APPLIED MICROBIOLOGY, Dec. 1972, p. 864-870
Vol. 24, No. 6

Printed in U.S.A.

Membrane Filter Technique for Enumeration of *Pseudomonas aeruginosa*

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Received for publication 31 May 1972

A membrane filter procedure for the quantitation of *Pseudomonas aeruginosa* (mPA procedure) has been developed. Through the use of inhibitors and an elevated incubation temperature, the level of background microbial flora was decreased approximately 10,000-fold. Using *P. aeruginosa* cells suspended in sea water and held for 24 hr, between 70 and 100% of the "viable" cells could be recovered by the mPA procedure. Assay variability was found to be insignificant. The recoveries of *P. aeruginosa* from surface (fresh and salt) waters, potable waters, and sewage by the mPA procedure exceeded those obtainable by current methods. Subsequent to its development and evaluation, the mPA procedure was used at three other laboratories for the enumeration of *P. aeruginosa* in potable and recreational waters and in sewage samples. It was found amenable to routine use, and confirmation of typical colonies approached 100%.

Pseudomonas aeruginosa is recognized as a human pathogen responsible for fatal septicemias in infants and in adult patients debilitated by burns, malignancies, or old age (18). Particularly with respect to these groups, the isolation of this organism in relatively large numbers from potable waters and in hospitals has been a cause of concern (2, 12). In addition, the presence of *P. aeruginosa* in surface waters may be related to the high incidence of otitis externa which occurs during the swimming season (14). The evaluation of the public health significance of *P. aeruginosa* in potable and recreational waters requires the development of suitable enumeration procedures for the organism (4) and appropriate epidemiological investigations.

Several selective media for the isolation and cultivation of *P. aeruginosa* have been developed (7, 11, 15). According to Drake (7), most of these media require large inocula and do not yield quantitative recovery of the organism. He proposed a liquid medium which, in conjunction with the most-probable-number (MPN) technique, would permit the quantification of *P. aeruginosa* in the presence of large numbers of coliforms and other bacteria in water samples. This medium subsequently was used to quantitate *P. aeruginosa* in the feces of healthy adults and in samples from rivers and sewage (13a, 18). The Drake technique, significantly modified, has been incorporated into the 13th edition of *Standard Methods for the Examination of Water and Wastewater* (1). Both techniques require confirmatory isolation of *P. aeruginosa* from questionable tubes, and, at times, the growth of other microorganisms may inhibit the reproduction of *P. aeruginosa* and produce false negative tubes (16). In addition, MPN procedures are more cumbersome and less precise than direct enumeration methods utilizing pour or spread plates or membrane filters (6, 9, 20). Membrane filter procedures permit the concentration of a small number of cells present in a large volume of water and the use of colonial characteristics for differentiating *P. aeruginosa* from other organisms.

The present study was designed to develop a membrane filter method for the enumeration of *P. aeruginosa* (mPA technique) which satisfies the following criteria: (i) accuracy—the recovery of at least 75% of the "viable" *P. aeruginosa* cells from estuarine and fresh water samples artificially seeded with the organism and stressed by storage in these suspending media; (ii) selectivity—the reduction of "background organisms" in naturally polluted water samples by at least three orders of magnitude (1,000-fold); (iii) specificity—when assaying field samples, at least 90% of those colonies designated as *P. aeruginosa* should verify as such; and no more than 10% of those colonies not designated as *P. aeruginosa* should, in actuality, be this organism; (iv) precision—that, with field sam-

ples, the distribution of D^2 value estimates of assay variability, calculated according to Eisenhart and Wilson (8), approximates that expected by chance; and (v) comparability—that the accuracy and sensitivity of the method be equal to or greater than existing methods.

MATERIALS AND METHODS

Cultures. The preliminary work was done with a strain of *P. aeruginosa* provided by C. Houston of the University of Rhode Island (strain SP1). The other test strains were isolated in this laboratory from fresh water (numbers 10 and 6) and estuarine water (numbers E and PC). Strains 10, E, and PC were both pyocyanogenic and fluorescent. Strain 6 produced fluorescein but was apyocyanogenic. Cultures were stored at 6 C on Pseudomonas P agar slants (Difco, Detroit, Mich.).

Recovery medium. The initial approach to this problem utilized the ability of *P. aeruginosa* to metabolize a wide assortment of organic and inorganic compounds which bacteria with more restrictive nutritive requirements are unable to use. A large number of compounds were screened as potential sole sources of carbon, energy, or nitrogen, or all three, for growth. Essentially quantitative recovery of three strains and a clearly recognizable colony type were obtained when using a chemically defined medium containing NH_4NO_3, KH_2PO_4, $CaCl_2$, $MgSO_4$, $MnCl_2$, $FeSO_4$, asparagine, proline, and glycerol. However, less than 75% of the organisms stressed by being held in estuarine water for 24 hr at 6 C were recovered. Therefore, a richer medium containing more complex ingredients was considered in conjunction with the antibiotics sulfapyridine, kanamycin, nalidixic acid, and actidione to suppress background flora. The maximum concentrations of the antibiotics which did not inhibit *P. aeruginosa* were determined by using a modification of the gradient plate technique of Szybalski (19). A sterile membrane filter was placed on the gradient plate, and a heavy inoculum was placed on the filter as a single streak perpendicular to the gradient.

The medium (mPA agar) was prepared by adding the ingredients (in g/100 ml: L-lysine hydrochloride, 0.5; NaCl, 0.5; yeast extract [Difco], 0.2; xylose, 0.25; sodium thiosulfate, 0.68; sucrose, 0.125; lactose, 0.125; phenol red, 0.008; ferric ammonium citrate, 0.08; and agar, 1.5) to distilled water, autoclaving the mixture at 121 C for 15 min, cooling the medium to 55 to 60 C, adjusting its *p*H to 7.2 ± 0.1, and adding the dry antibiotics (sulfapyridine, 17.6 mg [Nutritional Biochemicals]; kanamycin, 0.85 mg [Bristol-Myers]; nalidixic acid, 3.7 mg [Cal Biochemicals]; and actidione, 15.0 mg [Upjohn] per 100 ml of medium) thereto. Minimal quantities of lactose, sucrose, and xylose and a *p*H indicator (phenol red) are included in the medium so that those coliforms which grow in the presence of the inhibitors can be differentiated from the nonfermentative *P. aeruginosa*. The H_2S indicator system permits the differentiation of *P. aeruginosa* from most *Salmonella* and *Proteus* species. The ferric ammonium citrate, sodium thiosulfate, and phenol red also are essential for the development of the tan-to-brown color characteristic of *P. aeruginosa* colonies on mPA medium. The inhibitors used are those to which *P. aeruginosa* is insensitive relative to most gram-negative organisms. Actidione was incorporated into the medium late in the study after fungal overgrowth had been observed in the assay of some field samples. The concentration used was shown not to affect the recovery of *P. aeruginosa* from field samples. The elevated incubation temperature of 41.5 C prevents the growth of most pseudomonads other than *P. aeruginosa* as well as many of the organisms indigenous to the aquatic environment. The medium was dispensed in 3-ml quantities to sterile 50 by 12 mm petri plates—contamination through the use of unsterilized antibiotics was not a problem. An agar medium was used since variable recoveries were obtained when liquid media were used to impregnate filter pads. Poured plates of the medium were stored at 6 C for 1 month without affecting recovery or selectivity.

Membrane filters, through which the water samples were passed, were placed on the surface of mPA agar plates and incubated at 41.5 ± 0.5 C to suppress background organisms including most other *Pseudomonas* species. Incubation for 48 hr was required for the appearance of distinctive *P. aeruginosa* colonies. Typically, the colonies were 0.8 to 2.2 mm in diameter and flat in appearance with light outer rims and brownish to greenish-black centers.

The primary and confirmatory media used in the Drake (7) and "Standard Methods" (1) MPN procedures, to which the mPA method was compared, were as given by the authors. In the Drake MPN procedure, tubes of medium no. 10 (7) were inoculated with portions of the sample and incubated at 30 C for 4 days. The tubes were examined daily, and all turbid tubes were confirmed by streaking on acetamide agar. According to Drake (*personal communication*), acetamide agar is prepared as follows: acetamide, 1.0 g; NaCl, 0.5 g; K_2HPO_4, 0.13 g; KH_2PO_4, 0.07 g; phenol red, 0.0012 g; distilled water, 100 ml. The medium is sterilized at 121 C for 15 min. Purple coloration around the colonies is accepted as confirmation for the presence of *P. aeruginosa*. In addition, whenever growth was obtained on the acetamide media, the presence of *P. aeruginosa* was verified by the method of Brown and Scott Foster (3). The tables of Halvorson and Ziegler (13) were used to calculate the 10-tube MPN estimates.

Test suspensions. Cultures were prepared by inoculating tubes of Trypticase soy broth (BBL) with strains of *P. aeruginosa* and incubating for 20 hr at 37 C. Test suspensions were prepared from these cultures by dilution in phosphate-buffered saline (PBS: NaH_2PO_4, 0.58 g; Na_2HPO_4, 0.25 g; NaCl, 8.5 g; distilled water, 1,000 ml) and adding samples of the appropriate dilutions to the suspending solutions. Dilution blanks and suspending solutions were equilibrated to room temperature before use, since suspension of the cells in cold diluent is known to result in lowered recovery of *P. aeruginosa* (10). The number of organisms in the test suspension was determined from duplicate Pseudomonas P agar plates (100 by 15

mm) spread with 0.1-ml quantities of the appropriate dilutions. The plates were counted after 24 hr of incubation in an air incubator at 37.0 ± 0.5 C.

Field samples. Field samples were collected from fresh and estuarine surface waters in sterile containers and assayed with 4 hr of collection. All samples were collected approximately 12 inches (30.48 cm) below the surface.

Membrane filtration. Appropriate volumes of the test suspensions or water samples used in evaluating the experimental media were passed through membrane filters (47 mm diameter, 0.45 μm pore size). When the portion of the water sample to be filtered was less than 20 ml, it was brought to at least that volume with PBS before filtration. After filtration, the filter funnel was rinsed with two 20-ml portions of PBS to wash residual organisms onto the membrane. The glass filter holders were sterilized for 2 min in an ultraviolet sterilizing apparatus (Millipore Filter Corp). The membrane filters were obtained presterilized from the manufacturer.

Verification of colonies. Verification of colonies as *P. aeruginosa* was accomplished by two procedures. The first was a combination of eight tests based on the work of Sutter (18), Stanier et al. (17), and Cowan and Steel (5). The eight tests were: (i) Gram stain; (ii) growth on nutrient agar at 41.5 ± 0.5 C in a water bath; (iii) production of a slime layer on gluconate medium; (iv) characteristic fruity odor; (v) production on pyocyanin or pyorubin on Pseudomonas P agar; (vi) gluconate test; (vii) the oxidation-fermentation test (O-F) with maltose and glucose; and (viii) production of fluorescein on Pseudomonas F agar (Difco). With nonpigmented or nonfluorescent microorganisms, or both, which met all the other criteria, motility and polar flagellation (5) were determined. In practice, the OF test indicated motility as well as the OF pattern. The second procedure was the method of Brown and Scott Foster (3), in which the isolate is transferred to a milk agar plate—when transferring from isolated colonies, a single streak on a portion of the plate is sufficient. After incubation for 24 hr at 35 C, *P. aeruginosa* hydrolyzes the casein and produces a yellowish-green to green difusible pigment. After parallel testing of 198 colonies yielded comparable results, only the latter procedure was employed.

RESULTS

The accuracy of the mPA method was determined by comparing the recoveries obtained by this procedure to those observed when *P. aeruginosa* test suspensions also were plated on Pseudomonas P agar. The suspensions, whose initial cell densities varied from 10^5 to 10^6 per ml, were prepared in filtered estuarine water and stored at 25 C for periods up to 24 hr. The average recovery of the five strains of *P. aeruginosa* by the mPA method relative to that on the spread plates was 92% (Table 1). It can be seen that the relative recoveries varied from strain to strain and, with some strains, over the interval during which the organisms were exposed to estuarine water.

TABLE 1. *Effect of exposure to estuarine water on the recovery of P. aeruginosa by the mPA procedure*

Exposure[a] time (hr)	Percent recovery on mPA/spread plate[b] for strain					Avg
	10	SP1	6	E	PC	
0	57	109	77	91		83
2.5	77	108	105	114	84	97
6.0	81	100	104	102	86	94
24	70	109	103	103	57	88
Average	71	105	97	102	76	92[c]

[a] Number of hours following dilution of culture in membrane filter sterilized estuarine water (salinity, 21‰; temperature, 6 C).

[b] Averages obtained from three replicate plates or filters.

[c] Average of individual values.

The selectivity of the mPA method was such that a 10,000-fold reduction in the background microbial flora was obtained relative to that observed when the antibiotics were omitted and the plates were incubated at 35 C. This 99.99% reduction was obtained with polluted, fresh, and salt water samples whose initial background cell densities were 10^4 to 10^8/100 ml.

The specificity of the mPA method was examined by determining (i) the percentage of typical colonies which verified as *P. aeruginosa* and (ii) the percentage of "other colonies"—those which did not possess the typical colonial characteristics—which, in fact, were not *P. aeruginosa*. The 313 colonies examined were isolated from polluted field samples from a variety of sources. In each instance, all the colonies on a given plate were tested. As can be seen from Table 2, 95% of the typical colonies verified as *P. aeruginosa*, and none of the "other colonies" were *P. aeruginosa*.

The precision of the mPA method was determined from D^2 values for assay variability as calculated from the following equation as given by Eisenhart and Wilson (8): $D^2 = [N\Sigma Xi^2 - (\Sigma Xi)^2]/\Sigma Xi$ where ΣXi is the summation of the plate counts $X_1, X_2 \cdots X_N$ and N (the number of replicate plates/sample) was 5. The D^2 values calculated from the examination of 24 polluted, fresh, and estuarine water samples are displayed in Fig. 1 along with the expected D^2 control limits for $P = 0.005$, 0.025, and 0.5. In the event of excessive variability among the five replicate determinations (plates)—hence, poor reliability of a single determination—the observed D^2 values should have exceeded the control limits more frequently than expected by

TABLE 2. *Verification of colonies observed on mPA*

Samples examined[a]		No. of typical colonies[b]		No. of "other colonies"[c]	
Source	No.[d]	Observed	Verified[e]	Observed	Verified[e]
River	2	39	38	7	0
Pond	1	15	14	5	0
Reservoir	1	16	16	6	0
Sewage	2	63	63	7	0
Estuary[f]	2	52	50	3	0
Bay[g]	2	66	64	18	0
Paper mill effluent	2	14	13	2	0
Total	12	265	258	48	0
Percent verified			95		0

[a] With the exception of the paper mill effluents from Maine, the samples were collected in Rhode Island.

[b] Flat colonies, 0.8 to 2.2 mm in diameter, with light outer rims and dark-brownish to greenish-black centers.

[c] All colonies other than those described in *b* as "typical."

[d] Indicates the number of samples collected from the same source but at different locations (sampling stations).

[e] Verified as *P. aeruginosa*.

[f] Salinity, 4‰.

[g] Salinity, 21‰.

chance alone. By chance alone, 1 D^2 value in 40 would have been expected to exceed the P = 0.025 control limit of 11.1, and half the values should have exceeded the P = 0.5 control limit of 3.2. It can be seen that all 24 values were below the P = 0.025 limit. However, only 4 of the D^2 values exceeded the P = 0.5 limit of 3.2.

Twenty-nine water samples collected from a variety of sources were assayed in parallel by the Drake (7), Standard Methods (1), and mPA procedures (Table 3). The Standard Methods procedure was markedly insensitive relative to the other two methods except when sewage samples were assayed, in which case the recoveries were comparable to those with the Drake method and about half those with the mPA technique. In general, the *P. aeruginosa* densities as determined by the Drake method were approximately half those by the mPA procedure. However, with the Narragansett Bay seawater samples, indeterminate values were obtained by the Drake method when the *P. aeruginosa* densities, as determined by the mPA procedure, were well above the theoretical sensitivity of the Drake method (0.9/100 ml).

Subsequent to its development and evaluation, the mPA technique was applied by workers at three other laboratories to potable and recreational waters and to sewage samples collected at various stages of treatment. Once these workers had familiarized themselves with the procedure and had learned to recognize colonies typical of *P. aeruginosa*, verification on the milk agar medium of Brown and Scott Foster (3) was attempted with typical and atypical colonies from a number of the samples. The workers at all three laboratories found the procedure amenable to routine use. Verification of typical colonies as *P. aeruginosa* approached 100%, and only at one of the three laboratories were any atypical colonies (16%) verified as *P. aeruginosa* (Table 4).

DISCUSSION

The mPA procedure satisfied all the predetermined acceptability criteria for a primary, selective-differential method for the enumeration of *P. aeruginosa* in water. Although the average recovery of 92% exceeded the criterion for accuracy—recovery of 75% of the "viable" cells stressed by 24 hr contact with seawater—, the observed strain to strain variability suggests that, at times, the recoverable population could be underestimated by as much as 30%. The rationale for the arbitrary requirement for 75% recovery of *P. aeruginosa* is that enumeration of all the viable cells from a water sample, though desirable, is not essential when *P. aeruginosa* is assayed either as a possible indicator of fecal pollution or as a weak pathogen potentially

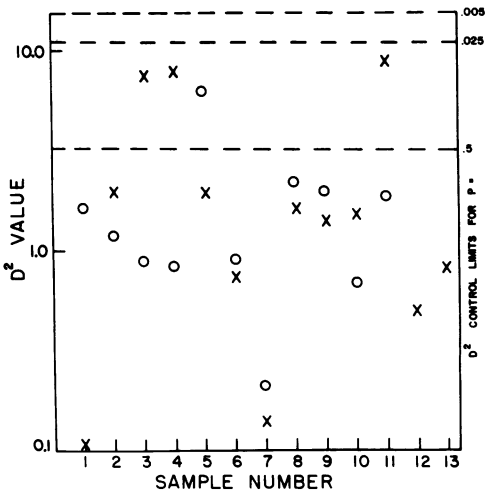

FIG. 1. *Precision of the mPA procedure for P. aeruginosa as estimated from the dispersion of D^2 values. Symbols: ×, estuarine water sample; O, fresh water sample; ----, control limits when probability is as stated. Data from five replicate plates used to calculate D^2 value for each point.*

TABLE 3. *Comparative recovery of P. aeruginosa by the Standard Methods, Drake, and mPA procedures*

Samples examined as to			Verified *P. aeruginosa*/100 ml by procedure		
Source	Location	Station	Standard Methods	Drake[a]	mPA[b]
River	Blackstone	1	<2.2/100	22.	49.
		2	<2.2/100	25.	23.
	Mohassuck	1	<2.2/100	6.4	14.
		2	<2.2/100	3.0	13.
		3	<2.2/100	<0.9/100	1.4
Pond	30-Acre	1	<2.2/100	220.	357.
		2	<2.2/100	82.	200.
		3	<2.2/100	32.	140.
		4	<2.2/100	8.0	19.
Bay	Narragansett	1	<2.2/100[c]	<0.9/100	31.
		2	<2.2/100[c]	<0.9/100	25.
		3	<2.2/100[c]	<0.9/100	22.
		4	<2.2/100[c]	<0.9/100	15.
		5	ND[d]	<0.9/100	15.
		6	ND	<0.9/100	15.
		7	ND	<0.9/100	7.8
		8	ND	<0.9/100	2.3
Well	Rhode Island	1	<2.2/100	<0.9/100	4.5
		2	<2.2/100	<0.9/100	4.2
		3	<2.2/100	<0.9/100	4.0
		4	<2.2/100	<0.9/100	2.7
Untreated sewage	URI STP[e]	1	72,000	51,000	127,000
	Cranston STP	1	91,000	39,000	172,000
	Warwick STP	1	51,000	75,000	124,000

[a] Values obtained from tubes confirmed on acetamide media and verified by method of Brown and Scott Foster (3).
[b] Values obtained following verification of colonies by method of Brown and Scott Foster (3).
[c] Copious precipitate appeared on addition of seawater sample.
[d] No data; procedure discontinued.
[e] URI, University of Rhode Island. STP, sewage treatment plant.

TABLE 4. *Verification of typical and atypical colonies by other workers*

Location	No. of samples	No. of colonies		% Verified[a]	
		Typical	Atypical[b]	Typical	Atypical
Chicago area[c]	50	54	25	98	16
Philadelphia area[d]	13	11	2	100	0
Rhode Island[e]	63	207	30	100	0

[a] As *P. aeruginosa*.
[b] Those colonies whose typical appearance was in question.
[c] Recreational waters.
[d] Potable and raw waters.
[e] Sewage samples.

transmissible via the waterborne route. It is essential, however, that the method be highly reproducible.

The selectivity criterion for the evaluation of the mPA method assumes that the recreational and potable waters to be examined for *P. aeruginosa* generally would contain less than 10^3 background organisms per ml (those cells which could grow in 48 hr on the somewhat nutritionally restrictive mPA medium when the inhibitors are omitted and the plates are incubated at 35 C). The required 1,000-fold (99.9%) reduction in the level of these organisms, to be achieved by the combination of inhibitors and the elevated incubation temperature, would result in 50 colonies per filter when a 50-ml water sample is assayed. The differential characteristics of the medium then should permit the detection of a single *P. aeruginosa* colony on such a filter. The actual reduction (99.99%) exceeded this requirement, thereby permitting the detection of *P. aeruginosa* in the presence of 500,000 "background" organisms

deposited on the filter. It is possible that, under certain limited conditions, the selectivity of the medium may not be sufficient, i.e., tropical waters with large numbers of thermophiles.

The differential feature (specificity) of the mPA procedure, since it is dependent both on the operator's ability visually to identify typical colonies and on the nature of the bacterial population in the sample, also was examined at three laboratories in addition to our own. The specificity criterion (that, in the hands of a trained operator, no more than 10% false positives and 10% false negatives will be obtained) was satisfied at all four laboratories. Therefore, it may be assumed that verification of typical colonies will not be required routinely. However, verification of a number of typical and other colonies is necessary not only when an operator is being trained in the mPA procedure but also when the method is used at a new location. The above considerations notwithstanding, in the absence of verification, estimates of *P. aeruginosa* densities should be designated as "probable." Following verification by the method of Brown and Scott Foster (3), the estimates would be considered as "confirmed."

The results clearly demonstrate the precision (reproducibility) of the mPA procedure. If anything, the plate-to-plate variability over the number of samples examined was less than that expected by chance alone; that is, the D^2 values did not distribute equally on both sides of the $P = 0.5$ limit of 3.2. Eisenhart and Wilson (8) discuss this often neglected consideration in the interpretation of D^2 estimates; they cite an instance of subnormal variation in which, "... a minor alteration in the preparation of the medium may have been a factor." In the present case, the subnormal variability observed among the replicate mPA plates may have been due to an inherent deficiency of the medium when used to assay field samples. Thus, that part of the heterogeneous *P. aeruginosa* population, which had been "weakened" by stress and could therefore be expected to vary from plate to plate in its ability to be recovered on a less restrictive medium, could have been uniformly inhibited on mPA medium. This possible deficiency in the mPA procedure may not have been detected since the accuracy of the mPA procedure was examined with pure cultures of *P. aeruginosa*. For obvious reasons, the actual *P. aeruginosa* densities in field samples could not be determined.

The overall precision of the MPN procedures, when performed with some reasonable number of tubes per dilution, (i.e., 10) could not be expected on theoretical grounds alone to equal that of an acceptable membrane filter test. Therefore, the comparison of the mPA to the Drake and Standard Methods MPN procedures for the enumeration of *P. aeruginosa* was made on the basis of accuracy and sensitivity. The results clearly demonstrate that the observed insensitivity of the Standard Methods procedure renders it inferior to the other two methods. For the same reason, the Drake method should not be used in assaying seawater samples for *P. aeruginosa*. In addition to its greater accuracy as seen from the results of this study, the mPA procedure has other substantial advantages over the Drake method. As noted previously, the Drake method had considerably greater logistic requirements. Secondly, large volumes of water can be more readily assayed by the mPA method than by the MPN test. However, the sensitivity of the mPA test is decreased in the assay of samples such as fish homogenates, highly turbid waters, and bottom muds unless the nonbacterial particulates are first removed. Not only is there a reduction in the volume of water which can be passed through the filter, but also excessive quantities of brown to black particulate matter deposited on the filters interfer with the detection of the *P. aeruginosa* colonies.

ACKNOWLEDGMENTS

We acknowledge the aid of Thomas Carlin, Bacteriologist, and David Goff, Sanitary Engineer, Philadelphia Water Department; John Miescier, Bacteriologist, FDA, Food and Drug Administration, Northeast Regional Laboratory; and Harriet Kennedy, Laboratory Director, North-Shore Health Department, Evanston, Ill., in examining the mPA technique in their own laboratories.

LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13 ed., p. 710. Amer. Publ. Health Ass., New York.
2. Asay, L. D., and R. Koch. 1960. Pseudomonas infections in infants and children. New Engl. J. Med. **262**:1062-1075.
3. Brown, M. R. W., and J. H. Scott Foster. 1970. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. J. Clin. Pathol. **23**:172-177.
4. Clark, J. A. 1969. The detection of various bacteria indicative of water pollution by a presence-absence (P-A) procedure. Can. J. Microbiol. **15**:771-780.
5. Cowan, S. T., and K. J. Steel. 1970. Manual for the identification of medical bacteria. Cambridge University Press, p. 106-164.
6. Cowell, N. D., and M. D. Morisetti. 1969. Microbiological techniques—some statistical aspects. J. Sci. Food Agr. **20**:573-579.
7. Drake, C. H. 1966. Evaluation of culture media for the isolation and enumeration of *Pseudomonas aeruginosa*. Health Lab. Sci. **3**:10-19.
8. Eisenhart, C., and P. W. Wilson. 1943. Statistical methods and control in bacteriology. Bacteriol. Rev. **7**:57-137.
9. Geldreich, E. E., H. S. Jeter, and J. A. Winter. 1967.

Technical considerations in applying the membrane filter. Health Lab. Sci. **4**:113-125.
10. Gorrill, R. H., and E. M. McNeil. 1960. Effect of cold diluent on the viable count of *Pseudomonas pyocyanea*. J. Gen. Microbiol. **22**:437-442.
11. Goto, S., and S. Bromoto. 1970. Nalidixic acid cetrimide agar. Jap. J. Microbiol. **14**:65-72.
12. Grieble, H. G., F. R. Coulton, T. J. Bird, A. Tiogo, and L. G. Griffith. 1970. Fine particle humidifiers. New Engl. J. Med. **282**:531-535.
13. Halvorson, H. O., and N. R. Ziegler. 1933. Application of statistics to problems in bacteriology. J. Bacteriol. **25**:101-134.
13a. Hoadley, A. W. 1968. On the significance of *Pseudomonas aeruginosa* in surface waters. N. Engl. Water Works Ass. **82**:99-111.
14. King, E. O., M. K. Ward, and A. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluoracein. J. Lab. Clin. Med. **44**:301-307.
15. Price, R. J., and J. S. Lee. 1970. Inhibition of Pseudomonas species by hydrogen peroxide producing Lactobacilli. J. Milk Food Technol. **33**:13-18.
16. Stanier, R. Y., and N. J. Palleroni, and M. Douderoff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159-271.
17. Sutter, V. L. 1968. Identification of *Pseudomonas* species isolated from hospital environment and human sources. Appl. Microbiol. **16**:1532-1536.
18. Sutter, V. L., V. Hurst, and C. W. Lane. 1967. Quantification of *Pseudomonas aeruginosa* in feces of healthy human adults. Health Lab. Sci. **4**:245-249.
19. Szybalski, W. 1952. Gradient plate technique for study of bacterial resistance. Science **116**:46-48.
20. Woodward, R. L. 1957. How probable is the most probable number? J. Amer. Water Works Ass. **49**:1060-1088.