APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1980, p. 423-429
0099-2240/80/02-0423/07$02.00/00

Vol. 39, No. 2

Rapid Membrane Filtration-Epifluorescent Microscopy Technique for Direct Enumeration of Bacteria in Raw Milk

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Membrane filtration and epifluorescent microscopy were used for the direct enumeration of bacteria in raw milk. Somatic cells were lysed by treatment with trypsin and Triton X-100 so that 2 ml of milk containing up to 5×10^6 somatic cells/ml could be filtered. The majority of the bacteria (ca. 80%) remained intact and were concentrated on the membrane. After being stained with acridine orange, the bacteria fluoresced under ultraviolet light and could easily be counted. The clump count of orange fluorescing cells on the membrane correlated well ($r = 0.91$) with the corresponding plate count for farm, tanker, and silo milks. Differences between counts obtained by different operators and between the membrane clump count and plate count were not significant. The technique is rapid, taking less than 25 min, inexpensive, costing less than 50 cents per sample, and is suitable for milks containing 5×10^3 to 5×10^8 bacteria per ml.

Assessment of the bacteriological quality of raw milk by indirect methods of estimating bacterial numbers is unreliable, and procedures based on counting colony-forming units (CFU) are time consuming and expensive. There is a need for a rapid method, taking <30 min, capable of estimating directly numbers of bacteria in milk having counts from $<10^4$ to $>10^7$ colony-forming units per ml. Such a test would be of value for monitoring incoming milk in tankers and bulked (silo) milks during storage before processing or manufacture. This would permit better control and management of supplies and reduce the risk of poor quality milk products resulting from high bacterial counts in the raw material. The test could also be used for grading farm milk on the basis of hygienic quality. To attain the required level of sensitivity for low-count milk, concentration of the bacteria is essential.

Direct counting of aquatic bacteria concentrated on membrane filters stained with fluorochromes by using epifluorescent microscopy is widely used (7, 9, 16). The application of this procedure to milk has been impractical because of the small volume (ca 0.01 ml) which can be filtered through bacteria-retaining membranes before blockage of the pores by particulate matter in the milk, e.g., somatic cells and fat globules. Filtration of at least 2 ml of raw milk containing up to 2×10^6 somatic cells/ml is essential to achieve adequate sensitivity. Dilution of milk with a suitable surfactant (5, 10) or treatment with a proprietary protease-surfactant-stain mixture (6) has been reported to improve the filterability of milk, but these procedures were found to be unreliable for the volumes required. Treatment of samples contaminated with mammalian cells, e.g., infected urines, with a protease from *Aspergillus oryzae* and a surfactant was reported to minimize filter clogging (12) in a procedure for determining bacterial adenosine triphosphate. To permit filtration of milk, however, we found it necessary to modify the procedure. Optimum conditions for rapid filtration, and the suitability of various enzymes, membranes, and stains, including fluorochromes, had to be determined before bacterial cells filtered from milk could be readily counted. Preliminary results obtained during the course of these trials, using a two-stage protease-surfactant treatment, have been published elsewhere (4).

MATERIALS AND METHODS

Milk samples. Milk samples were taken from farm-refrigerated bulk tanks containing milk from two milkings, exfarm collection tankers on daily collection, and silo tanks containing up to 90,000 liters of milk stored for 1 to 2 days. Portions of some tanker milks were stored for 7 days at 5 ± (0.5)°C and analyzed daily. Dilutions, where required, were made in sterilized 0.25-strength Ringer solution (Oxoid Ltd.). Milk containing $>10^7$ somatic cells/ml was obtained by infusing healthy quarters of bovine udders with 0.1 to 1 μg of endotoxin (lipopolysaccharide B, *Escherichia coli* O55:B5; Difco Laboratories) and milking by hand 16 to 20 h later. This milk was diluted in milk from uninfused quarters to give somatic cell counts in the range 10^6 to 10^7/ml.

Reagents for membrane filtration. Before use, all reagents were filtered through 0.22-μm pore size

membrane filters (Millipore Corp.) to remove bacteria and particulate matter and were dispensed into sterile containers. All reagents, except enzymes, were stored for ≤3 weeks at 4 to 5°C.

Suspensions of enzymes in cold (ca. 4°C) deionized water were centrifuged at 12,000 × *g* for 30 min to remove insoluble material. The supernatant was filtered under pressure, dispensed in 0.5-ml amounts, and stored at −20°C until required. The centrifugation step was unnecessary for the enzyme trypsin (Difco), which is available as a freeze-dried soluble preparation.

Solutions of the surfactant Triton X-100 (BDH Chemicals Ltd.) in deionized water at 0.5% (vol/vol) and 0.1% (vol/vol) were used for treatment of milk samples and rinsing purposes, respectively.

The fluorescent stain used was acridine orange at 0.025% (wt/vol) and Tinopal AN (Ceiba-Geigy Ltd.) at 0.025% (wt/vol) in 0.1 M citrate-NaOH buffer (pH 6.6). A portion of stock solution of stain was filtered daily before use and stored in an opaque container.

Membrane clump count (MCC). Whole milk (2 ml), or where necessary a suitable dilution, trypsin (0.5 ml), and surfactant (2 ml) were incubated at 50°C for 10 min in a sterile, capped test tube. A Nucleopore polycarbonate membrane filter (pore size, 0.6 μm; diameter, 25 mm; Sterilin Ltd.) was mounted shiny side uppermost on a membrane filter holder (Millipore) held in a five-way filter manifold (B.P. Engineering, unit no. 1, Bayton Way, Exhall, Coventry, England) connected to a vacuum line at 100 kiloPascals (kPa). At all stages of the procedure the vacuum to the manifold was released before the addition of liquid to the filter units.

The filter unit was warmed by filtration of 5 ml of Triton X-100 that had been heated to 50°C, the treated milk was filtered, and 5 ml of warmed Triton X-100 was used to rinse the test tube and filter tower. Controls containing 2 ml of diluent in place of milk were included to ensure that reagents remained free from bacterial contamination. Filter towers and sintered-glass bases were soaked in Decon 90 (Decon Labs Ltd.), brushed under running tap water, and immersed in boiling deionized water for 5 min before use.

The membrane was overlaid with 2 ml of stain. After 2 min, the vacuum was reapplied, and the membrane was rinsed with 2.5 ml of citrate-NaOH buffer (0.1 M; pH 3) followed by 2.5 ml of 95% (vol/vol) ethanol. The stained membrane was air dried and mounted in nonfluorescent immersion oil (Fractoil; R. A. Lamb Ltd.) on a slide beneath a cover slip.

The mounted membrane was examined by means of a Leitz SM LUX microscope fitted with a Ploempak epifluorescent illuminator system and an HP2 filter unit. Clumps of orange-red-fluorescing bacteria were counted in fields of view taken at random by the scheme shown in Table 1. A clump was defined as any cell or group of cells separated by a distance equal to or greater than twice the smallest diameter of the two cells nearest each other (1).

The MCC per milliliter of milk was estimated from the mean clump count of orange-fluorescing bacteria per field and a microscope factor of 5,750 (i.e., one clump per field = an MCC of 5,750/ml if 2 ml of milk is filtered). On occasion, as indicated, clumps of green-fluorescing bacteria were also counted and included (total MCC).

Plate count (PC). Milk samples were plated (3) by using yeast extract milk agar (Oxoid), and colonies were counted after 3 days of incubation at 30°C.

Breed smear count. Breed smears (2, 3) of stored milks (0.01 ml) were prepared and stained by using Newman stain with a contact time of 8 min (13). The prepared smears were examined microscopically by transmitted light, and clumps of bacteria were counted as described for the MCC.

The Breed clump count (BCC) per ml of milk was estimated from the mean clump count per field and a microscope factor of 650,000. The somatic cell count per milliliter of milk was determined by counting somatic cells in similarly prepared smears.

Enzyme efficiency. In preliminary studies numerous crude and purified proteases and lipases were compared by determining the rate at which 2 ml of raw and pasteurized whole milk could be filtered after a standard treatment with enzyme in the presence of surfactant. The following enzymes were tested either singly or in combination: trypsin (Sigma T8128, T0134; Difco; Hopkin and Williams); α-, β-, δ-, and γ-chymotrypsin (Sigma); lipase (Sigma L312b, or L2253); phospholipase (Sigma, P9139) and proteases of microbial origin (Rhom and Haas, Rhozyme-41; Sigma P4755, P1512, P5005). For proteases, enzyme concentrations were usually selected on the basis of specific activity against casein. Selection of concentrations of lipases were based on estimates of the amounts present in crude trypsin.

Determining the agreement between operators, using the MCC and PC. Three operators each performed three replicates of both the membrane and plate counts, using the same samples of three farm and three stored tanker milks. The order of plating and membrane filtration for each operator were randomized. The operators all had some experience with the MCC.

Determining the relationship between MCC, PC, and BCC. Samples of tanker milk were stored at 5°C, and at daily intervals MCC, PC, and BCC were made, each in triplicate, by one operator. In addition, single membrane and plate counts were performed by three operators on a total of 90 samples, 30 each of farm, tanker, and silo milks.

Electron microscopy. Nuclepore membrane filters through which trypsin-surfactant-treated milk had been filtered were fixed for 2 min with 6% (vol/vol) glutaraldehyde either after the filtration proce-

TABLE 1. *Number of fields of view counted on membrane filters, with number of bacteria per field*

No. of fields counted	Avg no. of clumps per field
15	0–10
10	11–25
6	26–50
3	51–75
2	76–100
Repeat[a]	>100

[a] Sample diluted procedure repeated.

dure or after the staining and rinsing stage. After washing with filtered distilled water, the membranes were air-dried and a segment was removed and attached to a scanning electron microscope stub with double-sided adhesive cellulose acetate tape. After being coated with gold in an Edwards S150 sputter coater, the preparations were examined in an ISI Super IIIA scanning electron microscope with an accelerating voltage at 15 kV.

RESULTS

Enzyme efficiency. To attain the required level of concentration for accurate counting microscopically of bacteria in milk containing $<10^6$ bacteria per ml, the enzyme-surfactant treatment had to permit the rapid filtration of at least 2 ml of milk and subsequent passage of rinse and staining solutions. Also, the final preparation needed to be relatively free from debris, with the bacterial cells intact and clearly visible after staining.

The crude enzymes, trypsin, and lipase from the porcine pancreas and the crude microbial proteases all permitted filtration of 2 ml of raw whole milk. With pure enzymes, trypsin, lipase, or phospholipase from porcine pancreas, the treated milk blocked the membrane filter before all the liquid had passed through it. The pure chymotrypsins were also ineffective unless reinforced by the addition of pure trypsin (T0134). However, the combination of this latter enzyme with pure lipase (L2253) was not effective. Most of the enzymes which were effective with raw milk also permitted filtration of laboratory pasteurized milk.

It was observed that ineffective enzymes failed to lyse somatic cells and therefore the unlysed cells probably caused blockage of the membrane pores. The combination of trypsin and one or other of the chymotrypsins lysed all somatic cells but left large strands of debris on the membrane. These were probably deoxyribonucleic acid because addition of deoxyribonuclease (source, bovine pancreas; Boehringer Mannheim Corp.) removed the material.

On the basis of these results, the clarity of the final preparation and the cost, crude trypsin (Sigma T8128) was selected for routine use. Where high-speed centrifugation facilities are available, other crude pancreatic trypsins and Rhozyme-41 are alternatives. It was found that 2 ml of raw milk could be filtered by using a preparation of 2% (wt/vol) trypsin, but a 20% (wt/vol) solution was routinely used because it gave clearer preparations, made possible the filtration of high somatic cell count milk (ca. 5×10^6 cells/ml), and retained its activity for at least 3 weeks at −20°C. When freeze-dried, it was still active after 3 months. Preparation of enzymes in deionized water instead of buffer solutions was found not to impair the stability or performance of the enzymes.

Treatment of raw whole milk with the preparation of 20% crude trypsin and Triton X-100 for 10 min at 50°C permitted the rapid filtration of the mixture (4.5 ml) containing 2 ml of milk. The bacterial cells, which remained intact after the treatment, were retained and concentrated on the membrane.

Staining procedure. Initially, conventional stains such as methylene blue, periodic bisulfite toluidine blue (11), and phenolic aniline blue (8) were used to stain bacteria on membranes after filtration of treated milk, and the bacteria were visualized by transmitted light. The low and variable counts compared with the colony-forming units per milliliter determined by PC on the untreated milk suggested that not all the bacterial cells took up the stains used. In addition, counting was tiring because of the difficulty of distinguishing bacteria from debris. Scanning electron microscopy of membranes revealed the presence of intact bacterial cells, some of which were coated with debris (Fig. 1). After the application of fluorescent stain and subsequent washing, this material was no longer visible (Fig. 2).

After staining with acridine orange/Tinopal AN and microscopic examination using incident ultraviolet light, the bacteria fluoresced either orange-red or green and were easily distinguishable from the small amount of fluorescent debris on the filter (Fig. 3). Tinopal AN was included in the stain primarily to increase contrast, but it had the disadvantage that bacteria unstained by acridine orange, and therefore presumably devoid of nuclear material, appeared dark green or dark brown with the microscope filter system employed. Other fluorochromes, e.g., fluorescein, euchrysine-2GNX, and ethidium bromide were tried, but acridine orange/Tinopol AN was preferred because of rapidity of staining and clarity of the preparation.

Acridine orange staining characteristics of bacterial cells. In fresh milk 36 to 65% of the bacteria fluoresced orange-red, and the remainder fluoresced green. The total MCC (orange plus green) generally exceeded the PC, whereas the MCC (orange) was in close agreement (Fig. 4 and 5). In refrigerated bulked milk stored for 1 to 4 days, all cells fluoresced orange-red and there was good agreement between the MCC (orange) and PC (Fig. 4 and 5). Where laboratory storage of the tanker milk was prolonged (>5 days), an increasing proportion of the population fluoresced green, during which time the PC either increased only slightly, or remained stationary.

MCC and PC were made on pure cultures of

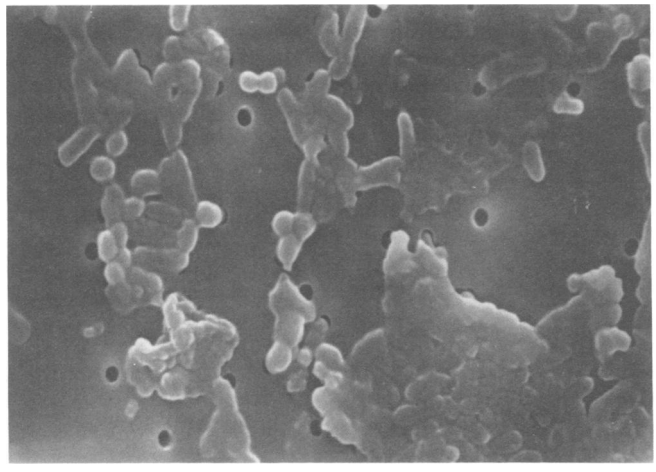

FIG. 1. *Preparation immediately after filtration of heated milk. Scanning electron micrograph of bacteria recovered from milk by membrane filtration. Magnification, ×3,400.*

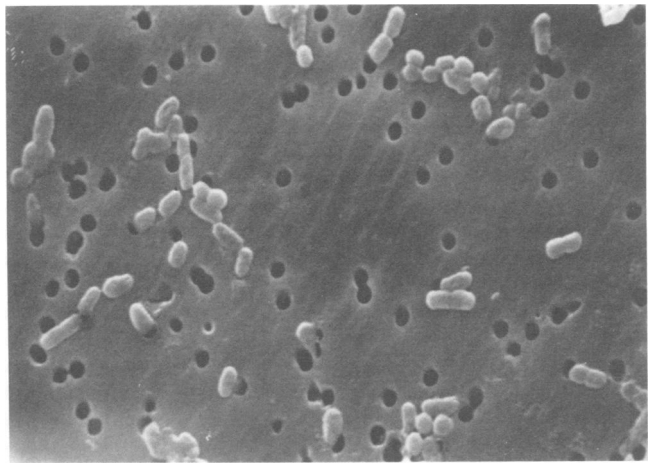

FIG. 2. *Preparation after staining with acridine orange/Tinopal AN and washing with buffer and ethanol. Scanning electron micrograph as in legend to Fig. 1.*

E. coli and *Bacillus subtilis* grown in nutrient broth (Oxoid). During the exponential phase of growth the majority of cells fluoresced orange-red, but after entering the stationary phase an increasing proportion fluoresced green. With both cultures the total MCC (orange plus green) was more closely related to the PC than that of the MCC (orange cells only).

Effect of trypsin/Triton X-100 treatment on recovery of bacteria. Quantities (0.01 ml) of high count raw milk (10^6 to 10^7 colony-forming units per ml) were suspended in 2 ml of diluent and heated with trypsin and Triton X-100 for 10 min at 50°C before being membrane filtered by the standard procedure. Controls of untreated milk suspended in 4.5 ml of diluent were filtered without enzyme-surfactant treatment. The MCC of the treated milk was 81% (range, 74 to 87%) of that of the untreated milk, showing that most of the clumps of bacterial cells which fluoresced orange-red in untreated milk reacted in the same way after treatment and were therefore counted.

In contrast, as determined by PC, less than 1% of the bacteria in the stored milk were viable after the treatment as compared with the untreated controls.

Agreement between operators using the MCC and PC. The mean MCC and PC for each of six milk samples (three farm milks A, B, and C, and three stored tanker milks D, E, and F) obtained by each of three operators is shown in Fig. 4. The agreement between the methods and between operators is good over a wide range of

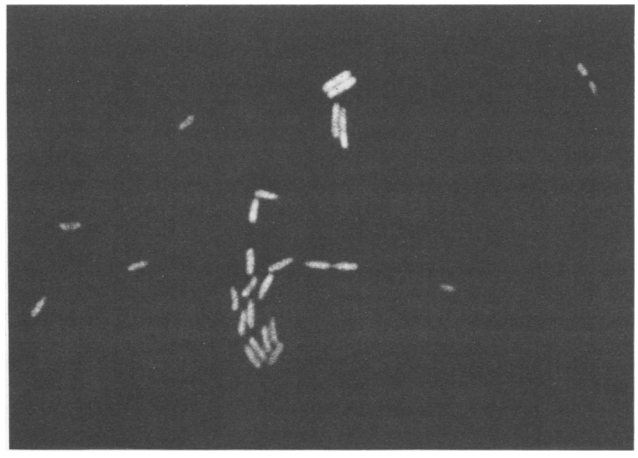

FIG. 3. *Bacterial cells recovered from milk by membrane filtration, stained with acridine orange and Tinopal AN, and revealed by epifluorescent microscopy. Magnification, ca. ×1,700.*

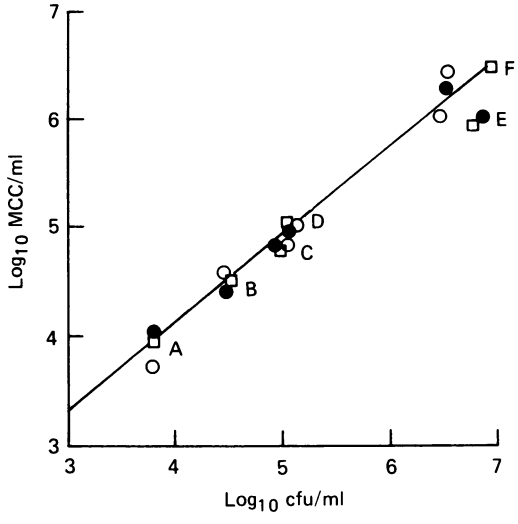

FIG. 4. *MCC and PC obtained by three operators for six milks. Farm milks (A-C), stored milk (D-F). Operators I (○), II (●), and III (□). Each point represents the mean of three determinations. Line represents fitted regression line (y = 0.98 + 0.78x).*

counts (Table 2). For both methods, the coefficient of variation (*CV*) between triplicate log counts within samples was ±1.6%. The *CV* between mean log counts obtained by different operators was ±2.2% for the MCC, and ±2.6% for the PC.

Relationship between MCC, PC, and BCC. For stored tanker milk tested at daily intervals the MCC and PC for any 1-day period were in close agreement (data not shown). Where a BCC could be made (>5 × 10^6 CFU/ml), these were also in good agreement with the other two counts.

The relationship between the MCC and the PC for the 90 samples of farm, tanker, and silo milks is shown in Fig. 5. There were no significant differences between the three relationships (Table 3), and overall a good correlation coefficient ($r = 0.91$) was obtained.

DISCUSSION

The major barrier to the filtration of whole milk through a bacteria-retaining membrane filter is the presence of somatic cells which, on average, approach 1,000 times the volume of bacterial cells and may occur in numbers as high

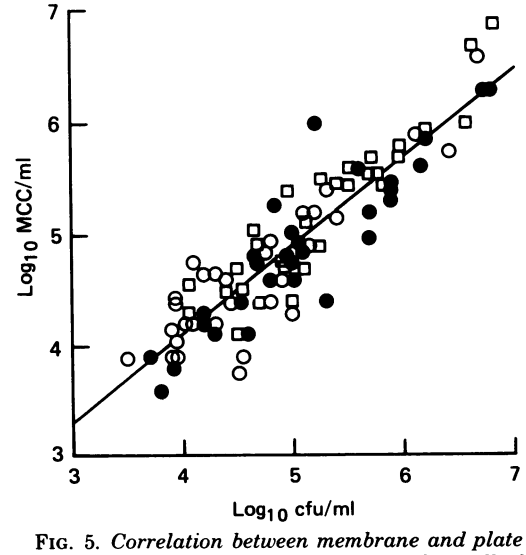

FIG. 5. *Correlation between membrane and plate counts for 30 samples each of exfarm (○), bulked tanker (●), and silo milks (□). Line represents fitted regression line (y = 0.89 + 0.8x).*

TABLE 2. *Mean $\log_{10}$ counts per milliliter obtained by three operators for six milk samples, using the membrane filter and PC methods*[a]

Operator no.	MCC[b]	PC[b]	Overall mean[c]
I	5.12	5.24	5.18
II	5.07	5.28	5.18
III	5.09	5.29	5.19
Overall mean	5.10	5.27	

[a] *n*-fold ratio, PC/MCC = 1.5.
[b] Difference ± standard error, based on the interaction between counting techniques and milk samples (5 df), was 0.18 ± 0.117 (not significant).
[c] Standard error difference of two overall operator means, based on the interaction between operators and milk samples (10 df), was 0.051.

TABLE 3. *Relationship between $\log_{10}$ MCC per milliliter (y) and $\log_{10}$ PC per milliliter (x) for exfarm, tanker, and silo milks*[a]

	a	SE	*b*	SE	df	*r*
Farm milks	1.07[c]	0.369	0.77[b]	0.078	28	0.88
Tanker milks	0.93[d]	0.369	0.78[b]	0.071	28	0.90
Silo milks	0.67	0.357	0.86[b]	0.067	28	0.92
Overall	0.89[b]	0.203	0.80[b]	0.040	88	0.91

[a] $y = a + bx$. SE, Standard error; *r*, correlation coefficient.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] $P < 0.05$.

as 500,000/ml in normal bulked milk or $>10^6$/ml in individual farm milks. For effective filtration, somatic cells must be lysed and the cellular material dispersed. Not all the proteolytic enzymes tested were suitable for the membrane filtration of milk, those unsuitable being incapable of rapidly lysing somatic cells when incubated in the presence of Triton X-100. Crude enzyme preparations were effective, whereas purified preparations were not, which suggests that more than one enzyme is involved. This suggestion is substantiated by the observation that, individually, pure trypsin and chymotrypsin were unsatisfactory, whereas when used in combination, filtration of raw milk was satisfactory. The matted strands of what may have been deoxyribonucleic acid overlying the membrane after this treatment did not hinder unduly filtration but reduced the clarity of the preparation. For enzymes of pancreatic origin, e.g., trypsin and chymotrypsin, a nuclease and possibly a lipase are required for efficient filtration of milk and clear preparations for the microscope. However, one of the microbial proteases (P4755) reported to be substantially free from ribonuclease and deoxyribonuclease permitted rapid filtration of milk and resulted in clear preparations. It is possible that at the high concentration used these latter enzymes may have been effectively active.

For practical purposes crude trypsin is satisfactory, but the preparations require centrifugation before use. A ready prepared soluble freeze-dried extract of crude trypsin is commercially available and has been found effective without prior centrifugation.

After membrane filtration, the bacteria in the milk are retained on the membrane but, as scanning electron microscopy shows, may be coated with debris, a factor possibly contributing to the poor uptake of conventional stains. The fluorochrome, acridine orange, can be used for differentiating deoxyribonucleic acid from ribonucleic acid by green as opposed to orange fluorescence in fixed or living mammalian cells (15) but its use for determining the activity of bacteria is debatable (7, 16). According to Hobbie et al. (7), the acridine orange-staining characteristics permit actively growing bacteria which fluoresce orange-red to be distinguished from inactive bacteria which fluoresce green. However, our observations with pure cultures indicate that some so-called "inactive" green-fluorescing cells are viable. The acridine orange-staining characteristics of mixed populations of bacteria in milk are presumably more complex than those of pure cultures.

There are a number of possible reasons why the total MCC (orange plus green) exceeds the PC for fresh milks. The presence of nonviable bacteria may account for this in part; viable bacteria unable to grow on yeast extract milk agar will be detected on the membrane but not in the PC; and the growth of some viable bacteria is inhibited in fresh raw milk (in such low-count milk, inhibition of colony formation sometimes occurs on plates prepared from the low dilutions of milk). Where bacterial counts on stored milks exceeded ca. 10^6/ml, the PC was slightly higher than the MCC, possibly because of the break up of clumps during preparation of dilutions or increased sensitivity of the aged bacteria to the trypsin/Triton X-100 treatment. For these reasons, the slope of the fitted regression line for the MCC versus the PC was less than 1.0 (Table 3). However, the correlation between the MCC and PC over the range from 10^3 to 10^8 bacteria per ml was good, and importantly, it was similar for farm, tanker, and silo milks.

For any new technique to gain acceptance it is advantageous if the results are similar or correlated with those of accepted standard methods. For raw milk, the MCC of orange-fluorescing bacteria meets this requirement because it is in close agreement with the standard PC. The

hygienic quality of milk is however more closely related to the total number of viable bacteria and not the number of viable clumps as measured by the PC. Thus the membrane single cell count (the total count of orange-fluorescing bacterial cells) should be a more realistic measure of the bacteriological status or activity in the milk. BCC yield the same information as the MCC but have the disadvantage of being inaccurate for milks with $<10^6$ bacteria per ml.

The membrane filter technique described, which takes less than 25 min to complete and is suitable for milks containing between 5×10^3 and 5×10^8 bacteria per ml, is both sufficiently rapid for monitoring silo and "accommodation" milk and sensitive enough for grading farm milk on the basis of hygienic quality. Agreement in counts between operators is good, and the variation is equal to or less than that of the PC. The technique is inexpensive, costing less than 50 cents per assay for disposable items. It has an advantage in that, once membranes are mounted in immersion oil, preparations are stable for at least 1 month, a factor which could prove important for centralized testing. Inter-laboratory trials are currently being conducted, and the possibility of automation, including counting by means of image analysis, is being investigated. Preliminary results show that the membrane filter technique also permits filtration of human milk, cream, reconstituted skim milk powder, and rinses of dairy plant and that it may prove useful for the direct enumeration of bacteria in these or any other materials that can be filtered after suitable treatment (14).

ACKNOWLEDGMENTS

We thank Ubaldina Rodrigues and Christine Veitch for their technical assistance, Rosemary Fulford for statistical analysis of results, and Brian Brooker for electron microscopy. We also thank Joan Betts of Cliffords Dairies for arranging the supply of commercial milk samples.

Part of the cost of the investigation was financed by the Joint Committee of the Milk Marketing Board and the Dairy Trade Federation.

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