula No. 2 (23).

Some staining formulations developed include fat solvent in the staining solution, thereby staining and defatting simultaneously (2, 5, 19, 23), thus making staining a "one-dip" procedure. This is certainly convenient if it can be done without sacrificing quality of the resulting stained smear. This method has, perhaps, been over emphasized, because even with the more involved procedures, the actual staining time is relatively brief compared with the time spent preparing smears and in microscopic examination of the stained smear. This is particularly true if a number of smears are stained at one time. Some authors (5, 10) have included in the formulations basic fuchsin, which stained bacteria blue against a pink background. The advantage of this method appears to be questionable (17) and such procedures have not been widely accepted. Comparative studies (1, 20, 26, 34) have shown that of the methylene blue stains, the aniline oil, the Levowitz-Weber, and the acid- and water-free procedures are most satisfactory and are recommended in the 11th Edition of Standard Methods (31).

Methylene blue, unfortunately, is a rather poor stain for nucleic acids and is not now generally used for this purpose in histology and cytology (8, 28). The first break from methylene blue is the procedure of Anderson, Gunderson, and Moehring (3), in which the methylene blue is polychromed. Polychroming is an oxidation process which converts the methylene blue to a mixture of related thiazine dyes such as the azures and thionin (8). These are generally considered to be more satisfactory nucleic acid stains than pure methylene blue (8, 28, 32). Polychroming is, however, the difficult way to obtain these dyes, considering that they are available commercially. Anderson (2) also recognized this and recently described a single-solution nuclear staining procedure using azure A in which defatting and fixing agents are incorporated into the dye solution. Olsen and Jezeski (25) treated milk smears with strong alkali before staining with crystal violet. Alkali treatment is supposed to increase dye uptake by proteins. Since both bacteria and milk contain protein, it is not clear why increasing dye uptake by proteins should be advantageous in staining bacteria in milk. The approach may ultimately prove satisfactory for reasons other than those given. Neither the Anderson nor the Olsen-Jezeski stain has been evaluated in published independent comparisons.

The author studied the use of a polysaccharide stain, the periodic acid-Schiff reaction, for selectively staining bacteria in milk (21, 22). This type of staining was recently reviewed by Kasten (14). In this procedure, the smear is treated with periodic acid which oxidizes structures of the type \( \text{OH OH} \), such as are found in polysaccharides, to \(-\text{CHO OHC-}\), splitting the carbon-carbon bond and forming two aldehyde groupings. The polyaldehydes thus formed are then reacted with Schiff's reagent, a colorless complex of bisulfite and basic fuchsin, to give a magenta stain. The Schiff reagent prepared from basic fuchsin, however, gave too light a stain to be practical. Many other dyes also form complexes with bisulfite which react with aldehydes in a similar manner (9, 13, 27), although, unlike the classic Schiff reagent, the complexes are not colorless. Of a number of other dyes tested in the periodic acid-Schiff procedure, toluidine blue and azure A were the most satisfactory. Toluidine blue was selected for use since it is cheaper and more stable in solution. This stain, at its best, was very good, but the dye-bisulfite solution was not stable more than a few days.

An alternative method of polysaccharide staining was developed which overcame the problem of instability of the Schiff-type reagents. In the revised procedure, the polyaldehyde is reacted with bisulfite and then the aldehyde-bisulfite complex is reacted with the dye. This gives results identical with those obtained by reacting the aldehyde with the bisulfite-dye complex and has the advantage that the reagents are stable for a long time. The toluidine blue dye solution is buffered to pH 4 and, therefore, stains both nucleic acids and polysaccharides (21, 22). The periodic acid procedure for polysaccharides is most advantageous for staining dead bacteria in nonfat dry milk. The author (22) found that, when bacteria are heated in milk, a large part of their nucleic acid content is extracted from the bacterial cells. Therefore, staining by the usual techniques, based on nucleic acid staining, is rather poor. Furthermore, the bacteria appear shrunken and are difficult to distinguish from particles of debris. When the periodic acid stain was used, no decrease in direct microscopic counts was observed after heating, and staining of the heat-treated bacteria was as
good as before heating, except in the case of gram-negative rods which were stained sufficiently by the periodic acid procedure to be counted, while they could not be seen at all when a standard methylene blue stain was used.

The direct microscopic count has not been used as a means of distinguishing between grades of nonfat dry milk because of the high interlaboratory variation in counts on duplicate smears observed in collaborative studies (29). With the periodic acid procedure, stained bacteria appear much larger and are much easier to identify positively. Contrast between bacteria and background is also much better. These features should make it easier for technicians to obtain reproducible results.

For bacteria in raw milk, use of the longer periodic acid procedure may not be justified. A good nuclear staining procedure such as that of Anderson (2) should be satisfactory. Another satisfactory procedure is to defat in xylene, fix a few minutes in 3:1 absolute ethanol-acetic acid until the smear appears clear, stain for 30 seconds in pH 4 toluidine blue (22), rinse lightly, and dry.

An effective method for staining gram-negative rods in heat-processed milk is still needed. Research on the development of new and improved stains for bacteria in milk should be encouraged.

REFERENCES


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