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Comparison of Two Culture Media for Selective Isolation and Membrane Filter Enumeration of *Pseudomonas aeruginosa* in water

ANNA TSORAEVA,* AND CLAUDIO RODRÍGUEZ MARTÍNEZ

Centro Nacional de Biopreparados, Apartado Postal 6048, Habana 6, CP 32600, Cuba.
Tel. 81-7024, 84-7133, Fax (537) 33-8439, E-mail: claudio@biocen.colombus.cu
*Corresponding author

ABSTRACT. *Pseudomonas aeruginosa* is generally considered to be an ubiquitous bacteria of water and soils, nevertheless in recent years particular attention has been paid to this microorganism because of its increasing significance as a human pathogen and its high resistance to most antibiotics. Nowadays, there are a variety of methods for the detection of *P. aeruginosa* using different culture media. The aim of this work was to compare the recovery of *P. aeruginosa* and the selectivity of a synthetic and an undefined culture media (m-Acetamide and m-Cetrimide) by membrane filtration method. Diluted suspensions (10^{-6}) of test bacterial cultures (ATCC collection) and simulated tap water samples (inoculated with highly diluted suspension of *P. aeruginosa* ATCC 27853) were analysed. The statistical analysis of simulated water samples data showed a significant difference ($p < 0.05$) in the enumeration of *P. aeruginosa* from water samples tested in m-Cetrimide and in m-Acetamide media, in favour of the latter. Overmore, m-Acetamide medium showed to be more selective and inhibitory for microorganisms other than *P. aeruginosa.* Sensitivity values were 96 and 100%, specificity values 95 and 100% and mean relative recovery frequency values 66.2 and 95.76 % for m-Cetrimide and m-Acetamide media, respectively. On the other hand, the advantage of m-Cetrimide medium was the faster growth of this microorganism.
Key words: *Pseudomonas aeruginosa*, culture medium.

RESUMEN. Aunque *Pseudomonas aeruginosa* se considera un integrante de la microbiota normal en suelos y agua, últimamente se le está prestando el interés particular debido a su creciente importancia como patógeno humano con alta resistencia a antibióticos. Actualmente existe una variedad de técnicas de detección de *P. aeruginosa* con empleo de diferentes medios de cultivo. El objetivo del trabajo fue comparar la efectividad, en cuanto a selectividad y recuperación de *P. aeruginosa,* de un medio sintético (m-Medio Acetamida) y otro indefinido (m- Medio cetrimida) mediante el método de filtración por membrana. Se realizó la evaluación microbiológica con altas diluciones (10^{-6}) de cepas testigos (colección ATCC) y con muestras de agua simuladas (inoculadas con *P. aeruginosa* ATCC 27853 en una concentración baja). El análisis estadístico ($p < 0.05$) de los resultados obtenidos demostró que el m-Medio acetamida es superior en cuanto recuperación de *P. aeruginosa* y es más selectivo ya que no permite el desarrollo de la microbiota acompañante. La sensibilidad del m-Medio cetrimida es de 96% y del m-Medio acetamida es de 100%, mientras que la especificidad es de 95 y 100%, respectivamente. Por otra parte los promedios de la frecuencia de recuperación relativa son de 66.2 y 95.76 %. Sin embargo, el m-Medio cetrimida lo aventaja en cuanto a la rapidez de desarrollo de ese microorganismo.
Palabras clave: *Pseudomonas aeruginosa*, medio de cultivo.

INTRODUCTION

The members of the specie *Pseudomonas aeruginosa* are very common in nature and can be isolated from a large variety of natural sources. It occurs in animal faeces, in feed, drinking water and also foodstuffs.[10,14,16] A number of strains are notorious for their nutritional versatility towards organic low molecular weight compounds in media totally devoid of organic growth factors. *Pseudomonas* are able to multiply on a wide range of substrates and may proliferate by utilising nutrients derived fom unsuitable materials used in the construction of water distribution systems and domestic plumbing installations.[10,12,14]

According to data published by Byrd and collaborators, *Pseudomonas* spp., compared with other Gram-negative bacteria, appear to possess the ability to remain viable and culturable, for unusually long periods of exposition to adverse conditions.[5] In recent years particular attention has been paid to this microorganism because of its increasing significance as a human pathogen and its high resistance to most antibiotics.

Examination of *P. aeruginosa* in most types of water

samples for is not recommended as a routine procedure. It is, however, of value in certain situations, namely: in the examination of bottled and mineral waters, water used in drinking vending machines, hydrotherapy, spa pool waters and swimming pool waters.[1,6,9,14,16,17]

Standardised media used for culturing *P. aeruginosa* usually contain potent inhibitory substances which not only suppress the competing bacterial biota, but exert an adverse effect on the bacteria to be isolated and/or enumerated, too.[2,6,9,14,16]

In 1989 Szita and Biro proposed a synthetic medium, free of inhibitory substances, for the isolation and enumeration of *P. aeruginosa.* In this culture medium the acetamide is the sole nitrogen and carbon source.[3,12,16]

Most bacteria are unable to utilise this organic compound while *P. aeruginosa* can split acetamide to ammonia and acetic acid, and thus allows to obtain of the nutrients required for its own growth. Szita and Biro, in a comparative study with raw milk samples in Z agar (with acetamide) and Cetrimide agar showed that the synthetic medium was more sensitive and selective.[16]

Actually, the Acetamide broth and agar are recommended for the confirmation test to detect *P. aeruginosa* in water samples. However, there are no antecedents of employment of these culture media for the isolation and enumeration of this organism in waters.

The aim of this work was to compare the recovery of *P. aeruginosa* and the selectivity of the synthetic and the undefined culture media (m-Acetamide and m-Cetrimide) by membrane filtration method.

MATERIALS AND METHODS

Preparation of culture media. The m-Cetrimide medium has the following composition: gelatine peptone (40.0 g/l); magnesium chloride (2.8 g/l); potassium sulphate (20.0 g/l); cetrimide (0.6 g/l); glycerol (10 ml/l). The m-Acetamide medium contains the following ingredients: acetamide (20.0 g/l); sodium chloride (10.0 g/l); potassium basic phosphate (2.78 g/l); potassium acid phosphate - (1.46 g/l); magnesium sulphate heptahydrate (1.0 g/l); phenol red (0.024g/l).[2]

Sterile cellulose absorbent pads (50 mm, Sartorius, Germany) were placed into the sterile polystyrene Petri dishes (54 mm, Nunc, Mexico) as a solid support for the membrane. 4 ml of the prepared membrane filtration culture media were dispensed aseptically into the Petri dishes and were used immediately.[11]

Triptone soy agar (Biokar, France) and *Pseudomonas* P agar (Merck, Germany) were used in this study as reference media and were dispensed in sterile glass Petri dishes (90 mm, Duran, Germany).

Samples. The bacterial strains included in this study consisted of *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 9027, *Escherichia* coli ATCC 25922, *Proteus mirabilis* ATCC 12453, *P. mirabilis* ATCC 7002, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, *Shigella flexneri* ATCC 12022 and *Enterobacter aerogenes* ATCC 13048. The spikes of the microorganisms were prepared by first culturing the reference microorganisms on Triptone soy agar slants (BioCen, Cuba). A sample of the growth from the prepared slants was added to a test tube with sterile 0.85 % (wt/vol) sodium chloride (NaCl, analytical grade; Merck, Germany), and the suspension was mixed thoroughly on a vortex mixer. Bacterial cell density was adjusted with additional saline to 50% transmittance at 580 nm (spectrophotometer PU 8620, PHILIPS) representing approximately 3.0×10^8 cells per ml. Decimal dilutions (10^{-6} for *P. aeruginosa* and 10^{-1} for other test microorganisms) of the suspensions were inoculated in culture media in order to determine their selectivity.

Thirty samples of tap water were taken during a month from five different sites of the water distribution system of the National Center of Biologicals (BioCen). *P. aeruginosa* detection was carried out on 100 ml of sample water divided into two equal volumes, each to be tested in one of the two culture media. Five samples were collected from the sites A, B, C and D and ten samples from the site E. The samples were spiked with 0.2 ml of 10^6 fold diluted suspensions of target microorganism (*Pseudomonas aeruginosa* ATCC 27853 standardised to 50 % transmittance) to prepare the simulated water samples.[7]

Two methods were used to inoculate the different culture media: the spread plate method[13,15] for reference culture media and the membrane filtration method[13,18] for test media. Each of the simulated water samples and the test strains spikes was filtered through a sterile 0.45 µm pore-size, white, gridded, cellulose nitrate membrane (50 mm; Sartorius, Germany). The membrane was then rinsed with 20 ml of sterile water and transferred to a absorbent pad saturated with m-Cetrimide or m-Acetamide media. The dishes were incubated at 37 ± 1 °C for 48 ± 2 h. At least five of each morphotype of suspect colonies were randomly selected from each Petri dish. The colonies were isolated and identified using the following tests: catalase, oxidase, mobility, glucose, lactose and sucrose oxidation/fermentation, arginine hydrolysis, pigment production in *Pseudomonas* P agar and gelatine hydrolysis.

RESULTS

Results processing. Growth on agar plates and membranes was monitored by counting the colonies formed (CFU) after an incubation period.

Statistical analyses were performed applying the Student's t test for paired data ($p < 0.05$) in order to compare the differences between the mean values of three CFU count replicates. The software used for the calculation was STATISTICA (version 4.2; Statsoft, Inc. 1993).

To evaluate the selectivity the Relative Growth Index (RGI) was calculated:

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$$\text{RGI} = \frac{\text{Test medium colony count (CFU)}}{\text{Control medium colony count (CFU)}} \times 100$$

The aim of the accuracy analyses was the detection and enumeration of a previously defined (group of) bacterial species, the target organism (s). All microbiological methods for water analysis were based on the use of selected cultural conditions which should allow the growth of the target organisms, while suppressing growth of all other organisms.

Normally, not all non-target organisms are completely inhibited. Therefore, specific additional reactions should lead to the unequivocal identification of colonies generated by target organism.

To evaluate the differential characteristics of the culture media the sensitivity (I) and specificity (E) was determined:

$$I = Tt / (Tt + Tn) \times 100\%$$

$$E = Nn / (Nt + Nn) \times 100\%$$

where Tt, typical colonies of target organisms (confirmed as *P. aeruginosa*); Tn, non-typical colonies of target organisms (confirmed as *P. aeruginosa*); Nt, typical colonies of non-target organisms (unconfirmed as *P. aeruginosa*); Nn, non-typical colonies of non-target organisms (unconfirmed as *P. aeruginosa*); Ct, total of typical colonies; Cn, total of non-typical colonies.[7]

The last calculated parameter (mean relative recovery frequency) is the mean of the percentage ratios, calculated for each water sample, between the number of *P. aeruginosa* grown on each medium and the highest number of this microorganism found in the sample with one of the two methods.[4]

$$\text{MRRF} = \frac{\Sigma(\text{M}Pa\text{ CFU/S}Pa\text{ CFU})}{\text{NS}}$$

Where MRRF, Mean relative recovery frequency; M*Pa*CFU, No. of *P. aeruginosa* colonies isolated with each medium; S*Pa*CFU, Maximum no. of *P. aeruginosa* colonies found in each sample; NS, No. of samples

RESULTS AND DISCUSSION

It was observed a moderate growth of *E. coli, E. aerogenes* and *S. typhimurium* in the m-Cetrimide medium (RGI < 10% in relation to the Triptone soy agar) while *S. aureus, P. mirabilis* and *S. flexneri* were inhibited (RGI < 0.001%). However in the m-Acetamide medium these microorganisms were totally inhibited in the 10^{-1} dilution.

These results confirm that to achieve enough inhibition in the m-Cetrimide medium, it is necessary to add different inhibitors, including antibiotics, such as nalidixic acid or fucidin and cephaloridine.[9,14] The relative growth indexes of P. *aeruginosa ATCC 27853* and *ATCC 9027* in respect to the traditional culture medium (*Pseudomonas* P agar) were: 79% and 67% for the m-Cetrimide medium and 93% and 76% for the m-Acetamide medium, respectively.

The average levels of P. *aeruginosa* ATCC 27853 counts in the m-Cetrimide medium, m-Acetamide medium and *Pseudomonas* P agar were 68.67 ± 5.51, 80.66 ± 1.53 and 86.5 ± 19.09, respectively. In case of *P. aeruginosa* ATCC 9027 the values were 80.00 ± 19.08, 90.00 ± 21.63 and 118.00 ± 7.07 (Fig. 1). More colonies grew out on the synthetic medium in comparison to the undefined one; this difference, however, was not statistically significant.

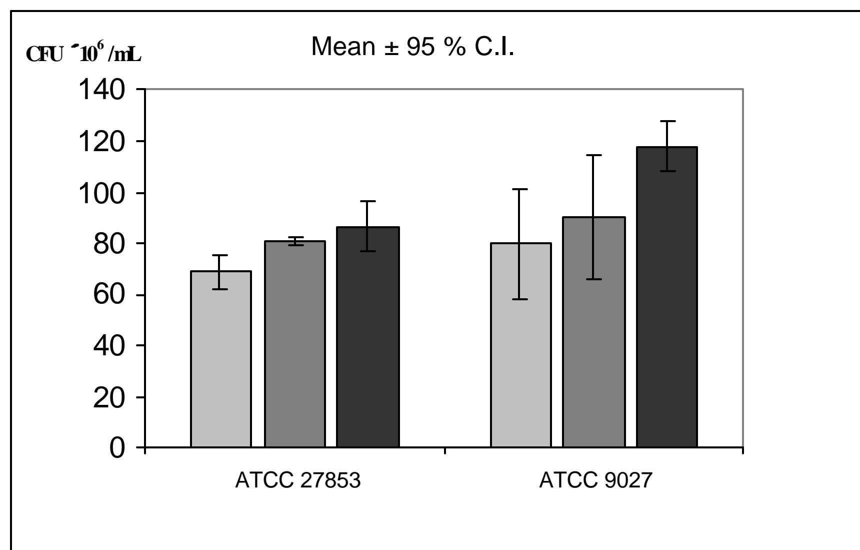

Fig. 1. Colony counts obtained with tenfold diluted suspensions *of Pseudomonas aeruginosa* ATCC 27853 and ATCC 9027 in different culture media.

Table 1. Comparison of the total colony count results in the m-Cetrimide and m-Acetamide media.

Site of sample	m-Cetrimide medium	m-Acetamide medium	Sign. dif.				
	No. of samples	Mean, CFU/50 ml	Standard deviation	No. of samples	Mean, CFU/50 ml	Standard deviation	p< 0.05
A	5	84.8	14.25	5	86.8	14.75	-
B	5	79.0	12.32	5	125.4	22.88	+
C	5	79.8	14.65	5	68.2	13.59	-
D	5	60.6	19.32	5	25.8	16.29	+
E	10	102.1	14.41	10	109.2	15.66	-
Total	30	84.73	20.22	30	87.46	36.97	-

High densities of non-target organisms on membranes may strongly affect the results of a microbiological analysis. This may be related to crowding effects, competition for nutrients, production of inhibitory substances, or neutralisation of pH changes by target organisms. Several authors have described the inhibitory effect of background organisms on coliform counts by membrane filtration.[6]

The possible negative effects of background microbiota can be quantitatively assessed by spiking the tap water samples with standardised suspensions of target organism (*P. aeruginosa* ATCC 27853).

The total colony counts for the simulated water samples is shown in the Table 1.

In general, there are no significant differences (p <0.05) among the total CFU counts in the two culture media. However all the colonies developed in the m-Acetamide medium were identified as *P. aeruginosa* with the help of additional biochemical tests, while in the m-Cetrimide medium the quantity of colonies of this microorganism reached only an average of 60% of the total colony number.

The CFU counts of *P. aeruginosa* from the same samples of water is shown in the Fig. 2. Comparison of results showed that mean CFU counts of the samples from A, B and E sites in the m-Acetamide medium were significantly higher, as shown by 95 % confidence intervals. These data showed that there was a superiority in favour of the synthetic culture medium when it is spiked with the tap water samples, and this fact may be due to a higher selectivity of the m-Acetamide medium. Media used for the microbiological examination of water varies, and some may not be adequate, especially if the heterotrophic bacteria are numerous in the sample.

Sensitivity of a method defines how far all target organisms that are able to develop colonies under the test conditions, do indeed give characteristic reactions, whereas the specificity describes the extent to which colonies of non-target organisms do not exhibit these properties.[8]

The specific colony characteristics are: blue colour for colonies of *P. aeruginosa* in m-Cetrimide medium and pink colonies in m-Acetamide medium.

In m-Cetrimide medium after 24 h of incubation at 37° C three types of blue colonies were observed: 898 opaque colonies of intense blue colour, 36 colonies of the same type, but with the yellowish centre and 29 transparent clear blue colonies (Table 2). The biochemical tests confirmed

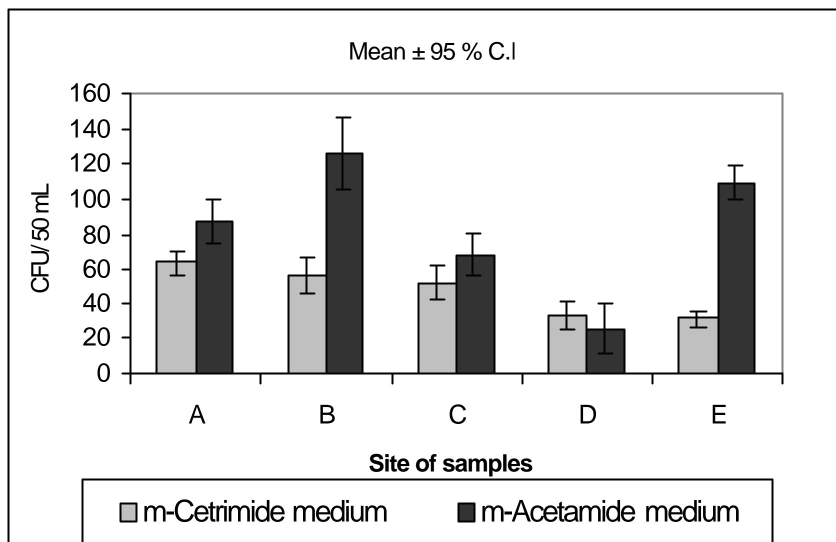

Fig. 2. Recovery of *P. aeruginosa* from simulated tap water samples in m-Cetrimide and m-Acetamide media.

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Table 2. Evaluation of the differential characteristics of the m-Cetrimide and m-Acetamide media

	Typical colonies	Non-typical colonies	All colonies
	m-Cetrimide medium		
Target organisms	898	36	934
Non-target organisms	29	558	587
All organisms	927	594	1521
	m-Acetamide medium		
Target organisms	1532	-	1532
Non-target organisms	-	-	-
All organisms	1532	-	1532

Table 3. Efficiency of methods (m-Cetrimide and m-Acetamide) for recovery from tap water samples (n = 30).

	CFU/ 50 ml of *P. aeruginosa*		Mean relative recovery frequency, %
	Mean	95 % C.I.	
m-Cetrimide medium	44.66	38.85 – 50.49	66.20
m-Acetamide medium	87.47	73.66 – 101.27	95.76

that only the first two types of colonies corresponded to *P. aeruginosa*.

In m-Acetamide medium colonies of pink colour of different sizes were observed after 36 h of incubation. The big colonies emitted greenish fluorescence under the UV light (364 nm). The results of the additional set of tests allowed to corroborate that all the observed colonies were of *P. aeruginosa*. However, the small colonies in the *Pseudomonas* P agar acquired a blue colour and the big colonies - white. This observation agrees with the discovery of Manaia and collaborators in relation to the existence of non-pigmented *P. aeruginosa* strains that can be missidentified.[9] Appearance of the two colony types in the spiked water samples may be explained by biofilm forming in the water distribution system.

The culture medium that is used thoroughly in Cuba for water quality assurance in biotechnological and pharmaceuthic industries is Cetrimide medium. Care must be taken to confirm the strains which grow on selective media but lack pigmentation.

There were observed several important biochemical reactions to confirm *P. aeruginosa* simultaneously in m-Acetamide medium: the oxidase reaction (because only oxidative bacteria can use acetate as a sole carbon source), the acetamide assimilation and fluorescence of green colour under the light of 364 nm.

The sensibility of the m-Cetrimide medium was 96% and of the m-Acetamide medium - 100%, while the specificity was of 95 and 100%, respectively.

The next table (Table 3) shows the mean *P. aeruginosa* CFU counts per 50 ml obtained with the two media and their capacity to recover the highest number of cells per sample. The average level of this microorganism in the m-Acetamide medium is approximately twice of that in the m-Cetrimide medium. Moreover, a comparison between the relative recovery percentages shows that the first medium shows up the highest levels of target microorganism more often. These results demonstrate that the m-Acetamide medium estimates better the quantity of *P. aeruginosa* cells in water samples.

The appearance of visible colonies occurs later in the m-Acetamide, but being more selective and specific medium it shows advantages in comparison with other media, due to avoiding numerous additional biochemical tests to confirm the obtained results.

It was demonstrated that the m-Acetamide medium (developed at laboratory scale for the membrane filtration method) have a higher selectivity in comparison with the other media tested.

The results of enumeration of *P. aeruginosa*, spiked tap water samples with the test media, indicated that the highest bacterial colony counts were achieved by using the synthetic medium.

The development of visible colonies of this microorganism was faster in the m-Cetrimide medium.

However, the overall results (sensibility, specificity and mean relative recovery frequency) indicated that the m-Acetamide medium offers a greater guarantee of efficiency than the other method tested for *P. aeruginosa* detection in tap water.

Taking in account results obtained at laboratory scale it could be considered that m-Acetamide medium was more appropriate for the detection of *P. aeruginosa* in water.

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