Pseudomonas aeruginosa strains resistant to antibiotics and heavy metals, producing biosurfactant, pyocyanin and biofilm from surfaces hospital environment.

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Original article

Abstract

Seven strains of *Pseudomona aeruginosa* isolated from hospital environmental surfaces were characterized in order to test their ability to produce to different antibiotic resistance and heavy metal concentration, biosurfactants, pyocyanin, biofilms, as well as to measure their tolerance, growth on various carbon sources, antimicrobial resistance and resistance to heavy metals. hemolytic profile, all strains produced mono and dirhamnolipids. When using the method known as Kirby Bauer, strains showed resistance to antibiotic (ampicillin, amoxicillin, cefotaxime, ceftazidime, amikacin and imipenem). The Minimum Inhibitory Concentration (MIC) was evaluated, showing tolerance to metals in decreasing order (As^{5+}>As^{3+}>Zn^{2+}>Pb^{2+}>Fe^{3+}>Cd^{2+}>Cu^{2+}>Cr^{6+}). Strains formed biofilms and produced pyocyanin. Thus, resistant strains could lead to hospital outbreaks because of antibiotic resistance; which also interfere with the treatment program and increases costs to health care institutions. This article could lead to further epidemiological evaluations in clinical environments, due to the potential risk associated with opportunistic and antibiotic resistance strains.

Key words: antibiotics resistant, heavy metals, biosurfactants, biofilm, hospital-Environmental, pyocyanin, *Pseudomonas aeruginosa*.

Introduction

P. aeruginosa is a versatile bacterium widely distributed in the environment and which has already been isolated from soil, water, animals, humans, devices and

hospital surfaces (Lee et al. 2006). This bacteria has been associated with nosocomial infections in immunosuppressed patients. Due to its ability to acquire resistance to antibiotics and heavy metals it can survive to adverse conditions (Lister et al. 2009). Furthermore, it is capable of producing a wide variety of secondary metabolites in which pyocyanin and biosurfactants are included. In addition, it has the ability to form biofilms on various surfaces which confers the strain virulence. (Roszak 1987; Santos-Burgoa 1994; Grosso-Becerra 2014; Tsiry et al. 2014; Ruvalcaba-Ledezma et al. 2014).

The biosurfactants reduce the surface and interfacial tension. They are environment-friendly, biodegradable, and resistant to a wide range of salinities, temperatures and pH environments. The most studied are the rhamnolipids produced by *P. aeruginosa* (Maier and Soberon-Chavez 2000). Pyocyanin (1-hydroxy-5-metilfenazina) is a secondary metabolite with the ability to oxidase and reduce other molecules. Both pyocyanin and biosurfactant synthesis are affected by growing conditions, source of carbon and nitrogen and oxygen level. Both metabolites act as virulent factors in *P. aeruginosa* as they play an important role in its pathogenesis (Laabei et al. 2014). The biosurfactant and pyocyanin of *P. aeruginosa* act as precursors in the production of biofilms, which is known as a community of microorganisms attached to a surface. This ability is important for chronic colonization of human tissues, which lead to persistence on medical implants (Vallet et al. 2004) and polluting hospital environmental surfaces; it confers resistance to various antibiotics used in the clinic, as well as to heavy metals, antiseptics and disinfectants (Bridier et al. 2011).

Heavy metals are ubiquitous and persistent environmental contaminants. They are introduced into the environment through anthropogenic activities such as mining, contaminating water reservoirs, and lead to alter the macro and microbiological communities. Bacteria have developed several mechanisms to counteract stress to heavy metals (Teitzel et al. 2003). The bacterial plasmids generally contain genes that confer resistance to high concentrations of metals and they are readily transferred from one cell to another by horizontal transmission of genetic material. Thus, they contribute significantly to the short-term adaptation of microbial communities in metal-contaminated environments (Unaldi et al. 2003; Shoeb and Ahmed 2006). Actually, different genes for resistance to heavy metals have been described and are contained on operon (Marrero et al. 2010).

Different reports about the ability of *P. aeruginosa* strain for antibiotics resistance and heavy metal can be found (Abdul-Sada 2009, De Bentzmann and Plésiat 2011). Nevertheless, there are few studies where the resistance to antibiotics, heavy metals and production of biosurfactants were evaluated together (Singh and Cameotra 2013). In this study different strains of *P. aeruginosa* obtained from hospital environments were characterized in order to measure their ability to produce biosurfactants and pyocyanin, as well as to form biofilms and measure their ability to resist antibiotics and heavy metals.

Materials and Methods

Study design and P. aeruginosa strains

An observational descriptive study was conducted in seven strains of *Pseudomonas aeruginosa* were isolated from hospital environmental surfaces and identified as: H15, H16, H17, H18, H19, H20 and H21.

Susceptibility to different antibiotics and heavy metals

Antimicrobial resistance to ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg) cefotaxime (30µg), ceftazidime (30µg), amikacin (30 µg) imipenem (10 µg) was determined by the Kirby Bauer method (Cerezo 1983; Clinical and Laboratory Standards Institute 2012). The susceptibility to heavy metals and metalloids was evaluated by the use of supplemented LB agar with increasing concentrations of metallic salts (Pb $(NO_3)_2$, Cd $(NO_3)_2$, Cu $(NO_3)_2$, Zn $(NO_3)_2$, $K_2Cr_2O_7$ $(NO_3)_2$, Fe $(NO_3)_3$, NaH_2AsO_4 and $NaAsO_2$) from 100 mmol l^{-1} stock solution. The concentrations of the metallic salts were 0.5, 2, 4, 6, 10 and 20 mmol l^{-1}. The optical density of each strain was adjusted and replanted in all concentrations, incubated at 35 °C for 7 days following the methodology of Raja and Omine (2012).

Biosurfactant and pyocianin assays

The ability to produce biosurfactant was determined according to the methodology known as Rakeshkumar et al. (2012). Foam production in broth PPGA was evaluated after 24h of being incubated with constant stirring at 30 °C. Partial purification was done by solvent extraction and quantification of rhamnolipids was done by using the orcinol method (Toribio Jimenez et al. 2011). Biosurfactant detection was performed by thin layer chromatography on preparative plate's Silica gel 60 F254 0.25 mm (Merck) (Sulbarán et al. 2005).

Emulsification index method (IE_{24}) was applied on partially purified biosurfactants (PPB) and the supernatant following the protocol of Cooper and Goldenberg (1987). All experiments were performed in duplicate and were included as a positive control for *P. aeruginosa* PAO1.

The stability the PPB was evaluated in regards the effect of temperature, 100 and 120 °C, for 1h. Different agar plates containing Luria-Bertoni agar were prepared with different NaCl levels (0 to 10% w/v) and different levels of pH (2.0, 5.0, 9.0 and 12.0) by using HCl or NaOH according with the method, as described by Nythya and Pandian (2010).

Production of pyocyanin.

The pyocyanin quantification in all strains was conducted by the method described by Essar et al, (1990).

Hydrocarbons growth assay

Each strain was inoculated into 5 mL of saline minimal medium (Minimal medium pH 7, containing 22 mM $KHPO_4$, 40 mM KH_2PO_4, 19 mM NH_4Cl, 17mM NaCl and 1.5 mM $MgCl_2$) supplemented with 2% (v/v) of a carbon source (cyclohexane, toluene, and diesel oil) and incubated for 48h at 30 °C (Raza et al. 2006). Growth was monitored by measuring the absorbance of the culture at 600 nm in a spectrophotometer Genesys 20 (ThermoScientific). As negative control,

we used SMM without carbon source for growth and as a positive control; the MS medium was used being supplemented with 2% (w/v) glucose.

Ability to form biofilms

Biofilm generated by each strain of *P. aeruginosa* were evaluated using the following crystal violet assay methodology (Peeters et al. 2007).

Results

Seven native strains, identified as *P. aeruginosa* were isolated from hospital surfaces. All the strains were able to cause hemolysis on blood agar, producing foam in PPGAS broth and showed surface activity by the drop collapse method (see Figure 1A). Only the H15 strain was able to emulsify to 65% toluene, 60% cyclohexane, 55% vegetable oil and 26% diesel, the strain H21 emulsified 15% vegetable oil, the other strains did not show this capability. The crude extracts showed activity at temperatures of 120°C, at salinity concentration of 10% NaCl, and in the interval of pH measured (2.0 to 11.0).

The strains were able to produce biosurfactants, the H15 strain produces 0.8 mg ml^{-1} of rhamnolipid. All strains produced mono and dirhamnolipids only shown by the H15, H20 and H21 strains in monorhamnolipid TLC and dirhamnolipids type than *P. aeruginosa* PAO1 (see Figure 1B), and these strains were able to grow on glucose, toluene, cyclohexane, petroleum and diesel as the only carbon source.

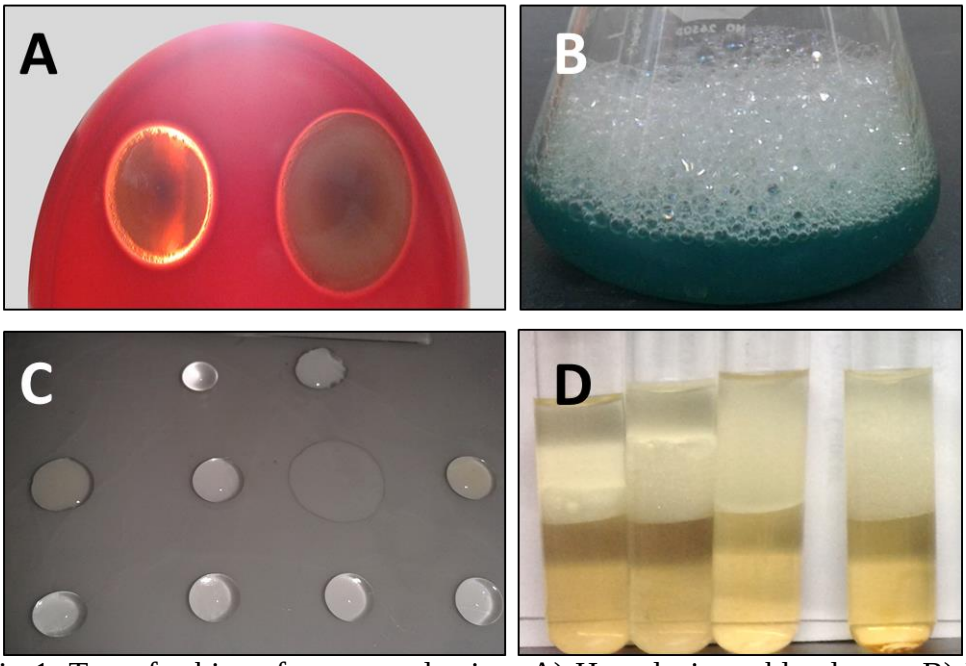

Fig 1. Tests for biosurfactant production. A) Hemolysis on blood agar. B) Foam production in PPGAS broth. C) Drop collapse, D) Emulsification index (IE_{24}).

Production of pyocyanin was registered in all strains, with varying concentrations ranging from 1.29, 1.51, 2.87, 2.9, 8.69, 8.9 and 9.56 µg ml^{-1} for strains H20,

H21, H16, H17, H19, H18 and H15 respectively. In addition, they were able to form biofilms under the conditions tested. The profile of antibiotic resistance in 100% of the strains showed that those were resistant to ampicillin, amoxicillin and 90% of cefotaxime, 80% to cefotaxime and ceftazidime, and 100% sensitive to amikacin and imipenem respectively.

As it observed in the analysis of MIC to heavy metals and metalloids, changes were observed in all strains of *P. aeruginosa* in colonial morphology in the presence of metal. Most of the colonies were mucoid and were capable of emitting fluorescence in the presence of Pb, Cd and Zn. The same resistance profile As^{5+}>As^{3+}>Zn^{2+}>Pb^{2+}>Fe^{3+}>Cd^{2+}>Cu^{2+}>Cr^{6+} (> 20, 10, 10, 6, 4, 4, 2 and 2 mmol l^{-1}, respectively) was observed.

Discussion

This paper describes for the first time the identification of native *P. aeruginosa* strains isolated from hospital environmental surfaces resistance to antibiotics and heavy metals with the ability to produce biosurfactants and pyocyanin, form biofilm and show commonly, such strains are founded at polluted soils and water.

The observed strains showed capacity to produce biosurfactants, with stability at 120 °C, 10% salinity, and pH ≤ 11.0. Supernatant from H15 strain presented a IE_{24} of 62.5% toluene, 60% cyclohexane, 55% vegetable oil and 26% diesel, in agreement with the findings of Abdel-Mawgoud et al. (2009) which reports that *P. aeruginosa* strain is capable of producing biosurfactant BS20, showing stability at 120 °C for 10 min, and showing activity at NaCl concentration of 6%, and pH 13.0, thus a IE_{24} of 59% and 66% for kerosene and diesel. Vanavil *et al.* (2013) reported a strain of *P. aeruginosa* NITT6L can produce biosurfactant and emulsified vegetable oil, with 0.3 mg ml^{-1} rhamnolipid. In this study, 0.8 mg ml^{-1} was produced by the strain H15, and the types of biosurfactants of both strains were mono and dirhamnolipids (Figure 2).

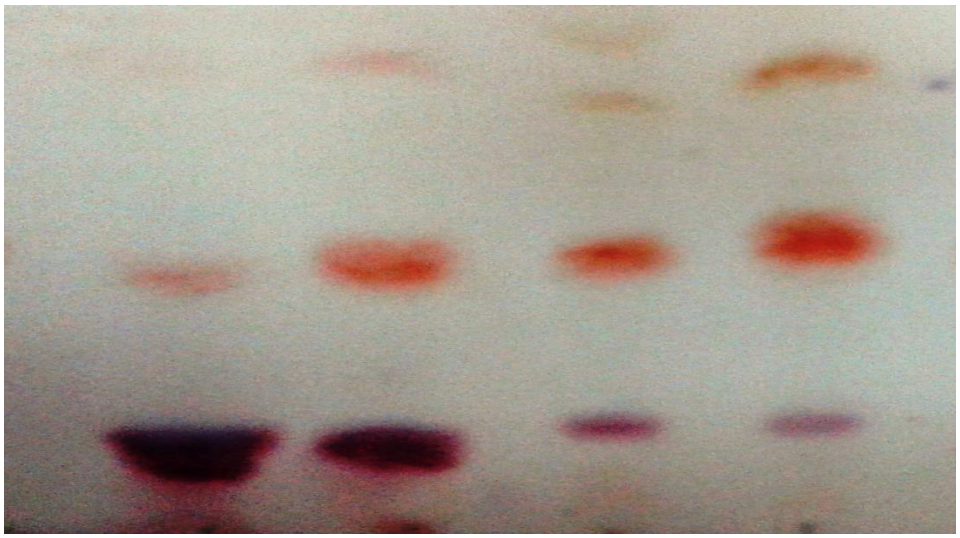

Fig 2. Detection of rhamnolipids by thin layer chromatography (TLC). Up to down: HAAs (Chains of fatty acids); Mono-RhL (Monorhamnolipids); Di-RhL: (Dirhamnolipids). From left to right: Strains PAO-1, H15, H20, H21.

The strains were capable of producing pyocyanin. Variation in the difference in production between the strains could be due to variations in any of the regulatory genes (*phzR* or *phzI*) biosynthetic (*phzABCDEFG*) biotransforming (*phzS* or *phzM*) or any of its negative regulators (Mavrodi et al. 2001). Allen et al. (2005) reported that pyocyanin induces apoptosis in neutrophils and deteriorates the defenses of neutrophils in vivo studies for what is considered a virulence factor.

The native strains were able to form biofilms this exopolysaccharide provides protection against extreme environments. As reported by Teitzel and Parsek (2003) the biofilms confer bacteria stress resistance to antibiotics, heavy metals and disinfectants which increased from 2 to 600 times compared to the individual cells.

P. aeruginosa strains may be resistant to antibiotics through intrinsic or acquired resistance mutations or expression of beta-lactamase (Pitout et al. 2005). The autochthonous strains were resistant to ampicillin, amoxicillin, cefotaxime, ceftazidime, imipenem and amikacin. This statement matches with Livermore's findings (2002), where antimicrobial resistance analyzed in hospitals in the UK were described, and reported to show 28% resistance to ciprofloxacin, 18% to gentamicin, 16% to piperacillin, 14% and 7% imipemen and amikacin.

In the hospitals of Mexico, the resistance to antibiotics was also shown. Castillo Vera et al. (2006) describe that *P. aeruginosa* strains were resistant to 21 antibiotics at Infectious Disease Hospital of the Mexican Social Security Institute. In addition, outbreaks of *P. aeruginosa* strains were reported; all reports in hospitals in Mexico by resistant strains increased morbidity and mortality in patients infected with the bacteria.

In this study, strains produced biosurfactants, pyocyanin, form biofilms and show resistance to antibiotics, heavy metals and metalloids. This behaviour is probably because the bacteria are under selective pressure by the presence in the environment of antibiotics and/or toxic substance. These multirresistance selectable factors should also be noted. *P. aeruginosa* is easily adapted to different ambient conditions, so it is important to note that the bacteria with this property can lead to the spread of these genes within hospitals and can remain so for long periods in the hospital environment which can potentially make them more pathogenic in some cases. We did not find reports of *P. aeruginosa* on hospital surfaces, resistant to antibiotics, heavy metals and metalloids that can produce rhamnolipids, pyocyanin and form biofilms. Bodour *et al*. (2003) reports production association of rhamnolipids with heavy metal resistance in two strains of *P. aeruginosa* isolated from soil contaminated with Pb. Furthermore, it has been reported that rhamnolipids are capable of removing metals and ions in soil Cd, Cu, Pb and Zn, due to their ability to form complexes.

In this study, only the H15 strain emerges as a candidate for evaluating the bioremediation strategies of sites contaminated with heavy metals and for the production of rhamnolipids. The results provide a basis for proposing microbial ecology studies in hospital settings, to determine the molecular mechanism of resistance to antibiotics and heavy metals, to evaluate the ability to acquire and

transfer resistance genes and associated biosurfactant production and pyocyanin with biofilm formation, since the rhamnolipids are involved in the maintenance of biofilms and this is considered a virulence and antibiotic resistance mechanism.

Proposal to avoid the persistence of nosocomial infections

According to Ruvalcaba et al, 2013 and Corona et al. (2014), a proposal could be to carry out research projects from an epidemiological point of view. The inclusion of a longitudinal descriptive study in order to evaluate the environmental aero microbiological quality, microbial viability, among other critical test (sampling of air in cultivate boxes added with selective agar for evaluate surveillance of the environmental aero microbiological quality) are critical to get representative results.

An air sampler "Andersen" can be used in order to simulate the presence of viable strains at different levels of the respiratory human system. In agreement with the author, we insist in the necessity of executing environmental epidemiology in all the hospitals, in order to get the best quality of inner hospital attention and thus reduce the risk of suffering a nosocomial infection with such strains. (Ruvalcaba et al, 2013) *Pseudomas aeruginosa* strains isolated from hospital environmental is considered as biological indicators of environmental pollution. (Ruvalcaba et al, 2014)

This is the first report of *P. aeruginosa* strains isolated from hospital environmental surfaces as a potential source of infection for hospitalized patients because it can give a cross-contamination between the hospital environment and critical patients' increasing morbidity, mortality, and is a risk for hospital outbreaks strains resistant to various antibiotics used in the clinic and their ability to persist in different environments by heavy metal resistance, producing biosurfactants, pyocyanin and biofilms, more virulent which makes their treatment to be expensive and not effective.

Conclusions

The presence of virulent microbiological agents resistance to antibiotics, heavy metals and metalloids, produced biosurfactants, pyocyanin, form biofilms in the hospital, may enhance the adverse effects on public health and hospital environmental surroundings, hence the importance, as they impact on human health in the family economy and health care.

Finally the ecological-environmental meaningful findings species of *Pseudomonas aeruginosa* of surfaces hospital environment that presents high resistance to antibiotics and heavy metals is also meaningful because apart from their presence in the surfaces is considered microbiological indicators of environmental pollution in hospital.

This research could lead to further epidemiological evaluations in clinical environments, due to the potential risk associated with opportunistic and antibiotic resistance strains.

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