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Pseudomonas aeruginosa in public water supply

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Abstract

Water is indispensable for life and its quality is directly related to public health. The objective of this study was to investigate the presence of *Pseudomonas aeruginosa* in public water supply in municipalities in São Paulo State, Brazil. Analyses were carried out at the Adolfo Lutz Institute between February and December 2016, and included physicochemical (chlorine, pH, color, turbidity and nitrate), and microbiological parameters (total coliforms and *Escherichia coli),* as well as *P. aeruginosa,* with susceptibility tests to antimicrobial agents and biofilm production capacity by the strains isolated. In total, 251 water samples were evaluated and 19 (7.6%) presented *P. aeruginosa.* No significant differences were observed between the physicochemical parameters found in the positive and negative samples for this bacterium, but the samples containing total coliforms were also those with the highest positivity for *P. aeruginosa.* All samples with *P. aeruginosa* reported chlorine concentrations between 0.2 and 2.0 mg/L, as required by Brazilian legislation, demonstrating this bacterium's resistance to conventional water treatment processes. Although not resistant to the antimicrobials tested, most strains isolated were classified as strong biofilm producers, emphasizing the need for further studies involving water supply quality.

Key words: biofilm, *pseudomonas aeruginosa*, public health, water, water supply

INTRODUCTION

Water is a fundamental resource for life, and its availablity in adequate quantity and quality are worldwide concerns (PAHO 2001).

Conveyance by water of harmful chemicals and biological agents, such as bacteria, viruses, protozoa and helminths, can be responsible for numerous diseases (Santos *et al.* 2013).

In Brazil, water intended for human consumption, and distributed through a system or alternative collective supply, must meet the drinking water standards defined in the MS Consolidation Ordinance No. 5 of 09/28/2017, in order not to offer risks to public health (MS 2017). For microbiological parameters, drinking water quality surveillance is based on determination of total coliforms and *Escherichia coli* (MS 2017), a bacterium indicative of recent fecal contamination, which is an indicator of the possible occurrence of other microorganisms (Silva *et al.* 2005).

Bacteria belonging to the genus *Pseudomonas*, especially *P. aeruginosa*, are distributed very widely (soil, water and decomposing organic matter), and frequently responsible for infections in the urinary

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and respiratory tracts, and of the skin and in the bloodstream, mainly in immunocompromised patients (Kaye & Pogue 2015).

In addition to resistance to several antibiotics (Neves *et al.* 2011), *P. aeruginosa* can metabolize a wide variety of compounds, and proliferate in waters with low concentrations of dissolved compounds, showing its ability to adapt in environments with low nutrient availability (Guerra *et al.* 2006). The species can also produce biofilms – microbial communities comprising sessile, mono- or multi-species cells adhering to surfaces and embedded in a matrix of extracellular polymers (exopolysaccharides) (Burmolle *et al.* 2010). Bacteria are significantly better protected within this matrix from adverse environmental conditions, natural immune defenses, antimicrobial compounds, radiation and dehydration (Bjarnsholt 2013).

P. aerugionosa has been isolated relatively frequently in bacteriological examinations of chlorinated, non-chlorinated and mineral waters (D'Aguila *et al.* 2000; Peil *et al.* 2015). Therefore, in view of its importance and its non-inclusion in routine control analyses for potable water quality monitoring, this study's general objective was to investigate the presence of *P. aerugionosa* in public water supply and its susceptibility to antimicrobial agents, as well as the biofilm production capability of the strains isolated.

METHODS

This study was carried out at the Adolfo Lutz Institute, Bauru Regional Laboratory Center (IAL – CLR Bauru), using water samples from the Water Quality Surveillance Program for Human Consumption (PROÁGUA) from public supply systems, etc, in three municipalities – Dois Córregos, Itapuí and Pirajuí – in the midwest of São Paulo State, Brazil. The three municipalities have estimated populations of 26,706, 13,475 and 24,762, respectively (IBGE 2016). The municipalities' public water supplies are managed by Municipal Sanitation and Supply Services. Dois Córregos draws its supplies from both ground- and surface-water sources, while Itapuí and Pirajuí use only groundwater.

Water samples were collected monthly from February to December 2016 by the Municipal Sanitary Vigilance, following the American Public Health Association (APHA) standards, as described in the Handbook of Collection, Conservation and Transportation of Water Samples of the Sanitary Surveillance Center (CVS 2004). The sampling plan took account of population density, critical and non-critical points, fixed points (places supplying a large number of consumers or susceptible consumers) and variable – i.e., random – points, seeking spatial and temporal representativeness, following the criteria established in the National Guideline of the Environmental Health Surveillance Sampling Plan, defined by the Ministry of Health (MS 2006).

P. aeruginosa was investigated using the membrane filtration method (Millipore, 045 μm) and m-PA-C agar, and the isolated colonies were confirmed on milk agar, following the method described in Standard Methods for the Examination of Water and Wastewater (APHA 2017).

The strains isolated were submitted to disc-diffusion tests against fourteen antimicrobials – amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), levofloxacin (5 μg), meropenem (10 μg), netilmicin (30 μg), piperacillin-tazobactam (100/10 μg), polymyxin B (300 U), ticarcillin clavulanic acid (75/10 μg), and tobramycin (10 μg) – and the results reading and interpretation were performed according to the Clinical Laboratory Standards Institute methods (CLSI 2011).

Biofilm production capacity was evaluated in polystyrene microtiter plates, following Stepanović *et al.* (2007), with minor modifications. Initially the isolates were cultured in brain and heart infusion broth (BHI) at 35 °C/24 h. After growth, the culture was diluted to 1.5×10^8 CFU (0.5 MacFarland scale) and 200 μl aliquots of that dilution were distributed in quadruplicate to the wells of the plate and incubated at 35 °C/48 h. The plate wells were then washed three times with buffered saline

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(PBS, pH 7.2) and stained with 1% violet crystal for 15 minutes. After three washes with distilled water, the plate was left at room temperature for 10 minutes and then the biofilm resuspended with 200 µl of 33% acetic acid for 10 minutes. Absorbance at 570 nm was determined on an ELISA reader (Biotek, Epoch 2 model, Winooski, Vermont, USA) and a blank (uninoculated BHI) used to correct the absorbance value.

After correction, from the mean of four replicates of OD (optical density) and according to the ratio of OD to ODc (white optical density – cut-off value), the samples were classified as non-producing, weak, moderate or strong biofilm producers, being: $OD \leq ODc$ not producing; $ODc < OD \leq (2 \times ODc)$ weak producer; $(2 \times ODc) < OD \leq (4 \times ODc)$ moderate producer; $OD > (4 \times ODc)$ strong producer. As a positive control, the standard strain *Pseudomonas aeruginosa* ATCC 9027 was used.

Laboratory data from PROÁGUA, including the physicochemical (residual chlorine, pH, color, turbidity and nitrate) and microbiological parameters (total coliforms and *Escherichia coli*) from the water were also used to study possible correlations between them and the presence of *P. aeruginosa.* The free residual chlorine and pH were measured at the time of collection, and the other parameters were analyzed in the Center for Chemical and Bromatological Sciences of the IAL – CLR Bauru, using the methodologies described by APHA (2017): single-wavelength spectrophotometry for color determination, nephelometry for turbidity, UV spectrophotometry with direct reading of absorbance at 205 nm for nitrate, and chromogenic substrate (Colilert®) to evaluate both total coliforms and *E. coli*.

Statistical analysis of the association between the presence of *P. aeruginosa* and the parameters studied was carried out using Statistica 10.0 software (Statsoft, Tulsa, OK, USA, 2011), considering a significant difference when $p < 0.05$.

RESULTS AND DISCUSSION

Of the 251 water samples evaluated, 19 (7.6%) were positive for the presence of *P. aeruginosa,* which was found in samples from all three municipalities. There was no significant relationship with the sampling month. However, it was significant that samples reporting total coliforms also showed the highest positivity for *P. aeruginosa* – Table 1.

The results disagree with those from other studies that have shown that *P. aeruginosa* produces a substance called 'Pseudocin' that has a bacteriostatic effect on the growth of *E. coli, Aerobacter aerogenes, Citrobacter freundii* and *Klebsiella sp.* (Coelho *et al.* 2010), and can interfere in colorimetric analysis by inhibiting their growth in culture media (Vasconcelos *et al.* 2006).

Table 2 shows the physicochemical determined in the samples against presence and absence of *P. aeruginosa*. In general, no significant differences were observed between the values found in the positive and negative samples.

All water samples that reported positive for the presence of *P. aeruginosa* had free residual chlorine concentrations between 0.2 and 2.0 mg/L, as recommended in the ordinance (MS 2017), confirming this species' resistance to conventional water treatment processes.

D'Aguila *et al.* (2000) and Mulamattathil *et al.* (2015) have also reported the presence of *P. aeruginosa* in chlorinated waters, and Guerra *et al.* (2006) have demonstrated its resistance to free residual chlorine at different concentrations.

All of the strains isolated proved sensitive to all of the antimicrobials, except ticarcillin/clavulanic acid, to which resistance was indicated as intermediate.

The capacity to form biofilm is one of the most important of these bacteria's virulence factors, contributing to the colonization of both biotic and abiotic surfaces (Garcia-Contreras 2016). Of the 19 strains of *P. aeruginosa* tested, 10 (52.6%) had strong biofilm production capability and only 2 (10.5%) were classified as non-producing (Table 3).

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Table 1 | Proportions of microbiological parameters observed in samples

Parameter	Proportional positivity to *P. aeruginosa*	p^a
Month		0.859
February	2/25 (8.0%)	
March	2/18 (11.1%)	
April	3/25 (12.0%)	
May	3/25 (12.0%)	
June	3/24 (12.5%)	
July	0/18 (0.0%)	
August	1/25 (4.0%)	
September	1/25 (4.0%)	
October	1/16 (6.3%)	
November	2/25 (8.0%)	
December	1/25 (4.0%)	
City[b]		**0.001**
Dois Córregos	2/98 (2.0%)[b]	
Itapuí	11/63 (17.5%)[a]	
Pirajuí	6/90 (6.7%)[a,b]	
Total coliforms		< **0.001**
Negative samples	6/220 (2.7%)	
Positive samples	13/31 (41.9%)	
E. coli		0.618
Negative samples	19/248 (7.7%)	
Positive samples	0/3 (0.0%)	

[a]Chi-square test.
[b]Percentages followed by different letters indicate significant differences after the multiple comparisons test, according to the Marascuilo procedure (Marascuilo & McSweeney 1967).

Table 2 | Physicochemical parameters observed vs the presence and absence of *P. aeruginosa*

	P. aeruginosa				
	Negative samples		**Positive samples**		
Parameters analyzed	**SD**	**Median (Range)**	**SD**	**Median (Range)**	p^a
Free residual chlorine (mg/L)	0.7 ± 0.6	0.5 (0.0–4.7)	0.7 ± 0.5	0.6 (0.2–1.8)	0.731
pH	7.3 ± 0.5	7.3 (6.0–8.3)	7.4 ± 0.4	7.6 (6.3–7.9)	0.181
Apparent color (CU)	2.6 ± 4.8	<2.0 (<2.0–24.0)	2.1 ± 4.4	<2.0 (<2.0–11.0)	0.621
Turbidity (NTU)	0.6 ± 1.1	0.3 (0.1–12.0)	0.4 ± 0.6	0.2 (0.1–2.0)	0.072
Nitrate (NO_3-N) (mg/L)	2.2 ± 2.5	0.8 (0.0–8.9)	1.4 ± 1.4	0.9 (0.0–4.7)	0.726

[a]Mann-Whitney test.

Table 3 | Biofilm production capability classification of *P. aeruginosa* strains

Biofilm production capability	N	%
Strong	10	52.6%
Moderate	3	15.8%
Weak	4	21.1%
Non-producing	2	10.5%

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Statistical analysis showed no significant differences in the microbiological/physicochemical parameters in the water samples with presence of *P. aeruginosa* and the biofilm production capability of the strains isolated (Tables 4 and 5, respectively).

Table 4 | Presence of total coliforms versus biofilm production capacity of *P. aeruginosa* strains isolated

Positivity to total coliforms	*P. aeruginosa* with strong/moderate biofilm production capability	p^a
Negative samples	4/6 (66.7%)	0.911
Positive samples	9/13 (69.2%)	

[a]Chi-square test.

Table 5 | Physicochemical parameters versus biofilm production capability of *P. aeruginosa* strains

	Biofilm production capability				
	Non-producing/Weak		Moderate/Weak		
Parameter	SD	Median (Range)	SD	Median (Range)	p^a
Free residual chlorine (mg/L)	0.5 ± 0.3	0.5 (0.2–0.9)	0.9 ± 0.6	0.6 (0.2–1.8)	0.398
pH	7.2 ± 0.5	7.4 (6.3–7.8)	7.6 ± 0.2	7.6 (7.1–7.9)	0.096
Apparent color (CU)	7.0 ± 6.1	10.0 (<2.0–11.0)	<2.0	<2.0	0.134
Turbidity (NTU)	0.8 ± 0.9	0.2 (0.1–2.0)	0.2 ± 0.1	0.2 (0.1–0.6)	0.759
Nitrate (NO_3-N) (mg/L)	1.3 ± 1.5	0.6 (0.3–4.0)	1.5 ± 1.4	0.9 (0.0–4.7)	0.819

[a]t-Student or Mann-Whitney test.

Biofilms are a major challenge to science and health. It is estimated that approximately 95% of all microorganisms in water distribution systems are present as biofilms adhering to pipe surfaces (Moritz *et al.* 2010). Several studies have also shown that sub-inhibitory concentrations of chlorine may further stimulate biofilm production due to stress (Suman *et al.* 2013).

CONCLUDING REMARKS

The *P. aeruginosa* found in treated public water supplies in three Brazilian cities showed no resistance to the antimicrobials tested but most strains were classified as strong biofilm producers. The free residual chlorine concentrations established in current legislation were insufficient for the bacterium's total elimination, demonstrating its ability to resist conventional water treatment processes.

Effort is needed to reduce the spread of pathogenic bacteria with biofilm production capabilities, and finding treatments capable of altering the bacterial phenotype without inducing and selecting genetic modifications that might lead to resistance is critical to achieving good results in controlling these pathogens.

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