APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1984, p. 433–434
0099-2240/84/080433-02$02.00/0

Vol. 48, No. 2

Membrane Filter Staining Method: Bacterial Plate Counts in 24 H

KIN-MEN TSE AND CARRIE M. LEWIS*

Glenmore Waterworks Laboratory, Engineering Department, City of Calgary, Calgary, Alberta T2P 2M5, Canada

Received 29 September 1983/Accepted 21 May 1984

We describe a technique to stain bacterial colonies on membrane filters. The procedure yielded reliable and reproducible bacterial plate counts in 24 h. The procedure can be applied to treated and untreated water samples requiring prompt analysis.

In 1979, Taylor and Geldreich (3) introduced m-SPC medium, providing the first accepted membrane filtration method to monitor bacterial levels in finished drinking water supplies. Since inoculum volumes larger than 1.0 ml could be used, the new procedure provided more sensitive and statistically reliable results than the pour plate procedure with standard plate count (SPC) agar (1). Haas et al. (2) demonstrated that membrane filtration plate count and SPC data are equivalent in the long term. However, the 48-h incubation period required by the m-SPC method remained a drawback.

In this paper we introduce a method for staining bacterial colonies on membrane filters. When the staining technique is applied to membranes incubated on m-SPC agar for 24 h, reliable and reproducible bacterial colony counts are obtained.

Samples of untreated surface supply reservoir water and chlorinated distribution system water from Calgary, Alberta, Canada, were used for this study. All samples were processed within 1 h of collection. m-SPC agar was prepared as described by Taylor and Geldreich (3). The cooled agar was poured into loose-fitting 60-by-15-mm petri dishes. Analyses were performed by using the guidelines set forth in *Standard Methods for the Examination of Water and Wastewater, 15th ed.* (1), except that the filtration procedure was modified as follows: 18 to 24 h before use, m-SPC plates were inverted in a 35 ± 2°C incubator to dry any condensation on the agar surface. The sample inoculum volume was adjusted to yield 20 to 200 CFU per filter. The membrane filter pore size selected for these experiments was 0.45 μm, (type HA; Millipore Corp.), as minimal colony spreading had been observed on these filters in previous tests. Replicate inoculated filters (n = 34 to n = 68) were placed on dried m-SPC agar plates. After 24 h of incubation at 35 ± 2°C, approximately half of the replicates (designated s-SPC) were removed from the incubator, the filters were stained, and CFU were enumerated. After 48 h of incubation, the remainder of the replicates was removed from the incubator, and CFU were enumerated. Colonies were enumerated with the aid of a Zeiss DR stereomicroscope at a magnification of ×25. Incident fluorescent light was used to count CFU in m-SPC preparations; direct halogen light was used with s-SPC preparations.

Membrane filters were stained with a solution of 100 mg of bromcresol green and 20 mg of methyl red in 100 ml of isopropyl alcohol. While the membrane filter was still in place on the agar surface, 5 drops of stain were applied near the center of the filter with a Pasteur pipette. After the stain had spread over the entire membrane, the filter was transferred from the agar medium to an absorbent pad (e.g., Whatman filter paper). The stained filter was allowed to air dry until the filter was a pale pink color (generally less than 2 min), at which time the colonies were enumerated. The membrane was not allowed to dry completely, as the filter curled and made colony counting difficult.

Data used for analysis came from membrane filtrations with colony counts ranging from 20 to 200 CFU to provide meaningful statistical comparisons. For consistency between trials, data are reported as CFU · 100 ml^{-1}.

Table 1 presents a comparison of the s-SPC and m-SPC procedures. For chlorinated distribution system water (trials D1, D2, and D3), the s-SPC colony counts were 86, 56, and 73% of the corresponding m-SPC counts. For two of the three trials, the coefficients of variation of the s-SPC data were smaller than those of the m-SPC data. In trial D3, the two coefficients of variation were nearly equal.

The means of the untreated reservoir water s-SPC colony counts were 98 and 97% of the mean m-SPC counts in trials R1 and R2, respectively. Both of these trials had s-SPC coefficients of variation lower than the corresponding m-SPC value.

For trials D1 and R1, bacterial colonies were enumerated before and after the filters, which had been incubated for 24 h, were stained. This verified that the staining of filters incubated for 24 h yielded higher CFU counts than simply enumerating CFU on unstained filters. The chlorinated distribution system mean colony counts for unstained filters and stained filters were 179.0 and 196.6 CFU · 100 ml^{-1}, respectively. The scatter plot of these results (Fig. 1) shows that consistently more colonies were enumerated after the filters were stained. The untreated reservoir mean colony counts of unstained and stained filters were 2,463 and 2,501 CFU · 100 ml^{-1}, respectively. More colonies were enumerated in 23 of the 25 replicates.

The s-SPC staining technique colors bacterial colonies dark blue and the membrane filter pink. With this technique, even minute bacterial colonies can be easily distinguished from debris collected on the filter and from irregularities of the membrane itself. This technique overcomes the difficulties encountered by Taylor and Geldreich (3) in their efforts to enhance colony visibility on the membrane filter. The staining technique does not require incorporation into the agar medium of dyes or stains potentially toxic to the bacteria. Colored membrane filters, themselves possibly inhibitory to bacterial growth, also are not required for the procedure. In addition, removing condensation from the agar surface before applying the inoculated membrane filter, combined with the use of loose-fitting petri dishes, virtually eliminates the problem of colony spreading. Colonies which have been stained with the s-SPC procedure cannot be picked for subculture. If subculture is desired, duplicate plates must be prepared for this purpose.

* Corresponding author.

TABLE 1. Comparison of CFU enumerated from s-SPC and m-SPC preparations of chlorinated and untreated water samples[a]

Prepn	CFU enumerated from:													
	Chlorinated distribution system water samples in trial:									Untreated reservoir water samples in trial:				
	D1			D2			D3			R1			R2		
	$\bar{x}$	cv	*n*	$\bar{x}$	cv	*n*	$\bar{x}$	cv	*n*	$\bar{x}$	cv	*n*	$\bar{x}$	cv	*n*
s-SPC	196.6	14.1	25	184.2	15.4	21	65.5	16.9	17	2,463	9.3	25	2,501	27.6	19
m-SPC	227.7	18.5	25	329.1	33.3	19	90.2	16.1	17	3,562	19.0	29	3,664	29.7	22
s-SPC/m-SPC ratio (%)		86.3			56.0			72.6			98.5			97.2	

[a] $\bar{x}$, Mean (CFU · 100 ml^{-1}); cv, coefficient of variation (%).

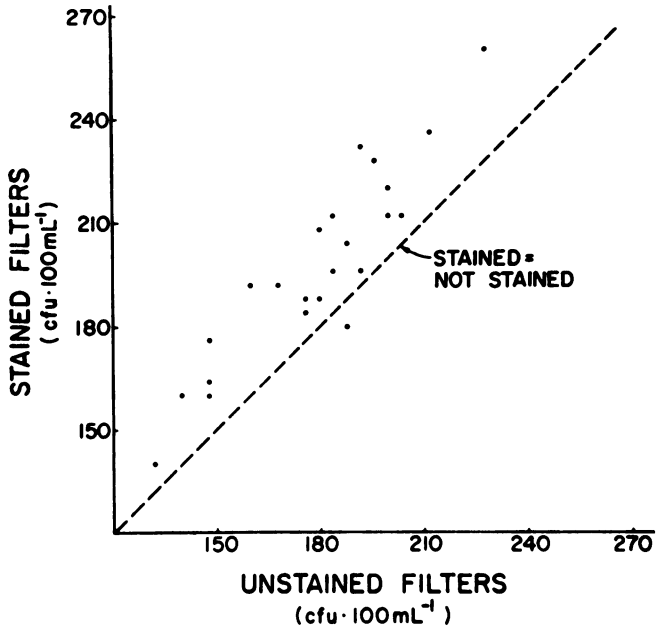

FIG. 1. Colony enhancement with s-SPC staining. Scatter plot of colonies enumerated after filter staining (*y*-axis) and before filter staining (*x*-axis). Results are from replicate membrane filtrations of chlorinated distribution system water incubated for 24 h.

When the staining technique is applied to m-SPC preparations incubated for 24 h, bacterial colony counts in chlorinated water are ca. 75% those of standard m-SPC preparations, whereas bacterial colony counts in untreated water are ca. 95% those of standard m-SPC preparations. The stained filter replicates consistently yielded a lower coefficient of variation than did their corresponding m-SPC replicates. These results indicate that the s-SPC technique yields reproducible bacterial colony counts, although at a somewhat lower level than does the m-SPC technique.

The s-SPC method is conveniently run in parallel with a total coliform membrane filtration analysis. This combination of tests can be applied to samples for which results are required within a 24-h period.

We thank Erika Hargesheimer and Read Seidner for critical reading of the manuscript and Barbara Kropelnick for typing.

LITERATURE CITED

1. **American Public Health Association.** 1980. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
2. **Haas, C. N., M. A. Meyer, and M. S. Paller.** 1982. Analytical note: evaluation of the m-SPC method as a substitute for the standard plate count in water microbiology. J. Am. Water Works Assoc. **74**:322.
3. **Taylor, R. H., and E. E. Geldreich.** 1979. A new membrane filter procedure for bacterial counts in potable water and swimming pool samples. J. Am. Water Works Assoc. **71**:402–405.