APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1978, p. 36-42
0099-2240/78/0036-0036$02.00/0
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Vol. 36, No. 1
Printed in U.S.A.

Improved Medium for Recovery and Enumeration of *Pseudomonas aeruginosa* from Water Using Membrane Filters

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Received for publication 16 January 1978

A modified mPA medium, designated mPA-C, was shown to recover *Pseudomonas aeruginosa* from a variety of water sources with results comparable to those with mPA-B and within the confidence limits of a most-probable-number technique. Enumeration of *P. aeruginosa* on mPA-C was possible after only 24 h of incubation at 41.5°C, compared with 72 h of incubation required for mPA-B and 96 h of incubation for a presumptive most probable number.

Many methods have been described for the selective enumeration of *Pseudomonas aeruginosa* in water (1, 4-6, 9, 13), but none has been widely accepted. Recovery of *P. aeruginosa* by most-probable-number (MPN) techniques is good (1, 4, 5, 9), but the precision of this method is poor (8). In addition, MPN techniques are cumbersome, not easily adapted to analyzing large volumes of water, and require extended incubation periods. MPN techniques for *P. aeruginosa* are further complicated by the variety of recommended asparagine broth formulations (1, 9), incubation conditions (1, 5, 6, 13), methods of interpretation of presumptive positive tubes (1, 4-6, 9, 13), and confirmatory procedures (1, 4-6, 9, 11).

Conversely, the precision of membrane filter (MF) techniques is well established, with certain limitations, including turbidity of the water and variation between brands of membrane filters (1, 4, 8). However, with the exception of mPA medium (4, 6, 13), the specificity of MF media for *P. aeruginosa* has been poor (1, 6), especially when the water contains a large heterogeneous bacterial flora and a relatively small *P. aeruginosa* population.

The original mPA medium described by Levin and Cabelli (13) resembles a modified formulation of xylose-lysine-deoxycholate medium (14) made more selective by incorporating the antibiotics kanamycin, nalidixic acid, sulfapyridine, and Actidione. This medium, when incubated at 41.5°C for 48 h, was moderately selective for *P. aeruginosa* and allowed differentiation of colonies of *P. aeruginosa* by their distinct appearance. The validity of mPA medium for the quantitative recovery of *P. aeruginosa* from a variety of water sources with membrane filters was confirmed by Carson et al. (4) and Dutka and Kwan (6). These investigators also noted certain problems with the preparation of the basal medium. Carson et al. suggested that the pH be adjusted above 6.2 before autoclaving and readjusted to 7.1 after autoclaving to promote the development of well-defined typical colonies. Dutka and Kwan demonstrated that 4 days of incubation on mPA was required for optimal recovery of *P. aeruginosa* from a stressed environment. They also modified the original mPA formulation by adding 0.15 g of magnesium sulfate per 100 ml and by reducing the sodium thiosulfate content from 0.68 to 0.5 g/100 ml to achieve slightly better recovery and colony definition. This medium was designated mPA-B.

Our initial experiences with the mPA medium originally described by Levin and Cabelli (13) were not satisfactory. Using pure cultures of *P. aeruginosa,* we observed poor recovery and a confusing range of colony morphology. This multiplicity of colonial types was compounded, as noted also by Carson et al. (4), when sewage effluent and surface waters were examined. Other laboratories which were using the original mPA formulation were contacted, and they reported similar problems. The report of Dutka and Kwan (6), describing a modified mPA medium, mPA-B, and the ability of Carson et al. (4) to improve the performance of mPA by pH adjustment encouraged us to reexamine mPA, particularly mPA-B.

During our initial evaluation of mPA-B, the final concentration of sulfapyridine was inadvertantly reduced from 176 to 1.76 μg/ml. We observed that *P. aeruginosa* colonies were countable after only 24 h of incubation at 41.5°C in the medium with reduced sulfapyridine, whereas

P. aeruginosa colonies were not evident until 48 h of incubation and not optimally countable until after 72 h of incubation in mPA-B with a high sulfapyridine concentration. This improved growth response was not observed by altering the concentrations of kanamycin, nalidixic acid, or Actidione. An investigation was undertaken to assess the effect of eliminating sulfapyridine on the accuracy, selectivity, specificity, and precision of mPA-B for *P. aeruginosa*. This paper describes our evaluation and further modification of mPA-B, which resulted in the development of mPA-C, an MF medium for the quantitative recovery of *P. aeruginosa* within 24 h.

MATERIALS AND METHODS

Culture sources. *P. aeruginosa* ATCC 15442, one strain isolated from water, and two clinical isolates were used for all pure culture and "spiked" sample studies. The *P. aeruginosa* cultures were grown at 35°C for 16 to 18 h in brain heart infusion (BHI) broth (Difco) on an Eberbach oscillating shaker and stored in NSB medium (2). The densities of the NSB cultures were determined by spread plate, using BHI agar (Baltimore Biological Laboratory [BBL]).

Spiked samples. Five water samples from the Humber River and five water samples from outdoor swimming pools located within metropolitan Toronto were used for spiking. These samples were selected because pretesting with mPA-C and asparagine broth indicated that they had no *P. aeruginosa* population. A 0.25-ml amount of each NSB culture (total, 1 ml) was added to 1 liter of each sample, which had been held at 4°C, to provide a calculated spike of 15 *P. aeruginosa* cells per ml. The spiked samples were held for 4 h at 4°C before comparative studies were done.

Field samples. Water samples were collected from each of the following sites: unchlorinated secondary sewage effluent (North Don sewage treatment plant), five samples over a 4-week period; whirlpool baths in health clubs, six samples over an 8-week period; polluted river water, five samples over a 4-week period. Each sample was pretested on mPA-C within 4 h of collection to determine the approximate *P. aeruginosa* density and then stored overnight at 4°C before comparative analyses were done.

Media. The asparagine medium described in *Standard Methods for the Examination of Water and Wastewater* (1) was used throughout this study for presumptive enumeration of *P. aeruginosa* by the MPN technique. The presumptive MPNs of the spiked samples were maximal after incubation for 48 h at 39°C, whereas incubation for 96 h at 39°C was used for the MPNs of the effluent study and all natural samples.

The basal ingredients of mPA-C and mPA-B were as described by Levin and Cabelli (13) with the modification of Dutka and Kwan (6) (in grams per 100 ml): L-lysine hydrochloride (0.5), sodium chloride (0.5), yeast extract (0.2), xylose (0.125), sodium thiosulfate (0.5), sucrose (0.125), lactose (0.125), phenol red (0.008), magnesium sulfate (0.15), ferric ammonium citrate (0.08), Oxoid agar no. 4 (1.2). Initially, the basal media were dissolved by boiling, and then the pH was adjusted to 7.4; after autoclaving (121°C for 10 min), the pH was 7.2. Autoclaving was later found to be unnecessary, and the pH was simply adjusted to 7.2 after boiling. The media were tempered to 55°C before the antibiotics were added and then poured into 50-mm plastic petri plates. The antibiotics were prepared as liquid concentrates which were frozen until needed. The antibiotics were added to mPA-B to provide final concentrations of (in micrograms per milliliter): kanamycin (Bristol Laboratories), 8; nalidixic acid (Winthrop Laboratories), 37; sulfapyridine (Sigma Chemical Co.), 176; and Actidione (The Upjohn Co.), 15. Only kanamycin and nalidixic acid were added to mPA-C. Enumeration of *P. aeruginosa* on mPA-B was done after 72 rather than 96 h of incubation at 41.5°C, as preliminary experiments indicated no improvement in colony definition with the additional incubation time. Counting on mPA-C was completed after incubation for 24 h at 41.5°C.

Membrane filters and sample analysis. The initial pure culture work was done with Millipore HAWG 047SG membrane filters, lot 27901-5. Field and spiked samples were filtered through Sartorius filters (no. 11456, lot Z5008715). All enumeration procedures with membrane filters were done with five replicates per sample. A five-tube MPN was used for comparison.

Verification of isolates. Twenty-five suspect *P. aeruginosa* colonies from each replicate set of filters on mPA-C and mPA-B were confirmed as outlined in Fig. 1. In addition, a total of 100 atypical colonies from each medium were also verified. Every MPN tube was examined for growth and fluorescence (Blak-Ray, long-wave UV light). All tubes exhibiting growth were subcultured to cetrimide agar and incubated for 24 h at 35°C for primary confirmation based on pyocyanin production and fluorescence. All apyocyanogenic isolates were further examined on King medium A (12) and according to prescribed methods (Committee on Continuing Education, American Society for Microbiology, "Identification of Glucose Non-Fermenting Gram-Negative Rods," 1975), as shown in Fig. 1.

Statistical considerations. The precision of the mPA technique for quantitative recovery of *P. aeruginosa* from a variety of water types was determined by plotting the dispersion of D^2 values (7).

RESULTS

Table 1 illustrates the accuracy of the mPA media for the recovery of pure cultures of *P. aeruginosa* after growth in BHI broth by comparison with a nonselective medium, BHI agar (BBL). The mean *P. aeruginosa* counts are equivalent on mPA-C and mPA-B incubated at 41.5°C and BHI agar incubated at 35°C. Interestingly, BHI agar did not perform as well when incubated at the higher temperature.

Table 2 compares the selectivities of mPA-C and mPA-B with the MPN for the recovery of *P. aeruginosa* from spiked water samples. Overall, mPA-C recovered 83.7% of the spiked *P. aeruginosa* populations from the swimming pool

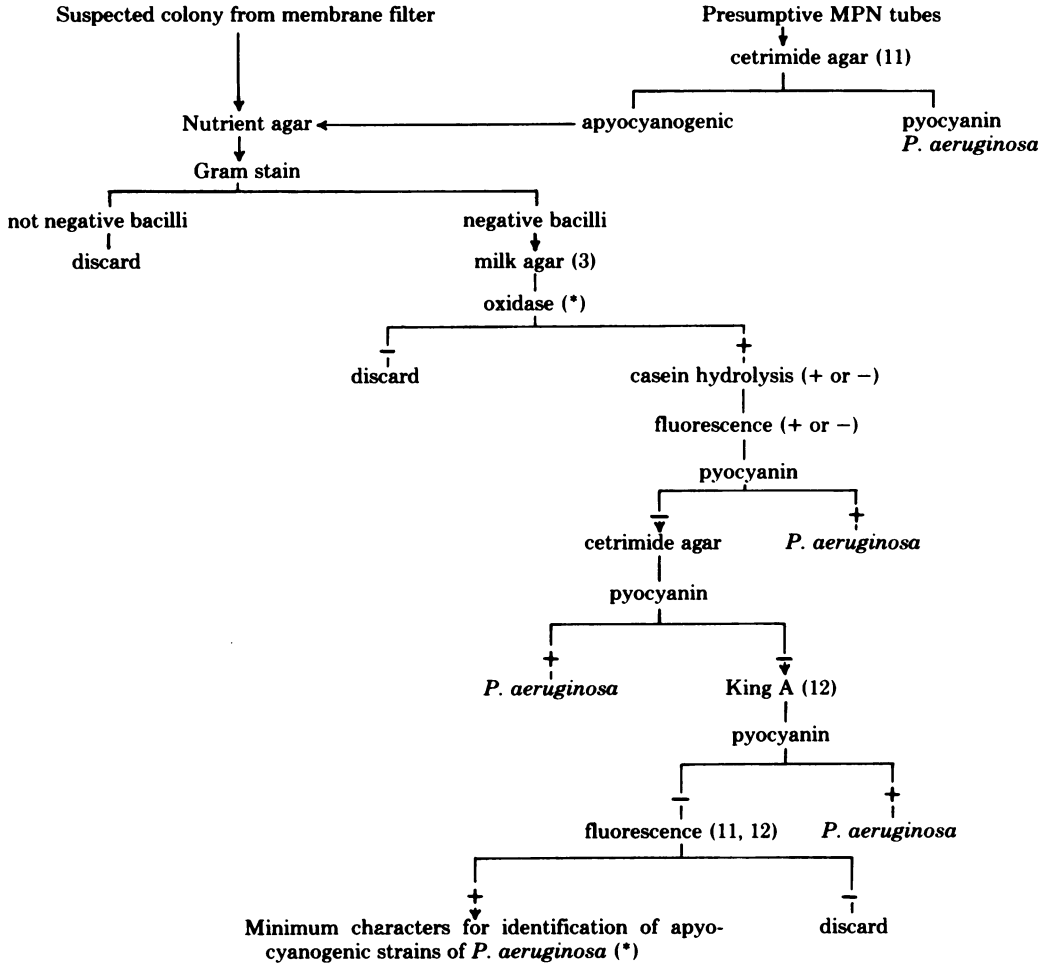

FIG. 1. *Procedure for confirmation of P. aeruginosa. Numbers in parentheses refer to Literature Cited. (*) Committee on Continuing Education, ASM, "Identification of Glucose Non-Fermenting Gram-Negative Rods," 1975. Fluorescent isolates which grew at 42°C but whose biochemical profiles did not otherwise match the minimum characters for identification of apyocanogenic strains of P. aeruginosa were regarded as unidentified fluorescent pseudomonads (10, 11).*

water samples and 99.4% of the spikes from the Humber River samples. mPA-B recovered 86.1 and 99.8% of the spiked samples, respectively. The MPN recovered 100% of the spiked populations. The BHI agar counts indicated that the swimming pool samples had virtually no background bacterial population capable of growth under the specified incubation conditions and that the Humber River samples had only a small natural flora. A comparison of the mean counts shows that mPA-C and mPA-B were equivalent in their ability to recover *P. aeruginosa* from these spiked samples and that the counts obtained were within the 95% confidence limits of the MPN.

Table 3 demonstrates the selectivities of mPA-C, mPA-B, and the MPN for the quantitative recovery of *P. aeruginosa* from natural waters. The results of the comparative study with secondary sewage effluent illustrate the selectivity of both mPA-C and mPA-B for *P. aeruginosa* when the organism is present in a large, heterogeneous bacterial population. Although the background flora was not eliminated, the non-*P. aeruginosa* colonies that did develop on the mPA media appeared for the most part as microcolonies, which did not interfere with the detection or enumeration of the relatively large, characteristic *P. aeruginosa* colonies. With the exceptions of effluent sample 2 and whirlpool sample 5, the mean counts of *P. aeruginosa* on mPA-C and mPA-B were com-

parable to each other and were within the confidence limits of the MPN for the three types of natural samples studied.

The precision of mPA-C and mPA-B is shown by D^2 values for assay variability from the examination of 24 naturally polluted and spiked water samples (Fig. 2). Only one value exceeded the $P = 0.025$ control limit of 11.1. Also, D^2 values were reasonably distributed around the $P = 0.5$ control limit of 3.2 for both mPA-C and mPA-B.

TABLE 1. *Accuracy of mPA media for recovery of pure cultures of P. aeruginosa*

Isolate	Mean count/ml[a] in:			
	BHIA[b] (35°C, 24 h)	BHIA (41.5°C, 24 h)	mPA-C[c] (41.5°C, 24 h)	mPA-B[c] (41.5°C, 72 h)
1	59.4	60.2	65.6	65.4
2	59.8	52.6	53.0	53.4
3	61.8	50.6	61.6	64.0
4	64.0	54.2	70.2	67.2
Overall	61.3	54.4	62.6	62.5

[a] Mean (arithmetic) of five replicates.
[b] BHIA, BHI agar.
[c] Refer to text for formulation.

The specificity of mPA-C and mPA-B for *P. aeruginosa* is illustrated in Table 4. Out of 773 typical colonies, 768 (99.4%) picked from mPA-C were confirmed as *P. aeruginosa*, with only 3 of 99 (3.0%) atypical colonies being verified. Similarly, 737 of 745 (98.9%) typical colonies picked from mPA-B were identified as *P. aeruginosa*, whereas only 3 of 98 (3.1%) atypical colonies were confirmed. Only 1 *P. aeruginosa* isolate from mPA-C and 4 isolates from mPA-B were apyocyanogenic. All isolates from both mPA media which fluoresced were confirmed as *P. aeruginosa*; no unidentified fluorescent pseudomonads (10, 11) were isolated.

A graphic comparison of the enumeration of

TABLE 2. *Recovery of pure cultures of P. aeruginosa from spiked water samples*[a]

Source	Sample no.	Mean count/100 ml[b] (% recovery) in:			
		BHIA[c]	mPA-C	mPA-B	MPN index[d]
Swimming pool	1	1,270	1,240 (82.7)	1,285 (85.6)	1,700
	2	1,160	1,220 (81.3)	1,285 (85.6)	3,500
	3	1,125	1,315 (87.7)	1,395 (93.0)	700
	4	1,133	1,250 (83.3)	1,200 (80.0)	790
	Mean	1,172	1,256 (83.7)	1,291 (86.1)	1,673
Humber River	1	3,020	1,375 (91.7)	1,540 (100)	1,300
	2	3,180	1,605 (100)	1,585 (100)	1,700
	3	3,520	1,585 (100)	1,500 (100)	1,300
	4	3,560	1,505 (100)	1,395 (93.0)	1,700
	5	3,160	1,390 (92.7)	1,465 (97.7)	1,700
	Mean	3,288	1,492 (99.4)	1,497 (99.8)	1,540
Overall		2,348	1,387 (92.4)	1,406 (93.7)	1,599

[a] Four cultures were combined for a spiked density of 15/ml.
[b] Mean (arithmetic) of five replicates.
[c] BHIA, BHI agar (BBL), incubated at 41.5°C for 24 h to measure total flora capable of growth at elevated temperature.
[d] As per *Standard Methods for the Examination of Water and Wastewater* (1), but with incubation at 39°C for 48 h.

TABLE 3. *Comparison of media for selective enumeration of P. aeruginosa from natural water samples*

Source	No. of samples	Mean count/100 ml[a] in:					
		BHIA[b]	mPA-C		mPA-B		MPN
			Total	*P. aeruginosa*	Total	*P. aeruginosa*	
Secondary sewage effluent	5	3.9×10^5	2.7×10^4	927	2.2×10^4	1,069	1,225
Whirlpool	6	2.1×10^5	1,738	1,344	1,515	1,244	2,942
River	5	ND[c]	2,503	1,735	2,070	1,839	2,412

[a] Arithmetic mean count of five replicates.
[b] BHIA, BHI agar (BBL).
[c] ND, Not done.

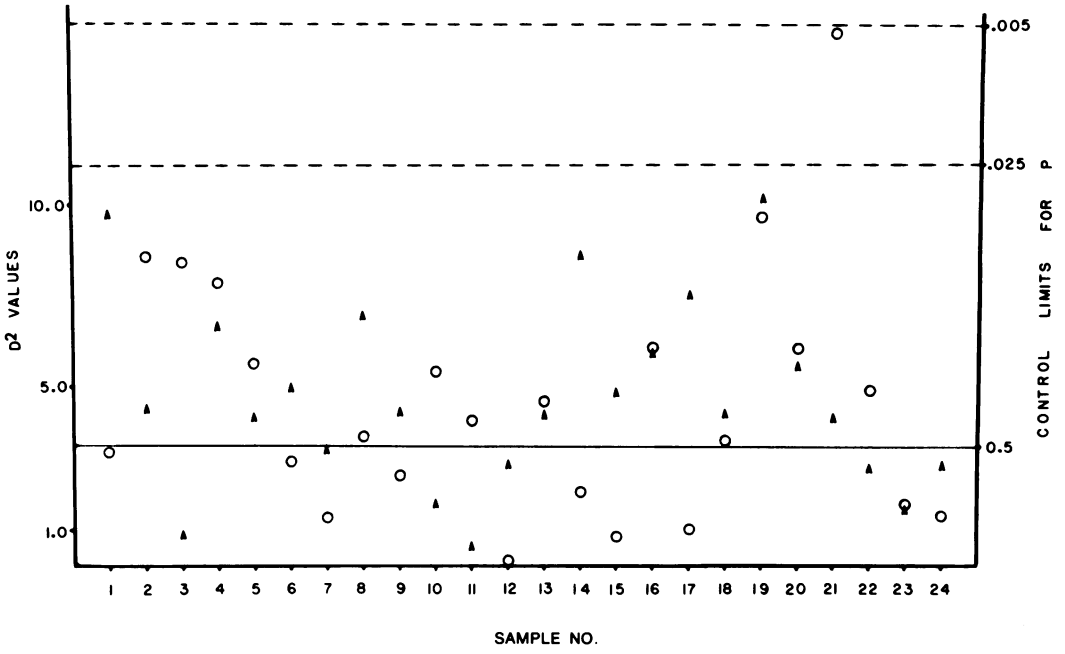

FIG. 2. *Precision of mPA procedure for P. aeruginosa as estimated by dispersion of D^2 values. Symbols: ○, mPA-C; ▲, mPA-B. Sample sources of: 1 through 5, secondary sewage effluent; 6 through 10, spiked river water; 11 through 14, spiked pool water; 15 through 19, natural whirlpool water; 20 through 24, natural river water.*

TABLE 4. *Verification of colonies observed on mPA media*

Samples examined		No. of colonies on:							
		mPA-C				mPA-B			
Source	No.	Typical[a]		Other[b]		Typical		Other	
		Observed	Verified[c]	Observed	Verified	Observed	Verified	Observed	Verified
Secondary sewage effluent	16	498	493			495	488		
Whirlpool	6	175	175	25	1	150	150		
River	4	100	100	74	2	100	99	98	3
Total	26	773	768	99	3	745	737	98	3
Percent verified			99.4		3.0		98.9		3.1

[a] Circular, raised colonies ≥1 mm in diameter, with entire or undulate margins, tan to dark-brown in color with dark-brown centers or pink with nucleated centers or surrounded by an amber halo.
[b] All colonies other than those described in footnote *a*.
[c] Verified as *P. aeruginosa*.

P. aeruginosa on mPA-C with MPN counts is presented in Fig. 3. With two exceptions, all *P. aeruginosa* counts from mPA-C, involving 24 samples of five different types of water, fell within the 95% confidence limits of the five-tube MPN.

DISCUSSION

The criteria outlined by Levin and Cabelli (13) were applied to our evaluation of the modified mPA media. The exclusion of sulfapyridine and the consequent reduction of incubation time did not adversely affect either the accuracy or the selectivity of mPA-C for *P. aeruginosa*. Both mPA-C and mPA-B recovered 100% of the pure broth cultures (Table 1). As shown in Table 2, mPA-C, with only 24 h of incubation, recovered 83.7 and 99.4% of the spiked *P. aeruginosa* populations from swimming pool and Humber River waters, respectively, compared

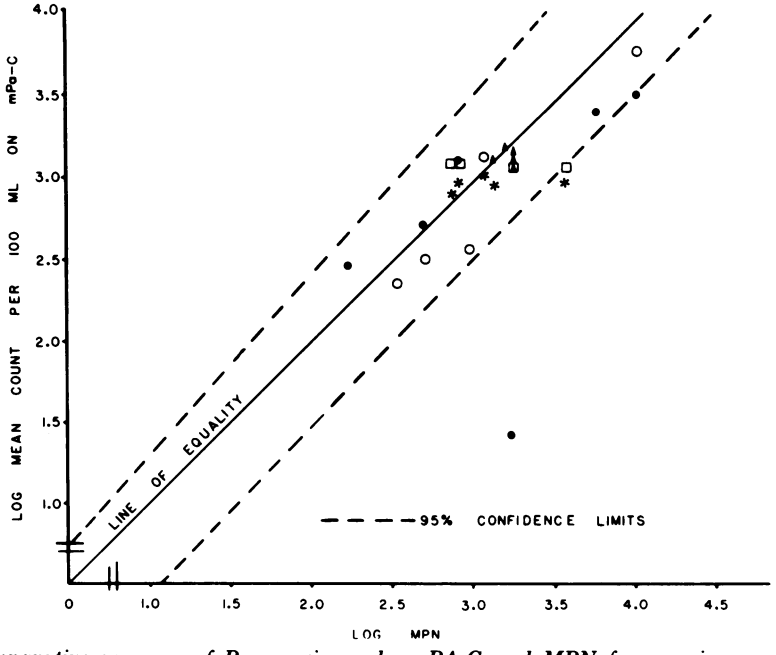

FIG. 3. *Comparative recovery of P. aeruginosa by mPA-C and MPN from various sources of water. Symbols: *, secondary sewage effluent; ●, whirlpool; ○, natural river water; □, spiked swimming pool; ▲, spiked river water.*

with 86.1 and 99.8% recovery by mPA-B after 72 h of incubation. These values compare favorably with the criterion of at least 75% recovery suggested by Levin and Cabelli (13).

The selectivity of mPA-C was shown to be equivalent to that of mPA-B (Table 3). The desirable reduction of background organisms by three orders of magnitude was not achieved by either medium; however, the background flora was restricted to pinpoint colonies, which, as shown by Table 4, did not interfere with the visualization of typical *P. aeruginosa* colonies. Ninety-nine percent of the typical colonies on mPA-C and mPA-B were confirmed as *P. aeruginosa,* whereas only 3% of other colony types were verified, indicating that the specificity of mPA-C and mPA-B for *P. aeruginosa* exceeded the recommendation of 90% verification of typical colonies and less than 10% confirmation of atypical colonies.

The D^2 values for assay variability (Fig. 2) approximate that expected by chance (7), demonstrating the precision of both the mPA-C and the mPA-B techniques. A comparison of the D^2 dispersion values reported by Levin and Cabelli on the original mPA investigation with the results of this study suggests that mPA-C and mPA-B are more precise than the original formulation. The D^2 dispersion values reported by Levin and Cabelli showed subnormal variability, which may have indicated a lack of statistical control caused by variations in medium preparation (7). Carson et al. (4) noted that the development of typical colonial morphology on mPA was pH dependent and that autoclaving the medium at a pH less than 6.0 might irreversibly alter the medium components responsible for the development of color characteristics. Similarly, the addition of 0.15% magnesium sulfate and the reduction of the sodium thiosulfate content to 0.5%, as recommended by Dutka and Kwan (6) for mPA-B, has improved colony definition making the detection of *P. aeruginosa* more reliable. Our practice of preparing the medium by boiling only may have eliminated some of the problems created by autoclaving, resulting in a more uniform mPA medium. As our media were always used within 1 week of preparation, the shelf life of the media, which might have been compromised by not autoclaving, was not considered a major problem. More significantly, the selectivity and specificity of mPA-C and mPA-B were not impaired by lack of autoclaving.

Actidione was omitted from mPA-C, in addition to excluding sulfapyridine, without any apparent adverse affects on the performance of the medium. Actidione was incorporated into mPA to inhibit fungal growth which may have developed during the prolonged incubation of the medium. Since mPA-C is incubated for only 24 h, fungal contamination was found not to be a

problem.

Comparability was one of the major concerns in the design of the mPA-C evaluation. *Standard Methods for the Examination of Water and Wastewater* (1) tentatively proposes an MPN technique which has been shown to be insensitive and imprecise for recovering *P. aeruginosa* and an MF technique which is unreliable (9, 13). Thus, we were faced with a number of media formulations, confirmatory techniques, and incubation conditions used by other investigators from which to choose, none of which has been widely adopted. Based on some preliminary testing, the asparagine broth formulation used in *Standard Methods* (1) was adopted, but the tubes were incubated under the conditions used by Drake (5) and Hoadley and Ajello (10), 39°C for 96 h. Our experience with the asparagine medium recommended by *Standard Methods* (1) had shown that fluorescence was not the most reliable criterion for judging a presumptive positive tube; hence, every asparagine tube which showed growth was subcultured onto cetrimide agar, as outlined in Fig. 1. In view of our inability to achieve reasonable results with the original mPA formulation, but having had some success with mPA-B, the latter mPA formulation was used for comparison against mPA-C. Our results show that *P. aeruginosa* colonies can be counted on mPA-C after only 24 h of incubation with the same accuracy, selectivity, specificity, and precision as on mPA-B after 72 h of incubation. Furthermore, the number of *P. aeruginosa* cells recovered by both mPA-C and mPA-B, from a variety of water sources, fell within the confidence limits of the MPN.

mPA-C thus represents an alternative 24-h MF technique for enumerating *P. aeruginosa* from water, compared with the extended incubation periods required for mPA-B and the MPN.

ACKNOWLEDGMENTS

Our thanks to D. A. Schiemann for his advice and encouragement throughout this investigation. We also thank W. Van der Kolk for preparing the figures.

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