APPLIED MICROBIOLOGY, Dec. 1975, p. 935-942
Vol. 30, No. 6

Printed in U.S.A.

Factors Influencing Detection and Enumeration of *Pseudomonas aeruginosa* by Most-Probable-Number and Membrane Filtration Techniques

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Received for publication 19 September 1975

Most-probable-number (MPN) and membrane filtration (mF) techniques were evaluated with respect to selectivity, sensitivity, and efficiency in recovering *Pseudomonas aeruginosa* strains in hospital fluids and extramural water environments. Known numbers of cells of a naturally occurring strain of *P. aeruginosa* maintained in distilled water or cells subcultured on Standard Methods agar were added to test samples containing various types and levels of background microbial contaminants. Environmental samples containing unknown numbers of *P. aeruginosa* strains also were tested. Asparagine and acetamide broths were employed as presumptive media in MPN tests, and mPA and Pseudosel agars were used in mF assays. Statistical analyses of data showed the superiority and comparability of the asparagine-MPN and mPA-mF systems. Greater precision and accuracy were consistently obtained in either assay technique by the use of naturally occurring cells as test organisms. The type of filter and nature of diluents employed, as well as pH of assay media, were found to greatly influence both recovery and development of characteristic colonial morphology in the mPA-mF system.

In recent years, considerable attention has been directed to the role of opportunistic gram-negative bacterial saprophytes in the hospital environment in hospital-acquired infections. The recognition that many of these organisms are capable of growth or prolonged survival in a wide variety of fluids (3, 6, 10 to 14, 20, 23) has led to a reassessment of these environments as major reservoirs of contamination and potential sources for transmission of pathogenic microorganisms (3, 7, 11, 13, 19, 20, 21, 24, 26).

Particular attention has been directed to *Pseudomonas aeruginosa* because of its gradual emergence as a significant human pathogen. A variety of selective media have been employed in attempts to improve isolation and identification of hospital strains from both clinical specimens and environmental sources (8, 15-17, 25). Most-probable-number (MPN) and membrane filtration (mF) techniques (1, 4, 8, 18) have also been advocated to assess the organism's ecological distribution and the role of specific types of fluid environments as reservoirs and/or routes of transmission, and to examine the quality of control measures employed. These methods have largely been developed and evaluated using laboratory-cultured strains of *P. aeruginosa* and other gram-negative bacteria to test the efficiency of recovery and the sensitivity or the selectivity of a particular medium.

We previously reported (23) the use of broth containing asparagine or acetamide as sole carbon and nitrogen sources, modified from the media of Drake (8) and Drake and Hoff (9), to confirm *P. aeruginosa* colonies from hospital air and surface samples replicated on Pseudosel agar (Baltimore Biological Laboratory, Cockeysville, Md.). We also reported their use as presumptive and confirmatory media, respectively, in an MPN system to enumerate *P. aeruginosa* in fluids from respiratory therapy equipment (10, 21) and in a wipe-rinse assay procedure for determining the presence of the organism on the hands of hospital staff (22). A strain of *P. aeruginosa*, detected in the reservoir of a mist therapy unit, was isolated in pure culture in distilled water without subculture on conventional laboratory media (10). Cells of this strain were shown to exhibit altered physiological responses after subculture (5, 10). Results of preliminary tests with these naturally occurring cells to evaluate the efficiency of the asparagine-acetamide MPN enumeration sys-

tem showed the system to be highly accurate and sensitive even when high levels of background contaminants were present. This study was undertaken to compare the efficacy of certain selective media for detecting *P. aeruginosa* and to examine their effectiveness in MPN and mF quantitative assays, using both naturally occurring and subcultured cells as test organisms.

MATERIALS AND METHODS

Media for presumptive assays. For MPN systems, asparagine broth prepared as previously described (10) and the acetamide medium of Hedberg (15) containing both carbon and nitrogen sources (NaCl, 5.0 g; $MgSO_4$, 0.2 g; $NH_4H_2PO_4$, 1.0 g; K_2HPO_4, 1.0 g; acetamide, 20 g; and distilled water, 1 liter) were utilized. Media were dispensed in 9-ml amounts in screw-cap tubes (125 by 16 mm). The pH determinations (Orion model 801 digital pH meter) of autoclaved asparagine and acetamide broths showed ranges of 7.1 to 7.3 and 6.6 to 6.8, respectively. Positive presumptive tubes were determined by fluorescence under long-wave ultraviolet light after incubation at 37 C for 48 h.

For mF assays, a medium for membrane filtration of *P. aeruginosa* (mPA agar) was initially prepared according to the formula of Levin and Cabelli (18), with the pH of the basal medium adjusted to 7.2 ± 0.1 after autoclaving and before the addition of antibiotics. In later experiments pH adjustments ranging from 5.5 to 7.6 were made before or after autoclaving. Media were dispensed in 3-ml amounts in sterile petri dishes (50 by 12 mm) with a snap-lid closure, or in 5- or 10-ml amounts in dishes (60 by 15 mm) with a standard lid closure (Falcon, Becton-Dickinson & Co., Rutherford, N.J.). Prepoured dishes (3 ml) of mPA agar prepared at the Environmental Protection Agency, Northeast Water Supply Research Laboratory, Narragansett, R.I. (pH 7.2; Panpeha, Pan-pH Paper, Schleicher and Schuell Co., Keene, N.H.) also were used. Detection of *P. aeruginosa* was determined on the basis of colonial appearance and pigmentation after 48 h at 41.5 C. Pseudosel agar was prepared according to the manufacturer's specifications and dispensed in 10-ml amounts in 60- by 15-mm plates. Presumptive evidence of *P. aeruginosa* was determined by the appearance of pigmented, ultraviolet-fluorescing colonies after 48 h at 37 C.

Test organisms and assay procedures. Cells of a naturally occurring strain (10) of *P. aeruginosa* (strain NOPs) were maintained at 25 C by monthly transfers in sterile commercial distilled water (Cutter Laboratories, Inc., Berkeley, Calif.). Test suspensions of strain NOPs were prepared by making 10-fold dilutions of cells from the commercial distilled water maintenance culture in buffered distilled water (1) equilibrated to room temperature. Phosphate-buffered saline (18) was used as the diluent in one series of tests. For subcultured cell preparations, inocula of strain NOPs were spread over the surface of a Standard Methods (SM) agar plate (BBL) and incubated at 37 C for 18 to 24 h (strain NOPs-sub). Strain SP-1, a laboratory-maintained strain of *P. aeruginosa* supplied by Northeast Water Supply Research Laboratory, also was grown on SM agar. Subcultured cells were aseptically harvested, washed twice, and diluted 10-fold in buffered distilled water or phosphate-buffered saline.

One-milliliter aliquots of test suspensions were inoculated directly into asparagine or acetamide broth (five-tube MPN series). One milliliter in approximately 10-ml of buffered distilled water or phosphate-buffered saline rinse fluid was passed through gridded membrane filters (Millipore, 0.45 μm and 0.22 μm, Millipore Corp., Bedford, Mass.; Sartorius, 0.45 μm, Sartorius Co., Inc., San Francisco, Calif.; or Gelman, 0.45 μm, Gelman Instrument Co., Ann Arbor, Mich.) and plated on mPA or Pseudosel agar. Control values of strains NOPs, NOPs-sub, and SP-1 were determined from pour plates in SM agar or membrane filters incubated on SM agar at 37 and 41.5 C.

To assess recovery of *P. aeruginosa* (strains NOPs and NOPs-sub) from environmental samples containing various background levels of other gram-negative microorganisms, surface waters, fluids exhausted from hemodialysis systems, and fluids from respiratory therapy equipment were initially screened for the presence of *P. aeruginosa* by inoculating aliquots into asparagine broth (three-tube MPN series). Test samples were refrigerated during the 48-h MPN-screening incubation and then diluted 100-fold beyond the highest dilutions showing fluorescence in asparagine broth to obtain background contaminants presumably either free of *P. aeruginosa* strains or at such low levels as to be undetectable in the test systems studied. Known numbers of naturally occurring (NOPs) or subcultured (NOPs-sub) cells were then added to the environmental samples as well as to distilled water cultures of naturally occurring cells of *P. cepacia* (6) and assayed by the MPN and mF techniques. Final cell concentrations in test samples ranged from 5.0×10^1 to 2.5×10^5/ml for naturally occurring cells and 6.6×10^1 to 1.6×10^5/ml for subcultured cells.

In the final series of tests, environmental samples containing unknown numbers of *P. aeruginosa* strains among other background flora were assayed. Samples collected from hospital sources included fluids from water treatment systems, hemodialysis and respiratory therapy units, and a burn unit physical therapy machine.

Verification of P. aeruginosa. The two highest dilution series showing fluorescence in asparagine or acetamide broth, as well as the next two dilutions negative for fluorescence, were selected. A 0.1-ml portion from each tube was inoculated into the confirmatory acetamide broth medium of Favero et al. (NaCl, 5.0 g; $MgSO_4$, 0.5 g; K_2HPO_4, 1.4 g; KH_2PO_4, 0.7 g; acetamide, 10.0 g; phenol red, 0.012 g; and distilled water, 1 liter [10]). Confirmatory tests were determined by alkalinization of the medium in 24 h at 37 C; the addition of 1.5% agar (slants) was found to facilitate reading of positive tubes. Presumptive colonies on mPA and Pseudosel agars also

were inoculated into confirmatory acetamide medium. Additional tests to complete identification of *P. aeruginosa* included demonstration of pyocyanin production in *Pseudomonas*-P broth (10) and growth at 42 C on heart infusion agar slants. Standard MPN tables (1) were used to calculate viable counts of *P. aeruginosa* in MPN assays.

RESULTS

Data comparing two MPN and two mF systems for recovery of known numbers of *P. aeruginosa* from various sources of water containing levels of background contaminants ranging from 10^2 to 10^7 microorganisms/ml were subjected to statistical analysis. The relevant statistics derived from paired *t* tests of the differences between results of sample assays and the known levels, as well as those from linear regression analyses, are presented in Table 1 for naturally occurring cells. The low *t* values for the two mF techniques indicated that these systems, in general, produced superior results to those achieved with the MPN systems when tested on naturally occurring *P. aeruginosa*. Membrane filtration with subsequent incubation on either mPA or Pseudosel agar gave almost identical results. Of the two MPN systems the results achieved with asparagine broth were superior to those obtained using acetamide.

Additional analyses presented in Table 1 reflect the precision of the various assay systems tested. The asparagine MPN and the two mF systems were highly accurate, with mean recoveries ≥95% for naturally occurring organisms. For the acetamide MPN system, counts of *P. aeruginosa* fell outside the 95% confidence limits of expected MPN values in 44% of the samples tested. Sufficient data were not available to statistically analyze the specific effect of various levels of background contamination on recovery of *P. aeruginosa*. However, the absence of significant differences between paired samples, the high correlation coefficients, the slope values approximating 1, and the intercept values near 0 demonstrated by the mPA and Pseudosel agar systems suggested that background contaminants were not a complicating factor.

The results of similar statistical tests on data from subcultured *P. aeruginosa* (strain NOPs-sub) are presented in Table 2. Overall, the mPA system appeared to produce the best results, but differences between the mF and MPN systems were not as clear-cut as with data from naturally occurring cell systems. The accuracy of all systems in recovery of subcultured cells was reduced. The mPA and Pseudosel mF systems showed mean recoveries of 83 and 77%, respectively. Values for the asparagine and acetamide MPN systems fell outside the 95% confidence limits of expected MPN values in 45 and 60% of test samples. Results of other tests comparing recovery of strains NOPs-sub and SP-1 indicated that strain differences were not a major factor in determining recovery efficiency of subcultured cells.

Based on these findings, the best mF system (mPA agar) was compared with the best MPN system (asparagine) in the recovery of *P. aeruginosa* strains from fluid samples collected in the hospital environment and from river water and sewage. Results of statistical tests similar to those described previously are presented in Table 3. The two systems were

TABLE 1. *Statistical results of paired t and linear regression tests performed on data comparing four recovery systems for naturally occurring P. aeruginosa*[a]

Statistic	5-Tube MPN systems		mF systems[b]	
	Asparagine broth (19)[c]	Acetamide broth (9)	mPA agar (19)	Pseudosel agar (16)
Mean $\log_{10}$ number of organisms/ml	3.76	2.55	3.63	3.64
Standard deviation	1.12	0.98	1.08	1.22
Standard deviation of paired difference[d]	0.2608	0.5326	0.0762	0.1590
Paired *t*	−2.06	2.74[e]	0.42	−0.35
Correlation coefficient	0.97	0.89	1.00	0.99
Slope	1.01	0.75	1.00	1.05
Intercept	0.08	0.28	−0.02	−0.18

[a] APHA buffered distilled water used as diluent.
[b] Millipore 0.45-μm membrane filter; 10 ml of agar in 60- by 15-mm plates.
[c] Sample size.
[d] Paired differences with control means.
[e] $P < 0.05$.

TABLE 2. *Statistical results of paired t and linear regression tests performed on data comparing four recovery systems for subcultured P. aeruginosa*[a]

Statistic	5-Tube MPN systems		mF systems[b]	
	Asparagine broth (15)[c]	Acetamide broth (5)	mPA agar (15)	Pseudosel agar (13)
Mean $\log_{10}$ number of organisms/ml	3.13	2.94	3.44	3.06
Standard deviation	0.90	1.57	1.07	0.94
Standard deviation of paired difference[d]	0.3459	0.4071	0.3430	0.8840
Paired *t*	5.30[e]	3.40[f]	1.89	1.55
Correlation coefficient	0.93	0.98	0.95	0.53
Slope	0.89	1.19	1.08	0.57
Intercept	−0.07	−1.29	−0.45	0.53

[a] APHA buffered distilled water used as diluent.
[b] Millipore 0.45-μm membrane filter; 10 ml of agar in 60- by 15-mm plates.
[c] Sample size.
[d] Paired differences with control means.
[e] $P < 0.01$.
[f] $P < 0.05$.

TABLE 3. *Statistical results of paired t and linear regression tests performed on data comparing the mPA membrane filtration and asparagine MPN systems*[a]

Statistic	Fluids from the hospital environment (8)[b]	River water and sewage (9)
Asparagine MPN system		
Mean $\log_{10}$ number of organisms/ml	3.18	2.14
Standard deviation	1.89	1.34
mPA membrane filtration system[c]		
Mean $\log_{10}$ number of organisms/ml	2.82	1.95
Standard deviation	2.01	1.35
Standard deviation of paired difference	1.355	0.1899
Paired *t*	0.76	2.65[d]
Correlation coefficient	0.76	0.99
Slope	0.81	0.99
Intercept	0.24	−0.18

[a] APHA buffered distilled water used as diluent.
[b] Sample size.
[c] Millipore 0.45-μm membrane filter; 10 ml of agar in 60- by 15-mm plates.
[d] $P < 0.05$.

comparable in assaying samples from hospitals. On samples of river water and sewage the asparagine MPN system gave significantly higher values than the mPA system, but was otherwise comparable when judged by linear regression analysis.

Reduction of background contaminants in the MPN systems was approximated from fluorescent negative tubes showing good turbidity but failing to confirm in acetamide or *Pseudomonas*-P broth. Comparison with total viable counts in SM agar showed values as low as 20 to 25% in asparagine and acetamide broths, although the majority of samples fell within a 90 to 99% range of reduction values. In the mF systems, selectivity was found to be much greater with mPA than with Pseudosel agar. Reduction in background levels on Pseudosel agar varied from 23 to 99%, with approximately one-third of the samples showing <90% reduction. However, although levels of background contaminants showed a consistent reduction ≥99%, colonies of *P. aeruginosa* were atypical in initial experiments with mPA agar. Colonies have been described by Levin and Cabelli (18) as typically flat, with brownish to greenish-black centers and light outer rims. In contrast, colonies of strains NOPs, NOPs-sub, and SP-1 were convex, consistently light (pale pink to pinkish-brown), and pigmented throughout, lacking a defined outer rim. With natural waters containing unknown strains of *P. aeruginosa*, despite the greater number and variety of strains examined, patterns of colonial morphology were markedly inconsistent.

Efforts to determine the cause of variability in pigmentation by examining various components of the basal mPA medium were unsuccessful. However, in examining dishes furnished by Northeast Water Supply Laboratory (3-ml volume in snap-lid dishes), differences both in the color of the media and in pH determinations of melted agar samples from different media batches were noted. Experiments were designed to measure the effect of

pH and volume of mPA medium on recovery or pigmentation of naturally occurring and subcultured cells of *P. aeruginosa*. Flasks of autoclaved basal mPA media were adjusted to pH values ranging from 6.8 to 7.6 before the addition of antibiotics and dispensed in 3-, 5-, or 10-ml amounts in snap-lid or standard-lid petri dishes. Results are shown in Table 4. These data, subjected to analysis of variance, showed the most significant ($P < 0.001$) single factor influencing recovery to be the type of culture. Overall, the level of recovery of naturally occurring cells was twice that of subcultured cells. The second most important factor ($P < 0.005$) was the pH of the basal mPA medium. Recovery values decreased with increasing pH for both naturally occurring and subcultured strains. The least important factor was the volume of plating medium, although significantly higher recovery ($P < 0.02$) was associated with the larger volumes of media. The only statistically significant ($P < 0.005$) interaction between factors showed that lower pH enhanced the superior recovery of naturally occurring over subcultured cells. The appearance of characteristic colonial morphology and pigmentation was not effected by pH alterations over the range tested.

To assess possible effects of the suspending menstruum, dilutions of strains NOPs and NOPs-sub were made in buffered distilled water and phosphate-buffered saline. The data in Table 5 show that the recovery of subcultured cells was significantly affected by the type of diluent. Recovery of naturally occurring cells was comparable under all conditions studied.

Additional experiments conducted to compare possible differences in availability of nutrients by filter type showed that mean ratios of mF/pour plate viable counts using 0.45-μm membrane filters (Millipore Corp.) were significantly higher than those obtained using 0.22-μm filters ($P < 0.01$) in recovery of naturally occurring cells. It was also subsequently learned that Northeast Water Research Laboratory routinely employed Sartorius (0.45 μm) filters and, although recoveries were shown to be comparable to those obtained with 0.45-μm membrane filters (Millipore Corp.), colonial morphology remained atypical on all types of filters tested.

Measurements of different batches of basal mPA media prepared at the Northeast Water Research Laboratory showed a pH range of 5.9 to 6.6 prior to autoclaving, whereas basal media prepared at the Phoenix Laboratories showed a range of 5.4 to 5.8. Paired samples of basal mPA medium were prepared, with one set adjusted (pH range of 5.4 to 6.5) prior to autoclaving. After autoclaving, both sets of flasks were readjusted to pH 7.1 ± 0.1, before the addition of antibiotics. Recoveries of naturally occurring cell suspensions were comparable on Sartorius or Millipore membrane filters (0.45 μm) incubated on mPA with pH

TABLE 5. *Effect of diluent on recovery of P. aeruginosa under four culture conditions*[a]

Culture conditions	Mean $\log_{10}$ no. of naturally occurring *P. aeruginosa*/ml		Mean $\log_{10}$ no. of subcultured *P. aeruginosa*/ml	
	PBS diluent	BDW diluent	PBS diluent	BDW diluent[b]
Pour plates in SM agar at 37 C	3.38	3.40	2.36	2.83
MF on SM agar at 37 C	3.45	3.45	2.52	2.83
MF on SM agar at 41.5 C	3.36	3.38	2.54	2.85
MF on mPA at 41.5 C	3.40	3.36	2.52	2.81

[a] Abbreviations: PBS, Phosphate-buffered saline; BDW, buffered distilled water; MF, membrane filters.
[b] Significantly greater than the number recovered from PBS diluent ($P < 0.05$).

TABLE 4. *Factors affecting comparative recovery of P. aeruginosa by mF techniques*[a]

pH[b]	Naturally occurring *P. aeruginosa*			Subcultured *P. aeruginosa*		
	10 ml of mPA, 60- by 15-mm plates	5 ml of mPA, 60- by 15-mm plates	3 ml of mPA, 50- by 12-mm plates	10 ml of mPA, 60- by 15-mm plates	5 ml of mPA, 60- by 15-mm plates	3 ml of mPA, 50- by 12-mm plates
6.8	96[c]	96	91	96	92	85
7.0	91	87	91	70	70	60
7.2	91	87	87	50	56	33
7.4	87	87	87	44	22	19
7.6	87	82	82	24	16	11

[a] Millipore membrane filters (0.45 μm); APHA buffered distilled water used as diluent.
[b] pH of basal mPA medium adjusted after autoclaving, before addition of antibiotics.
[c] Percentage of recovery calculated from control counts from membrane filters on SM agar.

adjusted either before or after autoclaving. However, colonial morphology was markedly affected. Colonies on mPA autoclaved at pH 6.0 or less were atypical, whereas those on media autoclaved at pH 6.2 to 6.5, irrespective of the pH after autoclaving, showed a well-defined center of dark-brown to greenish-black pigmentation with a pale outer periphery.

A final series of tests was conducted to compare recovery and colonial morphology of naturally occurring and subcultured cells of *P. aeruginosa* on various types of filters. Paired samples were assayed on mPA media adjusted only after autoclaving (pH 7.2) and on media adjusted both before and after autoclaving (pH 6.5 and 7.2, respectively). Results presented in Table 6 show that the lowest recoveries were obtained on 0.22-μm membrane filters (Millipore Corp.), under all conditions studied. With Gelman filters, viable counts for both types of cell systems were significantly lower than those obtained on Millipore or Sartorius 0.45-μm membrane filters when pH adjustments of basal media were made before autoclaving. Additional analyses performed on the data for Millipore and Sartorius membrane filters showed that, overall, significantly greater ($P < 0.01$) recoveries were obtained with subcultured than with naturally occurring cells, with or without pH adjustment, although the accuracy for both types of cells showed ≥90%, efficiency. Despite the higher recoveries ($P < 0.01$) obtained for either type of cell system on media without pH adjustment before autoclaving, mean recovery values ≥90% were noted even with pH adjustment. Optimum development of pigmentation on all types of filters again required pH adjustment before autoclaving.

DISCUSSION

Critical evaluation of methods for isolation or quantitation of gram-negative bacterial pathogens has been hampered to some degree by the obvious requirement of simulating their characteristics in natural ecological niches in the laboratory environment. That physiological changes do occur upon introduction into these artificial systems has been strikingly demonstrated in studies showing altered responses of laboratory subcultured organisms to various types of disinfectants (3, 5, 11) and differences in growth characteristics (6, 10).

Moreover, such techniques typically require the use of physical or chemical agents (11, 16, 17, 18, 20, 23, 25) either to inhibit substantial fractions of the total microbial flora or to enhance the growth or development of distinguishing characteristics that enable rapid detection of specific organisms. Whether the restrictions imposed by the use of selective media, on which injured or stressed populations may be unable to recover, further compounds the problems of interpreting results obtained in in vitro environments has been questioned by Hoadley and Cheng (16). They also observed that the nature of the diluent used affected recovery of indicator bacteria on selective or nonselective media; phosphate buffer was more effective than distilled water, tap water, or peptone water for recovery of *P. aeruginosa*. Data presented here not only confirm that recovery of subcultured cells is highly dependent on the type of diluent used, but show that significant differences may be obtained with phosphate buffer in distilled water versus phosphate buffer in saline. However, with naturally occurring cells the type of diluent did not appear to be as critical.

Using antibiotics in mPA agar, Levin and Cabelli (18) obtained average recoveries of 83% for subcultured *P. aeruginosa* strains after immediate exposure in estuarine waters. As reported here, recoveries of strains NOPs-sub and SP-1 on mPA agar were comparable, averaging 83 and 81% after immediate exposure in surface waters and in a variety of hospital environmental fluids. The selectivity obtained with mPA agar suggests that this medium may also be effective for routine isolation by direct inoculation of clinical as well as environmental samples. Lambe and Stewart (17) found that Pseudosel agar (0.03% cetrimide), although relatively nonselective for *P. aeruginosa*, facilitated early detection by enhancing pigmentation of clinical isolates. Mean recovery of subcultured cells using the Pseudosel-mF system was fair (77%), but the relatively poor reduction in background contaminants rendered interpretation of fluorescent colonies difficult. Significantly lower recoveries (<50%) also were obtained with subcultured cells in the asparagine and acetamide MPN media, without added inhibitors. The higher mean recoveries noted for naturally occurring cells in both mF (>95%) and MPN (>75%) systems may reflect their greater resistance to injury when cultured and suspended in nutritionally restrictive environments prior to plating on selective or minimal media. Similarly, results obtained with subcultured cells may reflect the cumulative stress obtained in shifting from growth on nutrient-rich media (SM agar) to suspension in minimal aqueous environments (buffered distilled water and environmental fluids) and subsequent transfer to selective or minimal media.

The results of studies to determine the basis

TABLE 6. *Factors affecting comparative recovery of P. aeruginosa on mPA agar*

Filter type[a]	Naturally occurring *P. aeruginosa*				Subculture *P. aeruginosa*			
	pH unadjusted[b]		pH adjusted[b]		pH unadjusted[b]		pH adjusted[b]	
	Mean[c]	SD[d]	Mean	SD	Mean	SD	Mean	SD
M-45	2.15	0.04	2.81	0.04	2.67	0.09	3.45	0.03
S-45	2.14	0.04	2.82	0.07	2.64	0.08	3.45	0.02
G-45	2.11	0.03	2.68[e]	0.07	2.60	0.03	3.23[e]	0.31
M-22	1.36[e]	0.10	1.67[e]	0.14	2.30[e]	0.08	2.86[e]	0.12

[a] M, Millipore; S, Sartorius; G, Gelman; 45, 0.45 μm; 22, 0.22 μm.

[b] The pH of the basal medium was unadjusted prior to autoclaving or adjusted to 6.5; both were adjusted to 7.2 after autoclaving.

[c] Mean of $\log_{10}$ number of organisms per milliliter, $n = 10$.

[d] SD, Standard deviation.

[e] $P < 0.01$.

of variability in colonial morphology on mPA agar show that a variety of other factors may influence detection or enumeration of organisms in mF systems. The use of 0.22-μm membrane filters for enumeration has been reported (2, 20). However, viable counts were significantly lower for either naturally occurring or subcultured cells of *P. aeruginosa* on 0.22-μm than on 0.45-μm filters incubated on mPA agar. Presswood and Brown (W. G. Presswood and L. R. Brown, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, E54, p. 10) obtained significantly higher counts of fecal coliform bacteria on Gelman than on Millipore (0.45-μm) membrane filters; data obtained in this study showed just the reverse for *P. aeruginosa*. These observed growth limitations may be related to altered rates of nutrient diffusion through membranes of different average pore diameters, or to differences in the composition or use of additives in membrane filters. Higher mean recoveries of subcultured cells were noted in studies examining the specific effect of pH and filter type (Table 6) than were obtained in comparing MPN and mF systems (Table 2) or the effects of pH adjustment after autoclaving (Table 4). Whether these discrepancies reflect real differences in control values obtained using pour plate versus mF techniques, or whether the addition of background contaminants did in fact influence recovery of subcultured cells, could not be determined.

The development of typical colonial morphology on mPA agar was shown to be pH dependent for both types of organisms; production of characteristic pigmentation required pH adjustment prior to autoclaving. It would appear that alteration of medium components responsible for development of color characteristics may occur during autoclaving at a low pH (≤6.0); pH adjustment after autoclaving did not reverse this effect. Sartorius membrane filters were superior in development of characteristic colonial morphology; pigments on Gelman filters tended to be more diffuse than on either Millipore or Sartorius filters. The nature of the growth menstruum of test organisms (environmental fluids, distilled water, or SM agar) or the prior length of subculture maintenance did not appear to be major determining factors affecting pigment production, although Lambe and Stewart (17) reported better pigmentation on Pseudosel agar with strains repeatedly subcultured than with original isolates.

The asparagine-MPN system has proven effective in microbiological surveys to detect or enumerate *P. aeruginosa* in a variety of hospital environments (10, 21, 22, 23) despite the limitations of sample size and procedural difficulties inherent in MPN systems. mF techniques which rely on development of typical colony characteristics may be subject to considerable variation, dependent to some extent on sample sources and differences in the physiological condition of test strains or on the quality of assay media and procedures. Both types of systems require attention to a variety of other factors that may influence the specificity or reproducibility of results. The asparagine-MPN and mPA-mF techniques used in this study were highly sensitive, as evidenced by their ability to detect levels of naturally occurring *P. aeruginosa* as low as 50 organisms/ml among background contaminants of 4.6×10^4/ml. Overall, greater precision and accuracy were consistently obtained in either type of system when naturally occurring cells were used as test organisms. The studies reported here indicate that the mPA-mF as well as the asparagine-MPN techniques afford promise as effective tools for detecting and

quantitating *P. aeruginosa* for epidemiological purposes.

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