Modified Pseudomonas agar: new differential medium for the detection/enumeration of *Pseudomonas aeruginosa* in mineral water

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Keywords: Cetrimide; Mineral water; Nalidixic acid; *P. aeruginosa*; Pyocyanin

Abstract

Pseudomonas aeruginosa has been implicated as a foodborne and waterborne pathogen and is now considered a primary infectious agent. In the present study, the survival of *P. aeruginosa* inoculated in mineral water was evaluated by drop counts on Pseudomonas Agar Base (PAB), PAB with CN supplement X107, PAB with cetrimide, PAB with nalidixic acid, and these media with added $FeSO_4$. Initial counts, before starvation, were the same in all media tested. Following this period, *P. aeruginosa* became sensitive to PAB with added cetrimide. The addition of $FeSO_4$ did not improve the recovery of stressed *P. aeruginosa* but gave colonies a typical dark brown colour being easily differentiated from other species that can grow at 42 °C. The modified Pseudomonas agar medium was also tested with several *P. aeruginosa* strains, other species of *Pseudomonas*, and other genera. Only *P. aeruginosa* strains (pyocyanin positive) produced the typical colonies. Our results demonstrate that Pseudomonas agar with ferrous sulphate, used for the differentiation of *P. aeruginosa* colonies, and nalidixic acid, used as an inhibitor of Gram-positive bacteria, might be a useful medium for the detection of injured *P. aeruginosa* in mineral water.

1. Introduction

Pseudomonas aeruginosa, as opportunistic bacteria, expresses virulence factors which are related to serious infections in humans, especially in immunocompromised individuals, and special precautions may be required to limit the exposure of these susceptible populations (Stiles, 1989, cited in Warburton et al., 1994a,b). It is widespread in natural and industrial environments and is able to grow in water (Vachée and Leclerc, 1995).

Contaminated water has frequently been described as the vehicle of infection (Havelaar et al., 1985). *P. aeruginosa* has been found in some mineral water in various countries, namely Brazil, Canada, France, Germany, Spain, the United States, and others (Hunter, 1993; Warburton et al., 1994a,b). Given the possibility of *P. aeruginosa* being a contaminant of bottled water, batches or brands of bottled water in which these organisms have been detected have been withdrawn from retail outlets in Europe (Manaia et al., 1990).

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Examination of drinking water for *P. aeruginosa* is not recommended as a routine procedure, but it is an indicator of good manufacturing processes and suitability for drinking of bottled water (Warburton et al., 1994a,b). According to the European Directive for mineral water (Anonymous, 1980), a natural mineral water shall be free of *P. aeruginosa* in any 250-ml sample.

Conventional methods for the detection of *P. aeruginosa* use cetrimide as a selective agent. Pseudomonas Agar Base (PAB) medium is a modification of King's A medium (King et al., 1954) which uses magnesium and potassium salts to enhance production of the pigment pyocyanin. The addition of the supplement (200 mg/l cetrimide, 15 mg/l nalidixic acid) makes the medium selective for *P. aeruginosa* (Goto and Enomoto, 1970). Pathogenic bacteria in water may be injured by exposure to sub-optimal temperature, salinity or toxic chemicals, or by starvation (McKay, 1992). Injury may result in an increased sensitivity to selective agents such as antibiotics and surface active agents (Ray and Johnson, 1984), and cells may also develop a requirement for some specific compounds for repair and subsequent growth. The use of selective media for the detection/enumeration of pathogens under stress conditions, such as those impaired by the aquatic environment, may therefore lead to their underestimation, which might represent, in some cases, a serious hazard to the public health.

In this study, a modified version of Pseudomonas agar was developed to improve *P. aeruginosa* recovery from mineral water. The performance of the new medium was also evaluated.

2. Materials and methods

2.1. Starvation experiments

2.1.1. Water samples

The mineral water used was collected at a still mineral water bottling plant in Portugal.

Sterilized glass bottles (Schott, Duran, Germany; 100 ml) containing filtered (0.2-μm pore size Nuclepore filters) and heated (60 °C; 20 min) mineral water (sterile water) were used (Colbourne et al., 1988; Moreira et al., 1994). Heating mineral water after filtration was necessary in order to obtain sterile water, since without that step, sterilisation could not be achieved due to the existence of autochthonous bacteria that could pass through 0.2-μm pore size membrane filters (Jones et al., 1999). Sterilization of the water was needed since modified media were to be used and it was necessary to ensure that only *P. aeruginosa* would be counted.

2.1.2. Bacterial strains and growth conditions

A loop of *P. aeruginosa* ATCC 10145 and *P. aeruginosa* ATCC 27853 was inoculated separately into 20 ml of Tryptic Soy Broth (Lab M, Bury, England) and incubated at 37 °C for 24 h. They were transferred to fresh TSB (20 ml) at a final concentration of 1% (v/v) and incubated as above. Each culture was harvested by centrifugation ($3000 \times g$ for 10 min; PSELECTA, CENTROMIX) at ambient temperature, the supernatant fluid discarded, and the pellets resuspended in 20 ml of sterile mineral water. This washing procedure was repeated three times with sterile mineral water before inoculation into glass bottles.

Four hundred microliters of cellular suspension was inoculated into sterilized glass bottles (Schott) containing 100 ml of sterile mineral water and shaken vigorously to mix.

The bottles were aseptically sampled immediately after the addition of the suspension and at regular intervals during storage at room temperature (ca. 20 °C). Bottles were wrapped in aluminium foil to exclude light.

Samples were serially diluted in Maximal Recovery Diluent (LAB M) and viable counts were determined by the drop count technique (Miles and Misra, 1938) on Pseudomonas Agar Base (PAB; LAB M), PAB with CN supplement X107 (200 mg/l cetrimide; 15 mg/l nalidixic acid) (LAB M), PAB with cetrimide (200 mg/l), PAB with nalidixic acid (15 mg/l) (Sigma, St. Louis, USA), PAB with added $FeSO_4$ (0.05% w/v) (Sigma), PAB with CN supplement and $FeSO_4$ (0.05% w/v), PAB with cetrimide (200 mg/l) and $FeSO_4$ (0.05% w/v), and PAB with nalidixic acid (15 mg/l) and $FeSO_4$ (0.05% w/v). All plates were incubated at 41.5 °C for 24 and 48 h. Since autoclaving oxidizes Fe^{2+} to Fe^{3+}, $FeSO_4$ was added to PAB as a filter-sterilized solution (Rayman et al., 1978).

Presented data are mean values obtained from three independent experiments. The error bars on the figures

indicate the mean standard deviations for the data points.

2.2. Measures of performance of the new medium

2.2.1. Specificity

2.2.1.1. P. aeruginosa strains. In total, 43 *P. aeruginosa* strains were tested. *P. aeruginosa* 1688, 1718, 1728, 1742, 1801, 1802, 1916, 1919, 2280, 2282, 2283, 2284 and 2285 were randomly chosen from the collection of cultures of the University of Surrey (UK). One *P. aeruginosa* was supplied from Serviços Microbiologia da Escola Superior de Biotecnologia, Porto (Portugal); two strains of *P. aeruginosa* were supplied by Laboratório Nacional de Investigaç ão Veterinária (LNIV) do Porto (Portugal); *P. aeruginosa* ATCC 10145 (1144), *P. aeruginosa* ATCC 27853 (1143), *P. aeruginosa* LMG 1242^t, and *P. aeruginosa* isolated from a clinical sample were supplied by the Departamento de Microbiologia da Universidade de Coimbra (Portugal); *P. aeruginosa* ESB1, NE44, NE45, NE46, NE47, NE49, NE50, NE51, NE52, NE53, NE54, NE55, NE56, NE63, NE64, NE65, NE67, NE68, NE69, NE70, NE71, NE73, NE75, and NE77 were supplied from the culture collection of Escola Superior de Biotecnologia.

2.2.1.2. Other Pseudomonas spp. *P. fluorescens* ATCC 13225, *P. putida* LMG 2257 (*Arthrobacter sidepsulatus*) were supplied by the Departamento de Microbiologia da Universidade de Coimbra; *P. oleovarans* LMG 2229^t, *P. alcaligenes* DSM 50342^t, *P. pseudoalcaligenes* DSM 50188^t, *P. fragi* LMG 2191^t, *P. mendocina* LMG 1223^t, *P. stutrezi* LMG 11199^t were supplied from the cultures collection of Escola Superior de Biotecnologia.

2.2.1.3. Other microorganisms. *Salmonella typhimurium* 7738/32, *Yersinia enterocolitica* Ger 0:3 P-NS+, *Escherichia coli* NCTC 9001 were obtained from Leatherhead Food Research Association at Leatherhead (UK); *Staphylococcus aureus* NCTC 08532 was supplied by Serviços de Microbiologia da Escola Superior de Biotecnologia, *Bacillus cereus*, *Streptococcus faecalis*, *Proteus vulgaris* were supplied from the culture collection of Escola Superior de Biotecnologia.

2.2.1.4. Experimental conditions. A loop of all strains was inoculated into 20 ml of Tryptic Soy Broth and incubated at 37 °C for 24 h. These cultures were transferred to fresh TSB (20 ml) at a final concentration of 1% (v/v) and incubated as previously discussed. They were all streak-plated on Pseudomonas Agar Base and Pseudomonas Agar Base with 0.05% $FeSO_4$ (v/v). Plates were incubated at 37 and 41.5 °C for 24 and 48 h, and the colour of the colonies were recorded.

2.2.1.5. Pyocyanin production. Culture of *P. aeruginosa* were streak-plated on Pseudomonas Agar Base with 0.05% $FeSO_4$ (w/v) and K_2HPO_4 (9 mM) (Merck, Darmstadt, F.R. Germany), as a way to correlate the colour of the colonies with the production or non-production of pyocyanin since K_2HPO_4 supresses its production (Burke et al., 1990). Plates were incubated at 41.5 °C for 24 and 48 h and the coloured colonies were recorded.

Two milliliters of HCl (0.1 M; PRONALAB; Lisboa, Portugal) was added to all colonies of *P. aeruginosa* grown on Pseudomonas Agar Base with 0.05% $FeSO_4$ (w/v) and the appearance on the colonies of a red-purple colour was recorded as a positive indication of the production of pyocyanin (Singleton and Sainsbury, 1997).

2.3. Statistical analysis

Statistical analysis was done with the ANOVA methodology using the Statview^{TM} package (Abacus Concepts, Berkeley, CA) using the starvation time as independent variable. Bacterial counts were converted to $\log_{10}$ cfu/ml.

3. Results and discussion

Enumeration of *P. aeruginosa* ATCC 10145 (1144) and ATCC 27853 (1143) during starvation in mineral water was performed in selective and non-selective media. Similar results were obtained for both strains. Therefore, results presented thereafter are only relative to *P. aerugnosa* 10145.

After inoculation into the mineral water, the counts of *P. aeruginosa* dropped by 2–3 logs, but over 2 days, the counts increased and remained constant for the

Table 1
Viable counts ($\log_{10}$ cfu/ml) of *P. aeruginosa* ATCC 10145 (1144) starved in sterile mineral water

Time (days)	PA		PA + $FeSO_4$		PA + CN		PA + CN + $FeSO_4$		PA + NA		PA + NA + $FeSO_4$		PA + C		PA + C + $FeSO_4$	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	5.4	0	5.5	0	5.4	0	5.5	0	5.5	0	5.3	0	5.3	0	5.4	0
1	3.7	0	3.8	0.17	3.7	0	3.8	0.17	3.7	0	3.7	0	4	0	4.06	0.12
2	2.83	0.15	2.93	0.15	2.53	0.06	2.46	0.45	2.76	0.25	2.46	0.45	2.6	0.26	2.7	0.35
3	3.1	0.04	3.13	0.06	3.2	0	3.06	0.06	3.1	0	2.83	0.06	3.23	0.06	3.26	0.06
4	5.06	0.05	5.1	0	4.4	0	4.3	0	5.07	0.06	5	0	4.46	0.06	4.43	0.06
5	5.46	0.20	5.53	0.23	4.2	0.4	4.23	0.45	5.5	0.2	5.5	0.2	4.5	0.2	4.5	0.17
6	5.83	0.32	5.8	0.2	4.73	0.15	4.66	0.06	5.83	0.25	5.8	0.2	4.76	0.25	4.76	0.21
7	5.83	0.11	5.93	0.15	4.36	0.15	4.33	0.06	5.9	0.1	5.83	0.12	4.9	0.17	4.9	0.17
9	5.86	0.05	5.9	0.1	4.3	0.1	4.3	0	5.9	0.1	5.86	0.06	4.86	0.15	4.9	0.1

Enumeration was made on PAB, PAB with $FeSO_4$, PAB with CN, PAB with CN and $FeSO_4$, PAB with nalidixic acid (NA), PAB with nalidixic acid and $FeSO_4$, PAB with cetrimide (C) and PAB with cetrimide and $FeSO_4$.

duration of the experiment (Table 1). An initial sharp decrease in the number of survivors of *P. aeruginosa* (Moreira et al., 1994) and of other contaminant organisms (Kersters et al., 1996) had already been recorded. This has been attributed to a requirement for a period of physiological adaptation to stress conditions (Matin et al., 1989; Roszak and Colwell, 1987) after which the mortality rate decreases. According to Watson et al. (1998), the maintenance of the viability relatively constant of starved *S. aureus* in water could be attributed to cryptic growth, where most of the cells die, providing nutrients for the survival of the remaining cells.

On the first days of the starvation experiment, no significant differences were observed between counts on the different media. On the subsequent days, the presence of cetrimide decreased counts (Table 1). It was therefore demonstrated that cetrimide was the selective compound responsible for the inhibition of starved cells since lower counts were obtained on all PAB media with cetrimide than on PAB, or PAB with nalidixic acid only.

The differences in viable counts observed during starvation using different recovery media can be safely attributed to sublethal lesions in a large proportion of the population induced by stress factors in water since the initial counts were the same on all media. Cetrimide, a quaternary ammonium compound, apparently acts by disrupting the bacterial cell membrane with a resulting increase in permeability of the membrane. Gram-negative bacteria are generally less susceptible to biocides than Gram-positive species, mainly because the outer membrane acts as a protective barrier. Cetrimide is generally not active against *P. aeruginosa*. In the present study, after starvation, cells become sensitive to this compound probably as a result of outer membrane damage.

These results support the earlier observation that one of the characteristics of injured microbes is loss of resistance to selective agents (Hurst, 1977; Barcina et al., 1997).

Reactive oxygen species such as superoxide and hydrogen peroxide can damage DNA, proteins, and membranes (Foster and Spector, 1995). Organisms can encounter potentially lethal levels of these intermediates from normal aerobic metabolism (Foster and Spector, 1995), or by inactivation of catalase (Rayman et al., 1978). According to Rayman et al. (1978), $FeSO_4$ could be used in plating media to enhance the recovery of stressed microorganisms because it is a compound that is capable of degrading H_2O_2 and it is not toxic to the organisms. However, in our experiments, there were no significant differences between counts in media with and without $FeSO_4$ ($P < 0.0001$). Interestingly, this experiment led to the discovery that in the presence of $FeSO_4$, colonies of *P. aeruginosa* appeared to be dark brown on PAB. In order to verify the possibility of PAB with $FeSO_4$ being used as a new differential medium for the enumeration of *P. aeruginosa*, other various organisms were plated on this medium. As can be observed in Table 1, *P. aeruginosa* strains were the only organisms that gave the characteristic appearance. In this way, Pseudomonas agar without

Table 2
Colour of the colonies of microorganisms grown on PAB with iron, PAB with iron and K_2HPO_4, and when acid was added to dark brown colonies on PAB with iron

	Dark brown colonies on PAB + iron	Dark brown colonies on PAB + iron + K_2HPO_4	Red-purple colonies after addition of acid
P. aeruginosa LMG 1242[t], 1742, 1718	–	–	–
Other 40 *P. aeruginosa*	+	–	+
Other *Pseudomonas* spp.	–	–	–
Other genera	–	–	–

cetrimide with ferrous sulphate might be a new culturing medium that enhances the enumeration of *P. aeruginosa* from water samples. As, during starvation, counts of *P. aeruginosa* on PAB with and without nalidixic acid were the same, nalidixic acid can be added to PAB to improve the inhibition of the accompanying microbial flora (Goto and Enomoto, 1970). The incubation temperature of 42 °C may also increase its selectivity since most autochthonous bacteria of mineral water are psychrotrophic (Mavridou, 1992).

P. aeruginosa produces a number of pigments, two of which are pyocyanin and pyoverdin (Burke et al., 1990). Of all the organisms that were investigated, *P. aeruginosa* strains were the only ones presenting dark brown colonies in the presence of ferrous sulphate (except *P. aeruginosa* LMG 1242[t], 1742, 1718; Table 2), independently of the temperature of incubation and of the media being evaluated, and since pyocyanin is the only factor present in *P. aeruginosa* that is absent in all other microorganisms tested, this pigment is probably responsible for the colonies colour.

In order to determine if the dark brown colour of the colonies was related to the production of pyocyanin, *P. aeruginosa* was spread-plated on PAB with $FeSO_4$ and K_2HPO_4. Phosphate is a crucial nutrient in the regulation of secondary metabolite production, with higher levels inhibiting their formation (Whooley and McLoughlin, 1982). According to Burke et al. (1990), K_2HPO_4 suppresses the production of pyocyanin. In the presence of K_2HPO_4, colonies did not present the dark brown colour. These results demonstrated that pyocyanin was probably responsible for the dark brown colour of colonies when iron was present.

This was reinforced by the fact that when acid (which turns pyocyanin into a red-purple colour) was added to *P. aeruginosa* colonies, no change of colour was observed in the colonies of the three strains that did not produce the characteristic dark brown colour, suggesting that none or low amounts of pyocyanin were present. Only dark brown colonies became red-purple in the presence of acid (Table 2).

Typical dark brown colour might be given by iron stored within the cell. In the presence of iron, pyocyanin can be reduced by *P. aeruginosa* to leucopyocyanin, which in turn can reduce ferric iron into iron-transferrin complexes. To transport iron into cells, the iron-transferrin complex binds to a cell-surface receptor and is internalized via a coated vesicle; the iron is subsequently released within the cell and may be stored as an iron-ferritin complex (Singleton and Sainsbury, 1997).

This study demonstrated that cells of *P. aeruginosa* become injured in mineral water which makes its accurate detection or enumeration unreliable on the selective media commonly used. Pseudomonas Agar Base with added nalidixic acid (15 mg/l) and $FeSO_4$ (0.05%) was proven to be an efficient selective and differential medium for the detection of *P. aeruginosa* (pyocyanin positive) in mineral water.

Acknowledgements

This work was financed by an EEC FAIR project CT-96-1037-'Improving methods and protocols used to insure the microbiological quality of bottled mineral waters'.

Financial support for author Ramalho was provided by a PhD fellowship issued by PRAXIS XXI (JNICT-BD/11496/97).

The authors acknowledge Sociedade das Águas de Luso and Danone Water Technology Center for their collaboration.

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