



The Brazilian Journal of INFECTIOUS DISEASES

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Original article

Biofilm production by clinical isolates of *Pseudomonas aeruginosa* and structural changes in LasR protein of isolates non biofilm-producing



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ARTICLE INFO

Article history:

Received 28 January 2018

Accepted 13 March 2018

Available online 28 March 2018

Keywords:

Pseudomonas aeruginosa

Biofilm

Multiresistance

Quorum sensing (QS)

ABSTRACT

Introduction: Biofilm production is an important mechanism for the survival of *Pseudomonas aeruginosa* and its relationship with antimicrobial resistance represents a challenge for patient therapeutics. *P. aeruginosa* is an opportunistic pathogen frequently associated to nosocomial infections, especially in immunocompromised hosts.

Objectives: Analyze the phenotypic biofilm production in *P. aeruginosa* isolates, describe clonal profiles, and analyze quorum sensing (QS) genes and the occurrence of mutations in the LasR protein of non-biofilm producing isolates.

Methods: Isolates were tested for biofilm production by measuring cells adherence to the microtiter plates. Clonal profile analysis was carried out through ERIC-PCR, QS genes were by specific PCR.

Results: The results showed that 77.5% of the isolates were considered biofilm producers. The results of genotyping showed 38 distinct genetic profiles. As for the occurrence of the genes, 100% of the isolates presented the *lasR*, *rhlI* and *rhlR* genes, and 97.5%, presented the *lasI* gene. In this study nine isolates were not biofilm producers. However, all presented the QS genes. Amplicons related to genes were sequenced in three of the nine non-biofilm-producing isolates (all presenting different genetic similarity profile) and aligned to the sequences of those genes in *P. aeruginosa* strain PAO1 (standard biofilm-producing strain). Alignment analysis showed an insertion of three nucleotides (T, C and G) causing the addition of an amino acid valine in the sequence of the LasR protein, in position 53.

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<https://doi.org/10.1016/j.bjid.2018.03.003>

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Conclusion: The modeling of the resulting LasR protein showed a conformational change in its structure, suggesting that this might be the reason why these isolates are unable to produce biofilm.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen frequently associated to nosocomial infections, especially in immunocompromised hosts. This pathogen has also been historically associated to lung infections in patients with cystic fibrosis.¹ This microorganism uses several virulence factors to aid in its pathogenicity that will favor infection.² Among these factors one can highlight the production of biofilm, which is configured as a set of bacterial cells coated by polysaccharide layer, acting as a protection factor of the bacteria against the attack of the antimicrobial and host immune system, favoring the chronicity of the infection.¹

The formation of biofilms causes considerable problems in the medical and industrial area, as these structures provoke greater resistance to the treatment with antibiotics and biocides, besides reducing the action of the host immune responses.² Due to its complexity and the impact of its development, microbial biofilms have been the subject of several studies trying to elucidate how these structures are formed and managed to interact within this community to mitigate the consequences of their formation.³

Biofilm formation is regulated through quorum sensing systems.⁴ The quorum sensing (QS) is a mechanism of cellular recognition and regulation of expression of many bacterial cell genes, mainly mediated by three interconnected systems, the Las, Rhl and Pqs systems.⁴ QS is, therefore, important in the formation and maintenance of biofilms by allowing the recognition of the population density, whatever the environmental conditions in which the cells are found, provide the necessary changes to guarantee their survival, through the production of small molecules of communication, the N-acyl-homoserine-lactones (AHL).⁵

Therefore, the QS plays an important role among the regulation systems of expression of virulence factors that favor bacterial pathogenicity, including biofilm formation.⁵ In *P. aeruginosa*, one of the main regulators of QS is the LasR protein, which needs, in order to regulate QS, to bind to its autoinducer, the molecule N-(3-oxododecanoyl)-homoserine lactone (OdDHL).⁶

The *lasR* gene codes the transcription factor, which is responsible for the activation of numerous target genes, most of them related to QS in *P. aeruginosa*. *lasR* mutations are diverse as well as the phenotypes generated by them.⁷ Moreover, mutations in *lasR* gene were recently suggested to be generated during infection, as a result of the strength of *lasR* transcription.⁷

In this work, we aimed to investigate biofilm production in multidrug resistant (MDR) and multidrug susceptible (MDS) clinical isolates of *P. aeruginosa* from different sites of

infection and colonization, and describe their clonality. In addition, to sequence PCR products of QS genes for investigating the occurrence of mutations in the LasR protein of non-biofilm producing isolates.

Methods

Characterization of study

This was an experimental, laboratory-based study in which 40 clinical isolates of *P. aeruginosa* stored in the bacterial collection of the Laboratory of Bacteriology and Molecular Biology of the Universidade Federal de Pernambuco (UFPE) were randomly selected, 20 multidrug-susceptible (MDS) and 20 multidrug-resistant (MDR) were analyzed. The study was approved by the Research Ethics Committee of UFPE, registered at CEP/CCS/UFPE under number 009/11. Isolates resistant to at least three different antimicrobial classes, mainly aminoglycosides, penicillins, cephalosporins, carbapenems, and fluoroquinolones were considered multidrug-resistant.⁸ These isolates were collected from November 2012 to November 2013, from several sites of infection and colonization (Table 1). The identification and analysis of the antimicrobial susceptibility was performed by the automated system (Phoenix – BD[®]) and sent to the Laboratory of Bacteriology and Molecular Biology of the Universidade Federal de Pernambuco, where they were kept frozen in 20% glycerol at –20 °C. The bacteria were reactivated in BHI (Brain Heart Infusion) broth and incubated for 48 h at 37 °C, and then seeded on ceftrimide agar and incubated at 37 °C for 24 h for further analysis.

Phenotypic characterization of biofilm production

Biofilm production was evaluated according to Stepanovic et al.,⁹ with modifications. Briefly, 50 g/L sucrose was added to

Table 1 – Source of clinical isolates of *P. aeruginosa*.

Source	Number of isolates
Blood	10
Tracheal secretion	19
Catheter tip	3
Bronchial lavage	1
Wound secretion	2
Surgical fragment	1
Urine	1
Nasal swab	1
Rectal swab	2
Total	40

Table 2 – Sequences of quorum sensing gene primers.

Primer	Sequence	Amplicon size (pb)
<i>lasI</i>	5'-CGTGCTCAAGTGTCAAGG-3' 5'-TACAGTGGAAAAGCCCAG-3'	295
<i>lasR</i>	5'-AAGTGGAAAATTGGAGTGGAG-3' 5'-GTAGTTGCCGACGACGATGAAG-3'	130
<i>rhlI</i>	5'-TTCATCCTCCTTAGTCTTCCG-3' 5'-TTCCAGCGATTCAGAGAGC-3'	155
<i>rhlR</i>	5'-TGCATTTTATCGATCAGGGC-3' 5'-CACTTCCTTTTCCAGGAGC-3'	133

BHI broth. The categorization of biofilm production was performed according to the biomass adhered to the bottom of the plate according to the values of the optical densities read at 570 nm. The BHI broth was used as negative control (CN) and the *P. aeruginosa* strain PAO1 was used as the positive control (CP), since this strain is recommended as a positive control for biofilm assays.

Detection of QS genes

DNA extraction from the isolates was performed using the Brazol kit (LGC-Biotechnology) according to the protocol provided by the manufacturer. DNA was quantified by spectrophotometry (Ultraspec 3000; Pharmacia Biotech) over the wavelength range 260–280 nm. PCRs were performed to detect the following QS genes: *lasR*, *lasI*, *rhlI*, and *rhlR*.^{10,11} The parameters for the amplification were 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1.5 min. The PCR products were stained with blue-green (LGC Biotecnologia – São Paulo), submitted to 2% agarose gel electrophoresis and visualized under UV light. The primers are presented in Table 2. The *P. aeruginosa* strain PAO1 was used as the positive control (CP) since this strain is recommended as a positive control for biofilm assays.

Sequencing of the QS genes and bioinformatics analysis

Three clinical isolates of *P. aeruginosa* were randomly selected from the non-biofilm producers for sequencing of the amplicons obtained after QS genes PCR. PCR products were purified with the DNA Wizard[®] SV gel and PCR clean-up System kit (Promega), according to the manufacturer's protocol and quantified by spectrophotometry. Purified amplicons were submitted to sequencing and the nucleotide sequences were confronted with sequences available in databases using the BlastN and BlastX BlastNetwork algorithms available in the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide sequences obtained from each isolate were analyzed and translated into amino acids.¹² The multiple sequence alignment was performed in the Clustal Omega program.^{13,14}

Analyses of the amino acid sequences for the three-dimensional modeling of the LasR protein were carried out to assess possible structural differences between these proteins of the non-biofilm-producing isolates and the biofilm-producing strain PAO1. In the PDB database (RCSB Protein Databank) (<http://www.rcsb.org/pdb/home/home.do>), BlastP was performed between the mature peptide sequence

and the structural models deposited in the database, aiming at the selection of the most similar structures to *P. aeruginosa* LasR protein, which could be used as a model for three-dimensional modeling of the protein. Furthermore, the three-dimensional structures of the candidate proteins were solved through comparative modeling. Multiple alignment between the model structures and quality evaluation were performed using the Protein Structure & Model Assessment tool of the SWISSMODEL Workspace platform (<http://swissmodel.expasy.org/>).¹⁵ Consequently, the quality assessment was based on QMEAN data, which compares the model to deposited structures of the same size and Z-score.^{16,17} The structures had the various visualization modes generated in the NOC 3.0 program. Using Rampage, were obtained the Ramachandran graph of the structure and its G-factor.¹⁸

Molecular typing of the isolates

The 40 isolates were submitted to molecular typing through technical enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR) to identify the clonal profile of the strains. The ERIC-PCR reactions were prepared in a total volume of 25 µL per tube, comprising: 100 ng genomic DNA, 10 pmol of the primers (ERIC-1 [5'-ATG TAAGCTCCTGGGGATATACAC-3']; [5'-AAGTAAGTGACTGGGGTGAGCG-3']), 1× buffer, 200 µM deoxyribonucleotide triphosphate, 1.5 mM MgCl₂ and 1.0 U of Taq DNA polymerase enzyme. The amplification parameters used: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 8 min. After the 30 cycles, a final elongation step of 16 min at 72 °C was performed. The PCR products were stained with blue-green (LGC Biotecnologia – São Paulo), and submitted to 1.5% agarose gel electrophoresis, visualized under UV light and photodocumented for later analysis of clonal profiles.¹⁹

Results

Biofilm production

In the quantitative technique for measuring biofilm production, described by Stepanovic et al.,⁹ a phenotypic test considered a “gold standard” for biofilm detection, 77.5% (31/40) of the isolates were considered biofilm producers, being distributed in the following categories: 42.5% (17/40) weakly

Table 3 – Phenotypic biofilm analysis according to detected biofilm genes in *P. aeruginosa*.

Adherence profile	Number of samples	Occurrence of QS genes			
		<i>lasI</i>	<i>lasR</i>	<i>rhlI</i>	<i>rhlR</i>
Non-adherent	9	9	9	9	9
Weakly adherent	17	17	17	17	17
Moderately adherent	11	11	11	11	11
Strongly adherent	3	2	3	3	3

adherent, 27.5% (11/40) moderately adherent, and 7.5% (3/40) strongly adherent, as can be observed in Table 3.

Detection of QS genes

In the present study, a high occurrence of QS genes was found, 100% of the isolates presented the *lasR*, *rhlI* and *rhlR* genes and 97.5% presented the *lasI* gene, observed in Table 4. In this study, nine clinical isolates of *P. aeruginosa* (P1A, P9A, P1B, P5B, P7B, P9B, P11B, P19B, and P20B), all presenting different genetic similarity profile, were non-biofilm producers. However, they all presented the genes *lasI*, *lasR*, *rhlI*, and *rhlR*.

Sequencing of the QS genes

When comparing the *lasR* gene sequences of three non-biofilm producing isolates (P1A, P7B and P20B), it was evidenced, through the alignment of sequences with the biofilm-producing strain *P. aeruginosa* PAO1 (default biofilm producing strain), the insertion of three nucleotides (T, C and G) between the positions 1558327–1558330 in the *lasR* gene sequences (Table 4). When aligning these three sequences with the strain of *P. aeruginosa* PAO1 by NCBI BlastP tool, it was observed that the insertions led to the addition of the amino acid valine at LasR protein sequence, at position 53, frame 2.

Bioinformatics analysis

The comparative modeling of the LasR variant was based on the use of a known model (with experimentally determined structure), aligned with the sequences for which the tertiary structure was desired. Furthermore, this analysis allowed a visualization of the general architecture of these carbohydrate binding proteins. In this study, the models were constructed from the model template 2uv0 and 3jpu, both of *P. aeruginosa*, with 1.8 Å and 2.3 Å resolution, respectively, available in the Protein Data Bank (PDB), which shows the best similarity with the proteins of the analyzed species. Thus, these sequences were submitted to homology-based computational modeling, presenting 100% identity values after alignment against the model and strains *P. aeruginosa* PAO1 and P1A isolate (non-biofilm producer). Three-dimensional modeling demonstrated that the insertion of the amino acid valine into the protein sequence of the P1A isolate generated a change in the protein structure, reducing the antiparallel beta sheets and altering the structure of the alpha cleavage (Fig. 1A and B).

The reliability analysis of the proposed models presented values within the reliability standards, with QMEAN scores varying within the spectrum -0.66 to 0.37 (values closer to one represent models with higher quality). Although negative Z-scores are an indication of a good model, the plot of the model should also be considered in the graph of the scores of all protein structures deposited in the PDB, which must be located inside the gray (light or dark) and black areas. Based on this analysis, the different proteins modeled are within the area referred to the desired quality standards, and the LasR protein was modeled for the clinical isolate of *P. aeruginosa* P1A, which is non-biofilm producer, plotted more externally to the graph (Fig. 2A and B).

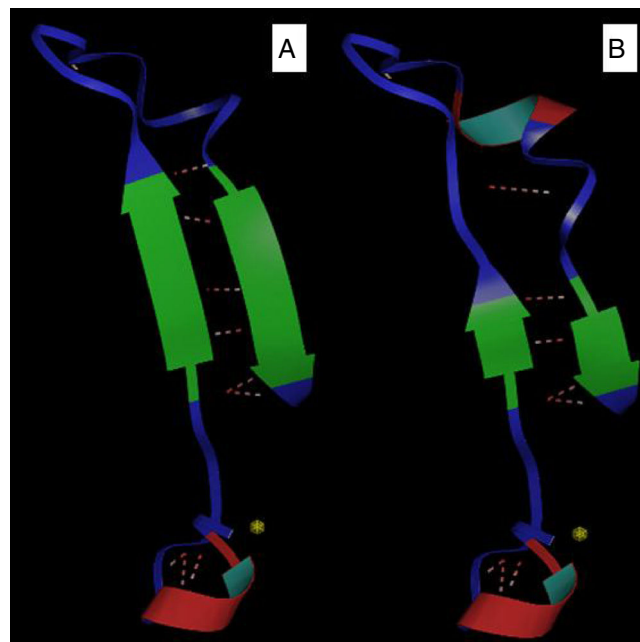


Fig. 1 – Three-dimensional models of the *P. aeruginosa* LasR protein. (A) *P. aeruginosa* PAO1 (standard biofilm producing strain) and (B) P1A (clinical isolate of non-biofilm producing *P. aeruginosa*).

The distribution of the amino acid residues in the Ramachandran graph, which evaluates the stereochemical quality of the structure, revealed that 98% and 93.3% of the residues were in the most favorable energy regions for a good quality model for *P. aeruginosa* PAO1 (standard biofilm producing strain) and *P. aeruginosa* P1A (clinical non-biofilm producing isolate), respectively (Fig. 3A and B). In the graph of *P. aeruginosa* P1A, there are amino acid residues in the energetically unfavorable regions, due to the presence of the amino acid valine, differing from the results described by Chowdhury et al.,²⁰ in which no amino acid residue was in the most unfavorable energy regions of the Ramachandran chart.

Molecular typing

The genotyping of clinical isolates of *P. aeruginosa* by ERIC-PCR showed 38 distinct genetic profiles, with similarity ranging from 24% to 100% (Fig. 4).

Discussion

The biofilm quantification test proved to be effective in the detection of biofilm production by the clinical isolates analyzed, since it was able to detect biofilm production by *P. aeruginosa* strain PAO1, used as positive control in the test. Similar data were observed by Lima et al.,²¹ showing that 75% of the clinical isolates of *P. aeruginosa* were biofilm producers. Additionally, in a study conducted by Perez et al.,²² in which biofilm production was observed in 68% (50/74) of the clinical isolates of *P. aeruginosa*, being distributed in the following categories: 96% (48/50) weakly adherent and 4% (2/50) moderately

Table 4 – Analysis of the gene and protein sequence of <i>P. aeruginosa</i> PAO1 strain and clinical isolates of non-biofilm producing <i>P. aeruginosa</i> .		
Isolate	Nucleotide sequence	Amino acid sequence
PAO1	ATGGCGAGCGACCTTGGATTCTCGAAGATCCTGTTGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGGCAACTAC	MASDLGFSKILFGLLPKDSQDYENAFIVGNYFrame + 1
P1A	ATGGCGAGCGACCTTGGATTCTCGAAGATCCTGTTGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCG**G*CAACTAC	MASDLGFSKILFGLLPKDSQDYENAFIV#GNY
P7B	ATGGCGAGCGACCTTGGATTCTCGAAGATCCTTTCGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGTC**G*CAACTAC	MASDLGFSKILFGLLPKDSQDYENAFIV#GNY
P20B	ATGGCGAGCGACCTTGGATTCTCGAAGATCCTGTTGGCCTGTTGCCTAAGGACAGCCAGGACTACGAGAACGCCTTCATCGTCGTC**G*CAACTAC	MASDLGFSKILFGLLPKDSQDYENAFIV#GNY
* Regions where nucleotide insertions (T, C and G) occurred, respectively, in clinical isolates of non-biofilm producing <i>P. aeruginosa</i> .		
# Regions where amino acid valine insertion occurred, in clinical isolates of non-biofilm producing <i>P. aeruginosa</i> .		

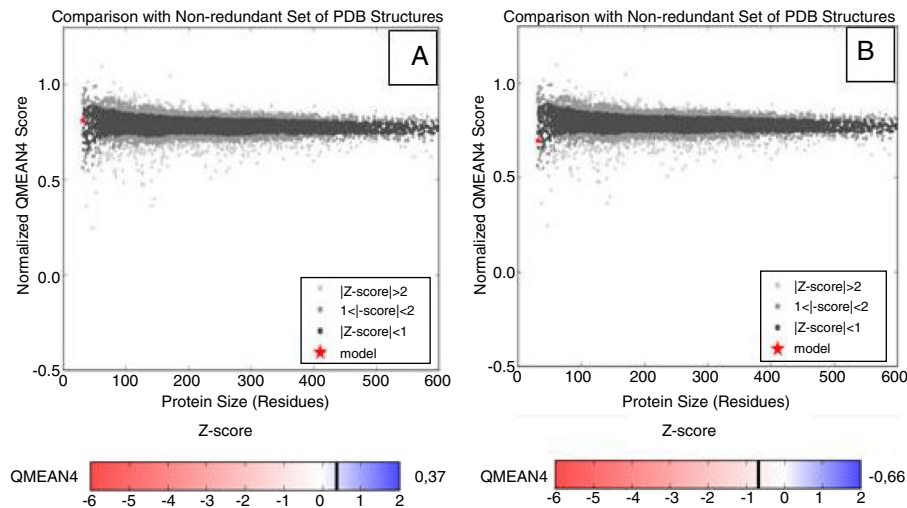


Fig. 2 – Representative Z-score graphs of the generated models, compared to X-ray (gray) and magnetic (dark gray) crystallography models deposited in the PDB. The dots in red refer to the generated models for the *P. aeruginosa* LasR protein. (A) *P. aeruginosa* PAO1 (standard biofilm producing-strain) and (B) *P. aeruginosa* P1A (clinical isolate non-biofilm producer), obtained through comparative modeling.

adherent. In the study of Perez et al.,²³ biofilm production was present in 93.4% (85/91), being distributed in the following categories: 60% (51/85) poorly adherent, 25.9% (22/85) moderately adherent, and 14.1% (12/85) strongly adherent.

Furthermore, among the isolates classified as biofilm producers in the present study, 48.4% (15/31) were MDR and 51.6% (16/31) were MDS, suggesting that, for these isolates, biofilm production was not relevant for antimicrobial resistance, differing from the results found by Perez et al.,²² where all isolates of *P. aeruginosa* producing metallo- β -lactamases (MBL) produced biofilm.

As for the occurrence of the genes of QS, our data are similar to those found by Perez et al.,²³ where 90.1% (82/91) of the analyzed isolates presented all the genes. By contrast, the results reported by Karatuna et al.²⁴ showed that QS genes detection were not as high, as those reported in our study

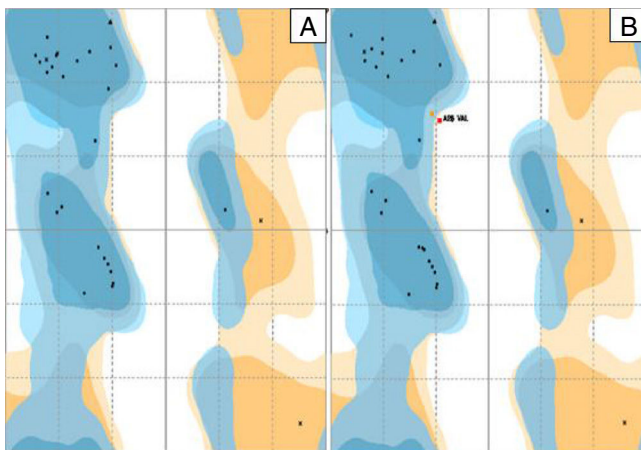


Fig. 3 – Dispersions of the amino acid residues in the Ramachandran graph. (A) *P. aeruginosa* PAO1 (standard biofilm producing strain) and (B) *P. aeruginosa* P1A (clinical isolate non-biofilm producing).

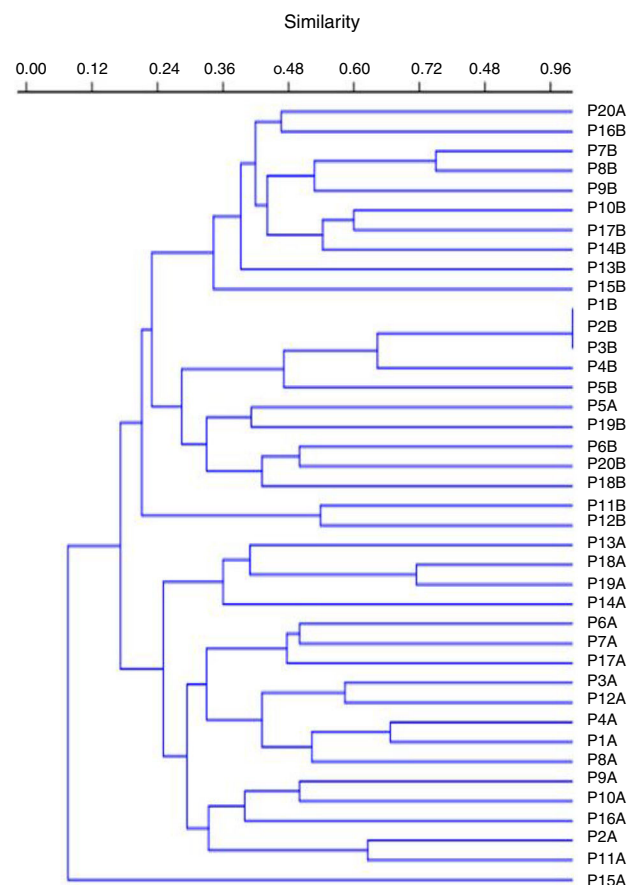


Fig. 4 – Dendrogram constructed by analysis of the results of the Enterobacterial Repetitive Intergenic Consensus-based PCR generated by the PAST software for 40 clinical isolates of *P. aeruginosa*.

detection (81.25% for the *lasI* and *lasR* genes, 68.75% for the *rhlI* gene, and 62.5% for the *rhlR* gene).

In the present study, the *P. aeruginosa* isolate unassembled only for the *lasI* gene was a strong biofilm producer. In the same way, in the study conducted by Perez et al.,²³ an unassembled isolate for the *lasR* gene, was biofilm producer. These facts can be explained by the *in vivo* compensation for the presence of the *rhlI/rhlR* QS system, as observed in *P. aeruginosa lasR*-mutant which showed no reduction in its virulence during a corneal infection process.¹⁰

In a study conducted by Sabharwal et al.,²⁵ in India, the occurrence of *lasI*, *lasR*, *rhlI*, and *rhlR* genes of *P. aeruginosa* was 75% for the *lasI* and *lasR* genes, 41.6% for the *rhlI* gene, and 58.3% for the *rhlR* gene. Accordingly, similar data were found in the study by Aboushleib et al.,²⁶ in Egypt, where the QS genes occurrences were 48% for the *lasI* gene, 40% for the *lasR* and *rhlI* genes, and 36% for the *rhlR* gene. Analysis of these studies revealed that genotypically positive and phenotypically negative isolate sequences appear to carry several mutations in the QS genes.

To the best of our knowledge, despite the report of numerous *lasR* mutations, the mutation reported herein had never been described elsewhere. In the study carried out by Senturk et al.,²⁷ four isolates of *P. aeruginosa* were non-biofilm producers, despite the presence of these QS genes. The authors attributed the lack biofilm production to the mutations detected by sequencing, although they did not report the types of mutations found. Bjarnsholt et al.²⁸ evaluated the occurrence of mutations in the QS genes of 238 *P. aeruginosa* isolates obtained from cystic fibrosis patients. Mutations in the *lasR* and *rhlR* genes were found and these isolates showed decreased virulence, similar to those found in the present study in which isolates that had mutations in the *lasR* gene were non-biofilm producers.

By contrast, Aboushleib et al.²⁶ found mutations in the *lasI*, *lasR*, *rhlI*, and *rhlR* genes in both biofilm producer and non-producer isolates.

Moreover, in the study by Feltner et al.,²⁹ the *lasR* gene of 2583 cystic fibrosis isolates of *P. aeruginosa* was sequenced, and 580 sequences encoded proteins that differed from conserved LasR proteins. The authors showed that about one-sixth of 31 LasR variant proteins were functional, including three with nonsense mutations. In addition, in *lasR*-null isolates, LasR-dependent were, nevertheless, expressed. The remaining 23 isolates showed non-functional LasR variants. Although the findings did not describe the mutation found in our study, it is possible that this mutation did also generate a non-functional LasR variant, which would explain the lack of biofilm production.

In addition, the *lasR* gene is located at the position 1558171–1558890 of the genome of *P. aeruginosa* and is franchised by genes *PA1429* and *rsaI*,⁷ responsible for cytoplasmic LasR protein coding. This protein belongs to the family of transcriptional regulators LuxR-type and is related to the regulation of transcriptional regulatory region, autoinducer region (area where the connection with the autoinducers occurs) and the region of the DNA-binding residues.³⁰ The mutation described in our study was in the first two regions, which could therefore prevent biofilm formation.

Bottomley et al.³⁰ observed through modeling that the region of insertion of the amino acid valine corresponds to the region of the beta 2 leaf. In addition, the analysis of the modeling reveals that the atomic interactions between the protein LasR and its autoinducer N-(3-oxododecanoyl)-homoserine lactone (OdDHL) occur in the N-terminal domain of the LasR protein, through hydrogen bonds. The amino acid residues found to be involved in the interactions with the autoinducer were tyrosine-56, arginine-61, aspartate-73, threonine-115 and serine-129. These residues bind concurrently and cause protein folding, leading to dimerization of LasR, thus allowing DNA binding to the promoter and consequent transcriptional activation of QS controlled genes. These amino acids, detected by computational analysis, provide information on the binding affinities of the amino acid residues to the specific ligands.³⁰ Based on this analysis, it can be suggested that the valine insertion in position 53 of the LasR protein prevented the binding of the amino acid tyrosine located in position 56 to form the hydrogen bond with the autoinducer, preventing activation of the protein biofilm formation.

Our results corroborate the findings of Bjarnsholt et al.,²⁸ in which amino acids tyrosine-56 and threonine-75 strongly interact with the autoinducer, and the occurrence of mutations in this region of the *lasR* protein would impair autoinducer binding.

A great genetic diversity was observed in the clinical isolates of *P. aeruginosa* analyzed in this study, as found in studies involving patients with severe or chronic infections, who present favorable conditions for the occurrence of non-lethal mutations.^{31,32} Similar data were also observed by Cavalcanti et al.,³³ who reported replacement of the SPM-1 *P. aeruginosa* clone by a variety of genetically distinct *P. aeruginosa* isolates in a university hospital in Recife, Brazil. Besides that, the presence of clones with phenotypic profiles of distinct biofilm production evidences the existence of regulation mechanisms of the expression of QS genes related to biofilm production. Of concern is the high biofilm production by these isolates, even by multidrug sensitive isolates, which may make it difficult to treat the infections caused by them. The three-dimensional models were satisfactory in the evaluation of the regulation of the proteins related to biofilm formation, and may, therefore, be used in other analyses. However, further studies including a larger number of clinical isolates of *P. aeruginosa* will be necessary to elucidate the relationship between this mutation found and non-biofilm producing clinical isolates of *P. aeruginosa*.

Funding

This research was in part supported by National Council for Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education Personnel (CAPES).

Conflicts of interest

The authors declare no conflicts of interest.

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