Sumerianz Journal of Medical and Healthcare, 2021, Vol. 4, No. 1, pp. 49-54
ISSN(e): 2663-421X, ISSN(p): 2706-8404
Website: https://www.sumerianz.com
DOI: https://doi.org/10.47752/sjmh.41.49.54

Original Article Open Access

Analysis of Cuorum Sensing-Dependent Virulence Factors and Drug Resistance in *Pseudomonas Aeruginosa* Strains

José de Jesús Alba-Romero
División de Estudios de Posgrado e Investigación. Facultad de Ciencias Químicas. Unidad Gómez Palacio. Universidad Juárez del Estado de Durango, México
Laboratorio de Asesores Especializados de la Laguna, Gómez Palacio, Durango

Pablo Ruiz-Flores
Centro de investigación Biomédica. Universidad Autónoma de Coahuila, Torreón Coahuila

Graciela Castro-Escarpulli
Laboratorio de Investigación Clínica y Ambiental, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Ciudad de México, México

Sandra Isabel Hernández-González
División de Estudios de Posgrado e Investigación. Facultad de Ciencias Químicas. Unidad Gómez Palacio. Universidad Juárez del Estado de Durango, México

Aurora Martínez-Romero*
División de Estudios de Posgrado e Investigación. Facultad de Ciencias Químicas. Unidad Gómez Palacio. Universidad Juárez del Estado de Durango, México
Email: aurora.martinez@ujed.mx

José Luis Ortega-Sanchez
Unidad Regional Universitaria de Zonas Áridas. Universidad Autónoma Chapingo, México

Article History
Received: February 10, 2021
Revised: March 18, 2021
Accepted: March 23, 2021
Published: March 26, 2021

Abstract

The objective was to analyze the virulence factors dependent on Cuorum Sensing and drug resistance in strains of *Pseudomonas aeruginosa*. Virulence factors such as pyocyanin, beta-lactamase, biofilm, and antibiotic resistance were determined in 95 strains of P. aeruginosa isolated from hospitalized patients. Genus and species were identified by protein analysis by MALDI-TOF. 100% of the strains were resistant to at least one drug and the highest proportion was 32 strains resistant to 4 drugs and 5 resistant PAM strains. In the analysis of virulence factors, 98.8% produce at least one virulence factor and 48.9% are beta-lactamase producers. Therefore, it is concluded that *P. aeruginosa* strains isolated from clinical samples constitute a risk factor for hospitalized patients.

Keywords: Cuorum Sensing; bacterial resistance; pyocyanin; biofilm; beta-lactamase.

1. Introduction

Currently, Infections Associated with Health Care (HAI) represent a serious problem for the world population, causing about 12 million infections annually [1]. In Mexico, between 2-15% of hospitalized patients have this type of infection, which is caused by opportunistic pathogens once admitted to the hospital. Recently, *Pseudomonas aeruginosa* (*P. aeruginosa*) has been reported as the most prevalent microorganism, [2] *P. aeruginosa* has the ability to acquire plasmids and other mobile genetic elements that confer resistance to antibiotics [3]. Antibiotic resistance is a major concern of contemporary medicine. The emergence of resistant strains that cause nosocomial infections contributes substantially to the morbidity and mortality of hospitalized patients [4]. Bacterial genes encoding different beta-lactamase (BL), metallo-beta-lactamase (MBL), and extended-spectrum beta-lactamase (ESBL), can confer resistance to multiple classes of beta-lactam antibiotics [5]. Therefore, there is a pressing need for new antibacterials with new modes of action. Cuorum Sensing (CS), an extracellular communication system used by bacterial populations to coordinate the expression of the virulence gene, which has been intensively investigated during the last decade. This perspective focuses on recent advances in targeting the three major quorum sensing systems (*las*, *rhl*, and *pqs*) of a major opportunistic human pathogen such as *P. aeruginosa* and will specifically evaluate medical chemistry strategies devised to develop inhibitors. of CS from drug discovery [6]. CS is a cell-to-cell signaling mechanism that coordinates a range of behaviors at the population level [7]. CS is a mechanism by which the bacterium censes the microbial density present through the production of N-acyl homoserine lactone-like molecules, which function as autoinducers that bind with receptor proteins to form a complex that in turn favors the expression of genes that code for the production of more N-acyl-homoserine lactone synthase and the receptor itself, thus forming circuit mechanisms in *las* and *rhl* series, in which, consequently, the expression of virulence factors such as elastase, protease, pyocyanin will begin with which the bacteria will begin biofilm synthesis to contribute to

*Corresponding Author

its adhesion and infection progress [8]. Finally, there is a third molecule of CS, which is the so-called autoinducer-3 (AI-3), which involves signaling between taxonomic domains, that is, between bacteria and archaea (prokaryotic cells), and eukaryotes (organisms higher). In this system, the AI-3 molecule produced by the commensal gastrointestinal tract microflora, the host-produced epinephrine and norepinephrine interact with a two-component regulatory system to activate the transcription of genes involved in bacterial pathogenesis [9]. Many Bacteria use signal transduction mechanisms known as CS detection to control population density and regulate resource-intensive collective processes that would not benefit individual bacteria [10]. *P. aeruginosa* has become an important and frequent cause of nosocomial infections (NIs), such as pneumonia, urinary tract infections, and bacteremia, which appear in the form of outbreaks. Immunodeficient patients such as those admitted to burn units, intensive care units and cancer centers are particularly susceptible to serious or fatal infections [11]. Previously, it has been reported that the frequency of classical beta-lactamase (CBL) and ESBL-producing *P. aeruginosa* in isolated sinks, taps, incubators and hospital cribs it was 45% and 10%, respectively [12]. However, it is necessary to carry out studies with biological samples from patients with infection to characterize the microorganisms during their stay in the host and not only on inert surfaces. Recently, it has been shown that virulence factors depend directly on the presence of the CS phenomenon even though CS deficient strains are equally capable of causing infections in humans [13]. Health care associated infections are a A worldwide problem, first due to the economic deterioration that it constitutes for public health institutions due to the increase in hospital stay, constituting a loss of up to 450 million dollars [2]. Second, the complications for the patient in their state of health due to increased morbidity and in some cases death. Among the main microorganisms worldwide, the enterobacteria, mainly *Escherichia coli*, Gram positive microorganisms such as *Staphylococcus epidermidis*; and within the non-fermenters *P. aeruginosa*. For all the above, it is necessary to carry out new studies that help elucidate the emerging infection mechanisms by opportunistic microorganisms in order to generate sufficient knowledge that can serve as a start for the future design of strategies in order to reduce the prevalence of these diseases. Therefore, the objective was to analyze the virulence factors dependent on Cuorum Sensing and drug resistance in *Pseudomonas aeruginosa* strains.

2. Material and Methods

This project was submitted for evaluation by the Ethics and Research Committee of the Faculty of Chemical Sciences of the Universidad Juárez del Estado de Durango. An informed consent letter was made which was given in writing to the participants, where the objective of taking their biological sample was informed, so that the present work, despite being experimental, did not put the integrity of the samples at risk participants in the study. During the course of the experiment, the results were reported to the treating physician and the corresponding Direccion General de Epidemiologia was notified.

2.1. Isolation of Strains and Growth Conditions

Strains were isolated from urine samples, expectoration, bronchial lavage wounds, catheter tip, and dialysis fluid. All strains were isolated from samples of hospitalized patients at IMSS, ISSSTE, HGG SSD, Clínica Lerdo, Clínica Divina Providencia and Sanatorio San José, located in the Lagunera Region of Durango.

The clinical samples were seeded on Mac Conkey agar, Casman blood agar, and Biggy agar. CDC protocol was followed to include only infectious process samples.

95 strains of *P. aeruginosa* were analyzed from samples of hospitalized patients, which were classified according to the different anatomical sites, consisting of 12 urine samples, sputum 36, bronchial lavage 18, infected wounds 16, catheter tips 5, dialysis fluids peritonial 8 (Table 1). Plates of Mac Conkey agar, Casman blood agar and Biggy agar were inoculated overnight at 37°C. Positive cultures for *Pseudomonas*. The strains obtained were seeded in Luria Bertani broth (LB) overnight at 37°C, for the biofilm tests (37°C/16 h), BL and pyocyanin. Likewise, ATCC strains were counted as control strains.

The 95 strains of *P. aeruginosa* from samples of hospitalized patients were isolated on Mac Conkey agar, and later, they were identified by analysis of ribosomal proteins through MALDI-TOF mass spectrometry, the strains were processed in the Department of Medical Bacteriology of the National School of Biological Sciences of the Instituto Politecnico Nacional.

Table-1. Number of clinical samples from hospitalized patients that were used in the study

Positive clinical samples	
Urine	12
Expectoration	36
Bronchial lavage	18
Wounds	16
Catheter tip	5
Peritoneal dialysis fluid	8
Total samples analyzed	**95**

2.2. Phenotypic Characterization

The phenotypic characterization was carried out in the first instance by means of the selected isolates. The primary tests such as oxidase and catalase were used then, the automated method of MicroScan® (Dade International, Inc., West Sacramento, California., USA) was used in order to obtain the tests of drug susceptibility and the presence of BL.

2.3. Determination of Drug Sensitivity

Drug sensitivity was performed using the automated MicroScan® method (Dade International, Inc., West Sacramento, California., USA), determining the minimum inhibitory concentration (MIC) for each of the isolates from the different anatomical sites from of hospitalized patients

The MicrosScan4® system complies with CLSI guidelines, [14] and has specific features such as user selection of antimicrobial agents, independent MIC results, Combo panels that combine two technologies and provide a single result, panel selection depending on the workload, confirmation in the same BL combo panel, confirmation of resistance induced to clindamycin, detection of resistant methicillin with cefoxitin.

2.4. Determination of Drug Sensitivity

2.4.1. Determination of Pyocyanin

Once the *P. aeruginosa* strains were isolated, the pyocyanin was extracted from the LB broth grown overnight and reseeded in alanine-glycerol medium [15]. The cells were removed by centrifugation and the pyocyanin remained in the supernatant, which was extracted in $CHCl_3$, 5.0 mL of supernatant are mixed with 3.0 mL of $CHCl_3$. The pyocyanin was re-extracted in 1.0 mL of acidified water (0.2 M HCl) which will give a red-pink solution. For the quantification of pyocyanin in the solution, the absorbance at 520 nm was determined for each tube representing each simple [16]. The tests will be carried out in triplicate.

2.5. Biofilm Determination

The ability of the isolated strains to form biofilm was measured using polystyrene microtiter plates. Overnight cultures grown in LB broth were used and 1: 100 dilutions were prepared in fresh LB broth and then 0.1 mL of the freshly inoculated medium was dispensed into the wells of a 96-well plate.16 The plate was incubated at 37°C/8h without stirring. Biofilms were detected by staining the wells with 10 µL of crystal violet [0.1% (w/v) in H_2O], after staining the plate was incubated for another 15 min at room temperature and then exhaustive washing with distilled water to remove planktonic cells and residues from the dye. Ethanol (95%) was used to elute crystal violet from the biofilms and the absorbance of the solubilized dye was measured at 590 nm using an ELISA plate reader. The tests were carried out in triplicate. This protocol can be carried out in borosilicate test tubes as an option.

2.6. Determination of Beta-Lactamase

This test was performed by the automated method of MicroScan® (Dade International, Inc., West Sacramento, California., USA) simultaneously with the determination of drug sensitivity in the panel for NC44 Gram negative microorganisms. However, some strains were verified as part of the internal quality control by replacing the strain under study at 0.5 NF and adding a Cefinase disk.

Nitrocefin method. A drop of physiological solution is placed on a nitrocefin disk (Cefinase®, BD BBL, USA) supported on a slide. Rub the disk with a dense inoculum taken from a solid medium culture of *P. aeruginosa* to be tested. The same is done with both strains, one BL positive and the other BL negative. Incubate in a humid chamber for up to 24 h, with serial readings. The red staining = positive test (presence of BL) and yellow staining = negative test (absence of BL).

3. Results

Of the isolates, 95 produced biofilm, 87 produced pyokinin, and 47 strains produced BL. When determining drug resistance, 27 strains resistant to 3 drugs, 32 strains to 4 drugs, 25 strains to 5 drugs, 15 strains to 6 drugs, and 5 strains were PAM resistant and 0 strains were sensitive to at least one drug.

When analyzing the virulence factors in the 95 strains of P. aeruginosa (Figure 1), 95 biofilm-producing strains were found, 87 pyocyanin-producing strains and 47 strains were BL producers.

The MALDI-TOF MS results were corroborated by the automated MicroScan® system, which were 100% concordant between both methods.

The analysis of BL-producing strains by group of antibiotics is presented in Figure 2 and that of strains resistant to a group of drugs in Figure 3.

Figure-1. Main virulence factors identified in 95 strains of *P. aeruginosa*.

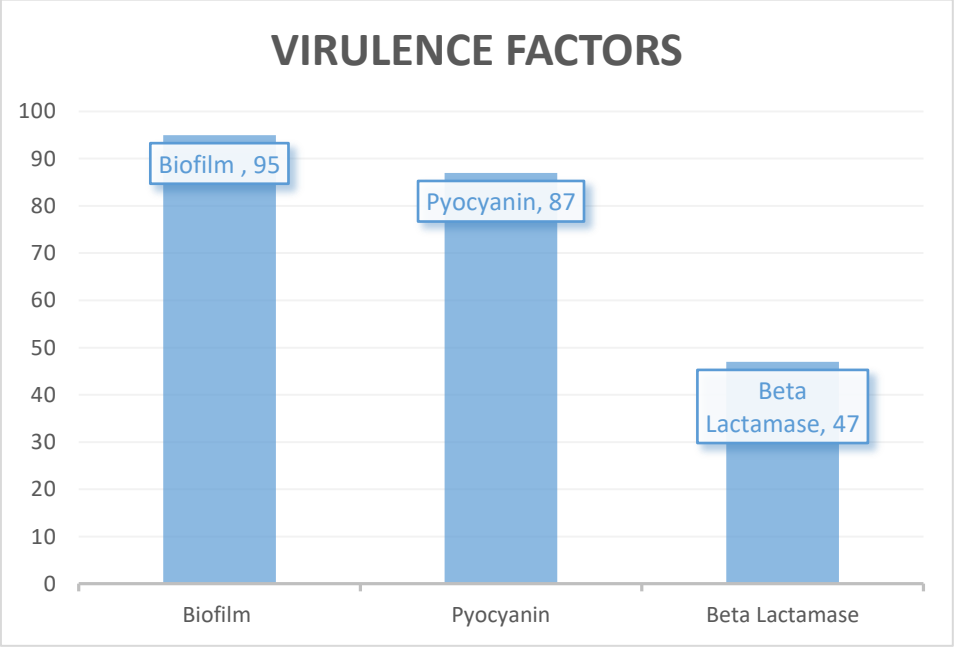


Figure-2. Analysis of Beta lactamase producing strains by group of antibiotics

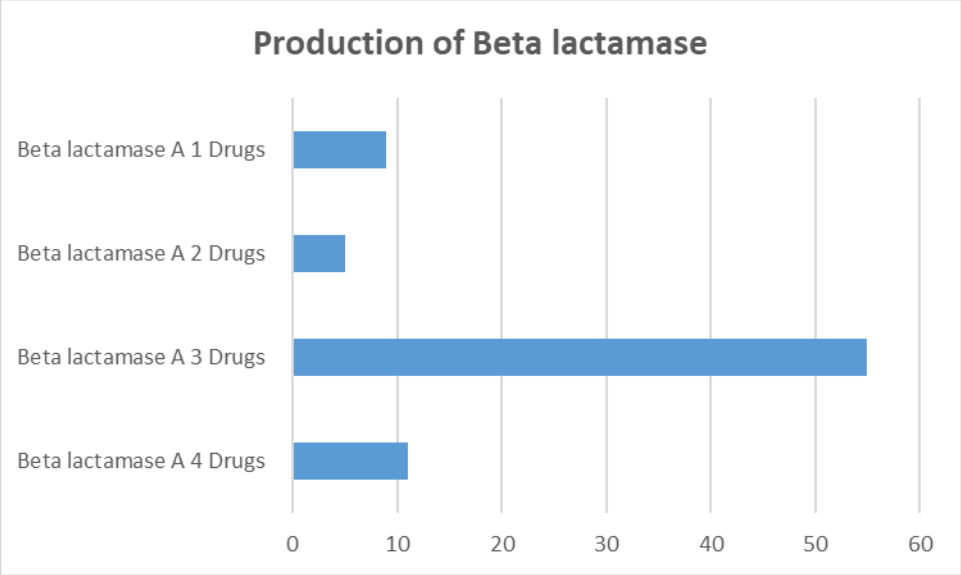
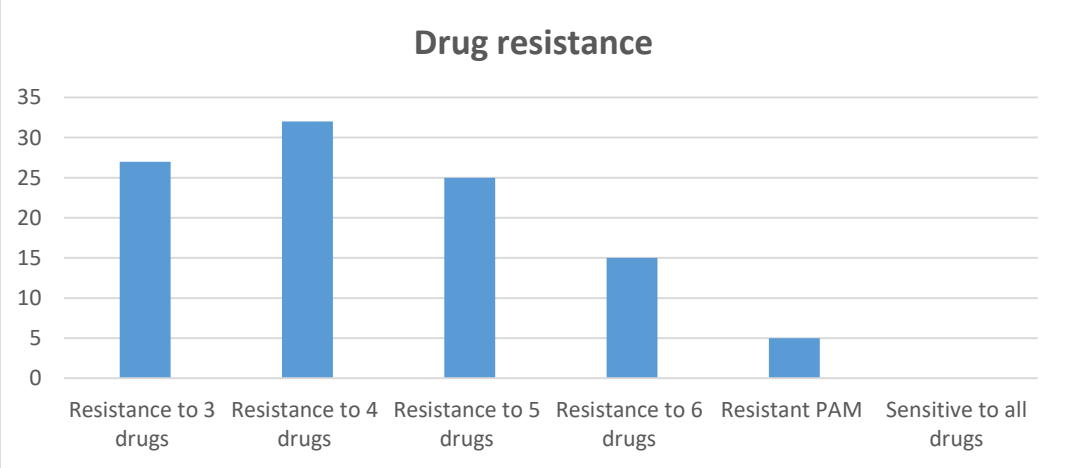


Figure-3. Analysis of drug group resistant strains



4. Discusion

In the present investigation, when analyzing the virulence factors dependent on Cuorum Sensing and drug resistance in 95 strains of *P. aerugionsa* obtained from clinical samples from different hospitals in the Lagunera Region of Durango, it was observed that one of the sampling hospitals specializes in respiratory tract diseases,

however, we could observe that *P. aeruginosa* is a microorganism that is present as a colonizing agent of the respiratory tract and produces disease or is associated as an opportunistic agent that endangers patients due to its high content of virulence factors dependent on an extracellular communication system called CS, described by Jack, *et al.* [16], Essar, *et al.* [17]. When analyzing the presence of virulence factors and drug resistance, it was found that most produce biofilm which is an exopolysaccharide that gives you a way to colonize different anatomical sites and even inherent surfaces, there is also a direct correlation with resistance to at least 4 drugs and the production of BL. An important aspect is that all the samples were obtained from infected patients or who had an association with a disease that originated upon admission to the hospital.

The analysis of virulence factors is a very relevant aspect since it is the epidemiological indicator of risk for every patient admitted to the hospital and that can be acquired by air currents, water droplets or by health care personnel. Liu, *et al.* [18], comment on the deficiency in policies, training, and supervision by the surveillance committee of infections associated with health care [18]. The search for and isolation of this type of microorganism, such as *P. aeruginosa*, constitutes a challenge. for health services due to its great variety of virulence factors that makes it one of the super bacteria as it is known today due to its high resistance to a large majority of antibiotics, among which the beta-lactam family stands out. The 95 strains obtained presented evidence in the production of biofilm, this aspect is relevant because it constitutes one of the main mechanisms of virulence and resistance to antibiotics, which coincides with that mentioned by Moore, *et al.* [19] and Jiménez-Pearson, *et al.* [20]. The identification of *P. aeruginosa* was carried out by analysis of ribosomal proteins using the MS MALDI-TOF system, resulting 100% coinciding with a phenotypic system such as MicroScan, MALDI ionization (matrix-assisted laser desorption/ionization), coupled to a TOF (time of flight) analyzer, is a gentle ionization technique used in mass spectrometry that allows the analysis of biomolecules (biopolymers such as proteins, peptides, and sugars) and large organic molecules (such as polymers, dendrimers, and other macromolecules) that tend to become brittle and fragment when ionized by more conventional methods, confirmed by Nadjm, *et al.* [21]. Currently, the MS MALDI-TOF system is used for the identification of non-tuberculous mycobacteria as described by Genc, *et al.* [22]. In this technique, the sample is mixed with the matrix in excess on a metal surface, in such a way that both crystallize when the solvent evaporates. This preparation is subjected to short laser pulses in high vacuum, which causes the energy absorption by the matrix to be converted into excitation energy and H^+ transfer to the sample (ionization), normally giving rise to monocharged species. That are analyzed by TOF [23]. This method is used to identify various microorganisms such as bacteria and fungi, mainly [24]. When carrying out the drug resistance analysis, 5 resistant PAM strains were observed, representing an alert signal for health services at the local, national and international level, as described by the appearance of *P. aeruginosa* strains MDR, XDR and PDR, which poses a significant therapeutic challenge [25]. The management of keratitis due to *P. aeruginosa* XDR and PDR would be extremely difficult due to to the shortage of safe and effective topical medications, in this regard [26] describe the clinical characteristics, risk factors, or *P. aeruginosa* XDR and PDR against keratitis [26].

5. Conclusion

The identification of the strains by the MicroScan phenotypic method and by the protein analysis by the MALDI-TOF mass spectrometry method, was 100% demonstrated that both methods are excellent. The strains of *P. aeruginosa* showed high levels of resistance in their entirety to at least one drug.

The production of virulence factors is high since 91% of the strains produce at least one virulence factor and 83% of the strains produce one or two virulence factors. Although the production of beta lactamase is relatively low, 45% of the strains are interfering with drug sensitivity and complications in the health-disease process. Infections by strains of P. aeruginosa acquired in the hospitals of La Comarca Lagunera, which are producers of virulence factors and resistant to drugs, are a serious problem due to the implications that it entails, for the patient during the days of hospitalization and even leading the patient to death, both in public and private institutions, treating a patient with infections by strains resistant to different drugs.

The epidemiological study of the strains involved in infections associated with health care are of vital importance due to the virulence factors that allow them to be resistant, extremely resistant and resistant PAM dependent on Cuorum Sensing, which is a communication mechanism. Intercellular that allows this type of bacteria to be highly dangerous due to its virulence factors. This is a problem at the regional, state, national and international levels.

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