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Short communication

Genotypic diversity and presence of β-lactamase encoding genes in *Pseudomonas aeruginosa* isolated from Brazilian soils

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ABSTRACT

Pseudomonas aeruginosa is considered an opportunistic pathogen widely distributed in the environment, which has a high genetic diversity among the isolates due to the frequent genetic recombination. This study determined the genetic relatedness among 61 *Pseudomonas aeruginosa* isolated from soil of different Brazilian regions by pulsed-field gel electrophoresis (PFGE), investigated the resistance profile and the prevalence of β-lactamase encoding genes in these isolates. It was observed a high genotypic diversity among the majority of the isolates (≥33.6%), which were differentiated in 55 PFGE-types. However, some few isolates presented a high similarity (≥80%) among each other, being allocated in B1-B8 subgroups. The majority of the isolates showed non-susceptibility to aztreonam (16 to ≥256 μg mL^{-1}) and 38% of the isolates had one or more of the target genes, being bla_{SHV}, bla_{GES} and bla_{VEB} the most prevalent. This work provided new information about the genotypic diversity and antimicrobial resistance mechanisms of *P. aeruginosa* isolated from soils of different states in Brazil.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium widely distributed in the environment, metabolically versatile and it is one of the most successful nosocomial opportunistic pathogen (Silby et al., 2011; El Zowalaty et al., 2015). Some studies have stated the persistence of multidrug-resistant clones in the hospital environment, showing little genetic diversity; however, other studies claimed that no specific clones were related to specific diseases or habitats and that some clinical isolates were indistinguishable from the environmental isolates (Pirnay et al., 2009; Stehling, et al., 2010). According to Cholley et al. (2011) and Feliziani et al. (2014), the diversity of *P. aeruginosa* isolates is probably due to the frequent genetic recombination observed in this species.

In the environment, *P. aeruginosa* has already been isolated from different sources, such as agricultural soils, plants, water and vegetables (Zanetti et al., 2013; Deredjian et al., 2014). *P. aergunosa* is considered a plant pathogen and the selective pressure exerted by different compounds, such as antibiotics, heavy metal and pesticides, can influence the presence of this pathogen in the environment (Fernandes et al., 2014; Holtze et al., 2006). The present study aimed to investigate the genotypic diversity of 61 *P. aeruginosa* isolates obtained from different soil samples from Brazil by pulsed-field gel electrophoresis (PFGE) and to evaluate if the resistance to aztreonam in these isolates was correlated with the presence of β-lactamases encoding genes.

2. Materials and methods

2.1. Soil sampling and obtaining of isolates

The soils samples were obtained from different cities and states of Brazil. In the collection of the soil samples, the surface layer was first removed and a total of 10 g of soil of three different points of each area was collected in a depth of 15 cm. Therefore, a single replicate based on three subsamples was used. The soil samples were classified in different types according to Santos et al. (2011). A total of 61 isolates of *P. aeruginosa* were included in this study, being 43 previously studied according to Pitondo-Silva et al. (2014) and Braz et al. (2016). Eighteen more isolates were posteriorly obtained using the methodology described by Mukherjee et al. (2011), using 1 g of each mixed soil sample and were included in the present study.

2.2. Molecular typing by pulsed-field gel electrophoresis (PFGE)

PFGE was performed for 61 soil isolates of *P. aeruginosa* according to Speijer et al. (1999), using 20U of *Spe*I restriction enzyme (*Bcu*I,

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Thermo Fisher Scientific) at 37 °C for 2 h. Electrophoresis was performed using the CHEF-DR III system (Bio-Rad, Richmond, CA) at 14 °C and a power level of 6 V/cm for 20 h with the switching interval ramping from 5 to 15 s during 10 h and after that, from 15 to 45 s during 10 h. *Salmonella* Braenderup H9812 digested with 40U of *XbaI* at 37 °C for 2 h was used as a molecular mass standard. Gels were analyzed by Bionumerics software package (Applied Maths). The similarity dendrogram was constructed using the unweighted-pair group method (UPGMA) settings with tolerance set to 1%.

2.3. Discrimination index (DI)

The DI for PFGE was assessed by Simpson's diversity index, as presented by Hunter and Gaston (1988).

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed according to CLSI (2015) using the disc diffusion assay for 17 different antimicrobials and the minimum inhibitory concentration assay (MIC) for aztreonam. The wild-type *P. aeruginosa* ATCC 27853 was used as a control in these experiments.

2.5. PCR detection and sequencing of the β-lactamases encoding genes

Genomic DNA of 61 isolates was extracted using the QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions. The amplification of the genes bla_{GES}, $bla_{CTX\text{-}M\text{-}Gp1}$, $bla_{CTX\text{-}M\text{-}Gp2}$, $bla_{CTX\text{-}M\text{-}Gp8}$, $bla_{CTX\text{-}M\text{-}Gp9}$, bla_{KPC}, bla_{SHV}, bla_{PER}, bla_{VEB}, and $bla_{OXA\text{-}48\text{-}like}$ was performed according to Dallenne et al. (2010). The amplicons were sequencing using the BigDye Terminator Cycle Sequencing Kit and analyzed by comparison with the sequences of the GenBank using the BLAST algorithm (Johnson et al., 2008).

3. Results and discussion

The soil samples were obtained between 2012 and 2013 and the majority was classified as red distroferric latosol (LVdf) (32), followed by dystrophic red-yellow argisol (PVad) (13), dystrophic yellow latosol (LAd) (12) and orthic quartzarenic neosol (RQo) (1). Among the soil samples, 32 were obtained in the Southeast region, 11 in the Center-West region, eight in two different regions, being North and Northeast and two in the South region. All GPS data are described in Fig. 1. The soil samples were obtained from different agricultural crops, such as corn, coffee, sugarcane, sunflower, cotton, manioc, soy, woods, beet, papaya, coconut, acerola, eucalyptus, pasture, citrus, beans, brachiaria and sorghum (Fig. 1).

The environmental isolates of *P. aeruginosa* were allocated into two major clusters designated as PFGE-A and PFGE-B with a genotypic diversity ≥33.6% and were typed in 55-PFGE types indicating the prevalence of diverse subtypes of *P. aeruginosa* in the soil of different agricultural cultures in the different Brazilian states. However, some isolates presented ≥80% similarity being allocated in B1-B8 subgroups (Fig. 1).

The PFGE-A cluster comprised only two (3.3%) isolates from different agricultural cultures and different Brazil regions, which exhibited a genotypic diversity above 34.8%. On the other hand, the PFGE-B cluster allocated fifty-nine (96.7%) isolates, being the largest cluster showing a genotypic diversity above 35.1%. However, some isolates presented a similarity ≥80% and were subdivided into PFGE-B1 to PFGE-B8 subclusters. The DI for PFGE was 0.996.

The PFGE-B2, PFGE-B3, PFGE-B4, PFGE-B7 and PFGE-B8 subclusters comprised only two (3.3%) isolates each, presenting a similarity of 100% among them and they were obtained from the same city, culture and year, suggesting that those isolates are derived from the same clone. In the same way, PFGE-B6 grouped four isolates (6.6%) with a similarity ≥92.6%, indicating that those isolates were clonally related and may probably have descended from a common *P. aeruginosa* subtype (Fig. 1).

On the other hand, some isolates have been obtained from the same crop and city; nevertheless, they remained outside of these subclusters with high similarity, as observed to the isolate S50, which it was isolated in Franca city, São Paulo state from a coffee culture in 2012 and showed genotypic similarity only 44.8% with PFGE-B2 subcluster.

The opposite was observed with the sub-clusters PFGE-B1 and PFGE-B5, which had a high genotypic similarity, 95.8% and 86.45% respectively, and isolates obtained from different cities and cultures. The B1 subcluster comprised an isolate from beet culture isolated in the Amazonia state and the other one was obtained from a coconut culture in the Acre state, approximately 1500 km away from each other. Also, B5 subcluster comprised isolates from different places and agricultural cultures, being one from pasture from São Pedro do Sapucaí city, Minas Gerais state, and the other one from a soy culture from Santa Juliana city also in Minas Gerais state, 789 km from each other.

Many authors have claimed that PFGE is an important epidemiological tool applicable to clinical isolates of *P. aeruginosa*, however, the study of Pappa et al. (2013) showed that PFGE was little applicable for environmental isolates from water, while other studies had found less genetic variation among the isolates of *P. aeruginosa* obtained from the open-ocean (Khan et al., 2007).

Isolates of *P. aeruginosa* from manure and soil were analyzed by Multilocus sequence typing (MLST) and they were not located in the same clonal complexes of those isolated from snake-related or environmental and clinical sources (Youenou et al., 2014). In the present work, PFGE was able to discriminate the strains and provided important epidemiological information regarding *P. aeruginosa* isolated from soil of diverse places of the country. Therefore, the results of the genetic relatedness by PFGE reinforce the great genetic diversity of the *P. aeruginosa* isolates from Brazilian soils, corroborating with the statement that the high frequency of genetic recombination observed in *P. aeruginosa* contributes to the diversity observed in this species (Cholley et al., 2011; Feliziani et al., 2014).

The majority of the environmental isolates of *P. aeruginosa* (95%) were classified as non-susceptible (intermediate or resistant) to aztreonam, presented a MIC ranging from 16 to ≥256 μg mL^{-1} and 49% to ticarcillin. Only two isolates (S129 and S132) were susceptible to aztreonam, showing a MIC of 8 μg mL^{-1} (Fig. 1). The eighteen isolates obtained in this study were susceptible to all other antimicrobials tested.

A total of 23 (38%) isolates presented one or more β-lactamase encoding genes, while 38 (62%) did not present any investigated gene, although they have presented different MICs for aztreonam. The bla_{SHV} was the most prevalent gene, being found in eleven isolates, followed by bla_{GES} (10), bla_{VEB} (5), $bla_{CTX\text{-}M\text{-}Gp1}$ (4), $bla_{CTX\text{-}M\text{-}Gp2}$ (3) and bla_{PER} (1), which are frequently related to clinical isolates of *P. aeruginosa*. (Fig. 1). The bla_{KPC}, $bla_{CTX\text{-}M\text{-}Gp8}$, $bla_{CTX\text{-}M\text{-}Gp9}$ and $bla_{OXA\text{-}48\text{-}like}$ genes were not found in these isolates. The amplicons were sequenced to confirm the identity of them and submitted to the GenBank database with the accession numbers MG188746 to MG188751.

The bla_{VEB} and bla_{PER} genes have already been found in clinical isolates of *P. aeruginosa* and they were associated with the resistance to oxyimino-cephalosporins, especially to aztreonam and ceftazidime (Bradford 2001) and a similar result was observed in isolates from Saudi Arabia, where a high level of resistant to aztreonam and the presence of bla_{GES}, bla_{VEB} and bla_{OXA} genes were detected (Tawfik et al., 2012). In Taiwan, clinical isolates of *P. aeruginosa* from blood samples were resistant to aztreonam and approximately 60% of these isolates carried out the genes bla_{GES}, bla_{VEB}, bla_{SHV}, bla_{PER}, bla_{BEL}, bla_{TEM}, bla_{OXA} and $bla_{CTX\text{-}M\text{-}groups}$ (Lin et al., 2012). However, in the present study just 38% of the isolates presented *bla* genes, suggesting the involvement of enzymes not yet identified or other mechanisms of resistance to aztreonam in the isolates from soil.