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Membrane Filter Method for the Isolation and Enumeration of *Pseudomonas aeruginosa* from Swimming Pools

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A membrane filter technique using black membrane filters, MacConkey agar and fluorescence under ultraviolet (UV) light was investigated for the quantitative isolation of *Pseudomonas aeruginosa* from swimming pools. Three thousand four hundred forty-five samples were collected from public swimming pools and enumerated by this method over a 6-month period. Fluorescent cultures were isolated from 222 specimens. Seventy-seven of these fluorescent cultures were selected for biochemical screening, with 75 (97%) being verified as *P. aeruginosa*. To further assess the specificity and sensitivity of this UV screening technique, a comparative study was made of some morphological and biochemical characteristics of fluorescent pseudomonads obtained from different sources. The sensitivity of the method was unimpaired by either colony types or biochemical variations of *P. aeruginosa*. The failure of the other two fluorescent species, *P. fluorescens* and *P. putida*, to grow and/or fluoresce on MacConkey agar at 37 C illustrates the specificity of this technique. Further studies are needed to compare the viability of *P. aeruginosa* on MacConkey agar to that of efficacious nonselective media.

The ability of *Pseudomonas aeruginosa* to survive chlorination of swimming pools (2, 8, 9) has led many investigators to regard it as a major etiological agent of otitis externa among swimmers (12, 17). However, it is difficult to evaluate the epidemiological significance of this pathogen in swimming pools without a quantitative recovery technique (15).

There have been many attempts to develop a selective medium for the isolation of *P. aeruginosa*, but none has gained acceptance for routine quantitative procedures. The only recognized quantitative technique for *P. aeruginosa* is a modification of Drake's most-probable-number (MPN) method (1). The criteria for presumptive isolation of the organism by this method are growth, production of a green or blue pigment, and/or ultraviolet (UV) fluorescence. However, in the absence of either pigmentation or fluorescence, or both, the bacterial growth in the tubes must be isolated and biochemically identified (7).

In recent years, the use of membrane filters (MF) for quantitative estimations of bacterial populations has essentially replaced MPN techniques. Membrane filter methods are less cumbersome and more precise than MPN, and they allow the isolation and enumeration of small numbers of cells from large volumes of fluids (10).

Only two MF techniques have been suggested for *P. aeruginosa* (7, 15). A modification of the Drake method (7), which is based on selectivity and the development of typically translucent, blue or blue-green colonies with a soluble diffused pigment, was found unsuitable for quantitation. Single cells did not produce pigmented colonies (1). When large numbers of *P. aeruginosa* were present, diffusion of the pigment into the surrounding medium made the distinction between pigmented and nonpigmented colonies difficult (1). The membrane filter procedure (mPA) technique of Levin and Cabelli (15) depends on the selectivity of the medium and the recognition of a specific colonial type. Typical colonies are flat in appearance with light outer rims and brownish to greenish-black centers.

We have reported the use of MacConkey agar and UV light for the rapid detection of *P. aeruginosa* (3). We report here an extension of this study that includes the possible application of MacConkey agar to a membrane filter technique for the isolation and enumeration of *P. aeruginosa* from swimming pools.

MATERIALS AND METHODS

Source of cultures. In our laboratory, swimming pool waters are routinely screened for coliforms by membrane filtration using M-Endo agar. As the mere presence of *P. aeruginosa* is considered a health

hazard (8, 9), we had performed only qualitative examinations for this organism: all noncoliform colonies on M-Endo agar were subcultured into Drake pseudomonas broth. These subcultures were incubated in a water bath at 35 C for 72 h and then examined for the presence *P. aeruginosa* (7).

To simplify this procedure for *P. aeruginosa* we compared results obtained using our routine method with results obtained using the Drake MF technique and our proposed MF-MacConkey method.

Isolation of P. aeruginosa from swimming pools by the MF-MacConkey technique. From each sample, duplicate 50-ml volumes were vacuum filtered through a black membrane filter (Millipore Corp. of Canada, Ontario, HAB GO47SO), pore size 0.45 μm. Using sterile forceps, each filter was transferred to an individual MacConkey plate (Difco). The duplicate plates were divided into two sets for overnight incubation at different temperatures in a humid atmosphere. One set was incubated at 37 C, and the other set was incubated at the more selective temperature of 42 C. After incubation, the filters were scanned under the UV lamp as previously reported (3). The fluorescent colonies were enumerated and reisolated onto fresh MacConkey agar plates. These pure subcultures were used for biochemical confirmation.

One hundred seventy-five suspected *P. aeruginosa* isolates obtained using the MacConkey agar fluorescent screening technique were selected for biochemical verification. Fifty cultures were isolated from plates obtained from the Clinical Bacteriology Laboratory, and an equal number was isolated from specimens received by the Hospital Infection Control Section of the Environmental Bacteriology Laboratory, Ministry of Health, Ontario (3). The remaining 75 fluorescent cultures were obtained by the MF-MacConkey method from samples collected from swimming pools.

P. fluorescens strains 1654, 1655, 1670, 1673, 1675, and *P. putida* strains 1544, 1572, 1638, 1639, 1671, were obtained from G. L. Gilardi of the Hospital for Joint Diseases and Medical Center, New York. *P. fluorescens* strains K129, K170, K175, K864, K958, and *P. putida* strains K89, K306C, K331, K355, K466, were received from M. J. Pickett of the University of California in Los Angeles. One strain of *P. putida* (VG) was obtained from A. Von Graevenitz of Yale University School of Medicine.

Verification of isolates. All strains of *P. fluorescens* and *P. putida* were streaked on duplicate plates of blood and MacConkey agars. One set of inoculated plates was incubated at room temperature and a duplicate set was incubated at 37 C. If no growth was observed after overnight incubation, the plates were left at room temperature for an additional 24 h. The viability and purity of the cultures were checked by their growth on the blood agar. Growth on the MacConkey plates was screened under UV light for fluorescence.

Cultures which fluoresced on MacConkey agar at 37 C were biochemically screened (3) for oxidase activity, and for their ability to oxidize gluconate, to reduce triphenyltetrazolium chloride (TTC), to hydrolyze casein, and to grow at 42 C, either on a beef agar slant or in Drake pseudomonas broth. These isolates were also examined for their appearance on Sabouraud maltose agar (SMA) (5) and for their colonial morphology on blood agar after overnight incubation at 37 C.

A limited study was conducted to evaluate the quantitative recovery of *P. aeruginosa* by membrane filtration using MF MacConkey agar, Tech agar (1), and mPA agar. *P. aeruginosa* isolates of each of the colony types A to E were inoculated into Trypticase soy broth. After overnight incubation at 37 C, serial dilutions of the broth cultures were prepared in phosphate-buffered saline and filtered onto each of the three media. The growth on the MF MacConkey agar and Tech agar was enumerated after 24 h of incubation. The filters on the mPA agar were examined after 48 h of incubation. All MF enumerations were determined by using a plate microscope (10).

RESULTS

The MF MacConkey technique was found to be more accurate than either Drake pseudomonas agar method or our routine procedure for the presumptive isolation of *P. aeruginosa* from swimming pool waters (Table 1). Ninety-seven percent of the cultures fluorescing on the MacConkey agar were verified as *P. aeruginosa*, compared with 50% of the suspicious colonies on Drake pseudomonas agar and 30% of the noncoliform organisms subcultured from M-Endo agar to Drake pseudomonas broth.

The results summarized in Table 2 illustrate the sensitivity of the MF MacConkey technique to detect low levels of *P. aeruginosa* from swimming pool waters. Seventy-seven percent of the 222 samples containing *P. aeruginosa* cells had fluorescent colony counts within the countable range for membrane filters of 1 to 80 (10). Background flora did not interfere with the detection of fluorescent colonies, but enumeration was often difficult when this background growth was confluent.

The selectivity of the MF-MacConkey technique was improved by incubating the plates at 42 C which reduced background flora. Although the *P. aeruginosa* counts at 42 C were comparable to those obtained at 37 C, the fluorescence of some *P. aeruginosa* cultures was not immediately obvious after incubation at the elevated temperature. We found that the fluorescence of these isolates could be readily demonstrated by allowing the culture to stand in a darkened area, at room temperature, for about 1 h.

The temperature of incubation appears to be the critical feature which enables *P. aeruginosa* to be distinguished from the other fluorescent pesudomonads by the MacConkey agar fluorescent technique. Table 3 illustrates that only 7 of the 21 strains of *P. fluorescens* and *P. putida* grew on the MacConkey agar after overnight incubation at 37 C. None of these strains fluo-

TABLE 1. *Evaluation of methods used to isolate Pseudomonas aeruginosa from swimming pools*

Method of isolation	No. pools tested	No. with suspected *P. aeruginosa*	No. with confirmed *P. aeruginosa*	Accuracy (%)
M-Endo agar (subcultured to Drake pseudomonas broth)	1,840	101	33	30
Drake MF-pseudomonas agar	710	118	57	50
MF-MacConkey agar	1,130	77	75	97

TABLE 2. *Enumeration of Pseudomonas aeruginosa in 222 swimming pool waters by the MF MacConkey technique*

Fluorescent colonies on black membrane filters (per 50 cm^3)	Samples with fluorescent colonies	
	No.	%
1-9	95	43
10-19	23	10
20-80	53	24
>80	51	23

resced at this temperature. Table 4 shows that all of the 150 *P. aeruginosa* cultures grew and fluoresced at 37 C. *P. fluorescens* and *P. putida* did grow (all 21 strains) and fluoresce (17 of 21 strains) on MacConkey agar when incubated at room temperature (Table 3). This observation demonstrates the suitability of MacConkey agar for use as a medium to detect fluorescent pseudomonads and emphasizes the selective role played by the 37 C incubation temperature. Some cultures, fluorescent on primary isolation, failed to fluoresce when subcultured. This problem occurred only when the MacConkey plates had been left overnight to dry at room temperature prior to inoculation. It is suggested, therefore, that freshly prepared or refrigerated McConkey plates, surface-dried in an incubator, be used when culturing for fluorescence.

Verification of fluorescent cultures as *P. aeruginosa* was based primarily on their oxidase activity and their growth at 42 C (16). Gluconate oxidation, TTC reduction, pigmentation, and casein hydrolysis were considered as secondary characteristics. Ninety-six percent of fluorescent cultures obtained from clinical and hospital sources were confirmed as *P. aeruginosa* using these characteristics (3). Of the 77 fluorescent cultures isolated by the MF MacConkey technique from swimming pool water samples, 75 (97%) were verified as *P. aeruginosa* by these primary features.

Pigmentation is often regarded as a primary characteristic of *P. aeruginosa* (5). Brown and Scott-Foster (4) reported a close relationship between pigment production and the ability of the organism to hydrolyze casein. The *P. aeruginosa* isolates examined in this study exhibited this same relationship (Table 4). On SMA, 43 of 50 (86%) clinical isolates were pigmented and 47 (94%) hydrolyzed casein; 47 of 50 (94%) hospitals isolates were pigmented on SMA and hydrolyzed casein. Although the cultures obtained from swimming pools had the same correlation between pigment production and casein hydrolysis, the percentage of strains demonstrating these traits was considerably less. Only 18 of 50 (36%) of swimming pool isolates produced pigment on SMA, whereas 19 (38%) hydrolyzed casein.

The 75 *P. aeruginosa* isolates from swimming pools were plated simultaneously on blood agar and SMA, to determine if a relationship between colonial morphology and pigmentation existed. Examination of *P. aeruginosa* colonial morphology revealed five distinct colonial types on blood agar (Table 5, types A to E). The detailed descriptions of these colonial morphologies are presented in Table 5. Hemolysis and a distinct "grape-like" odor are considered characteristic of *P. aeruginosa* (15). Colony types A and D (Fig. 1) would be regarded as typical for *P. aeruginosa* by these features; however, only 29 (38%) of the *P. aeruginosa* isolates from swimming pools developed either type A or D (Table 6). The relationship between colonial morphology and pigmentation is shown in Table 6. As illustrated, 19 of 26 (73%) pigmented cultures had either type A or D colonies on blood agar; however, of the 28 cultures with type A or D colonies, only these 19 (69%) produced pigment. The other colonial types were also capable of producing pigment on SMA, but with a much lower frequency.

The quantitative recovery of *P. aeruginosa* was found to be equivalent on MacConkey and Tech agars (Table 7) regardless of the colony type. The mPA agar allowed equivalent recovery only of the A and D colony types.

DISCUSSION

P. aeruginosa has become a common cause of nosocomial infections (17) and a major etiological agent of otitis externa among swimmers (12). These findings demonstrate the need for a rapid, sensitive, and accurate method for de-

tecting this species from various environments (7). Much of the emphasis has been focused on the development of highly selective or differential media (7). Most of these are designed to utilize or to promote some of the unique characteristics of the organism, including resistance to antimicrobial agents, colonial morphology, pigmentation, fluorescence, ability to grow at 42 C, and ability to oxidize gluconate with the development of slime (5). None has gained accept-

TABLE 3. *Effect of incubation temperature on the growth and detection of P. fluorescens and P. putida*

Test	*Pseudomonas fluorescens*										*Pseudomonas putida*									
	K129	K170	K175	K864	K958	1654	1655	1670	1673	1675	VG	K89	K306C	K331	K355	K466	1544	1572	1638	1669	1671
Growth on blood agar:																					
At RT[a]	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
At 37 C	+	+	+	−[c]	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−
Growth on MacConkey agar:																					
At RT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
At 37 C	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−
Fluorescence on MacConkey agar:																					
At RT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−
At 37 C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at 42 C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] RT, Room temperature.
[b] +, Culture demonstrates characteristic.
[c] −, Culture does not demonstrate characteristic.

TABLE 4. *Selected biochemical characteristics of Pseudomonas aeruginosa strains isolated from different environments*

Test[a]	Positive cultures					
	Clinical (50)[b]		Hospital (50)		Swimming pool (50)	
	No.	%	No.	%	No.	%
Fluorescence on MacConkey agar	50	100	50	100	50	100
Oxidase	50	100	50	100	50	100
Growth ca. 42 C	50	100	50	100	50	100
Reduction of triphenyltetrazolium chloride	43	86	47	94	41	82
Gluconate oxidation	45	90	47	94	46	92
Casein hydrolysis	47	94	47	94	19	38
Pigment on Sabouraud maltose agar	43	86	47	94	18	36

[a] All tests conducted at 37 C, unless otherwise indicated.
[b] Number of strains in parentheses.

TABLE 5. *Colonial types of Pseudomonas aeruginosa on blood agar after 24 h of incubation at 37 C*

Type	Size (mm)	Shape	Elevation	Surface	Edge	Hemolysis	Odor	Iridescence
A	1-2	Circular	Raised	Smooth, shiny	Entire	+	+	−
B	2-3	Circular	Raised	Smooth, shiny	Entire or lobate	−	−	−
C	≤0.5	Circular	Raised	Smooth, shiny	Entire	+ or −	−	+
D	2-3	Irregular	Effuse	Smooth, matte	Fimbriate	+	+	+
E	1-3	Mucoid	Flat	Smooth, shiny	Irregular	−	−	−

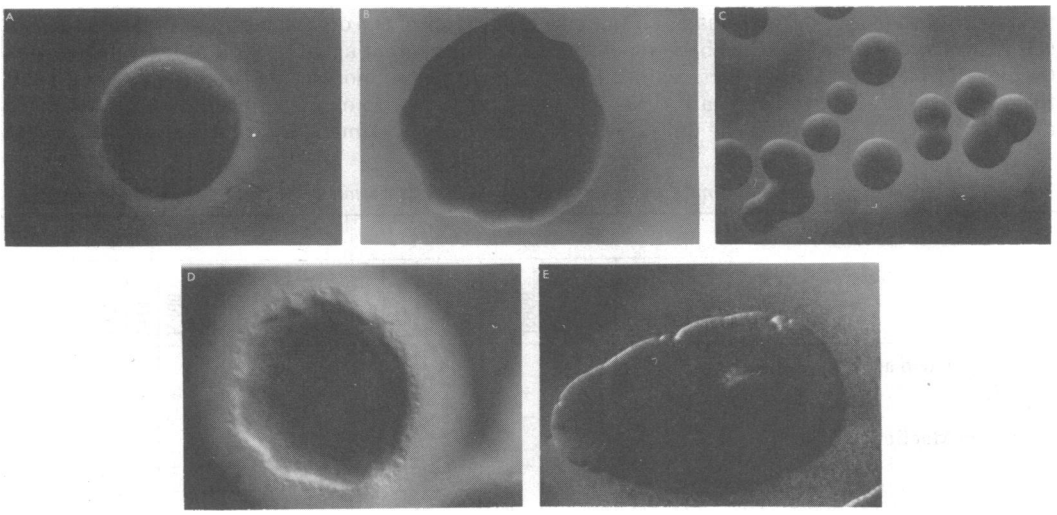

FIG. 1. *Colonial types A, B, C, D, and E of P. aeruginosa on blood agar after 24 h of incubation at 37 C.*

TABLE 6. *Relationship between colonial morphology and pigmentation of 75 Pseudomonas aeruginosa isolates from swimming pools*

Colony types on blood agar	No. of cultures with specific colonial type[a]	Pigment produced on Sabouraud maltose agar	
		No.	%
A	18	12	66
B	37	3	8
C	11	3	27
D	10	7	70
E	2	1	50

[a] Three cultures were found to produce multiple colonial types.

TABLE 7. *Comparative enumeration of Pseudomonas aeruginosa isolates by membrane filtration using MF-MacConkey agar, Tech Agar, and mPA agar*

Culture	Colony type	No. of cells/ml as determined on:		
		MF-MacConkey	Tech	mPA
1	A	3×10^9	1×10^9	4×10^8
2	E	2×10^8	3×10^8	No growth
3	B	6×10^6	4×10^7	No growth
4	C	2×10^7	1×10^7	No growth
5	D	4×10^7	1×10^8	No growth
6	B	3×10^6	3×10^6	No growth
7	D	7×10^8	1×10^9	No growth
8	D	3×10^9	5×10^9	5×10^9
9	E	4×10^8	2×10^9	No growth
10	A	3×10^9	3×10^9	3×10^9

ance for routine quantitative procedures. Either the media were too selective, thus requiring heavy inocula to initiate growth, or they were not sufficiently selective, therefore requiring biochemical confirmation of suspicious colonies (7). Although MacConkey agar shows promise as a quantitative technique, further studies are needed to compare the viability of *P. aeruginosa* in it and efficacious nonselective media.

MacConkey agar (6), a differential plating medium for gram-negative bacilli, is used routinely in many bacteriology laboratories. The observation that *P. aeruginosa* fluoresces on MacConkey agar (3) adds new potential to the routine use of this medium. Our results show that the incubation temperature is most important for differential detection of *P. aeruginosa* by fluorescence. Only *P. aeruginosa* fluoresced on MacConkey agar after incubation at 37 C; however, known strains of *P. fluorescens* and *P. putida* also fluoresced on the MacConkey medium if incubated at room temperature. The number of *P. aeruginosa* strains which do not fluoresce on MacConkey agar is unknown. In our preliminary investigation (3), we found only one *P. aeruginosa* out of 198 nonfluorescing cultures (<1%). On other media designed to enhance fluorescence, it has been estimated that 3 to 10% of the strains were negative (5, 11).

The observation that single colonies fluoresced led to the adaptation of the MacConkey agar fluorescent technique to the enumeration of *P. aeruginosa* from swimming pools by membrane filtration. The use of black membrane filters allowed the blue-green fluorescent colonies to stand out more brilliantly under the UV light when observed from above. In addition, when the cultures were viewed from the underside of the filter, clear fluorescent zones were apparent immediately under the colonies. This added feature was particularly helpful for quantitation from heavily contaminated specimens,

or when fluorescence of the colony was in doubt. The selectivity of this MF technique was improved by incubating the MF MacConkey plates at 42 C (15). Although this higher incubation temperature reduced background flora, it also suppressed the fluorescence of some *P. aeruginosa* isolates. Rapid recovery of the fluorescence occurred when such cultures were allowed to stand for 1 h at room temperature.

In comparison with other suggested screening techniques for *P. aeruginosa*, the fluorescent method appears to be the most reliable (13, 16), especially for isolations from swimming pools. Our results (Tables 1, 7) show that the use of MF MacConkey agar is as accurate as Tech agar and less cumbersome than Drake pseudomonas broth for the enumeration of *P. aeruginosa* in swimming pools. Many strains of *P. aeruginosa* are reported to be apyocyanogenic (13, 16). Sixty-six percent of our *P. aeruginosa* isolates from swimming pools failed to produce pigment on SMA (Table 4). All our isolates fluoresced on MacConkey agar on primary isolation. Lambe and Stewart (14) report that some *P. aeruginosa* cultures may not produce pigment on primary isolation.

The fluorescence was confined to the colony on the black filter and did not diffuse into the surrounding medium. As illustrated in Table 2, this feature makes quantitation by fluorescence possible when large numbers of colonies are present. In addition, single colonies fluoresced, whereas they may not be pigmented.

We observed a multiplicity of colonial types produced by *P. aeruginosa* (Table 5) and the erratic production of pigment by these isolates (Table 6). Levin and Cabelli reported complete agreement between a specific colonial type observed on their isolation medium and pigment production by these isolates when verified as *P. aeruginosa* on SMA. Our results (Tables 6, 7) suggest that the specific colonial type reported by Levin and Cabelli corresponds to our A and D colony types of *P. aeruginosa*. It would seem, then, that the application of either the Drake modified MF technique or the mPA technique of Levin and Cabelli to the enumeration of *P. aeruginosa* in swimming pools may produce conclusions that would not be entirely valid.

The inherent qualities of MacConkey agar make it a suitable medium for use in qualitative and, perhaps, in a quantitative screening technique for the detection of *P. aeruginosa* by fluorescence. Essentially all strains of the organism grow well on it (11, 16). The peptone in the medium, proteose-peptone, has been shown to be important for the development of fluorescence (5). It has no natural fluorescence under the UV light to interfere with the detection of true fluorescing colonies. Furthermore, since MacConkey agar is used routinely, it would be more economical to use it for an added test than to use an additional medium.

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