UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE MEDICINA

Centro de Investigación en Ciencias de la Salud y Biomedicina (CICSaB)

Caracterización epidemiológica de *Pseudomonas sp* provenientes del hospital de tercer nivel “Dr. Ignacio Morones Prieto”.

TESIS QUE PRESENTA

M. en C. NALLELY SARAI BADILLO LARIOS

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOMÉDICAS BÁSICAS

CO-DIRECTORES DE TESIS
DRA. PERLA DEL CARMEN NIÑO MORENO
DR. EDGAR ALEJANDRO TURRUBIARTES MARTÍNEZ

Enero del 2025

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Dra. Perla del Carmen Niño Moreno
Dr. Edgar Alejandro Turrubiartes Martínez

ASESORES INTERNOS
Dr. Fidel Martínez Gutiérrez
Dra. Esther Layseca Espinosa

ASESOR EXTERNO
Dr. Gerardo Martínez Aguilar

Enero de 2025

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Research Article

Interesting Cytokine Profile Caused by Clinical Strains of *Pseudomonas aeruginosa* MDR Carrying the exoU Gene

Nallely S. Badillo-Larios,[1] **Edgar Alejandro Turrubiartes-Martínez**,[1,2] **Esther Layseca-Espinosa**,[1,3] **Roberto González-Amaro**,[1,3] **Luis Fernando Pérez-González**,[3,4] **and Perla Niño-Moreno**[1,5]

[1]*Center of Research in Health Sciences and Biomedicine, Faculty of Medicine, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico*
[2]*Laboratory of Hematology, Faculty of Chemical Sciences, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico*
[3]*Faculty of Medicine, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico*
[4]*Central Hospital Dr. Ignacio Morones Prieto, San Luis Potosi, Mexico*
[5]*Genetics Laboratory, Faculty of Chemical Sciences, Autonomous University of San Luis Potosi, San Luis Potosi, Mexico*

Correspondence should be addressed to Perla Niño-Moreno; ncarmenp@uaslp.mx

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Pseudomonas aeruginosa is an opportunistic pathogen in HAIs with two facets: the most studied is the high rate of antimicrobial resistance, and the less explored is the long list of virulence factors it possesses. This study aimed to characterize the virulence genes carried by strains as well as the profile of cytokines related to inflammation, according to the resistance profile presented. This study aims to identify the virulence factors associated with MDR strains, particularly those resistant to carbapenems, and assess whether there is a cytokine profile that correlates with these characteristics. As methodology species were identified by classical microbiological techniques and confirmed by molecular biology, resistance levels were determined by the minimum inhibitory concentration and identification of MDR strains. Virulence factor genotyping was performed using PCR. In addition, biofilm production was assessed using crystal violet staining. Finally, the strains were cocultured with PBMC, and cell survival and the cytokines IL-1β, IL-6, IL-10, IL-8, and TNF-α were quantified using flow cytometry. Bacteremia and nosocomial pneumonia in adults are the most frequent types of infection. In the toxigenic aspect, genes corresponding to the type III secretion system were present in at least 50% of cases. In addition, PBMC exposed to strains of four different categories according to their resistance and toxicity showed a differential pattern of cytokine expression, a decrease in IL-10, IL-6, and IL-8, and an over-secretion of IL-1b. In conclusion, the virulence genes showed a differentiated appearance for the two most aggressive exotoxins of T3SS (*exoU* and *exoS*) in multidrug-resistant strains. Moreover, the cytokine profile displays a low expression of cytokines with anti-inflammatory and proinflammatory effects in strains carrying the exoU gene.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium and one of the most important pathogens in healthcare-associated infections (HAIs), causing 32,600 infections and 2,700 deaths in the United States in 2017 [1]. Classified as opportunistic pathogens involved in acute and chronic infections, the main reason for this versatility is the large list of virulence determinants. It possesses both intrinsic and acquired resistance to antibiotics and high metabolic flexibility because of its ability to use various carbon sources or electron acceptors [2]. Owing to these mechanisms, *P. aeruginosa* is responsible for several HCAIs, such as pneumonia, surgical infections, bacteremia, and urinary tract infections [3]. The challenge of treating an infection caused by *P. aeruginosa* is why the World Health

Organization (WHO) has included it in the "critical" category in its priority list of pathogens that urgently require research and development of new antibiotics [4].

According to a review in 2021, it is estimated that 7.1%–7.3% of HCAIs are caused by this pathogen, and the most common infection is pneumonia, followed by surgical site infection (SSI); however, its prevalence has increased over the last decade [5]. In addition, the available data on the Mexican situation are limited; in 2022, the RHOVE (Red Hospitalaria de Vigilancia Epidemiológica, in Spanish) found *P. aeruginosa* to be the second most common cause of HCAIs in the country [6]. The latest information provided by the IMSS (Instituto Mexicano del Seguro Social, in Spanish) in 2016 points to *P. aeruginosa* as the third cause, just below *Escherichia coli* and *Staphylococcus aureus* [7]. Another report pointed out that Mexico has the highest rate of infection caused by this pathogen [8].

As mentioned above, the complexity of the infections caused by *P. aeruginosa* has two faces: the most studied is the high rate of antimicrobial resistance, especially resistance to carbapenems, with 17%–52% of the cases worldwide [9], and the ultimate concern is multidrug-resistant (MDR) strains, representing 9% of cases in the USA in 2018 [10].

Another issue mentioned above is the ample list of virulence factors possessed by *P. aeruginosa*, some of which are enzymes, exoenzymes, toxins, and the ability to grow as a planktonic community as well as in biofilms [11]. In this respect, the arsenal includes factors that are associated with the bacterial surface and those secreted, which include exotoxin A, phospholipase, alkaline protease, pyoverdine, elastases, pyocyanin, and the type three secretion system injectosome, which can deliver four different toxins in the cellular host [2, 12].

The contribution of each factor to the deterioration of the cellular host is variable, and according to Moradali, environmental stress induces differential genomic expression, forming persistent, resistant, but less virulent phenotypes in chronic illness [2]. In acute infections, virulence factors, such as flagella and pili, are vital because they are involved in motility and initial attachment. The flagellum provides swimming motility in an aqueous environment, is essential for bacterial chemotaxis, and adheres to epithelial cells by binding its flagellum to the glycolipid asialo GM1, which can also initiate the inflammatory response. Pili is an important adhesin. It is also a decisive factor for motility, but it does so through a movement called twitching. Together, these two factors also create a highly coordinated form of motility called swarming, which is useful for semisolid surfaces [12].

P. aeruginosa produces lectins that bind with specific sugars in the initial attachment of the bacteria, particularly LecB (also named PII-L), which reduces ciliary beating of the airway epithelium and is linked to biofilm formation, serving as a structural protein in the matrix [13].

P. aeruginosa owns the exotoxin A, which is an ADP-ribosyl transferase related to attachment that enters the cell and inhibits host protein synthesis by inactivating eukaryotic elongation factor 2 [14].

Furthermore, this pathogen has several proteolytic enzymes, such as elastase A and B (LasA, LasB), secreted by the type 2 secretor system (T2SS), both capable of degrading host elastin. Specifically, LecB, also named "pseudolysin," is considered the most abundant protease capable of disrupting epithelial tight junctions [15].

Alkaline protease, also known as "aeruginosin," secreted by the type 1 secretor system, is a metalloendopeptidase that can damage the epithelium, interfering with fibronectin and laminin [16]. Phospholipase C, a lipolytic enzyme secreted by T2SS, has hemolytic activity capable of destroying the eukaryotic membrane and destabilizing phospholipids and sphingomyelin [17].

Other important factors include the pigments produced by *P. aeruginosa*, such as pyoverdine, pyochelin, and pyocyanin. The first one is associated with the characteristic fluorescent green of the species and is related to the acquisition of iron, which is highly efficient but requires enormous amounts of energy in its production. In most cases, active pyochelin is produced [18]. The other pigment, pyocyanin, which is responsible for the blue-greenish color of the colonies in culture, is a phenazine and secondary metabolite related to the decline in lung function because of its inflammatory effects [19].

Another secretor system that is important to review is the type three secretion system (T3SS), a syringe-like injectosome that can deliver toxins directly into the host cell [20] and has four toxin effectors: ExoY, ExoT, ExoU, and ExoS. The first two were the most abundant and were expressed in more than 89% of the isolates. ExoY is an adenylate cyclase capable of causing cell necrosis, endothelial disruption, and irreversible actin microtubule disassembly [21]. ExoT, the most prevalent of these effectors, has two main functions: acting as an exotoxin with GTPase-activating protein (GAP) and as an adenosine diphosphate-ribosyl transferase (ADPRT), which disrupts epithelial barriers [22]. However, neither of these toxins is thought to be sufficient to establish an infection specifically in the lungs [21, 22].

The two remaining molecules are less common but highly lethal. ExoS is a GAP found in 55%–72% of clinical isolates and is associated with chronic infections, capable of producing cell death and actin cytoskeletal disruption [23]. Finally, ExoU is less frequent (28%–42% of the isolates), it has phospholipase activity with cytotoxic effects that rapidly destroy the cell membranes of mammalian cells, and some studies have found that it is more frequent in isolates with high resistance [24].

This study aimed to identify the virulence factors associated with MDR strains, particularly those resistant to carbapenems, and assess whether there is a cytokine profile that correlates with these characteristics.

2. Methods

2.1. Bacterial Isolates. A total of 75 isolates of *Pseudomonas aeruginosa* from infections classified as HCAIs were obtained from a tertiary hospital (HCIMP in San Luis Potosi, Mexico) between May 2018 and June 2019 before approval

by the Research Committee and the Research Ethics Committee of the HCIMP. Informed consent was obtained from all participants or legal guardians.

Data collected from the medical records of the patients included sex, age, length of hospital stay, and outcome.

2.2. Bacterial Identification and Antimicrobial Susceptibility Testing. Species identification and antimicrobial susceptibility testing were performed using an automated VITEK 2 (BioMérieux SA. F-69280 Marcy l'Etoile, France). Additionally, PCR amplification was performed to confirm species identity using the primers described by Spilker et al., including primers Pa-gs for the genus and Pa-ss for the species (16S rDNA) [25].

The susceptibility profiles were confirmed using broth microdilution. Briefly, antibiotics were serially diluted 2-fold in 50 μL of Mueller-Hinton broth, mixed with 50 μL of bacteria at a density of 10^6 colony-forming units/mL, and incubated for 18 h at 37°C. Antimicrobial susceptibility testing was performed using the following antibiotics: amikacin, cefepime, gentamicin, piperacillin/tazobactam, aztreonam, ciprofloxacin, and meropenem. The results were interpreted according to guidelines recommended by the Clinical and Laboratory Standards Institute [26]. Additionally, isolates were classified as nonmultidrug-resistant (NMDR) or MDR (defined as acquired nonsusceptibility to at least one agent in ≥3 antimicrobial categories).

2.3. DNA Extraction. Genomic DNA was extracted using a boiling method as described previously, and one loopful of fresh bacteria (grown overnight on Brain-Heart Infusion agar plates) was collected and suspended in 200 μL of sterile DNase/RNase-free water and incubated at 94 °C for 5 min and −70 °C for 5 min. The bacterial suspension was then centrifuged at 13,000 rpm at 4°C for 3 min, and the supernatant was collected and stored at −20°C.

2.4. Virulence Factor Detection. Detection of the following virulence genes was carried out by conventional PCR: type 4 fimbrial biogenesis protein (*pilB*), alginate (*algD*), alkaline protease (*aprA*), elastase (*lasB*), exoenzymes for the type three secretion system (*exoS, exoU, exoT,* and *exoY*), exotoxin A (*toxA*), hemolytic phospholipase C (*plcH*), elastase B (*lasB*), pyoverdine (*pvdA*), a lectin (*lecB*), a phenazine (*phzM*), and flagellin (*flag*). Primers, temperature melting (Tm), and expected amplicons of genes associated with virulence used in this study are listed in Supplementary Table 1. Amplification was carried out in a 25-μL volume containing 23 μL of PCR Master mix (DreamTaq Green PCR master mix, Thermo Scientific), 1 μL of each primer (forward and reverse), 1 μL of template DNA, and nuclease-free water.

2.5. Biofilm Production. Biofilm formation was determined using crystal violet staining. Briefly, 200 μL of TSA broth inoculated with the strains at a concentration of 0.5 on the MacFarland scale was dispensed into a 96-well polystyrene microtiter plate and incubated at 37°C for 24 h. After incubation, the contents of each well were removed by gentle tapping and washed four times with 250 μL of phosphate-buffered saline (pH 7.2) to remove free-floating bacteria. Biofilms formed by adherent bacteria were fixed by air drying. Afterward, wells were stained with crystal violet (0.1%) for 10 minutes. The excess stain was washed three times with deionized water, the plates were dried, and the dye bound to the cells was resolubilized with 200 μL of absolute methanol per well. The optical density (OD) of the stained adherent biofilm was measured using an Epoch Microplate Spectrophotometer (Winooski, VT, USA) at a wavelength of 590 nm. The negative control wells contained inoculated sterile broth, and every experiment was performed in triplicate and repeated three times. Biofilm production was interpreted according to Stepanovic et al. using the following criteria: OD > ODc = no biofilm producer, ODc < OD = 2 (ODc) = weak biofilm producer, 2 (ODc) < OD = 4 (ODc) = moderate biofilm producer, 4 (ODc) < OD = strong biofilm producer, and ODc as three standard deviations (SD) above the mean OD of the negative control [27].

2.6. Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Peripheral blood from healthy donors was collected in heparin (with informed consent), and PBMCs were obtained using a standard gradient protocol (Ficoll-Paque™ Plus, Merck KGaA, Darmstadt, Germany). Briefly, blood was diluted with PBS in a 1 : 1 ratio, and 30 mL of the mixture was layered into 10-mL Ficoll-Paque PLUS in 50-mL tubes. The tubes were centrifugated at $1000 \times g$ for 20 minutes at 20°C without breakage. Buffy coats were collected, pooled, resuspended in PBS, and centrifuged at $400 \times g$ for 20 min without breaking. Cells were washed twice with sterile PBS, and after centrifugation ($400 \times g$, 30 min), a layer of PBMCs was obtained and collected, and cells were counted in a Neubauer chamber. Cell viability was evaluated using the trypan blue exclusion method (Sigma-Aldrich, USA). Cells were used only when viability was >98%. Cells were suspended in complete RPMI containing the RPMI 1640 medium supplemented with 10% fetal bovine serum (previously inactivated with heat at 56°C for 30 minutes) and 2 mM L-glutamine.

2.7. PBMC: Bacterial Coculture. Sixteen strains recovered from respiratory infections were selected and classified into four groups according to their susceptibility pattern (MDR and non-MDR) and the exotoxin genes detected (*exoU* and *exoS*). A concentration of 1×10^6 CFU/mL with PBMC at a concentration of 1×10^6 CFU/mL was seeded in 1-mL 24-well plates.

PBMC bacterial cocultures were incubated in complete RPMI as mentioned before at 37°C in 5% CO_2 for 24 h. PBMCs were used as negative controls, and PBMC with 5 μg/ml of phytohemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO) was used as a positive control for cell reactivity. Following incubation, 50-μL aliquots were recovered for the cell viability assay under each experimental condition. Before centrifugation at $400 \times g$ for 5 minutes, supernatants were collected, sterile filtered (0.22 μm), and stored at −80°C for further assays.

2.8. Determination of Cytokines. The levels of a panel of cytokines (TNF-α, IL-1beta, IL-6, IL-8, and IL-10) present in the coculture supernatants were determined using a Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD, Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) according to the manufacturer's instructions.

2.9. Statistical Analysis. Data were analyzed using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, California, USA, https://www.graphpad.com) and Python 3.12 (https://www.python.org) for Mac. The distribution of virulence genes among MDR and NMDR strains and their presence in carbapenem-resistant and fluoroquinolone-resistant strains were calculated using Fisher's exact test for each gene. The correlation between virulence genes and resistance rates (fluoroquinolones, carbapenems, and multidrug resistance) and the type of infection were calculated using the DataFrame.corr() method in Python. For the presence of virulence genes in biofilm production, the chi-squared test was used, as well the relationship between virulence genes and the infection involved. Differences observed in PBMC assays between groups (MDR/*exoU*, NMDR/*exoU*, MDR/*exoS*, and NMDR/*exoS*) were analyzed using a nonparametric test of variance (Kruskal–Wallis and Dunn's post-test). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Sample Collection. As shown in Table 1, seventy-five *Pseudomonas aeruginosa* strains associated with HCAIs were obtained in a year, corresponding to 28 respiratory tract infections, 18 bacteremia, 11 infections from the surgical site, 9 from skin and soft tissue, and 9 from ocular and urogenital infections.

Forty-three patients were males, and thirty-two were female. Most isolates were identified in adults between 36 and 60 years of age ($n = 27$). The mean length of hospital stays was 42.2 days (range, 3–243 days). Most patients were discharged because of clinical improvement ($n = 59$), and only five deaths were associated; however, no statistical difference was found.

3.2. Antimicrobial Susceptibility. Among the antibiotics tested (Table 2), different levels of resistance were observed: 24% and 27% were nonsusceptible to amikacin and ciprofloxacin, respectively. Aztreonam showed the highest resistance (48%, $n = 36$), followed by meropenem (43%, $n = 32$). The remaining strains presented approximately 30% nonsusceptibility, and 25 strains were classified as multidrug-resistant.

3.3. Virulence Genes in Infections, Biofilm, and Antibiotics. After the PCR analyses, 16 genes were screened. The genes were classified according to the associated infection, biofilm production, susceptibility to fluoroquinolones, carbapenems, and multidrug-resistant status to determine any association or correlation.

Classified by the type of infection, the most prevalent genes were *plcH, lasB,* and *algD, as shown in* Figure 1, which were present in at least 90 percent of the strains, followed by *exoT, exoY, aprA, phzM*, and *lecB*, which were present in 60 percent of the strains. However, this difference was not statistically significant.

The correlation between the type of infection and the virulence genes shown in Figure 2, with a slight positive correlation between toxA and skin and soft-tissue infection and a negative correlation between pillB and skin and soft-tissue infection as well as between *exoU* and bacteremia, was detected; nevertheless, any strong positive or negative correlation was found.

As expected, most strains were medium- or high-biofilm producers (Table 3). However, three genes showed significant differences in *exoY* and *toxA,* with the majority being the stronger producers with 29 (38.7%) and (37.3%) strains, respectively. The *lecB* gene was mainly present in moderate producers (27 strains; 36.0%). Interestingly, some trends were observed. *The pilB* and *exoU* genes had a higher presence in medium biofilm producers, whereas the rest of the genes were present in strong producers.

Later, it explored how the genes could be distributed among strains with different resistance patterns, and in the case of resistance to carbapenems and quinolones, multidrug resistance was interesting. As shown in Figure 3, statistical differences in two of the genes belonging to SST3, *exoS* and *exoU*, were found. Additionally (Figure 4), a negative correlation between exoS and the different resistance patterns was −0.36 and −0.50 complementary, and *exoU* in the same resistance patterns was positive between 0.33 and 0.57. The last one was relevant because of the association between exotoxin U and strains with nonsusceptibility and MDR and its relationship with cellular destruction.

3.4. Cell Viability after Bacterial Coculture and Cytokine Production. Based on the results of the resistance and frequency of genes, four groups were formed, two bearing the *exoU* gene (*exoU*/MDR and *exoU*/NMDR) and two bearing the *exoS* gene (*exoS*/MDR and *exoS*/NMDR). The first assay involved checking the cellular viability (Figure 5), and significant differences were observed. The *exoU*/MDR group had higher mortality rates than the control and *exoS*/NMDR groups.

As shown in Figure 6, some differences were observed in cytokine assays. The presence of the strains did not affect the secretion of TNF; however, IL-6, IL-8, and IL-10 showed lower levels of secretion in the *exoU*/MDR group than in the positive control group, and *exoU*/NMDR had lower secretion of IL-8 and IL-10. Interestingly, IL-1beta was higher in *the exoS/MDR group* than in the control group. These findings provide interesting insights into how the presence of *P. aeruginosa* affects the in-vitro profile of cytokines.

4. Discussion

P. aeruginosa is an opportunistic pathogen considered a public health problem in every country. Despite the

Table 1: The clinical and demographic characteristics of patients with *Pseudomonas aeruginosa* infection were included in the study ($n = 75$).

	n	%
Sex		
Male	43	57.3
Female	32	42.7
Age (years)		
Infants (0-1)	8	10.7
Children (2–10)	6	8
Adolescents (11–17)	6	8
Young adult (18–35)	15	20
Adults (36–60)	27	36
Senior (>60)	13	17.3
Length of stay (days)		
Mean	42.5	
SD	35.6	
Range	3–243	
Hospital discharge		
Improvement	59	78.7
No associated death	11	14.7
Associated death	5	6.7
Type of infection		
Respiratory tract	28	37.3
Bacteremia	18	24
Surgical site	11	14.7
Skin and soft tissue	9	12
Others	9	12

Table 2: Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* causing HIAs ($n = 75$).

Antibiotic	Susceptible *n* (%)	Nonsusceptible *n* (%)
Amikacin	57 (76.0)	18 (24.0)
Cefepime	51 (68.0)	24 (32.0)
Gentamicin	52 (69.3)	23 (30.7)
Piperacillin/Tazobactam	51 (68.0)	24 (32.0)
Aztreonam	39 (52.0)	36 (48.0)
Ciprofloxacin	55 (73.3)	20 (26.7)
Meropenem	43 (57.3)	32 (42.7)

attention being focused on antibiotic resistance, this bacterium is much more complex. It possesses intrinsic resistance, a large genome capable of harboring new resistant genes, and biofilm, a barrier that confers its physical protection. In addition, this pathogen carries an extraordinary repertory of virulence factors, which this study aimed to assess.

It is difficult to scrutinize patient demographics in Mexico because there is little data about how *P. aeruginosa* appears in HCAIs, and the most recent descriptive analysis was made by González-Olvera and Cols. in 2019. They found a mean of five strains per month compared with six strains per month in this study, comparing the population density of the municipalities of each study, and found that San Luis Potosi has good management of nosocomial infections (NI). In addition, the predominant type of infection was on the respiratory tract (ten percent more), while for González-Olvera, most of the cases were from the urinary tract. [28–30]. Another study focusing on *P. aeruginosa* was conducted by Elmouaden and Cols. in 2019, with a total of 87 strains classified as NI in two years but not classified according to the type of infection [31]. Aside from these studies to date, no other study has used the same approach as the present study. However, the epidemiological data are concordant, indicating that the highest rates of infection by *P. aeruginosa* are in the respiratory tract [5, 9].

Something remarkable about the strains in this study is the resistance rates detected: 42.7% of the strains in this study were resistant to carbapenems and 33.3% of MDR strains. These rates are part of the increasing global problem; in this respect, many studies have been conducted, but Hojarcada and Cols. resumed this in an excellent review. In some geographical areas, the rates of MDR are between 15% and 30%; in Europe, 13.7% are resistant to at least three antimicrobial groups. In the United States, the situation is not better; MDR pathogens cause 13% of severe HIAs [32]. In Mexico, the data are limited because of the lack of a national network, and some sources suggest that resistance has diminished in the last ten years. The most recent study was conducted by Garza-González in 2019 and did not include information about the state of San Luis Potosi; they found that 27.8% resistance to meropenem was considerably lower than that found in this study [33]. It is worth mentioning that the lack of information about the country's

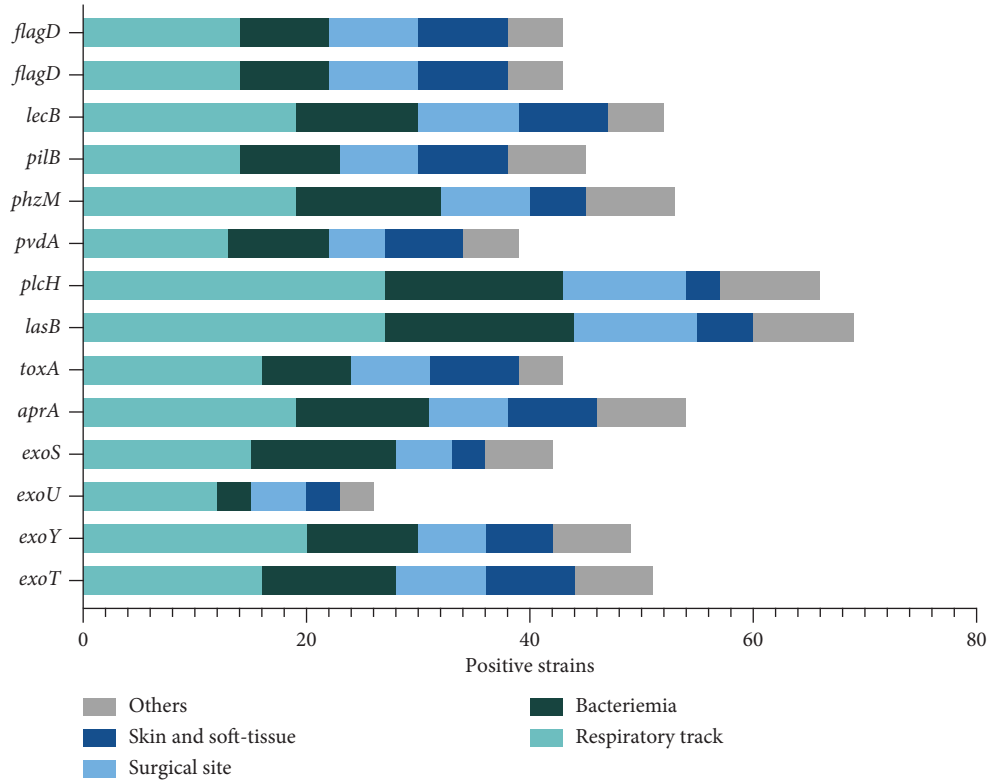

Figure 1: The frequency of virulence genes among *P. aeruginosa* strains according to infection-type comparisons among groups was determined using the chi-square test; $^{*}p < 0.05$ was considered statistically significant.

situation has been attributed to problems such as self-prescription, low quality of generic antibiotics, the use of antibiotics in agriculture, and corruption [34].

P. aeruginosa is a pathogen with great versatility in virulence, and this study attempted to find a correlation between virulence genes, type of infection, rates of resistance, and biofilm production. In the first respect, non-statistical differences were found, which may be because, as some authors point out, genetic differentiation or changes in gene expression occur in the transition from an acute to a chronic infection, which leads to the silencing of genes associated with acute infections when they progress to chronicity or biofilm production [35]. Furthermore, at the time of publication of this manuscript, most studies count virulence genes in all strains rather than counting them according to the type of infection; only one study with a classification similar to this one was found; Fazeli in 2014 found wound infection as the most frequent and *exoS* as the most prevalent gene; for this project, respiratory tract infections, *plcH*, and *lasB* genes are the most common ones, mentioning that discrepancies may be due to geographical differences [36].

Because of the behavior of the data and evidence from other studies, it was considered important to detect genes in different biofilm producers. Statistical differences were observed, and *exoY* and *toxA* were prevalent in strong producers, which is not the first report of an association between *exoY* and biofilm producers. Half of the Azimi strains in 2016 ($n = 150$) were producers and harbored *exoY* [37]. Similarly, in 2018, Asadpour found genes encoding exotoxin A in stronger producers, suggesting that biofilm-forming strains are more virulent, potent, and resistant [38]. According to the present study and in agreement with Bogiel T. et al., there may be a potential relationship between virulence factors and biofilm production rates, suggesting that biofilm producers are more virulent [39]. In a completely different scenario, Passos da Silva et al. recently demonstrated that *lecB* coding for a glycoprotein can bind the exopolysaccharide PSL and stabilize the biofilm matrix, which explains why this gene is present in 69.3% ($n = 52$) of their strains [13] and 45.3% ($n = 49$) in this study.

A classic scenario for bacteria is that they are considered simple life forms with a small space in their genome, and some hypothesize that a strain cannot harbor a plethora of virulence factors while containing many resistance-related genes. However, this has changed in recent years; the relationship between antibiotic-resistant genes and virulence factors might follow a Darwinian model, resulting in the emergence of virulent and resistant clones, and precisely, the *P. aeruginosa* large genome is an excellent candidate to acquire both characteristics [40, 41]. Since 2008, twenty-seven papers have reported a relationship between the *exoU* gene and resistance to carbapenems, including reports of community and nosocomial infections [42].

The evidence in this study suggests a possible relationship between virulence genes and resistance rates; nevertheless, there are very few studies looking for this relationship, and some have not found significant differences [37]. Although this study revealed differences, it found strains that harbored the *exoU* gene, were resistant to

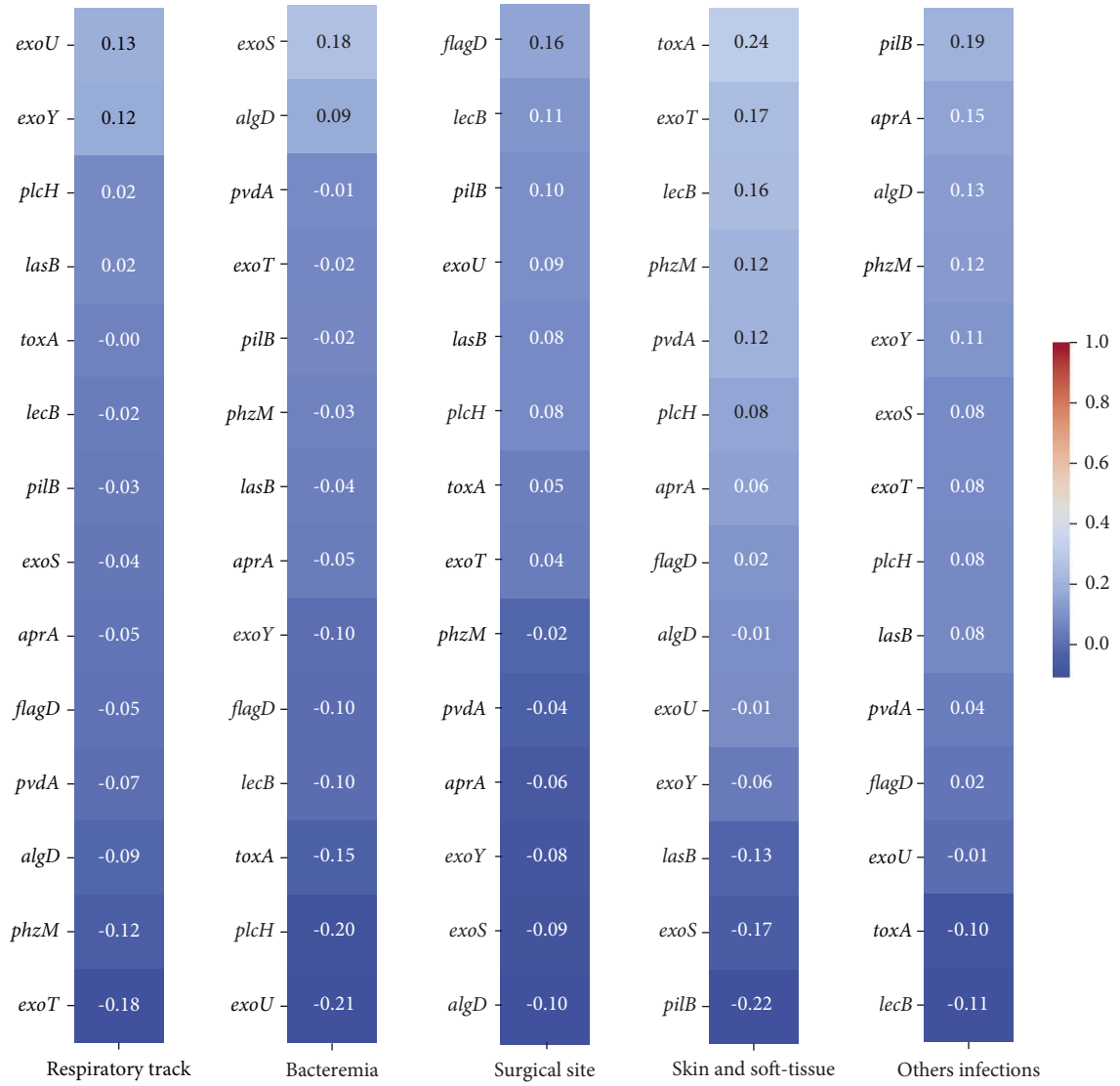

Figure 2: Correlation between the type of infection and virulence genes was calculated using the DataFrame.corr() method in Python.

Table 3: Relationship between virulence genes of *Pseudomonas aeruginosa* causing HIAs and level of biofilm production ($n = 75$).

Gene associated with virulence factor	Biofilm production		
	Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)
exoT	1 (1.3)	24 (32.0)	26 (34.7)
*exoY**	3 (4.0)	16 (21.3)	29 (38.7)
exoU	1 (1.3)	16 (21.3)	9 (12.0)
exoS	2 (2.7)	17 (22.7)	23 (30.7)
aprA	2 (2.7)	21 (28.0)	30 (40.0)
*toxA**	0 (0)	15 (20.0)	28 (37.3)
lasB	3 (4.0)	33 (44.0)	36 (48.0)
plcH	3 (4.0)	33 (44.0)	36 (48.0)
pvdA	1 (1.3)	18 (24.0)	19 (25.3)
phzM	1 (1.3)	23 (30.7)	32 (42.7)
pilB	2 (2.7)	20 (26.7)	17 (22.7)
*lecB**	3 (4.0)	27 (36.0)	22 (29.3)
flagD	1 (1.3)	18 (24.0)	21 (28.0)
algD	2 (2.7)	28 (37.3)	37 (49.3)

Comparisons between groups were performed using the chi-square test; * $p < 0.05$ was considered statistically significant.

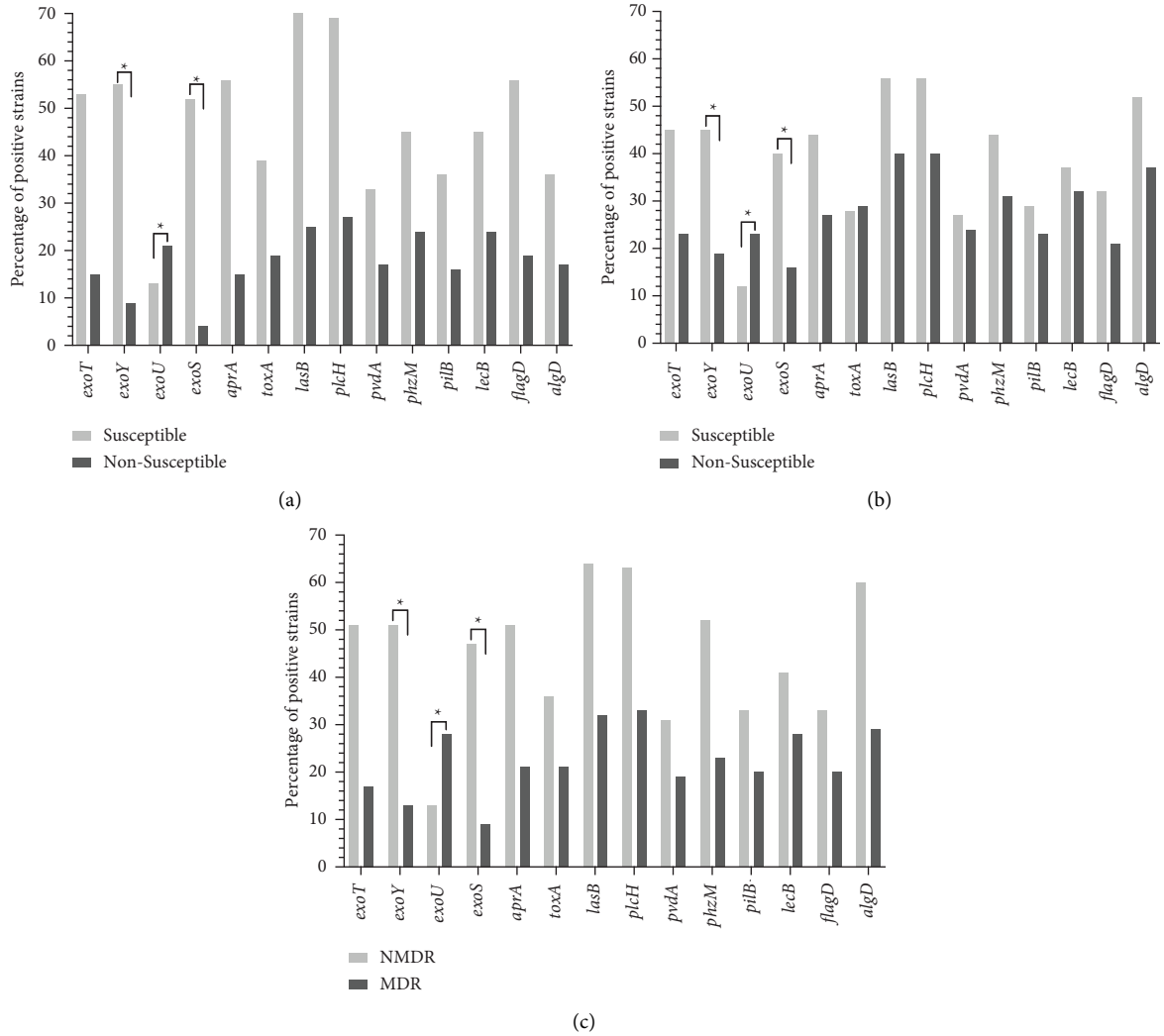

Figure 3: Frequency of the virulence genes between *P. aeruginosa* strains according to their susceptibility to (a) fluoroquinolones, (b) carbapenems, and status as (c) multidrug-resistant. Comparisons between groups were performed using Fisher's exact test; $p < 0.05$ was considered statistically significant.

fluoroquinolones and carbapenems, and were considered MDR. These findings are consistent with the data of Takata et al. and Subedi et al. in 2018, who found that the *exoU-positive* genotype is more frequent in nonsusceptible fluoroquinolones, carbapenems, and MDR strains [24, 43]. In 2019, Horna found that strains resistant to carbapenems and fluoroquinolones and classified as MDR also harbored the *exoU* gene [44]. Kainuma et al. found the same phenomenon and suggested that *exoU* carriers have a greater potential to spread within hospital units, which, if true, represents a major risk [45].

The final aim was to explore a possible relationship among the genes of T3SS, specifically *exoS* and *exoU*, multidrug resistance, and the production of cytokines (TNF, IL-1beta, IL-6, IL-8, and IL-10). The first approach was to determine the effects of these bacteria on cellular viability. As shown in Figure 5, *exoU*/MDR strains presented higher mortality than the other strains owing to their potent phospholipase activity, which causes necroptosis and cell lysis [46], and *the exoS* groups showed a higher survival rate. Some evidence points out that *exoS* may dampen the immune response, provoking cell death by apoptosis, which is slower than necrosis [47]. Information about how *P. aeruginosa* can affect the pattern of cytokine expression is limited; one of the most accurate studies was made by Christopher H. The Moody group related the effect of a recombinant *exoS* with the mRNA of cytokines from PBMC. They found a decrease in IL-10 and an increase in TNF, IL-6, IL-8, and IL-1beta [48, 49]. They worked with recombinant proteins. The present study attempted to evaluate whether these results are transferable to clinical strains, which is partial, and observed a significant increase in IL-1beta with a significant difference in the MDR group. A possible explanation is provided by Grandjean et al., who found that ExoS can convert prointerleukin (IL-1beta) into mature IL-1beta [50]. IL-10 (NMDR group) and TNF (MDR, NMDR group) levels also increased, but the difference was not statistically significant, which could be an indirect effect of ExoS through NF-κB [20].

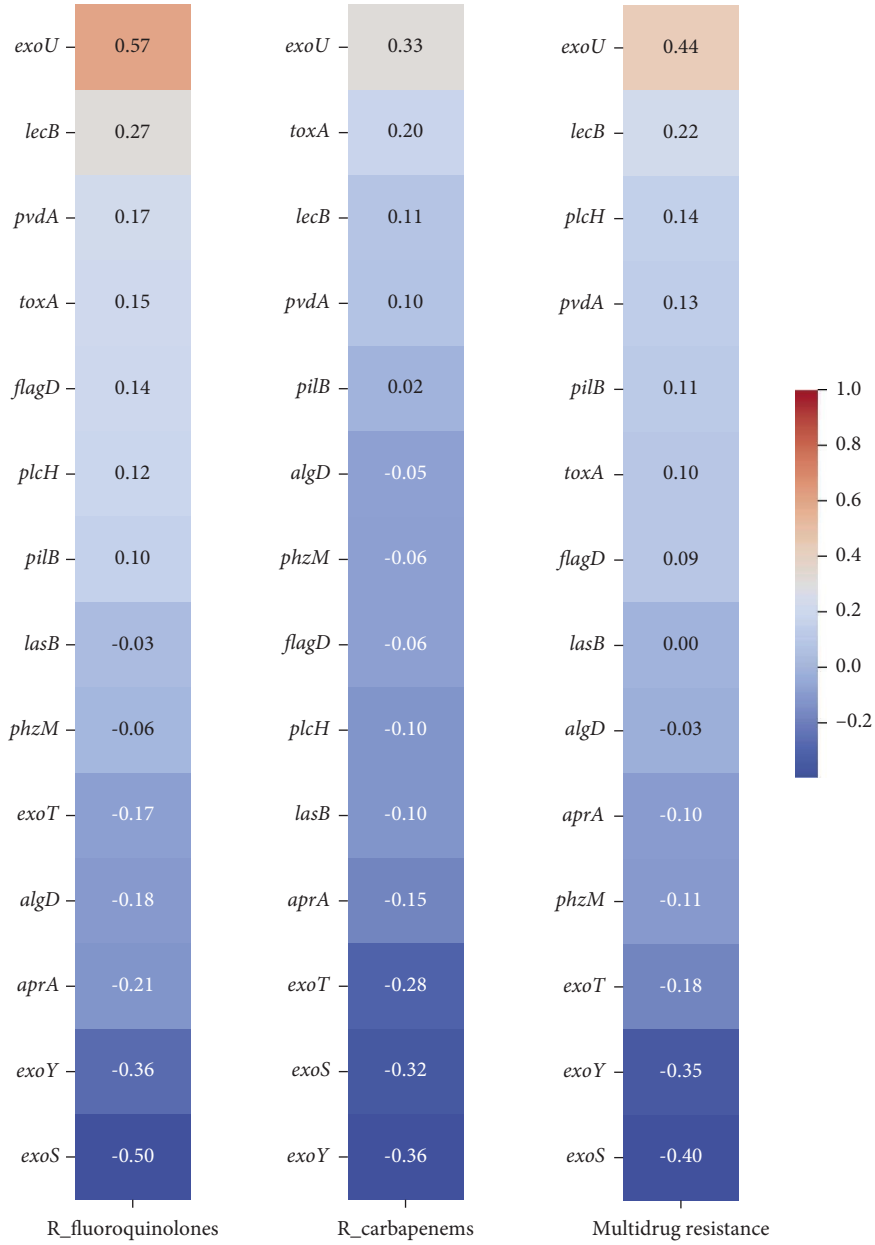

Figure 4: Correlation between virulence genes and resistance rates (fluoroquinolones, carbapenems, and multidrug resistance) was calculated using the DataFrame.corr() method in Python.

For the *exoU* group, the first finding was higher levels of IL-1beta, although nonsignificant, than those in the control group. Hardy et al. in 2022 showed that ExoU has a non-cytolytic function through the transitory activation of caspase-1 and proIL-1beta. Moreover, interesting findings emerged: IL-6, IL-8, and IL-10 levels decreased, but only in MDR strains. At the time of publication of this manuscript, there were three papers on the relationship between *exoU* and IL-8; one of them addressed the relationship with IL-6, and the other mentioned the effect of this gene on IL-10. All these studies reported that strains with *exoU* caused an increase in the cytokines tested. This discrepancy may be due to the articles being published before 2012 and the use of different cell lines. Today, considering the results of Hardy, a second look is taken at the relationship between these exotoxins and their effects on cytokines [51–54]. However, a limitation in this study is that these results are the product of an in-vitro model, and the results should be interpreted with caution due to the complexity of the study and the possibility that there are intracellular cytokines that could cross the membranes of the cells undergoing cell death and affect the cytokine pattern.

In conclusion, the appearance of the *exoU* virulence gene in MDR strains has increased in recent years, which is concerning because it is an indicator of strains with a more aggressive and difficult-to-treat infectious capacity. More importantly, the fact that these strains present a different cytokine profile than expected represents the possible

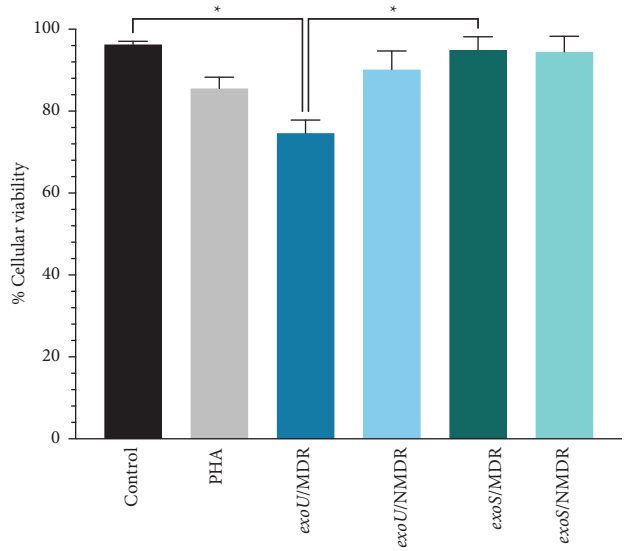

FIGURE 5: Cellular viability of PBMC incubated with *P. aeruginosa* strains. The mean values ± SD from four independent experiments are shown. The Kruskal–Wallis multiple comparison test was performed; $^{*}p < 0.05$ was considered statistically significant.

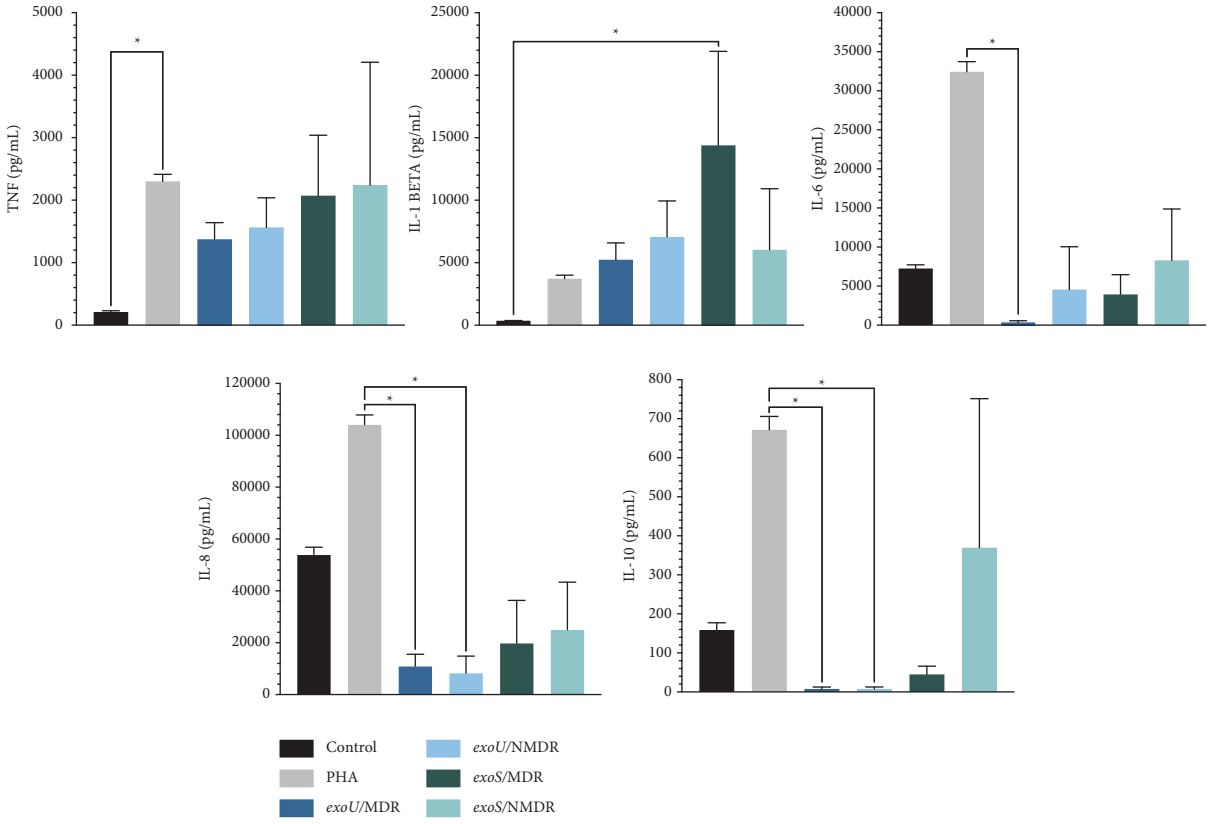

FIGURE 6: Cytokine profiles of PBMC stimulated with *P. aeruginosa* strains. Cytokine levels were evaluated using a flow cytometric multibead capture assay. The mean values ± SD from four independent experiments are shown. The Kruskal–Wallis multiple comparison test was performed; $^{*}p$ value <0.05 was considered statistically significant.

existence of unknown mechanisms of how these strains interact with host cells, which may represent a benefit for the progression of the infection.

Abbreviations

HAIs: Healthcare-associated infections
PBMC: Peripheral blood mononuclear cells
T3SS: Type three secretion system
MDR: Multidrug-resistant
WHO: World Health Organization
SSI: Surgical site infection.

Data Availability

The data used to support the findings of this study are restricted by the ethics board of the hospital "Dr. Ignacio Morones Prieto" in order to protect patient privacy. Data are available from the corresponding author to this research, Perla Niño-Moreno (ncarmenp@uaslp.mx), for researchers who meet the criteria for access to confidential data.

Ethical Approval

Strains were obtained from a tertiary hospital (HCIMP in San Luis Potosi, Mexico) from May 2018 to June 2019 before approval of the Research Committee (COFEPRIS 17 CI 24 028 093) and the Research Ethics Committee of the HCIMP (CONBIOETICA-24-CEI-001-20160427) by the registration number 38–18.

Consent

Informed consent was obtained from all participants or legal guardians.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

N.S.B.L. contributed to the data acquisition, interpretation, and drafting. E.A.T.M. interpreted the data and critically revised the manuscript. L. F. P. G. contributed to the data acquisition. E.L.S. contributed to the data acquisition and interpretation of the cytokine assay and critically appraised the manuscript. P.N.M. contributed to the data interpretation and critically revised the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of this work.

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Supplementary Materials

Consist of a list of the primers used in our research, including the sequence, the temperature of melting, and the length of the product of PCR expected for each gene included in the research. (*Supplementary Materials*)

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RESEARCH ARTICLE

Lippia graveolens HBK oleoresins, extracted by supercritical fluids, showed bactericidal activity against multidrug resistance *Enterococcus faecalis* and *Staphylococcus aureus* strains

Oscar de Jesús Calva-Cruz[a]*, Nallely S. Badillo-Larios[b]*, Antonio De León-Rodríguez[a], Eduardo Espitia-Rangel[c], Raúl González-García[d], Edgar Alejandro Turrubiartes-Martinez[b,d], Arnulfo Castro-Gallardo[e] and Ana Paulina Barba de la Rosa[a]

[a]IPICYT, Instituto Potosino de Investigación Científica y Tecnológica A. C., San Luis Potosí, Mexico; [b]CICSaB, Centro de Investigación en Ciencias de la Salud y Biomedicina, Universidad Autonoma de San Luis Potosí, San Luis Potosí, Mexico; [c]INIFAP, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Valle de México, Coatlinchán, Mexico; [d]Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, Mexico; [e]Centro de Investigación para los Recursos Naturales, Antigua Normal Rural de Salaices, Chihuahua, México

ABSTRACT

Objective: The aim of this work was to characterize *Lippia graveolens* oleoresins, obtained by Supercritical Fluid Extraction (SFE), from crops collected at different locations in Mexico. The antimicrobial effect of oleoresins was tested in reference strains and clinical isolates of susceptible and multidrug-resistant (MDR) strains of *Enterococcus faecalis* and *Staphylococcus aureus*.

Significance: The increasing of MDR strains is becoming a global public health problem that has led to the search for new treatments, and essential oils have resurged as a source of compounds with bactericidal functions. Oregano essential oil has attracted attention recently, however, this oil is mainly obtained by hydro-distillation (uses large amounts of water) or solvents extraction (potential contaminant). SFE has gained popularity as it represents an environmentally friendly technology.

Methods: *L. graveolens* oleoresins were obtained by SFE, total phenol contents were quantified by Folin-Ciocalteu method, the identification of compounds and thymol and carvacrol quantification was carried out by GC-MS. The antimicrobial activity was tested by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results: SFE showed higher yields compared with the hydro-distillation process. *L. graveolens* grown in different Mexican locations showed differences in oleoresin composition and a slightly different antimicrobial capacity against clinical isolates.

Conclusions: It was demonstrated that SFE is an efficient technology for extracting *L. graveolens* oleoresins. Additionally, the solvent-free extraction method and the observed antimicrobial effect increase the applications of these oleoresins in fields, such as cosmetics, food industry, medicine, amongst others.

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Antimicrobial activity; essential oil; gas chromatography-mass spectrometry; *Lippia graveolens*; multidrug resistance pathogens; oleoresins; phytochemical composition; supercritical CO_2

Introduction

The emergence of antimicrobial resistance is currently a major threat to global public health, reporting that multidrug resistance strains (MDRs) have caused ~700,000 deaths annually and are expected to increase to 10 million by 2050 [1]. The problem is raised because antibiotics are becoming ineffective against MDRs, in addition, the lack of novel synthetic drugs and the widespread occurrence of MDR has increased the interest in searching for natural compounds with bactericidal activity, therefore, the use of essential oils is becoming an alternative solution to fight MDRs [2–4].

Enterococci are microorganisms located mainly in the human gut and are also found in human feces. Once considered as bacteria of minimal clinical impact, Enterococci, particularly *Enterococcus faecalis*, and *Enterococcus faecium* have now emerged as one of the main causes of human clinical infections [5]. Actually, *E. faecalis* is responsible for twice as many healthcare-associated infections as those reported by *E. faecium*, both being the most common species associated with human disease [6]. Their importance is reinforced by their intrinsic and acquired resistance to several antibiotics, which makes them difficult to treat and sometimes are fatal, as in the case of infections caused by vancomycin-resistant Enterococci [7]. On other hand, *Staphylococcus* species are amongst the most frequently isolated bacteria in hospital settings and have been involved in many infections in humans, such as skin and soft tissue infections, surgical sites, and wound infections. From these, *S. aureus* has been detected in hospital wastewaters, specifically, methicillin-resistant *S. aureus* (MRSA) is a reason for concern because it has become one of the main causes of infection in both hospital and community settings [8,9].

Oregano essential oils contain up to 56 compounds in different proportions depending on the species, but two of them, thymol and carvacrol, are of great importance because they determine

CONTACT Ana Paulina Barba de la Rosa apbarba@ipicyt.edu.mx IPICYT, Instituto Potosino de Investigación Científica y Tecnológica A. C., Camino a la Presa San José 2055, San Luis Potosí, S.L.P., C.P. 78216, Mexico
*These authors contributed equally to this work.

the quality and price in the market [10–13]. Traditionally oregano oil has been used as an antiparasitic, antibacterial, antifungal, antiviral, and anti-inflammatory [14–19]. Reports also have indicated that oregano essential oils are used to treat liver and respiratory diseases as well as dyslipidemia in type-2 diabetic rats [20], the antimutagenic and antiproliferative activity have also been reported [21–23]. The microbicidal activity of *Origanum vulgare* essential oil has been tested in reference strains [24,25] as well as in some MDR bacteria [26], against pathogenic strains, such as *S. aureus, Escherichia coli*, and *Candida albicans* [27,28] and on biofilm-grown *S. aureus*, *Staphyloccoccus epidermidis*, and *Salmonella enterica* strains [29,30]. *Origanum glandulosum* essential oil was tested against uropathogenic *E. coli* including MDR strains [31].

Lippia graveolens HBK, belonging to the *Verbenaceae* family, is grown in Mexico and because of its aromatic characteristics, stronger and robust flavor, Mexican oregano has a high commercial value [11,32,33]. Mexico ranks as the second-largest producer of dry oregano with 4000 tons per year from which, 85% is exported to EUA, 5% to Europe, and the rest is for national consumption [34]. Mexico has nearly 40 species of oregano, some of which are endemic and distributed in several states in Mexico and the properties of each species are still unknown [35].

Currently, the main method used to obtain the oregano essential oil is steam distillation, which although efficient, involves the use of large amounts of water and high operating temperatures. This method could also modify the chemical profile of the essential oil due to the breakdown of some compounds by hot water vapor [36–38]. Supercritical fluid extraction (SFE) is a relatively faster extractive technique that is widely considered the most stable for the recovery and separation of essential oils and their derivatives for use in food, cosmetics, pharmaceuticals, and other related industries [39]. SFE has gained popularity for oleoresins extraction, since it represents one of the most environmentally friendly technologies and has several advantages over traditional extraction methods, such as the use of low temperatures and reduced energy expenditure [38,40–42]. The present research aims to use SFE for *L. graveolens* essential oil (oleoresins) extraction from samples collected at different climate areas in the Mexican territory: the main commercial cultivars are grown in semi-arid to the arid area (Chihuahua), a sample collected in the tropical area (Yucatan), and one cultivar growing as wild was collected in template area (Queretaro). Oleoresins were characterized by GC-MS and the bactericidal effect was evaluated against reference strains as well as clinical isolates of susceptible and MDR strains of *E. faecalis* and *S. aureus*.

Materials and methods

Plant material

L. graveolens sample from the north of the country (Chihuahua State) was provided by the Research Center for Natural Resources (CIReNa). Chihuahua is a semi-arid to the arid area located at 1413 m above mean sea level (mamsl) with annual average temperature and precipitation of 26 °C and 403 mm, respectively. The sample from the Center of Mexico was growing in wild at Peña-Miller (Queretaro State), located at 1450 mamsl with a mean annual temperature of 20 °C and 750 mm annual precipitation. The third sample was collected from Yucatan State, which presents tropical weather with annual mean temperature and precipitation of 27 °C and 900–1000 mm, respectively. *L. graveolens* samples were received as dry leaves and were kept in plastic containers. Samples were ground manually in a mortar and passed through 16 and 18 mesh screens and stored in dark containers until use. Residual moisture was determined by drying the samples in the oven at 135 °C (±2 °C) for 2 h [43].

Supercritical fluid extraction

Extractions were carried out in a semi-pilot multi-vessel botanical extraction system (SFE, Waters Corp., Milford, MA, EUA) using a 1 L extraction cell. The schematic instrument set-up of extraction is shown in Figure 1. The system consists of a high-pressure CO_2 pump (P-200 Waters Corp.) for CO_2 displacement. *L. graveolens* oleoresin extraction was carried out using 100 g of dry and ground plant material without filling the void volume with another material. SCF yield extraction was tested at different conditions of temperature (35 and 45 °C), pressure (25, 30, and 35 MPa), and 5 h. All extractions were carried out at a constant CO_2 mass flow of 30 g/min. The extracted oleoresin was stored at 4 °C in amber flasks. The extraction yield was calculated according to Equation (1) [44] where:

$$\text{Extract yield } (\%) = (\text{Extracted mass}/\text{sample mass}) \times 100 \quad (1)$$

Total phenolic contents

Total phenol contents were quantified using the Folin-Ciocalteu method. Oleoresins (1 g) was dissolved in 10 ml of ethanol, the mix was centrifuged and 20 µL of supernatant was mixed with 780 µL of deionized water and 50 µL of Folin-Ciocalteu reagent, mixed with a vortex and incubated at RT for 10 min. Then, 150 µL

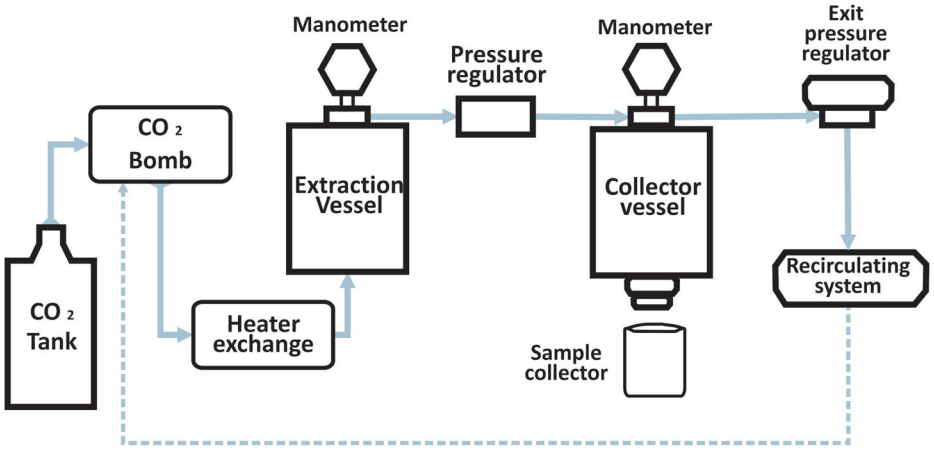

Figure 1. Schematic representation of supercritical fluids system used for *Lippia graveolens* oleoresins extraction.

of 20% Na_2CO_3 solution was added, mixed, and incubated for 2 h. The absorption of the solution was read at 765 nm in a UV-Vis spectrophotometer (Multiskan GO, Thermo Scientific Ltd., Vantaa, FIN). Total phenolic content was expressed as mg of gallic acid equivalents/g of oleoresin (mgEq/g). All determinations were carried out five times.

Gas chromatography (GC-MS) analysis

L. graveolens oleoresins were characterized by GC-MS 7820 A/5977E System (Agilent Technologies, Santa Clara, CA, USA) using a Zebron ZB5-MS (30 m × 0.25 mm × 0.25 μm) column (Phenomenex, Torrence, CA, USA). The operating conditions were as follows: 250 and 280 °C injection temperature and detector, respectively. The temperature program started with 10 min at 60 °C increasing 3 °C/min until reaching 240 °C, remaining at this temperature for 10 min. Helium flow was 1 ml/min and the mass range analyzed was from 40 to 400 *m/z* at a range of sample analysis of 1.0 s/s. The injector temperature was set at 250 °C in splitless mode. MS detector was operated under Electron Impact Ionization at 70 eV using the SIM and Scan mode. The identification of the compounds was carried out by comparing the spectrum obtained with the spectrum reported in the software library of the equipment (NIST) library database.

Thymol and carvacrol quantification

Thymol and carvacrol concentrations present in the samples were monitored by GC-MS (Agilent Technologies) using an HP-5MS capillary column and operating conditions as described above. Calibration curves for thymol (0.625–10 mg/L) and carvacrol (0.75–12 mg/L), both with $R^2 = 0.999$, were performed with analytical standard purchased from (Sigma–Aldrich™, St. Louis, MO, USA). Thymol and carvacrol present in oleoresins obtained from the different samples were quantified with respect to the control curve.

Bacterial strains and growth conditions

Reference strains *E. faecalis* (ATCC 29212) and *S. aureus* (ATCC 25923), as well as two clinical isolates of *E. faecalis* (B600 and A605), and two of *S. aureus* (A702 and A710) were kindly donated from the Human Genomic laboratory of the Research Center for Health Sciences and Biomedicine (CICSaB) from the Universidad Autónoma de San Luis Potosí (San Luis Potosi, Mexico). All bacterial strains were grown in Luria Broth (Condalab, Torrejón de Ardoz, Madrid, Spain) at 37 °C for 18 h. The susceptibility pattern was determined using the Kirby-Bauer diffusion testing method on Mueller–Hinton agar plates (Merck, Darmstadt, Germany) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [45]. *E. faecalis* strains were tested against the following antibiotics: penicillin (10 U), levofloxacin (5 μg), ciprofloxacin (5 μg), erythromycin (15 μg), linezolid (30 μg), and vancomycin (30 μg). While, *S. aureus* isolates were tested for penicillin (10 U), levofloxacin (5 μg), ciprofloxacin (5 μg), erythromycin (15 μg), linezolid (30 μg), cefoxitin (30 μg), oxacillin (1 μg), clindamycin (2 μg). Based on the results, the resistance phenotype was determined, strains were classified as susceptible (strains susceptible to all antibiotics tested), MDR, MRSA (Methicillin-resistant *Staphylococcus aureus),* or VRE (Vancomycin-resistant *Enterococcus*). Vancomycin (No. Cat. V0045000), and ciprofloxacin (No. Cat. 17850) were purchased from Sigma–Aldrich (Sigma–Aldrich).

Antimicrobial susceptibility activity

Determination of minimum inhibitory concentration (MIC)

MIC was determined by broth microdilution assay in 96 well U-shaped bottom microtiter plates according to CLSI [45]. Briefly, oregano oleoresins stock solutions (Chihuahua, Queretaro, and Yucatan samples) were prepared at 1000 mgEq/mL of total phenolic compounds, then serial 2-fold dilutions were prepared with Müeller-Hinton Broth to range from 500 up to 0.00097 mgEq/mL. From each dilution point, 50 μL were added to each well (from 1 to 10 wells). Afterward, 50 μL of bacterial suspension was added to each well. The bacterial suspension was prepared for a 1×10^5 CFU/mL concentration using 0.5 McFarland standard diluted to 1:1000 in saline solution (0.92%). Plate-wells 11 and 12 were used as control, which contained 100 μL oleoresins-free of bacterial suspension and 100 μL sterile buffered Müeller-Hinton Broth, respectively. Plates were incubated at 37 °C for 18–24 h and examined for bacterial growth. Absolute ethanol was used as a control. The first well without turbidity was determined as a MIC value [24].

Determination of minimum bactericidal concentration (MBC)

MBC was obtained by broth sub-culturing from dilutions of the MIC assay in Müller-Hinton agar plates that do not contain the oleoresin samples. Plates were incubated at 37 °C for 18 h and examined for bacterial growth. The MBC refers to the lowest broth dilution of the sample that inhibits the growth of the organism on an agar plate. All assays were performed in triplicate. Vancomycin and ciprofloxacin (Sigma–Aldrich) were used as susceptibility control for *E. faecalis* and *S. aureus*, respectively.

Statistical analysis

One-way Kruskal–Wallis test was carried out and significant differences among samples were determined by Dunn's *post-hoc* test at $p \leq 0.05$.

Results and discussion

Effect of pressure and temperature on L. graveolens oleoresin extraction yield

Oleoresin extraction yield was determined at different extraction conditions, data showed that higher temperatures and higher pressure increased the yield. Therefore, the selected conditions for *L. graveolens* oleoresins were 45 °C, 35 MPa, and 5 h of extraction, which gives a 4.1% yield (Table 1). Soto-Armenta et al. [42] tested different extractions conditions for *L. graveolens* oleoresins, with the maximum yield reported was 2.7% at 35 MPa, 60 °C under a dynamic extraction with a flow rate of 4.8 g CO_2/min. In this work, extractions were carried out at a constant CO_2 mass flow of 30 g/

Table 1. Supercritical fluids experimental conditions for oleoresins extraction from *Lippia graveolens*[a].

Experiment	Temperature (°C)	Pressure (MPa)	Yield[b] (%)
1	35	25	2.6
2	35	30	2.8
3	35	35	3.2
4	45	25	2.9
5	45	30	3.3
6	45	35	4.1

MPa: megapascals.
[a]Chihuahua sample was selected as representative sample for supercritical fluids extraction conditions.
[b]According to the formula described in M&M section.

min obtaining almost double of yield. SFE has been used for oleoresin extraction from *O. vulgare* reporting yields of 4.8% at 39.85 °C and 30 MPa with 1.5 h of extraction [46]. On the contrary, Alves-Rodrigues et al. [47] obtained only 1.32% at 313 °K (39.85 °C) and 20 MPa. Garcia-Pérez et al. [27] reported yields as higher as 13.4%, but with the use of ethanol as co-solvent, however, this also will increase the extraction costs.

Present results also showed that supercritical CO_2 extraction renders higher yields when compared with the traditional steam-distillation method, in which the maximum yield reported for *L. graveolens* essential oil ranged from 2.5 to 3% [11,36]. Yields of essential oils obtained from *O. vulgare* species ranged from 0.96 to 5.1% [48]. Busatta et al. [49] recovered 1.2% from species of *O. majorana* and *O. vulgare* grown in California, while Gong et al. [50], reported yields ranging from 0.1 to 0.7% (v/w) for species grown in China and Pakistan, and Daouk et al. [51] reported a yield value of 3% for *O. syriacum* essential oil recovery.

L. graveolens oleoresins characterization

Oleoresin obtained from *L. graveolens* growing as wild in template areas (Queretaro), was the sample with the highest total phenol contents 189 mgEq/g, Yucatan oleoresin, growing in a tropical area, presented the lowest content of 148 mgEq/g (Table 2). Differences in abundances of different compounds were observed; 23 compounds were detected in Chihuahua; 35 in Queretaro, and 30 in Yucatan oleoresins samples. Figure 2 shows an example of the chromatogram profile of each sample. Oleoresins samples contain several fatty acids, such as palmitic, palmitic ester, oleic acids, and some are sample-specific, such as lauric, myristic, behenic acid for Queretaro sample (Table 3).

Table 2. Total phenol contents *in Lippia graveolens* oleoresins.

Sample	Total phenolic* (mgEq/g)
Chihuahua	185 ± 3.0^{a}
Queretaro	189 ± 2.9^{a}
Yucatan	148 ± 1.2^{b}

*As mg of gallic acid equivalents/g of oleoresin. Means of 5 determination ± standard deviation. Different superscript letters indicate significant differences among samples, according to the statistical analysis described in M&M section.

L. graveolens oleoresins phytochemical composition

L. graveolens oleoresin composition extracted by supercritical CO_2 showed similar phytochemical composition as *O. vulgare* obtained under supercritical conditions [38,47,52]. In all samples, the most abundant compounds were thymol and carvacrol, but the amount and proportion of both compounds were different amongst samples (Table 4). Intrinsic factors, such as plant genetics or extrinsic factors, such as the climate, temperature, and light, in which the plant grows can direct the thymol/carvacrol accumulation [11,13,51,53,54]. Vokou et al. [55] indicated that the high presence of carvacrol, thymol, or the sum of both in essential oils is related to local climatic conditions, such as higher air temperatures. Different types of soils also affected the levels of thymol and carvacrol on essential oils; manure and vegetable compost soils promoted thymol synthesis, whereas potting mix, professional agriculture mixture, and nursery mixture soils promote the thymol/carvacrol chemotype [56].

Compounds detected in all L. graveolens samples

In addition to thymol and carvacrol, *L. graveolens* oleoresins collected in different areas, also share the presence of compounds, such as linalool, α-terpineol, β-caryophyllene, β-caryophyllene (humulene), caryophyllene oxide, and humulene epoxide (Table 5). It has been demonstrated that eucalyptol showed anti-inflammatory and antioxidant effects in various diseases, including respiratory, pancreatitis, colon damage, and neurodegenerative diseases [57]. The synergic of eucalyptol and terpinen-4-ol, have shown to have a stronger antimicrobial effect [58]. Terpineol has been an excellent bactericidal against *S. aureus* strains [6,10,59]. Humulene,

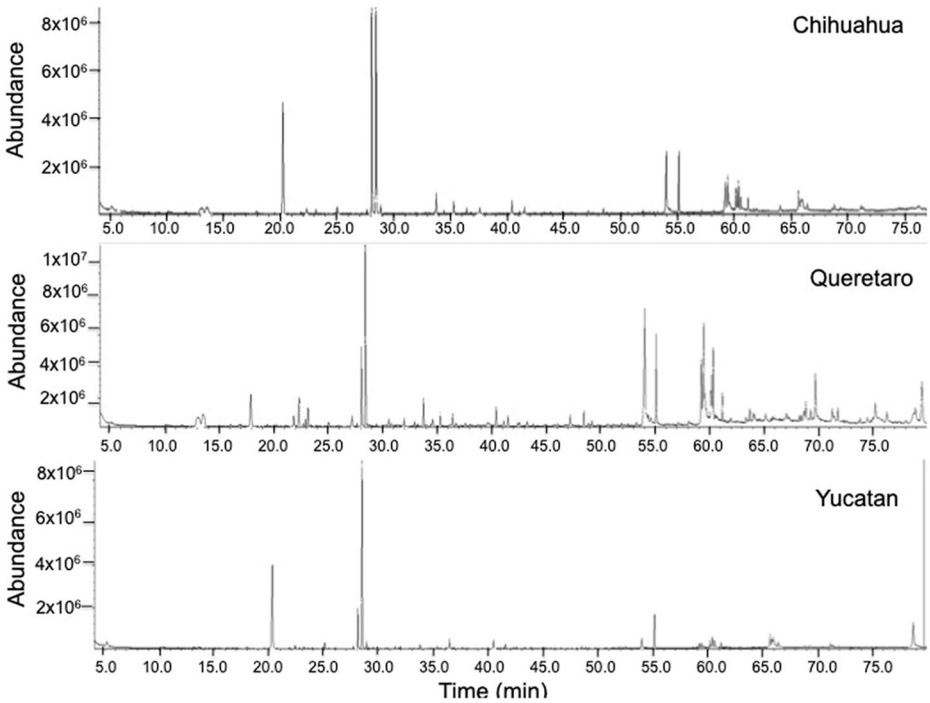

Figure 2. GC-MS profile of *Lippia graveolens* oleoresins obtained by supercritical CO_2. Samples were collected at different climate areas: arid to semi-arid (Chihuahua), template (Queretaro), and tropical (Yucatan).

Table 3. Fatty acid composition of *Lippia graveolens* oleoresins extracted with supercritical CO_2.

			Lippia graveolens samples		
RT (min)	CAS (#)	Compound	CHI	QRO	YUC
41.119	106332	Lauric acid (12:0)	nd	0.05	nd
54.059	57103	Palmitic acid (16:0)	0.91	1.27	0.06
47.202	544638	Myristic acid (14:0)	nd	0.13	nd
48.449	124061	Myristic acid, ethyl ester	nd	0.15	0.04
55.103	628977	Palmitic acid, ethyl ester	0.92	0.96	nd
59.238	506218	Linoelaidic acid (18:2)	0.43	0.50	nd
59.597	2027476	Oleic acid (18:0)	0.40	0.06	0.91
60.128	544354	Linoleic acid ethyl ester	0.25	0.21	nd
54.439	693710	Trans-13-Octadecenoic acid (18:1)	nd	0.60	nd
60.347	6114187	Ethyl oleic acid	nd	0.70	nd
60.352	6512998	Oleic acid, ethyl ester	0.37	nd	0.28
68.787	5090415	9-Oleic aldehyde	0.05	nd	nd
71.173	301020	Oleamida (18:1 amide)	0.05	nd	0.38
78.757	112845	Kemamide E (22:1)	0.71	nd	0.67
69.701	23470000	Palmitin 2-mono	nd	0.56	nd
71.267	112856	Behenic acid (22:0)	nd	0.13	nd
71.790	7770094	Tetradecanoic acid,2-hydroyx-1,3-propanedyl ester	nd	0.17	nd
60.536	629549	Palmitamide	nd	nd	0.15
61.201	111615	Stearic acid, ethyl ester	nd	nd	0.12
60.329	821170	Oleic acid, 3-hydroxypropyl ester	nd	nd	0.01

RT: retention time; CAS: chemical abstract service, a registry number assigned to each compound; *L. graveolones* samples from; CHI: Chihuahua; QRO: Queretaro; YUC: Yucatan; nd: non detected.
Numbers indicate the relative % of compound observed by GC.

Table 4. Thymol and carvacrol content for oleoresins from samples collected at different locations.

Sample	Thymol*	Carvacrol*	Percentage	T:C proportion
Chihuahua	154 ± 2.0^{a}	230 ± 2.9^{a}	38	1:1.5
Queretaro	30.9 ± 0.6^{c}	128 ± 2.1^{b}	16	1:4.2
Yucatan	33.7 ± 0.4^{b}	228 ± 3.0^{a}	26	1:6.8

T:C: thymol:carvacrol proportion.
*mg of thymol or carvacrol per g of oleoresin. Means of 5 determination ± standard deviation. Different letters indicated significant differences amongst samples, according to the statistical analysis described in M&M section.

a natural monocycle terpene, was able to inhibit hepatocellular carcinoma (HCC) cell proliferation and induced HCC apoptosis both *in vitro* and *in vivo* [60]. β-caryophyllene, a major plant volatile found in essential oils of many different spices and food plants, such as oregano, is a phytocannabionoid with a strong affinity to cannabinoid receptor type-2. The action of β-caryophyllene oxide could be partly based on its ability to disrupt the mitochondrial membrane potential and to activate the initiator of caspases, but other mechanisms could be involved [61]. β-caryophyllene, humulene, and β-caryophyllene oxide presented anticancer properties [61,62].

Compounds detected in at least two L. graveolens samples

Eucalyptol, terpino-4-ol, p-cyme-8-ol, anisole, hydroxyquinone, 3-ter-butylated hydroxyanisole, β-bisabolene, were detected in two samples (Table 5). β-bisabolene is a sesquiterpene that showed selective cytotoxic activity for mouse and human breast cancer cells [63]. Eucalyptol, a terpenoid oxide, has anti-inflammatory and antioxidant effects in various diseases, including respiratory, pancreatitis, colon damage, and neurodegenerative diseases [58]. The synergic of eucalyptol and terpine-4-ol, have shown to have a stronger antimicrobial effect [59]. *P*-tert butylated hydroxyanisole (BHA) is an antioxidant consisting of a mixture of two isomeric organic compounds, 2-tert-butyl-4-hydroxyanisole and 3-ter-butyl-4-hydroxyanisole and is used as food additive. BHA is also used in medicines, such as isotretinoin, lovastatin, and simvastatin [64].

Table 5. Phytochemical composition of *Lippia graveolens* oleoresins extracted with supercritical CO_2.

			Lippia graveolenes samples		
RT (min)	CAS (#)	Compound	CHI	QRO	YUC
13.063	527844	O-cymene	0.01	0.05	nd
13.541	470826	Eucalyptol	0.01	0.03	nd
17.951	78706	Linalool	0.01	0.50	0.02
20.320	1055261	Azulene 1,2,3-triphenyl	nd	nd	0.03
21.859	507700	Endo-borneol	nd	0.12	nd
21.876	4017929	3-caranol	nd	0.04	nd
22.331	562743	Terpien-4-ol	0.04	0.38	0.06
22.748	1197019	p-cyme-8-ol	nd	0.03	0.01
23.188	98555	α-Terpineol	0.05	0.24	0.02
25.006	1076568	Anisole, Thymol methyl ether	0.07	nd	0.06
28.068	89838	Thymol	3.81	0.90	1.11
28.429	499752	Carvacrol	4.64	3.68	8.94
30.550	91101	Syringol	nd	0.05	nd
31.465	6380285	Carvacrol acetate	nd	nd	0.05
31.957	2444282	Hydroxyquinone	nd	0.05	0.04
33.743	87445	β-Caryophyllene	0.32	0.03	0.07
33.746	13877913	β-Ocimene	nd	nd	0.03
34.418	58319065	Sesquithujene	0.03	nd	nd
34.546	13674163	Hydrocinnamic acid	nd	0.02	nd
34.585	2217609	Thymoquinol	nd	0.06	nd
35.287	6753986	α-caryophyllene, humulene	0.18	0.17	0.04
36.406	88324	3-tert-butylated hydroxyanisole	nd	0.11	0.22
37.582	495614	β-bisabolene	0.07	0.02	nd
40.415	1139306	Caryophyllene oxide	0.17	0.19	0.24
41.511	19888347	Humulene epoxide	0.04	0.10	0.12
42.372	131711	Trans-Z-α-bisabolene epoxide	nd	nd	0.01
42.572	948607	Pterin-6-carboxylic acid	nd	nd	0.01
54.602	20675961	Syringenin	nd	0.04	nd
68.672	480397	Galangin flavanone	nd	0.04	nd

RT: retention time; CAS: chemical abstract service, a registry number assigned to each compound; *L. graveolones* samples from; CHI: Chihuahua; QRO: Queretaro; YUC: Yucatan; nd: non detected.
Numbers indicate the relative % of compound observed by GC.

Compounds detected in only one L. graveolens sample

Oleoresin obtained from a sample grown in a semi-arid to the arid area (Chihuahua) was characterized by the presence of sesquithujene, belonging to the sesquiterpenoid family, which present a wide range of bioactivities [65]. Oleoresin extracted from *L. graveolens* grown in the tropical area (Yucatan sample) was

characterized by the presence of azulene 1,2,3-triphenyl, carvacrol acetate, β-ocimene, trans-Z-β-bisabolene epoxide, pterin-carboxylic acid (Table 5). Oleoresin from *L. graveolens* collected in template areas (Queretaro) showed several compounds, such as endo-borneol, 3-caranol, syringol, hydroxinnamic acid, thymoquinol, syringenin, and galangin flavanone (Table 5).

Carvacrol acetate exhibited anti-inflammatory activity in mice [66], while thymoquinonas have been reported protective roles against renal disorders, showed anti-inflammatory and antioxidant properties in animal and *in vitro* models [67]. Thymoquinol glycosides have been related to neuroprotective activity [68].

Galangin has been detected only in hydrolyzed methanolic extracts of *L. graveolens* of commercial samples [69]; however, using supercritical CO_2, this compound was extracted more safely for food uses. Galangin is an efficient free radical scavenger and also exerts a protective effect on macromolecules, such as DNA, lipids, and proteins against UVB-induced damage [70]. This compound showed marked inhibitory activity against penicillinase and β-lactamase activities and cause cytoplasmic membrane damage [71]. Galangin also showed a bacteriostatic effect against MSSA (methicillin-susceptible *S. aureus*), MRSA (methicillin-resistant *S. aureus*), and VISA (vancomycin-intermediate *S. aureus*), independent of the mechanisms of resistance [72]. Galangin has been proposed as a novel compound to develop a new generation of phytopharmaceuticals that may use alone or in combination with other antimicrobial agents to treat highly resistant microorganisms [71].

Antimicrobial effect

According to the results of the Kirby-Bauer diffusion test method, reference strains, *E. faecalis* B600, and *S. aureus* A702 are classified as susceptible. In contrast, *E. faecalis* A605 was classified as MDR and VRE while *S. aureus* A710 expressed a phenotype compatible with MDR and MRSA (Table 6).

To achieve precisely the antimicrobial properties of *L. graveolens* oleoresins, the MIC and MBC were performed using vancomycin and ciprofloxacin as susceptibility controls in *E. faecalis* and *S. aureus*, respectively. Due to the opacity of oleoresins was not able to read MIC, however, the MBC was correctly performed (Table 7). *E. faecalis* reference strain, and clinical isolates 29212 and B600, were inhibited by vancomycin below 0.25 and 4 μg/mL, while A605 was resistant to concentrations above 32 μg/mL corroborating their phenotype of resistance. When *L. graveolens* oleoresins were used, Queretaro extract showed the highest inhibitory power at 31 μg/mL for both reference strains and clinical isolates. When Chihuahua and Yucatan samples were used, it was needed up to 62 μg/mL to achieve a bactericidal effect for all *E. faecalis* strains.

For *S. aureus* strains, ciprofloxacin controls corroborated the phenotypic profile, being necessary <0.125 and 0.5 μg/mL to inhibit the growth of the reference and A702 strains, respectively. In contrast, A710 isolate needed more than 4 μg/mL to reach the bactericidal effect. When *L. graveolens* oleoresins were used, a lower concentration was needed to inhibit the growth of reference strain, 16 μg/mL of Queretaro sample, and 31 μg/mL of Chihuahua and Yucatan samples. Both *S. aureus* clinical isolates required 16 μg/mL of Queretaro sample and 62 μg/mL of Chihuahua and Yucatan samples.

It had been described that *O. vulgare* essential oil showed antimicrobial properties against ATCC strains as well as clinical isolates, such as *S. enteritica,* and *P. aeruginosa*, reporting MIC and MBC values of 100 and 150 mg/mL, respectively [73], values that

Table 6. Antibiotic resistance spectrum of strains.

Strain	Resistant phenotype	PEN	LVX	CIP	ERY	LZD	VAN	FOX	OXA	CLI
Enterococcus faecalis										
ATCC 29212	Susceptible	S	S	S	S	S	S	ND	ND	ND
B600	Susceptible	S	S	S	S	S	S	ND	ND	ND
A605	MDR, VRE	R	R	R	R	S	R	ND	ND	ND
Staphylococcus aureus										
ATCC 25923	Susceptible	S	S	S	S	S	ND	S	S	S
A702	Susceptible	S	S	S	S	S	ND	S	S	S
A710	MDR, MRSA	R	R	R	S	S	ND	R	R	R

PEN: penicilin; LVX: Levofloxacin; CIP: Ciprofloxacin; ERY: Erythromycin; VAN: Vancomycin; LZD: Linezolid; FOX: Cefoxitin; OXA: Oxacillin; CLI: Clindamycin; S: Sensible; R: Resistant; ND: non-determinate; MDR: multidrug resistant; VRE: vancomycin-resistant *Enterococcus*; MRSA: methicillin-resistant *Staphylococcus aureus*.

Table 7. Minimum bactericidal concentration (MBC) of *Lippia graveolens* oleoresins from samples obtained from three different Mexican locations.

			(μg/mL)*		
Strain	Resistant phenotype	Control	QRO	CHI	YUC
Enterococcus faecalis					
29212	Reference strain	<0.25	31	62	62
B600	Susceptible[a]	<4	31	62	62
A605	VRE	>32	31	62	62
Staphyloccocus aureus					
25923	Reference strain	<0.125	16	31	31
A702	Susceptible[b]	<0.5	16	62	62
A710	MRSA	>4	16	62	62

VRE: vancomycin-resistant *Enterococcus*; MRSA: methicillin-resistant *Staphylococcus aureus*. Oleoresins samples: CHI: Chihuahua; QRO: Queretaro; YUC: Yucatan.
*μg/mL total phenols (Gallic acid equivalent).
[a]Vancomycin was used as control.
[b]Ciprofloxacin was used as control.

are consistent with the low effectivity of essential oil in gram-negative bacteria. Da Silva et al. [74] tested seven genotypes of *L. gracilis* against the plant pathogen *Xanthomonas campestris*, MIC values ranged from 700 μg/mL to 1000 μg/mL, showing that thymol-rich phenotype presented the highest antimicrobial activity. MIC and MBC values for commercial oregano essential oil were 0.4 mg/mL against MRSA bacteria [26]. Cáceres et al. [2] tested the antimicrobial activity of several essential oils against *Escherichia coli* O157:H7 and O33, and *S. epidermidis* ATCC 122228 strain, being the chemotype thymol-carvacrol from *L. origanoides* and *Thymus vulgaris* the oils with higher antimicrobial activity with MIC values of 0.37–0.75 mg/mL. The Mexican oregano *Poliomintha longiflora* showed MIC and MCB values from 250 to 3000 mg/L [32], and a commercial oregano extract processed in Mexico showed a growth inhibition for different ATCC strains (*Acinetobacter baumannii*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis*, *S. aureus*, and *Streptococcus pyogenes*) with values higher than 0.5 μg/mL [75,76]. However, there are a few works that reported the microbicidal activity of *L. graveolens* oleoresins against MDR clinical isolates.

Although thymol and carvacrol mechanisms of action have not been completely elucidated, it appears that they can destabilize the cytoplasmic membrane by increasing its fluidity causing ions leakage and leading to a decrease in the pH gradient across the cytoplasmic membrane and disequilibrium of inorganic ions [26,77]. Zhou et al. [78] demonstrated that changes in membrane

fluidity of *S. aureus* could be a combined effect of the lipophilic properties of thymol and the increase of IoIS activity, which is a predicted Aldo-keto reductase (AKR) in *S. aureus*. Higher AKR activity leads to depletion of NADPH and consequently a decrease in glutathione causing a high susceptibility to hydroxyl free radicals, and oxidative damages resulting in a deficiency in lipid biosynthesis and thus increasing bacterial membrane permeability, subsequently cell death.

For carvacrol, has been claimed that its inhibitory potential for bacterial growth is due to its high hydrophobicity, then impacting the arrangement and stability of phospholipid bilayer creating holes and expansion of the membrane. Once the membrane is damaged, the internal pH decreases, and electrochemical potential dissipates, leading to a forced production of ATP [79]. Another factor that allows acting as a transmembrane ion exchanger is the –OH group present in carvacrol molecule, which after interacting and crossing the membrane, reaches the cytoplasm where it loses the acidic proton and o compensate for this loss, it binds intracellular $K^{\pm}$ and crosses back to extracellular; this contributes to the accumulation of $K^{\pm}$ in the extracellular space causing a detriment of the intracellular compartment [76]. This could explain the similarity in minimum bactericidal concentration for susceptible and MDR strains.

However, it is noteworthy that the Queretaro sample required a lower concentration to reach the same effect as Chihuahua or Yucatan sample, despite the high ratios of thymol: carvacrol present in the Chihuahua sample, this could be explained by the complexity of the Queretaro sample. In summary, the antimicrobial activity of *L. graveolens* could be the result of a synergistic mechanism among the diverse metabolites present, which contribute to potentiating the biological activity. One of the problems of essential oil is the lack of specificity of the mechanism of action, several studies propose microencapsulation as an alternative of administration, nevertheless, this reduces the potency of the essential oil as a bactericide [80,81]. For which, essential oils can be an excellent alternative as surface disinfectants, it is well-described that pure thymol and carvacrol present high antimicrobial effects alone and combined with different additives [74,82]. The uses proposed for essential oils are in the field of the food industry, dental, or even veterinary in the vast majority. However, the results of this study suggested that the Mexican oregano oleoresins represent an excellent alternative to be used as a disinfectant for medical surfaces in healthcare facilities with MDR bacteria problems, and further works should be addressed in that direction.

Conclusions

In this work, supercritical fluids extraction, the emergent and environmentally friendly technology were used to obtain *L. graveolens* oleoresins. Oleoresins yields were higher than the values reported by the hydro-distillation process. Data reported here contribute to increasing the knowledge of *L. graveoleons* species and could be important for future breeding programs for chemotypes selection with novel specific uses. Furthermore, *L. graveolens* oleoresins extracted with SCF grown under different climatic conditions presented differences in phytochemical composition and showed a slightly different antimicrobial capacity and higher than other studies. As far as we know, a few reports show the effect *of L. graveolens* oleoresins grown under different conditions against MDR strains from clinical isolates, and its potential should be further evaluated for application in modern medicine, food industry, and cosmetics due to their naturally high content of substances with antioxidant and antibacterial activity.

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Disclosure statement

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ORCID

Raúl González-García http://orcid.org/0000-0002-7020-3212

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Universidad Autónoma de San Luis Potosí
Posgrado en Ciencias Biomédicas Básicas

Centro de Investigación en Ciencias de la Salud y Biomedicina

Tesis Doctoral:
Caracterización epidemiológica de *Pseudomonas aeruginosa* provenientes de un hospital de tercer nivel.

Presenta:
M. en C. Nallely Sarai Badillo Larios

Director de Tesis:
Dra. Perla del Carmen Niño Moreno

Co-director de Tesis:
Dr. Edgar A. Turrubiartes Martínez

Asesores internos:
Dr. Fidel Martínez Gutiérrez
Dra. Esther Layseca Espinosa

Asesor externo:
Dr. Gerardo Martínez Aguilar
Unidad de investigación biomédica
IMSS delegación Durango

San Luis Potosí, SLP, Enero 2025

Jurado

PRESIDENTE DE SINODALES:
Dr. Mauricio Comas García

SECRETARIO DE SINODALES:
Dr. Fidel Martínez Gutiérrez

SINODAL:
Dra. Esther Layseca Espinosa

SINODAL:
Dr. Juan Carlos Muñoz Escalante

SINODAL EXTERNO:
Dr. Gerardo Martínez Aguilar

SINODAL SUPLENTE:
Dra. Mónica Raquel Calera Medina

Contenido

Introducción

Infecciones Asociadas a la Atención de la Salud

De acuerdo con la OMS, las **IAAS** se definen como aquellas **infecciones** que afectan a un paciente durante el proceso de asistencia en un **hospital** o Centro Sanitario, que **no** estaba **presente**, ni en período de incubación **al** momento de su **ingreso** y que pueden inclusive llegar a manifestarse después del alta del paciente.

RHOVE. Boletín Infecciones Asociadas a la Atención de la Salud, (2022).

Introducción

Infecciones Asociadas a la Atención de la Salud

- De 1 de cada 10 pacientes se ve afectado por las IAAS.
- Son más frecuentes **países de ingresos bajos y medios**.
- **136 millones de casos** en el mundo de infecciones por bacterias resistentes al año.
- **En el 2019 se notificaron 5 millones de muertes** en el mundo asociadas con la resistencia a los antimicrobianos.
- En Europa, cada año ocurren 9 millones de infecciones por bacterias resistentes que generan 25 millones de días adicionales de hospitalización y un costo estimado entre **13,000 y 24,000 millones de euros.**

"Key facts and figures." Accessed: Jul. 14, 2024. [Online]. Available: https://www.who.int/campaigns/world-hand-hygiene-day/key-facts-and-figures

Introducción

Infecciones Asociadas a la Atención de la Salud

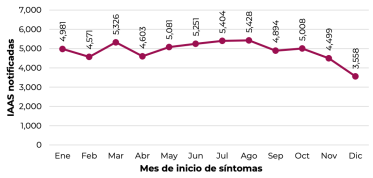
- Cada día, **1** de cada **31 pacientes** hospitalizados sufre al menos una IAAS.
- El 3% de los pacientes hospitalizados en 2015 presentaron una o más IAAS.
- Se calcula que en 2015 se produjeron 687,000 IAAS en hospitales de Estados Unidos.
- Alrededor de 72,000 pacientes con IAAS murieron durante su hospitalización.

"HAIs: Reports and Data | HAIs | CDC." Accessed: Jul. 14, 2024. [Online]. Available: https://www.cdc.gov/healthcare-associated-infections/php/data/index.html

Introducción

Infecciones Asociadas a la Atención de la Salud

- Norma Oficial Mexicana NOM-045-SSA2-2005, para la vigilancia epidemiológica, prevención y control de las Infecciones Nosocomiales.
- En el 2023 se tuvo una notificación de **58,604** IAAS con una media mensual de 4,884.

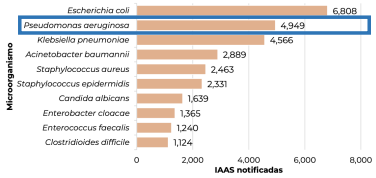


RHOVE. Boletín Infecciones Asociadas a la Atención de la Salud, (2023).

Introducción

Infecciones Asociadas a la Atención de la Salud

- Prolongan las estancias hospitalarias de 5.9 a 9.6 días e incrementa la probabilidad de morir (riesgo atribuible) hasta en un 6.9%.
- Implica aumentos en los gastos hospitalarios.
- A pesar de lo que dicta la **NOM,** menos de 10% de las unidades hospitalarias en todo el país, reportan periódicamente la incidencia de **IAAS**.

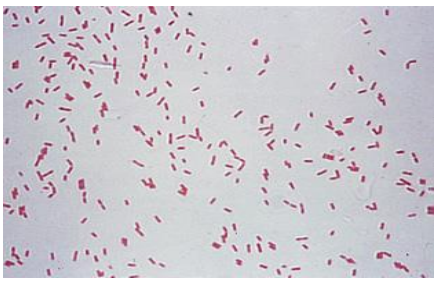


RHOVE. Boletín Infecciones Asociadas a la Atención de la Salud, (2022); Boletín Conamed OPS, Ing. Marlenne Rodríguez Salgado (2018)

Introducción

Pseudomonas aeruginosa

- Bacteria Gram negativa
- Móvil por flagelos polares
- Aerobia
- Oxidasa positiva
- Microorganismo ubicuo
- Patógeno oportunista



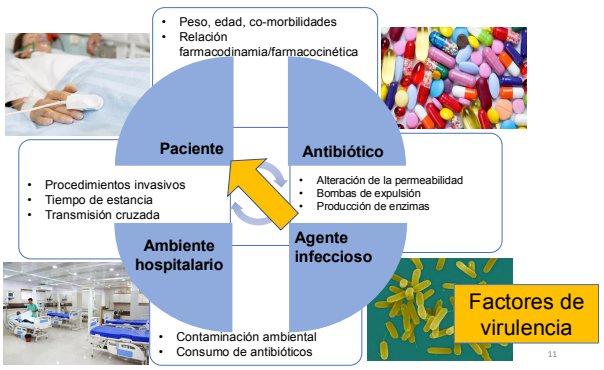
M. F. Moradali, S. Ghods, and B. H. A. Rehm, *Frontiers in Cellular and Infection Microbiology*, (2017).

Introducción

Factores de riesgo

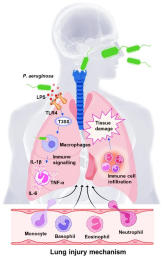
Introducción

Factores de riesgo

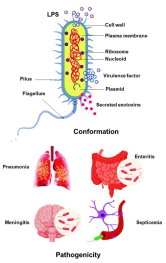


Introducción

Pseudomonas aeruginosa



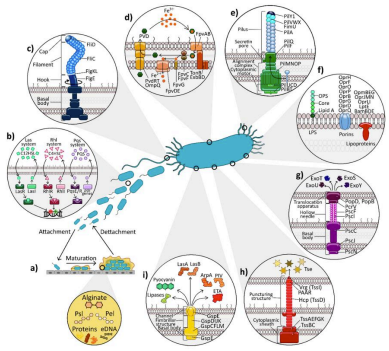
- Patógeno oportunista implicado en infecciones agudas y crónicas, con una amplia lista de factores de virulencia.
- Posee resistencia intrínseca y adquirida a antibióticos y una gran flexibilidad metabólica.
- Es responsable de varias IAAS, como neumonías, infecciones de sitio quirúrgico, bacteriemias e infecciones urinarias.



S. Qin et al., *Signal Transduction and Targeted Therapy (2022)*; M. F. Moradali, S. Ghods, and B. H. A. Rehm,*Frontiers in Cellular and Infection Microbiology*, (2017); M. W. Azam and A. U. Khan, *Drug Discovery Today*, (2019).

Introducción

Pseudomonas aeruginosa - Factores de virulencia



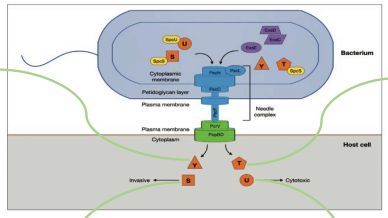
El arsenal que posee es amplio y contiene elementos que le permiten:

- Formar biopelícula
- Desplazarse
- Adquirir nutrientes del medio
- Adherirse a la superficie celular
- Seleccionar lo que ingresa al interior de la bacteria (AB)
- Ingresar toxinas al interior de la célula huésped
- Verter distintas sustancias (toxinas) al espacio extracelular

Jurado-Martín, M. Sainz-Mejías, and S. McClean, International Journal of Molecular Sciences, (2021)

Introducción

Sistema de secreción tipo 3



ExoY
- >89% de las cepas
- Adenilato ciclasa
- Desensamble de microtúbulos
- Necrosis celular

ExoT
- 92 - 100% de las cepas
- Impide fagocitosis
- Disrupción del citoesqueleto
- Inhibición de la citocinesis

ExoS
- 58 - 72% de las cepas
- Disrupción del citoesqueleto
- Impide fagocitosis

ExoU
- 24 – 42% de las cepas
- Fosfolipasa A2
- Citotóxica
- Proinflamatoria
- Activa NF-kB e IL-8

J. J. Yang, K.-S. C. Tsuei, and E. P. Shen, *Tzu Chi Medical Journal*, 2022

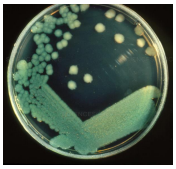
Pregunta de investigación

¿Existe una relación entre la frecuencia de genes de factores de virulencia y el perfil de resistencia en aislamientos de *Pseudomonas aeruginosa* causantes de infecciones en el Hospital Regional de Alta Especialidad “Dr. Ignacio Morones Prieto”?

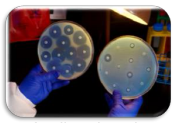
Hipótesis

La frecuencia de los genes relacionados con los factores de virulencia en cepas de *Pseudomonas aeruginosa* provenientes de aislamientos clínicos se encuentra asociado al perfil de resistencia.

Justificación

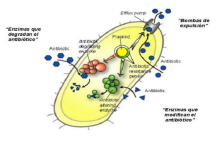


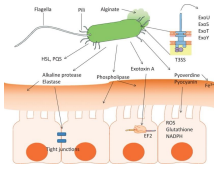
Patógeno oportunista



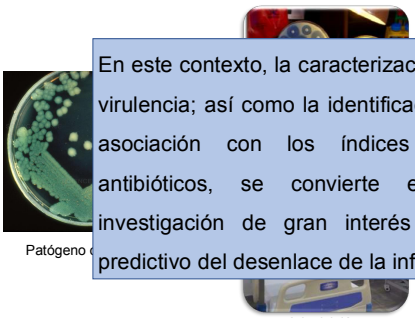
Amplia resistencia

Adquisición y complicación de IAAS

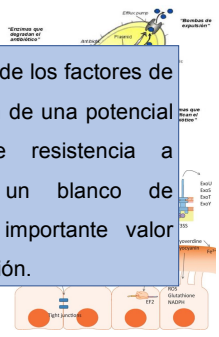




Justificación



Adquisición y complicación de IAAS



En este contexto, la caracterización de los factores de virulencia; así como la identificación de una potencial asociación con los índices de resistencia a antibióticos, se convierte en un blanco de investigación de gran interés e importante valor predictivo del desenlace de la infección.

Objetivos

Objetivo principal

Caracterizar molecularmente los factores de virulencia y los perfiles de resistencia de cepas de *Pseudomonas aeruginosa* provenientes del Hospital Regional de Alta Especialidad “Dr. Ignacio Morones Prieto”.

Objetivos

Objetivos particulares

- Determinar las características clínicas de los pacientes.
- Determinar el perfil de resistencia de los aislamientos.
- Identificar cepas productoras de biopelícula.
- Realizar la detección molecular de los factores de virulencia.
- Determinar el patrón de citocinas asociado a cepas que poseen los genes *exoU* o *exoS*.

Descripción del estudio

Tipo de estudio:
Descriptivo y observacional

Muestreo a conveniencia
Recolectando todos los aislamientos de *Pseudomonas aeruginosa* en un periodo de un año, provenientes del Hospital Regional de Alta Especialidad “Dr. Ignacio Morones Prieto”.

Criterios de selección:

Inclusión	• Todo aislamiento clínico de *Pseudomonas aeruginosa*. • Todo aislamiento con el debido consentimiento informado por parte del paciente
No inclusión	• Aquellos aislamientos clínicos de *Pseudomonas aeruginosa* no recuperados.
Eliminación	• Todo aislamiento clínico de *Pseudomonas aeruginosa* al que se le retire el consentimiento informado o que no pertenezcan a la clasificación de IAAS.

Aspectos éticos

Proyecto con riesgos mínimos que fue sometido al comité de Ética del Hospital Regional de Alta Especialidad
“Dr. Ignacio Morones Prieto”

Aprobado el 25 de abril de 2018 con número de registro 38-18

Se recolectó la primera muestra el 25 de mayo de 2018


SSLP
Hospital Central “Dr. Ignacio Morones Prieto”

San Luis Potosí, S.L.P., a 25 de abril de 2018

M. en C. Nallely Saral Badillo Larios
Investigador Principal:

Por este medio se le comunica que su protocolo de investigación titulado “Caracterización epidemiológica de Pseudomonas spp. provenientes del hospital Dr. Ignacio Morones Prieto”, fue evaluado por el Comité de Investigación, con Registro en COFEPRIS 17 CI 24 029 050, así como por el Comité de Ética en Investigación de esta Institución con Registro CONBIOETICA-24-CEI-001-20160427, y fue dictaminado como:

APROBADO

El número de registro es 38-18, el cual deberá agregar a la documentación subsecuente, que presente a ambos comités.

De igual forma pido sea tan amable de comunicar a los Comités de Investigación y de Ética en Investigación: la fecha de inicio de su proyecto, la evolución y el informe final pertinente.

*Se le recuerda que todos los pacientes que participen en el estudio deben firmar la versión sellada del formato de consentimiento informado.

Atentamente

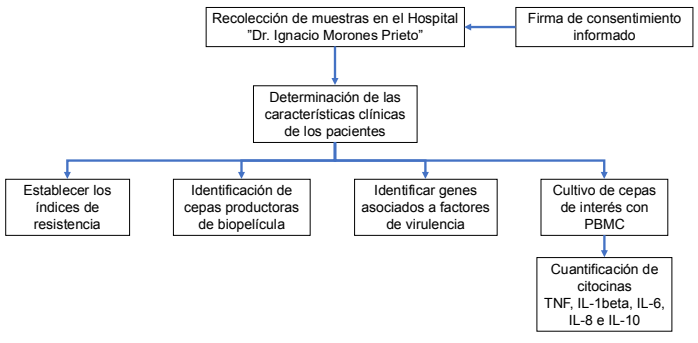
Dra. Mg. Sol Ptan Salazar Leal
Subdirectora de Educación e Investigación en Salud
Hospital Central “Dr. Ignacio Morones Prieto”

25 ABR 2018
DIRECCIÓN DE EDUCACIÓN E INVESTIGACIÓN EN SALUD

C.C.P. Archivo

Metodología

Estrategia experimental



Resultados

Resultados y discusión

Recolección de muestras y datos clínicos

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Firma de consentimiento informado

Determinación de las características clínicas de los pacientes

- Edad
- Sexo
- Área del hospital
- Tipo de infección
- Tiempo de estancia
- Resolución

Resultados y discusión

Demografía de los pacientes

25 de mayo de 2018 a 24 de Mayo de 2019

75 IAAS causadas por *Pseudomonas aeruginosa*

Tabla 1. Frecuencia de los tipos infecciones causadas por cepas MDR y NMDR. Chi cuadrada *p= <0.05

Tipo de infección	MDR n (%)	NMDR n (%)	Total
Tracto respiratorio	10 (13.3)	18 (24)	28
Bacteriemia	1 (1.3)*	13 (17.3)	14
Sitio quirúrgico	5 (6.6)	7 (9.3)	12
Piel y tejidos blandos	4 (5.3)	5 (6.6)	9
Otros	5 (6.6)	7 (9.3)	12

Badillo-Larios et al, International Journal of Microbiology, 2024

Tabla 2. Comparativa entre datos clínicos recuperados del expediente médico.

	n	%
Sexo		
Hombre	43	57.3
Mujer	32	42.7
Edad (años)		
Infantes (0 -1)	8	10.7
Niños (2 - 10)	6	8
Adolescentes (11 - 17)	6	8
Adultos jovenes (18 - 35)	15	20
Adultos (36 - 60)	27	36
Adultos mayores (>60)	13	17.3
Estancia (días)		
Media	42.5	
Desviación estándar	35.6	
Rango	3 - 243	
Resolución		
Dado de alta	59	78.7
Muertes no asociadas	11	14.7
Muertes asociadas	5	6.7

Resultados y discusión

Demografía de los pacientes

JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Virulence genes and antibiotic resistance of *Pseudomonas aeruginosa* isolated from patients in the Northwestern of Morocco

Chaimae Elmouaden[1,2], Amin Laglaoui[2], Latifa Ennanei[1], Mohammed Bakkali[2], Mohammed Abid[1]

[1] *Department of Research, Institut Pasteur du Maroc, Tangier, Morocco*
[2] *Biotechnology and Biomolecule Engineering Team, Department of Biology, Faculty of Science and Technology, Abdelmalek Essaadi University, Tangier, Morocco*

Table 3. Distribution of virulence factor genes in P. aeruginosa strains isolated from NI and CAI.

	Number of virulence factor genes				
	Five	Four	Three	Two	One
Strains from CAI n = 68	44 (64.7%)	20 (29.4%)	4 (5.9%)	0	0
Strains from NI n = 87	53 (60.9%)	28 (32.2%)	6 (6.9%)	0	0
Total n = 155	97 (62.6%)	48 (31%)	10 (6.5%)	0	0

NI: Nosocomial infections; CAI: Community-acquired infections; n: Number.

87 cepas clasificadas como IAAS en dos años, pero no se clasificaron según el tipo de infección.

Elmouaden, Chaimae, et al. , " The Journal of Infection in Developing Countries", (2019)

Resultados y discusión

Demografía de los pacientes en mundo

JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Virulence genes and antibiotic resistance of *Pseudomonas aeruginosa* isolated from patients in the Northwestern of Morocco

No existe ningún otro estudio con el mismo planteamiento que el presente. Sin embargo, los datos epidemiológicos son concordantes al señalar que las mayores tasas de infección por *Pseudomonas aeruginosa* son la que afectan el tracto respiratorio.

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Elmouaden, Chaimae, et al. , " The Journal of Infection in Developing Countries", (2019)

Resultados y discusión

Demografía de los pacientes en México

En nuestro estudio

Seis cepas por mes
Infecciones de tracto respiratorio

1,449,804 + 1,372,451 = 2,822,255

19vo estado con más población

Original Article

JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Antibiotic resistance, virulence factors and genotyping of *Pseudomonas aeruginosa* in public hospitals of northeastern Mexico

Eliab Misael González-Olvera[1], Rebeca Pérez-Morales[2], Alberto González-Zamora[3], Graciela Castro-Escarpulli[4], Ingrid Palma-Martínez[4], José de Jesús Alba-Romero[1,5]

Cinco cepas por mes
Infecciones de tracto urinario

927,784 + 904,866 = 1,832,650

25vo estado con más población

San Luis Potosí aparentemente tiene un buen manejo de sus infecciones nosocomiales

González-Olvera et al., 2019; *Número de Habitantes. Durango*, n.d.; *Número de Habitantes. San Luis Potosí*, n.d.

Resultados y discusión

Susceptibilidad a antimicrobianos

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Determinación de las caracteristicas clínicas de los pacientes

Establecer los índices de resistencia

Concentración mínima inhibitoria

Resultados y discusión

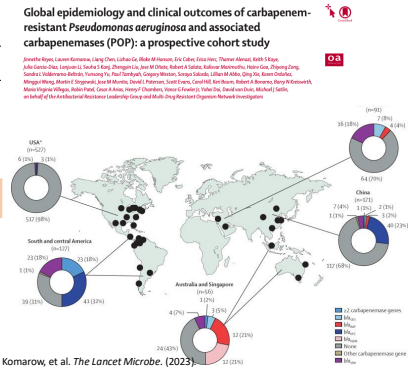
Susceptibilidad a antimicrobianos

Tabla 3. Patrones de susceptibilidad a antibióticos de Pseudomonas aeruginosa causante de IAAS (n=75).

Antibiótico	Susceptible n (%)	No-susceptible n (%)
Amikacin	57 (76.0)	18 (24.0)
Cefepima	51 (68.0)	24 (32.0)
Gentamicina	52 (69.3)	23 (30.7)
Piperacilina/Tazobactam	51 (68.0)	24 (32.0)
Aztreonam	39 (52.0)	36 (48.0)
Ciprofloxacino	55 (73.3)	20 (26.7)
Meropenem	43 (57.3)	32 (42.7)

Las comparaciones entre grupos se realizaron mediante el test exacto de Fisher, *Los valores P < 0,05 se consideraron estadísticamente significativos.

25 cepas se clasificaron como Multidrogorresistente.



Badillo-Larios et al, International Journal of Microbiology, 2024; Reyes, J., Komarow, et al. *The Lancet Microbe*. (2023).

Resultados y discusión

Susceptibilidad a antimicrobianos

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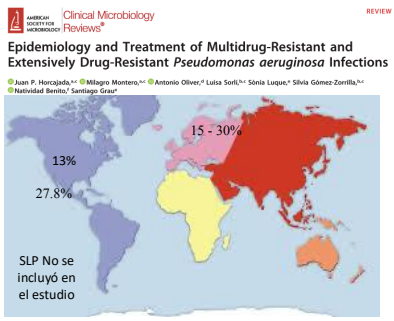


Badillo-Larios et al, International Journal of Microbiology, 2024; Horcajada, J. P. et al. *Clinical Microbiology Reviews*. (2019); Garza-González, E., et al. *PloS One*, (2019).

Resultados y discusión

Susceptibilidad a antimicrobianos

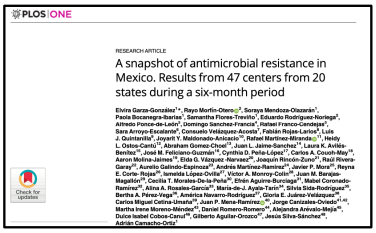
Estudio multicéntrico de resistencias bacterianas nosocomiales en México

Pseudomonas aeruginosa	sensible	intermedio	resistente	total	% resistencia
Amikacina	57	1	26	84	30.95
Ceftriaxona	3	0	5	8	62.50
Cefepima	46	6	32	84	38.10
Ceftazidima	46	6	32	84	38.10
Cefotaxima	1	0	0	1	0.00
Ciprofloxacina	55	2	19	76	25.00
Gentamicina	46	7	22	75	29.33
Imipenem	41	2	22	65	33.85
Meropenem	59	1	21	81	25.93
Moxifloxacino	17	2	0	19	0.00
Piperacilina	0	18	13	31	41.94
Piperacilina tazobactam	18	4	8	30	26.67

Gutiérrez-Muñoz J,et al. Rev Latin Infect Pediatria, 2017

Resultados y discusión

Susceptibilidad a antimicrobianos



Genus/species	Specimen (n)	AMK	AMC	AMP	SAM	AZM	CFZ	FEP	FOX	CRO	CIP	ETP	GEN	IMP	LVX	MEM	NIT	TZP	TGC	TOB	SXT
P. aeruginosa	All (1.995)	17.3	ND	ND	ND	12.8	ND	17.5	ND	ND	18.6	ND	16.7	29.7	22.4	27.8	ND	14.8	ND	17.5	ND
	RES (370)	14.9	ND	ND	ND	15.4	ND	7.2	ND	ND	13.2	ND	14.6	27.0	11.3	32.7	ND	7.5	ND	17.6	ND
	URI (342)	30.1	ND	ND	ND	ND	ND	28.4	ND	ND	35.9	ND	31.1	ND	ND	31.3	ND	19.1	ND	31.5	ND
	BLO (197)	7.7	ND	ND	ND	4.5	ND	17.4	ND	ND	11.36	ND	5.8	30.0	15.2	20.3	ND	8.8	ND	3.5	ND
	CSF (28)	10.0	ND	ND	ND	ND	ND	0.0	ND	ND	0.0	ND	0.0	0.0	0.0	40.0	ND	0.0	ND	11.1	ND

Garza-González, E., Morfín-Otero, R., PLoS ONE, 2019

Resultados y discusión

Factores de virulencia / tipos de infecciones

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Determinación de las características clínicas de los pacientes

Establecer los índices de resistencia

Reacción en cadena de la polimerasa

Electroforesis de productos de PCR

Lectura en luz UV

Tipos de infecciones

Prueba exacta de Fisher

Prueba de Correlación

IBM SPSS

python™

Resultados y discusión

Distribución de los genes en los tipos de infección

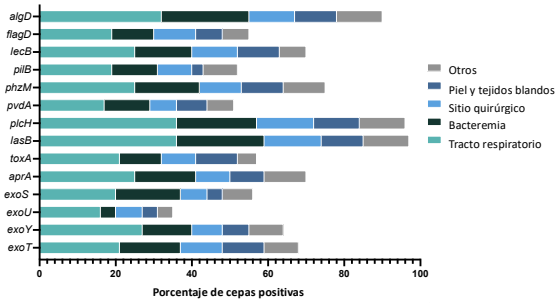


Figura 1. Frecuencia de genes de virulencia entre cepas de *P. aeruginosa* según el tipo de infección. Frecuencia de los genes de virulencia entre las cepas de *P. aeruginosa* según el tipo de infección. Las comparaciones entre grupos se realizaron mediante la prueba exacta de Fisher, * Los valores P < 0,05 se consideraron estadísticamente significativos.

Badillo-Larios et al, International Journal of Microbiology, 2024

Resultados y discusión

Genes de virulencia in distintas infecciones

Iran Red Crescent Med J. 2014 October; 16(10): e15722. DOI: 10.5812/ircmj.15722
Published online 2014 October 5. Research Article

Virulence Gene Profiles of Multidrug-Resistant *Pseudomonas aeruginosa* Isolated From Iranian Hospital Infections

Nastaran Fazeli [1]; Hassan Momtaz [1,*]
[1]Department of Microbiology, Shahrekord Branch, Islamic Azad University, Shahrekord, IR Iran

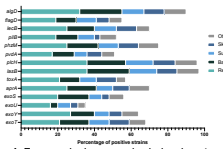


Figura 1. Frecuencia de genes de virulencia entre cepas de *P. aeruginosa* según el tipo de infección.

Table 6. Distribution of Virulence Genes in *Pseudomonas aeruginosa* Isolated From Clinical Infections in Humans [a]

Source of Isolation	*P. aeruginous* Isolates	*exoT*	*exoY*	*exoU*	*exoS*	*plcH*	*aprA*	*plcH*	*phzM*	*plcS*	*lasA*	*lasB*	*pvdA*	*pilB*	*toxA*	*algD*	*algU*	*plcH*	*nanI*
Wound infection																			
M	20	5	7	18	-	1	2	-	1	-	1	-	1	2	10	2	4	2	5	11	6	1
F	16	2	6	11	2	-	1	8	3	2	-	2	3	8	1	6	3	1	2	7	11	2
Respiratory infection																			
M	8	1	3	6	2	2	-	2	4	1	1	1	2	1	2	1	-	-	1	4	1	-
F	7	4	1	3	-	-	2	1	2	-	1	2	3	1	3	2	5	-	-	5	2	2
Urinary tract infection																			
M	6	3	-	2	1	-	1	3	3	1	1	1	-	2	1	-	4	-	3	-	2	-
F	3	1	1	1	-	2	-	-	1	-	1	1	1	-	-	1	2	-	-	-	-	-
Bed ulcer																			
M	10	7	4	7	3	4	-	4	6	2	1	5	-	2	4	-	8	-	7	4	-	6
F	6	2	-	2	-	1	2	1	1	2	-	2	1	1	2	1	-	-	2	2	1	2
Burn																			
M	14	10	5	11	4	7	4	6	9	3	8	7	3	6	7	3	9	2	10	8	6	8
F	12	2	6	8	2	4	3	4	7	2	6	6	5	2	5	2	1	3	2	5	3	3
Total																			
M	58	26	19	44	10	14	7	15	23	7	12	14	5	13	24	6	25	4	26	27	17	15
F	44	11	4	25	4	7	6	14	14	6	6	11	13	12	11	4	6	10	17	17	9	

[a] Abbreviations: M, male; F, female.

Las discrepancias pueden deberse a las diferencias geográficas.

Badillo-Larios et al, International Journal of Microbiology, 2024; N. Fazeli and H. Momtaz, *Iranian Red Crescent Medical Journal*, 2014

Resultados y discusión

Correlación de los genes en los tipos de infección

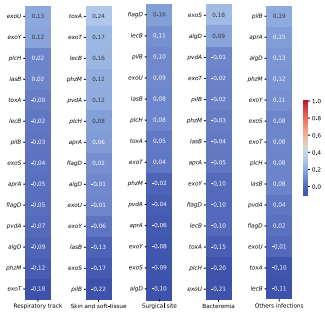


Figura 2. Correlación entre el tipo de infección y los genes de virulencia se calculó utilizando el método DataFrame.corr() de Python.

Badillo-Larios et al, International Journal of Microbiology, 2024

Resultados y discusión

Correlación de los genes en los tipos de infección

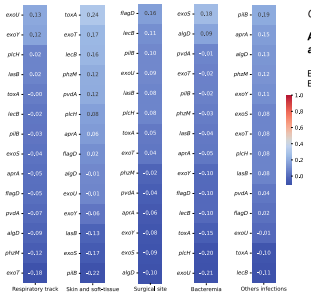


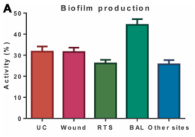
Figura 2. Correlación entre el tipo de infección y los genes de virulencia se calculó utilizando el método DataFrame.corr() de Python.

Original Article

JIDC THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Antibiotic resistance, virulence factors and genotyping of *Pseudomonas aeruginosa* in public hospitals of northeastern Mexico

Eliab Misael González-Olvera[1], Rebeca Pérez-Morales[2], Alberto González-Zamora[3], Graciela Castro-Escarpulli[4], Ingrid Palma-Martínez[4], José de Jesús Alba-Romero[1,5]



Nuestro estudio encontró las infecciones de tracto respiratorio como las comunes, un grupo de Durango cuantificó las ITUs como las más comunes, pero que las de mayor producción de biopélicula eran las asociadas a lavados bronco alveolares.

Badillo-Larios et al, International Journal of Microbiology, 2024; González-Olvera et al., 2019; *Número de Habitantes. Durango*, n.d.; *Número de Habitantes. San Luis Potosí*, n.d., M.

Resultados y discusión

Correlación de los genes en los tipos de infección

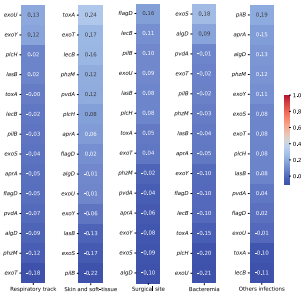
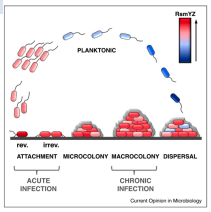


Figura 2. Correlación entre el tipo de infección y los genes de virulencia se calculó utilizando el método DataFrame.corr() de Python.

Valetini, 2021



En nuestros resultados no se encontró diferencia estadísticamente significativa, pero se ha descrito que la diferencia en la distribución de los genes en *P. aeruginosa* pudiera no ser relevante en los tipos de infección, pero si los cambios de expresión genética cuando pasa de una infección aguda (plantónica) a una crónica (biopelícula).

Badillo-Larios et al, International Journal of Microbiology, 2024; Valentini, D. Gonzalez, D. A. Mavridou, and A. Filloux. Current Opinion in Microbiology, 2018

Resultados y discusión

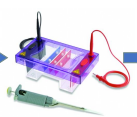
Factores de virulencia / Biopelícula

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

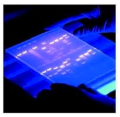
Determinación de las características clínicas de los pacientes

Identificación de cepas productoras de biopelícula

Reacción en cadena de la polimerasa



Electroforesis de productos de PCR



Lectura en luz UV

Producción de biopelícula

Medición por espectroscopia a 590nm

Prueba X^2

IBM SPSS

Resultados y discusión

Genes en cepas productoras de biopelícula

Tabla 4. Relación entre los genes de virulencia de *P. aeruginosa* causante de IAAS y el nivel de producción de biopelícula (n=75).

Gen asociado al factor de virulencia	Indice de producción de biopelícula		
	Bajo n (%)	Moderado n (%)	Fuerte n (%)
exoT	1 (1.3)	24 (32.0)	26 (34.7)
exoY*	3 (4.0)	16 (21.3)	29 (38.7)
exoU	1 (1.3)	16 (21.3)	9 (12.0)
exoS	2 (2.7)	17 (22.7)	23 (30.7)
aprA	2 (2.7)	21 (28.0)	30 (40.0)
toxA*	0 (0)	15 (20.0)	28 (37.3)
lasB	3 (4.0)	33 (44.0)	36 (48.0)
plcH	3 (4.0)	33 (44.0)	36 (48.0)
pvdA	1 (1.3)	18 (24.0)	19 (25.3)
phzM	1 (1.3)	23 (30.7)	32 (42.7)
pilB	2 (2.7)	20 (26.7)	17 (22.7)
lecB*	3 (4.0)	27 (36.0)	22 (29.3)
flagD	1 (1.3)	18 (24.0)	21 (28.0)
algD	2 (2.7)	28 (37.3)	37 (49.3)

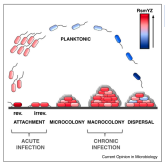
Las comparaciones entre grupos se realizaron mediante la prueba Chi cuadrada, *Los valores P<0.05 se consideraron estadísticamente significativos.

Azimi en 2016, encontró en hopitales de Iran que *exoY* era un gen con alta prevalencia en cepas altamente productoras de biopelícula.

Badillo-Larios et al, International Journal of Microbiology, 2024; S. Azimi et al., GMS Hyg Infect Control. 2016

Resultados y discusión

El paso a biopelícula cambia la expresión génica



Valetini, 2021

Infección aguda → Infección crónica

Caracterizadas por la formación de biopéliculas

Este cambio en el estilo de vida permite a la bacteria un ciclo de vida y una expresión de factores de virulencia flexible que es estrategico en el desarrollo de una infección.

Leila Asadpour en 2018, reportó que la frecuencia de los genes que codifican para *exoA* si era mayor para las cepas productoras de biopélicula.

Genes				Biofilm density
exo S	exoY	exoU	exoT	
+	+	+	+	W
+	+	+	+	M
+	+	–	+	W
+	+	–	+	M
+	+	–	+	S
+	–	+	+	M
–	+	+	+	W
–	+	+	+	M
–	+	+	+	S
–	+	+	+	N
–	+	+	+	S
–	+	–	+	M
–	–	+	+	M
–	–	–	+	W
–	–	–	+	S

W, weak; M, moderate; S, strong; N, negative.

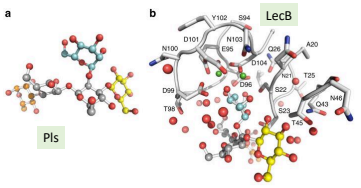
Jabalameli *en* 2012 observó que *exoY* es un gen común en cepas productoras de biopelícula.

M. Valentini, D. Gonzalez, D. A. Mavridou, and A. Filloux. Current Opinion in Microbiology, 2018; N. Fazeli and H. Momtaz, *Iranian Red Crescent Medical Journal*, 2014; F. Jabalameli *et al.*, *Burns*, 2012

Resultados y discusión

LecB es crucial para iniciar la biopelícula

Infección aguda → Infección crónica



The *Pseudomonas aeruginosa* lectin LecB binds to the exopolysaccharide Psl and stabilizes the biofilm matrix

Daniel Passos da Silva[1], Michael L. Matwichuk[1], Delaney O. Townsend[1], Courtney Reichhardt[1], Doriano Lamba[2], Daniel J. Wozniak[3] & Matthew R. Parsek[1,4]

Se demostró que LecB y Psl se unen y su interacción afecta a la estructura de la biopelícula y que *P. aeruginosa* puede utilizar LecB para adherirse a exopolisacáridos que contengan manosa, fucosa o a proteínas glicosiladas presentes en biopelículas establecidas de otras especies.

M. Valentini, D. Gonzalez, D. A. Mavridou, and A. Filloux. Current Opinion in Microbiology, 2018; D. Passos da Silva *et al.*, *Nature Communications*, 2019

Resultados y discusión

Factores de virulencia / Susceptibilidad a antibióticos

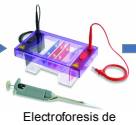
Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Determinación de las características clínicas de los pacientes

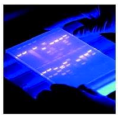
Establecer los índices de resistencia

Identificar genes asociados a factores de virulencia

Reacción en cadena de la polimerasa



Electroforesis de productos de PCR



Lectura en luz UV

Susceptibilidad a antibióticos

Prueba Exacta de Fisher

IBM SPSS

Prueba de Correlación

python

Resultados y discusión

exoU es mas frecuente en cepas resistentes a AB

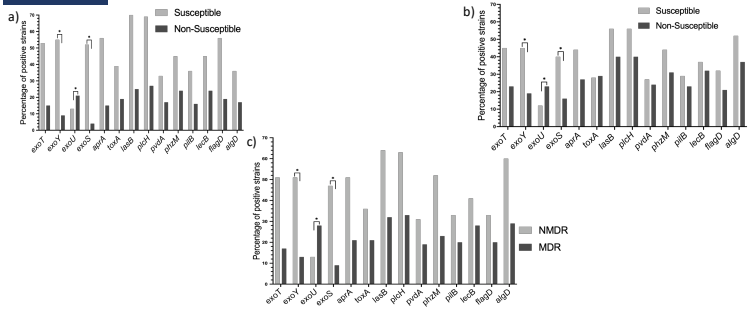


Figura 2. Frecuencia de los genes de virulencia entre *cepas de P. aeruginosa* según su susceptibilidad a (a) fluoroquinolonas, (b) carbapenemicos, y el estado de (c) multirresistente. Las comparaciones entre grupos se llevaron a cabo mediante la prueba exacta de Fisher, * Los valores P < 0,05 se consideraron estadísticamente significativos.

Badillo-Larios et al, International Journal of Microbiology, 2024

Resultados y discusión

Distancia entre los genes de resistencia y virulencia

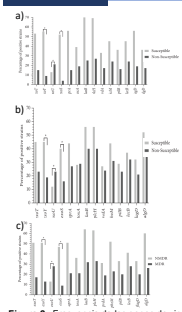


Figura 3. Frecuencia de los genes de virulencia entre *cepas de P. aeruginosa* según su susceptibilidad a (a) fluoroquinolonas, (b) carbapenemicos

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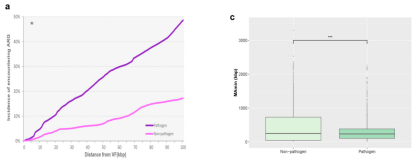
Coexistence of Antibiotic Resistance Genes and Virulence Factors Deciphered by Large-Scale Complete Genome Analysis

Yu Pan,[a,b] Jiaxiong Zeng,[a,b] Liguan Li,[a] Jintao Yang,[c] Ziyun Tang,[c] Wenguang Xiong,[a,c] Yafei Li,[a] Sheng Chen,[f] Zhenling Zeng[a,b,c]



Badillo-Larios et al, International Journal of Microbiology, 2024; Y. Pan *et al.*, *mSystems*, 2020;

Resultados y discusión

Correlación entre resistencia a AB y genes de virulencia

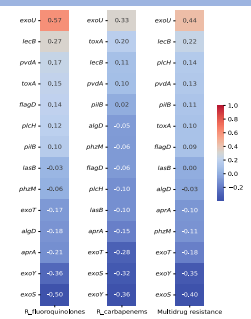


Figura 4. La correlación entre los genes de virulencia y las tasas de resistencia (fluoroquinolonas, carbapenemicos y multirresistencia a fármacos) se calculó mediante el método DataFrame.corr(en Python).

Badillo-Larios et al, International Journal of Microbiology, 2024

Resultados y discusión

Correlación entre resistencia a AB y genes de virulencia

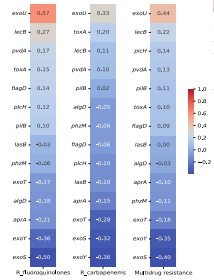
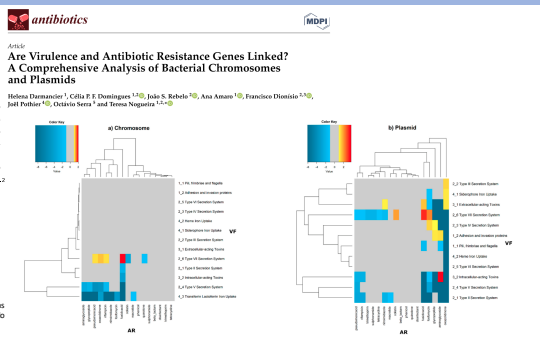


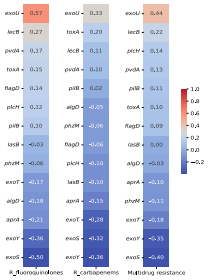
Figura 4. La correlación entre los genes de virulencia y las tasas de resistencia, se calculó mediante el método DataFrame.corr(en Python).



Badillo-Larios et al, International Journal of Microbiology, 2024; H. Darmancier *et al.*, *Antibiotics*, 2022

Resultados y discusión

Correlación entre resistencia a AB y genes de virulencia



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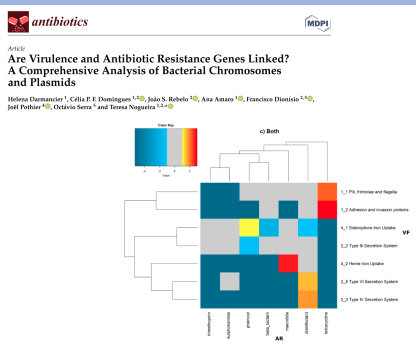


Badillo-Larios et al, International Journal of Microbiology, 2024; H. Darmancier *et al.*, *Antibiotics*, 2022

Resultados y discusión

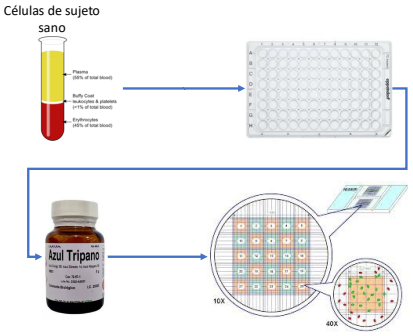
Evaluación de citocinas / Viabilidad celular

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Determinación de las características clínicas de los pacientes

Establecer los índices de resistencia

Cultivo de cepas de interés con PBMC



Resultados y discusión

exoU tiene relación con una mayor muerte celular

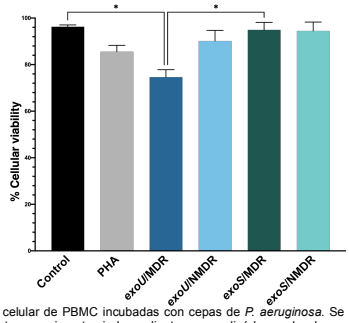
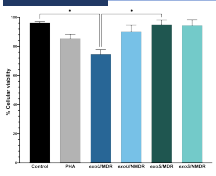


Figura 5. Viabilidad celular de PBMC incubadas con cepas de *P. aeruginosa*. Se muestran los valores medios ± DE de cuatro experimentos independientes; se realizó la prueba de comparaciones múltiples de Kruskal-Wallis *Los valores P<0,05 se consideraron estadísticamente significativos.

Badillo-Larios et al, International Journal of Microbiology, 2024

Resultados y discusión

exoS y su relación con el daño celular



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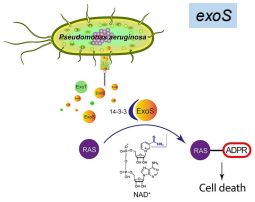


Review

Pseudomonas aeruginosa Cytotoxins: Mechanisms of Cytotoxicity and Impact on Inflammatory Responses

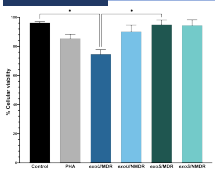
Stephen J. Wood [1,2], Josef W. Goldufsky [1], Michelle Y. Seu [1], Amir H. Dorafshar [2] and Sasha H. Shafikhani [1,3,4,*]

Las células intoxicadas con ExoS muestran signos tanto de apoptosis intrínseca dependiente de Caspasa-9 como de apoptosis extrínseca dependiente de Caspasa-8 mediada por receptores de muerte, y ambos dominios de ExoS contribuyen a la apoptosis inducida por ExoS.

Badillo-Larios et al, International Journal of Microbiology, 2024; S. J. Wood, J. W. Goldufsky, M. Y. Seu, A. H. Dorafshar, and S. H. Shafikhani, *Cells*, 2023.

Resultados y discusión

exoU una causa de muerte celular



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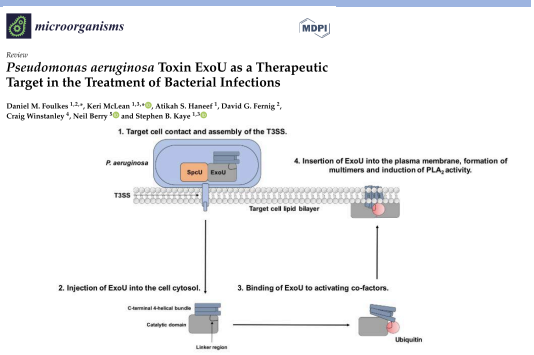


Badillo-Larios et al, International Journal of Microbiology, 2024; D. M. Foulkes *et al.*, *Microorganisms*, 2019.

Resultados y discusión

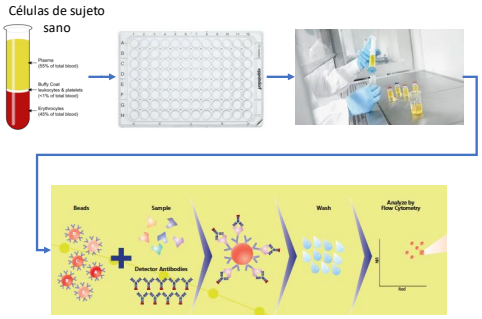
Evaluación de citocinas

Recolección de muestras en el Hospital "Dr. Ignacio Morones Prieto"

Determinación de las características clínicas de los pacientes

Establecer los índices de resistencia

Cuantificación de citocinas TNF, IL-1beta, IL-6, IL-8 e IL-10



Resultados y discusión

Evaluación de citocinas

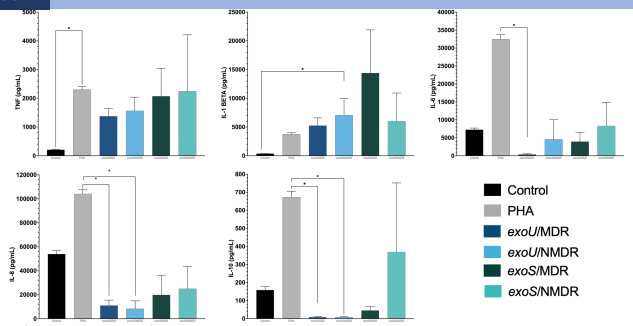


Figura 6. Perfiles de citocina de PBMC estimuladas con cepas de *P. aeruginosa*. Los niveles de citocinas se evaluaron mediante un ensayo de captura multiperla. Se muestran los valores medios ± desviación estándar de cuatro experimentos independientes. Se realizó la prueba de comparación múltiple de Kruskal-Wallis El valor *p <0,05 se consideró estadísticamente significativo.

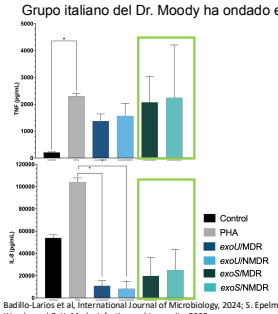
Badillo-Larios et al, International Journal of Microbiology, 2024

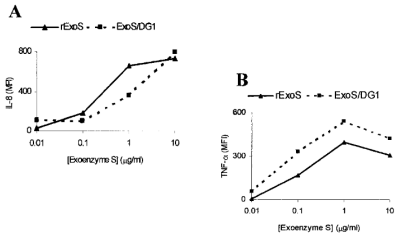
Resultados y discusión

exoS provoca el aumento de citocinas inflamatorias

Hay poca información acerca de cómo pueden variar las citocinas o el perfil inflamatorio a estar el contacto con esta especie bacteriana.

Grupo italiano del Dr. Moody ha ondado en la relación que existe entre la producción de ExoS y algunas citocinas.





Badillo-Larios et al, International Journal of Microbiology, 2024; S. Epelman *et al., Journal of Leukocyte Biology*, 2002; Epelman, T. F. Bruno, G. G. Neely, D. E. Woods, and C. H. Mody, *Infection and Immunity*, 2000

Resultados y discusión

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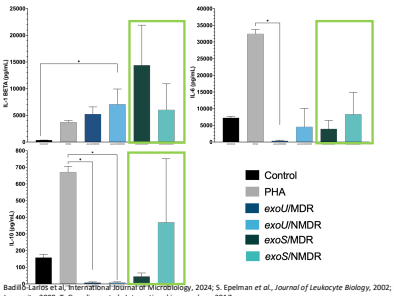


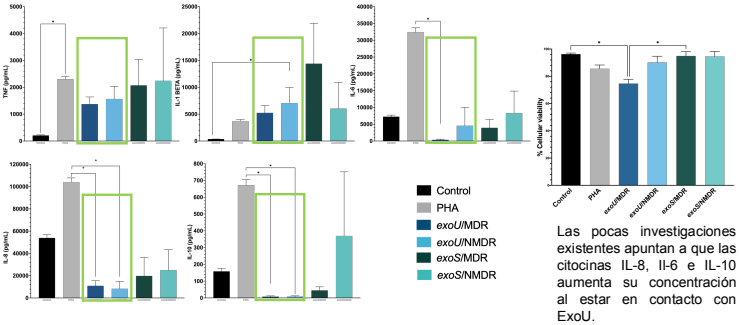
TABLE 1. Exoenzyme S induces Th1 and proinflammatory cytokines

Cytokine	Ratio of stimulated/unstimulated[a] (stimulated/L32)[b] band intensity after stimulation with ExoS/DG1 for various periods:			
	5 h	Day 1	Day 3	Day 5
IFN-γ	1.2 (0.06)	0.9 (0.1)	0.7 (0.04)	2.8 (0.3)
IL-2	1.8 (1.1)	2.5 (0.6)	3.2 (0.3)	2.4 (0.13)
TNF-α	18 (2.1)	5.2 (0.56)	3.3 (0.3)	2.5 (0.22)
IL-1α	9.6 (4.5)	107 (2.8)	9.9 (0.8)	3.4 (0.13)
IL-1β	21 (20)	67 (25)	28 (5)	12 (1.2)
IL-1RA	2.0 (18)	1.1 (9.4)	2.3 (8)	3.7 (6)
IL-6	– (5.54)	73 (3.1)	6.3 (0.15)	– (0)
IL-10	4.1 (0.27)	1.2 (0.11)	0.5 (0.12)	0.9 (0.17)
TGF-β1	1.0 (0.43)	1.2 (0.87)	0.7 (0.44)	2.2 (0.98)
TGF-β3	1.2 (0.53)	2.0 (0.69)	1.1 (0.27)	3.0 (0.37)
IL-12p40	11 (0.19)	6.0 (0.08)	1.3 (0.03)	1.2 (0.56)

Badillo-Larios et al, International Journal of Microbiology, 2024; S. Epelman *et al., Journal of Leukocyte Biology*, 2002; Epelman, T. F. Bruno, G. G. Neely, D. E. Woods, and C. H. Mody, *Infection and Immunity*, 2000; T. Grandjean *et al., International Immunology*, 2017

Resultados y discusión

exoU provoca una expresión dispar de citocinas

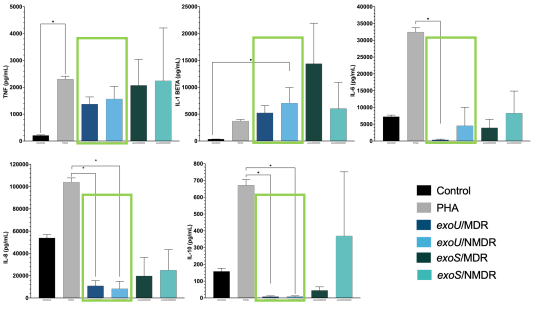


Las pocas investigaciones existentes apuntan a que las citocinas IL-8, Il-6 e IL-10 aumenta su concentración al estar en contacto con ExoU.

Badillo-Larios et al, International Journal of Microbiology, 2024; K. S. Hardy *et al.*, Infection and Immunity, 2022

Resultados y discusión

exoU provoca una expresión dispar de citocinas



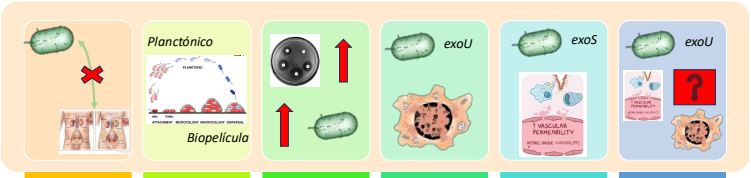
Las pocas investigaciones existentes apuntan a que las citocinas IL-8, Il-6 e IL-10 aumenta su concentración al estar en contacto con ExoU.

Las discrepancias:

- Muerte celular por el efecto citotóxico de ExoU
- Hardy et al en 2022 apunto a la existencia de una función no citotóxica de ExoU, que parece ser más un mecanismo de evasión inmunológico.

Badillo-Larios et al, International Journal of Microbiology, 2024; K. S. Hardy *et al.*, Infection and Immunity, 2022

Resumen de hallazgos



No hay una distribución específica de los genes por tipo de infección.	Cambio en la progresión de la infección a aguda y persistente.	Hay un correlación entre la resistencia y los genes de *exoU* y *exoS*.	Cepas portadoras de exoU y MDR ocasionan mayor muerte celular.	El gen *exoS* produce patrones levemente altos de citocinas.	El gen *exoU* está relacionado con un patrón citocinas distinto al esperado.

Conclusiones

- No hay correlación entre los tipos de infección que desarrolla el paciente y los genes de virulencia que posee *Pseudomonas aeruginosa.*
- La progresión de la infección a persistente acompañado de la producción de biopelícula, parece ser clave en la diferenciación de la existencia y expresión de ciertos genes.
- La muerte celular del hospedero aumenta cuando la célula esta expuesta a cepas poseedoras del gen *exoU.*
- Las cepas con el gen *exoS* generan un aumento en la expresión de citocinas en PBMC.
- Las cepas con el gen *exoU* generan un patrón distinto al esperado en la expresión de citocinas en PBMC, probablemente por un mecanismo de patogenicidad aún no descrito en su totalidad.

Limitaciones

- Falta de información sobre aspectos clínicos de los pacientes.
- No se realizó una descripción más a fondo de los mecanismos de resistencia.
- En el ensayo de citocinas se utilizaron cepas bacterianas de origen clínico, por lo que los efectos observados en las citocinas podrían reflejar la acción conjunta de todos los factores de virulencia.

Prospectivas

- Explorar los mecanismos de resistencia a antibióticos.
- Determinar los genes asociados a la resistencia a antibióticos.
- Establecer si existe alguna correlación entre los genes de virulencia y los genes de resistencia.
- Búsqueda de cepas de alto riesgo.
- Establecer los cambios en la expresión génica cuando la bacteria desarrolla la biopelícula.

Extras

Un artículo de colaboración:

DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY
2021, VOL. 47, NO. 10, 1546–1555
https://doi.org/10.1080/03639045.2021.2008417

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RESEARCH ARTICLE

***Lippia graveolens* HBK oleoresins, extracted by supercritical fluids, showed bactericidal activity against multidrug resistance *Enterococcus faecalis* and *Staphylococcus aureus* strains**

Oscar de Jesús Calva-Cruz[a,*], Nallely S. Badillo-Larios[b,*], Antonio De León-Rodríguez[a], Eduardo Espitia-Rangel[c], Raúl González-García[d], Edgar Alejandro Turrubiartes-Martinez[b,d], Arnulfo Castro-Gallardo[a] and Ana Paulina Barba de la Rosa[a]

[a]IPICYT, Instituto Potosino de Investigación Científica y Tecnológica A. C., San Luis Potosí, Mexico; [b]CICSaB, Centro de Investigación en Ciencias de la Salud y Biomedicina, Universidad Autonoma de San Luis Potosí, San Luis Potosí, Mexico; [c]INIFAP, Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias, Campo Experimental Valle de México, Coatlinchán, Mexico; [d]Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, Mexico; [e]Centro de Investigación para los Recursos Naturales, Antigua Normal Rural de Salaices, Chihuahua, México

Extras

Presentación en congresos:

Sociedad Mexicana Bioquímica
Oaxaca, Oaxaca
2019

Bioquímica clínica
León, Guanajuato
2022

Infectología
Boca del Rio, Veracruz
2023

Extras

Colaboraciones en curso:

- Clonas de alto riesgo de *Pseudomonas aeruginosa* st411 y st167. Primer Reporte en américa
- *Stenotrophomonas maltophilia* st293. Primer reporte en el continente americano
- *Enterococcus sp* vancomicina resistentes, reporte de un ST originarios de San Luis Potosí

Extras

Formación de recursos humanos:

- 6 años de experiencia docente en UCSLP
- 4 años de experiencia docente en UVM en la carrera de Q.F.B.T.
- 2 tesis dirigidas