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Original Article

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

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Abstract

Introduction: *Pseudomonas aeruginosa* is the second most prevalent opportunistic pathogen causing nosocomial infections in Mexico. This study evaluated antibiotic resistance, production of virulence factors and clonal diversity of *P. aeruginosa* strains isolated from patients undergoing nosocomial infections in public hospitals of northeastern Mexico.

Methodology: Ninety-two *P. aeruginosa* isolates from urine culture, Foley catheter, ear, wounds, respiratory tract secretions, scalp, blood culture, bronchoalveolar lavage, expectoration and cerebrospinal fluid causing nosocomial infections were analyzed. The isolates were identified by MALDI-TOF and antibiotic resistance profiles obtained by MicroScan®. The production of virulence factors was analyzed with spectrophotometric techniques and isolates genotyped by ERIC-PCR.

Results: Out of the 92 isolates, 26 (28.2%) were found to be multidrug resistant (MDR); 21 (22.7%) were classified as extremely drug resistant (XDR). Highest resistance rate was found for gatifloxacin (42%) while ciprofloxacin accounted for the antibiotic with the lowest resistance rate (2%). Bronchoalveolar lavage isolates produced the highest amounts of virulence factors: biofilm (44.4% ± 2.7%), elastase (58.5% ± 4.3%), alkaline protease (60.1% ± 5.0%); except for pyocyanin production. The ERIC-PCR assay showed 83 genetic patterns (90% clonal diversity) and 13 isolates had 100% genetic similarity, forming 4 real clones, 3 of these clones were obtained from different anatomical site and/or hospital.

Conclusions: Antibiotic resistance and virulence factors production was heterogeneous among samples analyzed. Genotyping of *P. aeruginosa* strains showed high genetic diversity in the studied isolates.

Key words: *Pseudomonas aeruginosa*; antibiotic resistance; ERIC-PCR; virulence factors.

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Introduction

Pseudomonas aeruginosa (Schroeter 1872) Migula 1900 is an opportunistic pathogen that is involved in nosocomial infections, mainly in pneumonias, urinary tract infections, bloodstream infections, surgical site infections, skin infections and immunodeficient patients [1,2] essentially due to the ability of this microorganism to persist within many sources in hospitals, thus infecting hospitalized patients. Its intrinsic ability to resist the action of many antibiotics even during the course of treating an infection, has led to the development of this remarkably difficult persistent pathogen [3]. In addition, the production of virulence factors such as elastase, alkaline protease, pyocyanin and biofilm provides it with the ability to degrade innate immune system proteins such as surfactant proteins A and D [4], break the extracellular matrix protein laminin [5], increase the host oxidative stress [6] and retard the penetration of antimicrobials [7], respectively. In *P. aeruginosa*, bacterial resistance to antibiotics can be attributed to its irrational use due to advertising strategies, lack of previous susceptibility studies or intake of food for human consumption of previously treated animals. The main mechanisms of

resistance are the acquisition of plasmids that carry genes and chromosomal genes that metabolize drugs or interfere with their mechanism of action. By the other hand, some strains of *P. aeruginosa* present β-lactamase activity that interferes with the action of drugs with lactam rings, this allows them to be very resistant to various antibiotics [8].

Accurate identification of *P. aeruginosa* is key for its adequate treatment and the corresponding techniques include conventional microbiological methods such as those based on pH changes and substrate utilization, MicroScan® [9] and Vitek2 [10]; molecular assays as PCR end point [11] and quantitative PCR protocols. However, the continuous growth of nosocomial infections and the necessity for immediate analysis have led to the development of rapid methods for the identification of isolates. In this regard, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) has shown to be a reliable technique [12]. Outbreaks due to *P. aeruginosa* represent a constant threat in hospitals and genotyping studies such as ERIC-PCR and PCR-Ribotyping [13] have been used in order to identify them and develop the adequate mechanisms for mitigating the spread of infection. In spite of previous studies reporting the characterization of *P. aeruginosa* isolates from different sites such as patients, hospital means, medical equipment and staff [14,15]; the analysis of isolates causing nosocomial infections from different biological samples has not been characterized appropriately. In Mexico, *P. aeruginosa* accounts for the second most prevalent opportunistic pathogen causing nosocomial infections and it generates large economic costs for health systems and patients [16]. In this regard, the aim of this study was to analyze the antibiotic resistance, production of virulence factors and genetic diversity of *P. aeruginosa* strains isolated of different biological samples from patients undergoing nosocomial infections in public hospitals of northeastern Mexico.

Methodology

Bacterial isolates

The clinical *P. aeruginosa* isolates were obtained from three public hospitals in Gómez Palacio, México from January 2016 to July 2017. Ninety-two bacterial isolates from different samples including urine culture (n = 28, 30.4%), Foley catheter (n = 1, 1.1%), ear (n = 4, 4.3%), wounds (n = 30, 32.6%), respiratory tract secretions (n = 11, 12%), scalp (n = 1, 1.1%), blood culture (n = 1, 1.1%), bronchoalveolar lavage (n = 10, 10.9%), expectoration (n = 4, 4.3%) and cerebrospinal fluid (n = 2, 2.2%) causing nosocomial infections in hospitalized patients according to the guidelines of the Centers for Disease Control and Prevention (CDC) [17] were studied. Clinical samples from different anatomical sites were seeded on Mac Conkey agar and CASMAN agar. The isolates were preliminarily identified by using conventional microbiological methods [18] and stored in Luria Bertani broth (Sigma-Aldrich Co, St. Louis, USA) with 10% glycerol at -70º C. *P. aeruginosa* PAO1 ATCC® 47085 was used as reference for antimicrobial susceptibility and virulence factors production tests.

MALDI-TOF-MS based identification

The confirmation of the identification of all isolates was performed by using MALDI-TOF MS as was previously described [19]. In brief, all isolates were activated in fresh Luria Bertani broth and grown overnight at 37° C. After that, isolates were grown on BHI agar 16 hours at 37º C and a sample of the growth was taken with a 1µL loop to the MALDI-TOF target plate. The sample was subjected to routine MALDI-TOF MS by adding 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix and air-drying at room temperature. MALDI-TOF MS analyses were carried out on VITEK® MS mass spectrometer (bioMérieux, Marcy l'Etoile, France and analyzed against the IVD database by MYLA Software (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was carried out by using the automated MicroScan® autoSCAN-4 System (Siemens, Sacramento, CA). A total of 21 antibiotics including amikacin, amoxicillin, ampicillin, cefazolin, cefepime, cefotaxime, cefotetan, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, gatifloxacin, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin/tazobactam, ticarcillin/clavulanic acid, tobramycin and trimethoprim were tested and minimum inhibitory concentrations (MIC) determined (dried Gram negative panel), in the same panel the presence of β-lactamase was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [20]. Categorization of *P. aeruginosa* isolates as MDR, XDR and PDR was done as previously reported [21]. MDR was defined as acquired non-susceptibility to at least one agent in ≥ 3 antimicrobial categories; XDR as non-susceptibility to at least one agent in ≥ 6 antimicrobial categories and PDR as non-susceptibility to all antimicrobial categories tested.

Elastolytic activity

The activity of elastase was determined by using elastin Congo red as substrate. In brief, cells were grown in LB broth at 37º C overnight, centrifuged at 15 000 *xg* at 4º C for 10 minutes and 250 µL of supernatant was added to 500 µL of assay buffer (30 mM Tris buffer, pH 7.2) containing 5 mg of elastin Congo red (Sigma-Aldrich Co, St. Louis, USA). The mixture was incubated at 37 ºC for 6 hours with constant rotation, centrifuged at 1200 *xg* for 10 minutes and its absorbance measured at 495 nm [22]. Each sample was analyzed in triplicate.

Alkaline protease activity

Alkaline protease activity was determined by adding 170 µL of supernatant of isolates grown overnight in Luria Bertani broth to 500 µL of assay buffer (20 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 8) containing 17 mg of hide remazol brilliant blue (Sigma-Aldrich Co, St. Louis, USA). Tubes were incubated at 37º C for 1 hour with constant rotation, placed on ice for 1 minute, centrifuged at 4000 *xg* for 5 minutes and absorbance of the supernatant measured at 590 nm [23]. Each sample was analyzed in triplicate.

Pyocyanin quantification

Pyocyanin was determined by growing isolates in glycerol alanine minimal medium for 24 hours. The cells were removed by centrifugation and pyocyanin in the supernatant was extracted into chloroform by mixing 2500 µL of supernatant with 1500 µL of chloroform. Pyocyanin was then re-extracted into 400 µL of acidified water (0.2 M HCl) which gave a pink-red solution. The absorbance was measured at 520 nm [24]. Each sample was analyzed in triplicate.

Biofilm production

Biofilm was tested using crystal violet staining technique. Briefly, 200 µL of freshly inoculated Luria Bertani were dispensed into a 96-well polystyrene microtiter plate and incubated at 37º C for 24 hours. Next, the content was removed and the plates washed thrice with 250 µL of saline solution. Later, 200 µL of 99% methanol were added for 15 minutes, and the wells were emptied and dried. Then, 200 µL of crystal violet were added to each well and after 5 minutes washed. Finally, the colorant was resuspended by adding 160 µL acetic acid glacial and read at 490 nm in a microplate reader [25]. Each sample was analyzed in triplicate.

Typing of P. aeruginosa using Enterobacterial repetitive intergenic consensus (ERIC)-PCR

DNA extraction was performed by using InstaGeneTM Matrix ® Kit (BioRad Laboratories, Inc., Hercules,USA) according to the manufacturer's instructions.

The enterobacterial repetitive intergenic consensus (ERIC)-PCR method used primers to ERIC sequence of bacterial genomic DNA [26] ERIC1 (5´-ATGTAAGCTCCTGGGGATTCAC-3´) and ERIC2 (5´-AAG TAA GTG ACT GGG GGT-3´). A typical ERIC-PCR reaction mix contained 5 µL of 10X PCR (20 mM Tris-HCl pH 8.4, 50 mM KCl), 7. 5 µL of dNTPs (2.0 mM), 3 µL of $MgCl_2$ (50 mM), 1 µL of each primer (0.1 µM) (ERIC1 and ERIC 2), 0.5 µL of Taq® DNA Polymerase, recombinant (Invitrogene), 2 µL (100 ng) of template DNA and molecular biology grade water was added to reach a final volume of 50 µL. DNA amplification was performed using TGradient thermocycler (Biometra, Goettingen Germany). PCR conditions were of 30 cycles as follows: initial denaturation at 94º C for 60 seconds, primer annealing at 52º C for 60 seconds, extension at 65º C for 8 minutes and final extension at 65º C for 16 minutes. Twenty-five microlitre aliquots from each of the PCR amplifications were separated by electrophoresis for 3 hours in acrylamide gel 12% (w/v) at 300 V, and then visualised by ethidium bromide staining (0.5 µg/mL). The DNA molecular weight marker VI (154-2176 bp) (Roche Applied Science, Indianapolis, IN, USA) was used to estimate product sizes and the bands were visualized in a Pro MiniBis photodocumentor (DNR Bio Imaging Systems, Neve Yamin, Israel). A negative reaction control was included and the double *Pseudomonas* strain was used as a positive control for each ERIC-PCR.

Construction of the dendrogram and genotypic resemblance

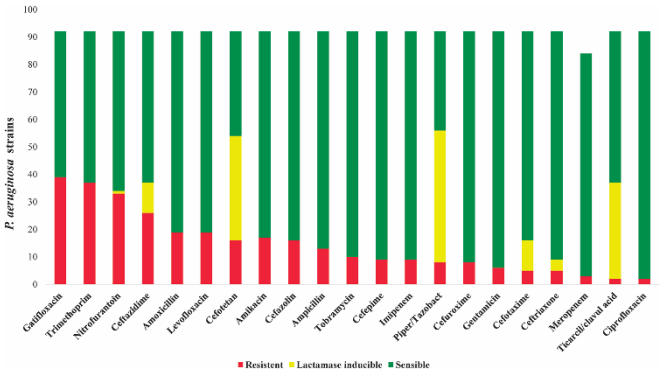
A dendrogram for cluster analysis of all strains isolated was constructed using the R ver.3.5.2 software [27] based on the 53-band pattern produced by the ERIC-PCR. The bands varied between 154 and 2176 bp. A data matrix derived from the analysis of images of the DNA band patterns was analyzed and constructed where the presence of a given band was coded as 1 and the absence of a given band was coded with 0. The Dice-Sörensen coefficient was used to calculate, compare and evaluate the similarity among the different isolates. The algorithm for constructing the grouping was the unweighted pair-group method arithmetic mean (UPGMA) through the Sequential Agglomerative

Hierarchical and No Overlap option (SAHN). The criterion to relate isolates or classify them within the same group was when the similarity pattern showed 75% or greater similarity. The representation of antibiotic resistance was mapped for each of the strains and annexed to the dendrogram to show the response pattern with respect to the similarity pattern shown in the dendrogram. A 2-way Mantel test was performed to validate the results of dendrogram [28].

Statistical analysis

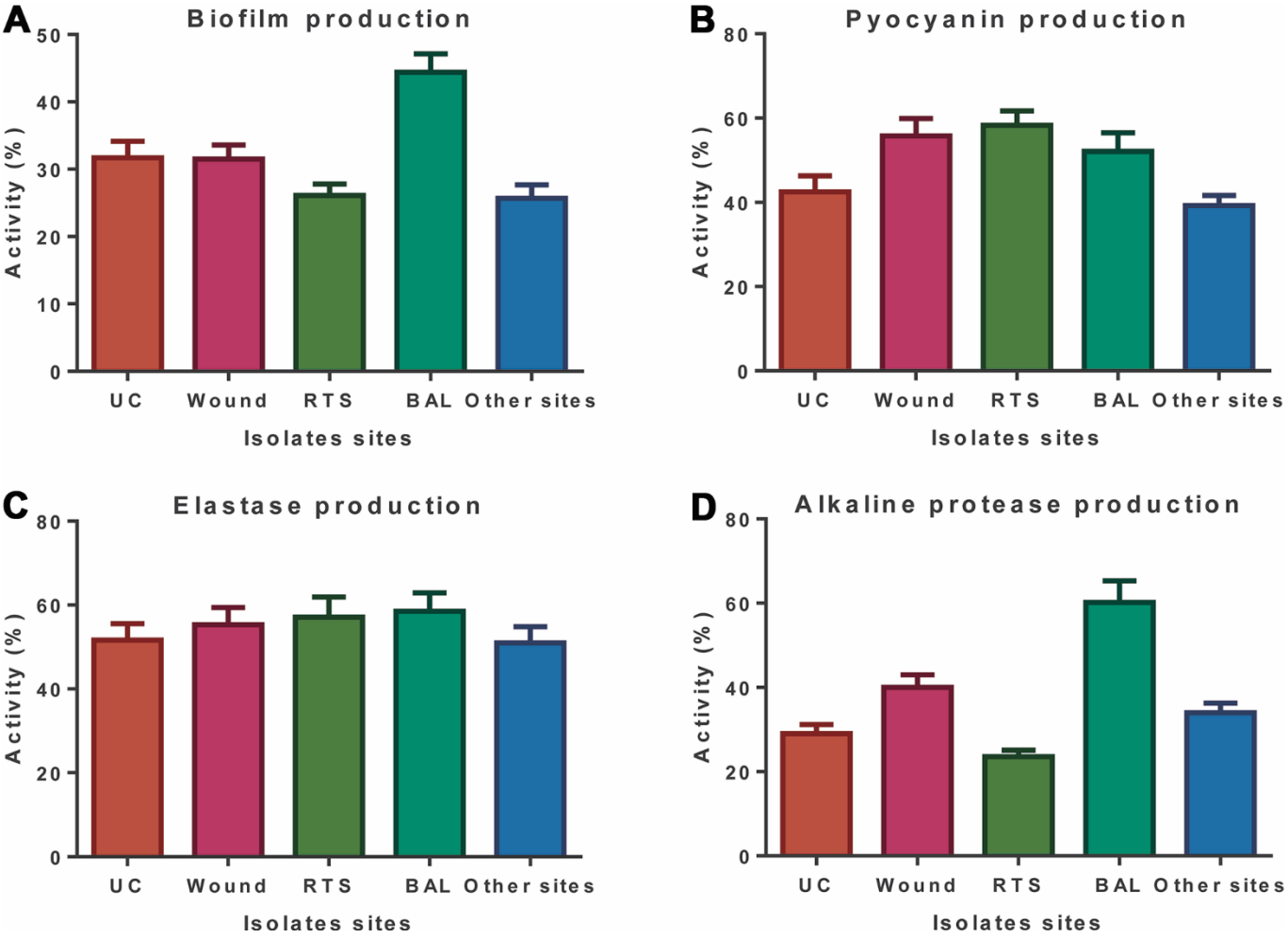
Data were analyzed using the GraphPad Prism software version 6 and ANOVA Kruskal Wallis was performed to compare the activity of virulence factors among groups. Results were considered as statistically significant if $p < 0.05$.

Figure 1. Antimicrobial susceptibility testing in *P. aeruginosa* isolated from several biological samples.



The strains showed more resistance to the antibiotic gatifloxacin and greater sensitivity to ciprofloxacin.

Figure 2. Virulence factors production of P. aeruginosa clinical isolates.



UC, urine culture; RTS, respiratory tract secretion; BAL, bronchoalveolar lavage, other sites included Foley catheter, ear, scalp, blood culture, expectoration and cerebrospinal fluid. ANOVA Kruskal Wallis was performed, and the differences were significant: biofilm $p = 0.0001$, elastase $p = 0.0002$, pyocyanin $p = 0.0001$ and alkaline protease $p = 0.0001$.

Results

MALDI-TOF identification

All 92 isolates were identified as *P. aeruginosa* by MYLA Software (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

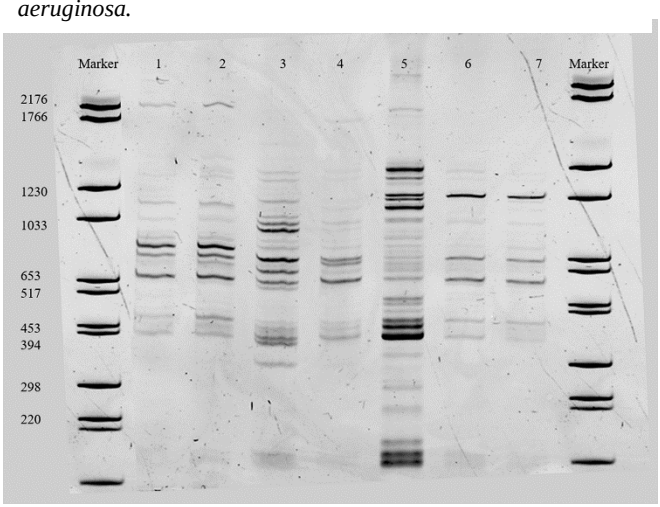
The general resistance is shown in Figure 1. Out of the 92 isolates, 26 (28.2%) of them were found to be multidrug resistant (MDR); 21 (22.7%) were classified as extremely drug resistant (XDR); 30 (32.7%) as resistant and 15 (16.4%) as susceptible. Isolates were more resistant to gatifloxacin (n = 39), trimethoprim (n = 37), nitrofurantoin (n = 33) and ceftazidime (n = 26), followed by amoxicillin, levofloxacin, cefotetan, amikacin, cefazolin, ampicillin and tobramycin (10 – 19 strains), whereas 3 – 9 isolates were resistant to cefepime, imipenem, piperacillin/tazobactam, cefuroxime, gentamicin, cefotaxime, ceftriaxone and meropenem; finally, only 2 isolates were resistant to ticarcillin/clavulanic acid and ciprofloxacin.

Additionally, β-lactamase activity was detected in some isolates susceptible to nitrofurantoin, ceftriaxone, cefotaxime, ceftazidime, however most of the isolates that expressed β-lactamase were susceptible to ticarcillin/clavulanic acid, cefotetan and piperacillin/tazobactam.

Production of virulence factors

We analyzed four virulence factors including biofilm, elastase, pyocyanin and alkaline protease production, which were analyzed according to the isolation site due to the importance of the microenvironment specificity. Bronchoalveolar lavage isolates produced the highest amounts of virulence factors: biofilm (44.4 ± 2.7%), elastase (58.5 ± 4.3%), alkaline protease (60.1 ± 5.0%); except for pyocyanin production where respiratory tract secretions isolates showed the maximum percentage of expression (58.3 ± 3.4%) (Figure 2). Urine culture and wound isolates produced equal amounts of biofilm 31.7 ± 2.4% and 31.5 ± 2.1%, respectively. Similarly, the production of pyocyanin in wound was 55.8 ± 4.0% and 52.1 ± 4.4% in bronchoalveolar lavage isolates, the lowest production of pyocyanin was in isolates from urine culture and other samples (Foley catheter, ear, scalp, blood culture, expectoration and cerebrospinal fluid). Elastase production was the most homogeneous virulence factor among all samples. The lowest production of alkaline protease was 23.5 ± 1.5% and corresponded to respiratory tract secretions isolates, while the highest production was from bronchoalveolar lavage isolates as previously mentioned.

Figure 3. DNA-banding profiles of representative *P. aeruginosa.*



Strains were isolated from several anatomical sites of infection and analyzed by gel electrophoresis following ERIC-PCR technique.

ERIC-PCR genotyping

ERIC-PCR fingerprinting revealed 83 different genetic patterns. The average amplicons per gel lane was 4. Over three- quarters of isolates had 2 to 6 bands per pattern. The detection of ERIC sequences by PCR produced 53 DNA fragments ranging from 154 bp to 2,176 bp with fragments of 1,030 bp and 490 bp found in 47.8% and 42.4% of the isolates, respectively (Figure 3). In isolate UC6, 20 bands (37.7%) could be recognized, however, 8 isolates presented only one band. The dendrogram of similarity obtained using the DNA bands of ERIC-PCR demonstrated the existence of high genetic diversity in *P. aeruginosa*, and four main clusters were formed with a high dissimilarity between clusters; approximately 99% between cluster A that contained the isolate UC28 and the other three clusters; 96.5% for cluster B that contained the isolates UC20 and W20; 94.5% for cluster C that contained the isolates W1 and W16; and cluster D that contained the majority of the isolates (n = 87, 94.6%). The dendrogram showed that there was no consistent pattern based on the anatomical sites of the isolates because the levels of similarity were very variable among them; in this respect 15 genotypes containing 40 of the isolates (43%) revealed a high similarity (between 75 and 100%). In cluster D, 13 of the isolates had 100% similarity, forming 4 real clones. The clones are a) Bal4 and Bal10; b) CSF2 and W3; c) Exp3, UC10, UC18 and W24; d) E3, Bal9, RTS1, RTS8 and UC19. Isolates of the clones a and b were obtained from the same hospital, the isolates of clone c were obtained from two different hospitals, while those of clone d were obtained from the

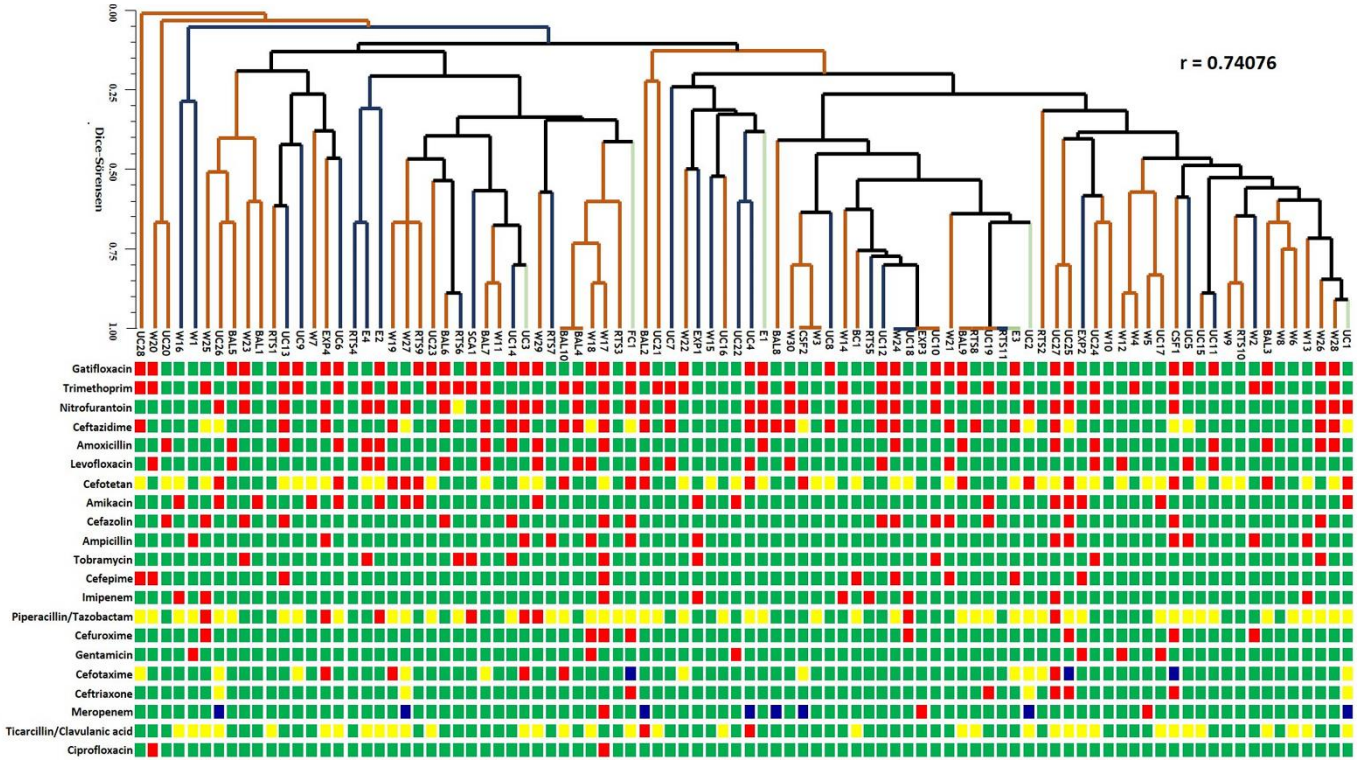
3 different hospitals. The response to antibiotic susceptibility was not the same in each clone. (Figure 4). Additionally, three groups can be recognized, e) UC1 and W28; f) UC11 and UC15; g) W4 and W12 that present a 90% similarity. Isolates with a level of genetic similarity equal to or greater than 90% also showed a similarity of about 70% in the response to antibiotics (Figure 4).

Discussion

Nosocomial infections are a public health problem in all countries, causing high costs for health systems and patients, also complicating clinical conditions. *P. aeruginosa* is an opportunistic pathogenic bacterium responsible for acute and chronic infections. Aproximately between 10-15% of nosocomial infections are caused by this pathogen and usually hard to treat due to specie intrinsic resistance; thus the appropriate organism identification enables the administration of specific antibiotics which may prevent complications and resistance generated as a consequence of indiscrminated usage of wide spectrum drugs. Currently the delay between sample receipt and pathogen identification is about 24 – 48 hours, which could be significantly shortened by an accurate direct method such as MALDI-TOF MS which has shown to be a powerful tool in microorganisms identification [29]. This method was used in our study for the identification of pathogens in the clinical isolates where also antibiotics resistance, virulence factors production and clonal relationship among *P. aeruginosa* isolates causing nosocomial infections in public hospitals of northeastern Mexico were analyzed.

The analyzed samples in this study were found in different anatomical sites such as urinary, respiratory and auditive tract, wounds from different parts of the body, in addition to bloodstream samples. *P. aeruginosa* was mainly isolated from the respiratory and urinary tract, as well as from wounds, in consistence with other studies where they have also been found in these sites and wounds of severely burnt patients [30]. Regarding antibiotic resistance, in this

Figure 4. Dendrogram representing the genetic relationships of *P. aeruginosa* strains using SAHN based on fingerprint analysis of DNA obtained by ERIC-PCR.



The abbreviations correspond to the origin and code of the strains, labeled with each of the sampling site of the isolate) UC, urine culture, RTS, respiratory tract secretions, BAL, bronchoalveolar lavage, E, ear, W, wound. The colors in the branches correspond to the isolation sites of the strains, Hospital 1, orange branches; Hospital 2, branches dark blue; and Hospital 3, green branches. The scale on the left represents the percentage of genetic similarity among the isolates. Matrix correlation was performed through a normalized Mantel statistic Z, approximate Mantel t-test t = 23.3921, p = 1. The squares below represent the resistance pattern of each strain to different medications. The green squares indicate sensitivity, the yellow squares indicate induction of beta-lactamase, the red squares indicate resistance and the dark blue squares are indeterminate data.

study the percentage of MDR isolates was higher (28.2%) than earlier studies conducted in Mexico [31] with only 6.8% of the isolates classified as MDR. Nevertheless, the XDR isolates in our study were lower (22.7%) than the previously mentioned study (72%). Furthermore, a low resistance rate of the *P. aeruginosa* isolates to ciprofloxacin and ticarcillin/clavulanic acid was also observed. Also, these data are consistent with other studies reporting a low rate of ciprofloxacin resistance (0%) in ear infections [32]. However, the findings indicate that variations in resistance profiles of *P. aeruginosa* isolates may be due to the differences in treatments. In this regard, the low resistance of ciprofloxacin found in our study might show the importance of indiscriminate usage of antibiotics even third generation cephalosporins, since the use of this fluoroquinolone has been decreased in recent years. Thus, antibiotic susceptibility surveillance in *P. aeruginosa* becomes a major concern for clinical staff. In other study Pérez *et al*., found that 3.8% of the isolates were PDR, 35.8% XDR and 30.2% MDR to imipenem, ciprofloxacin, ceftolozane/tazobactam and ceftazidime/avibactam; in our study no PDR isolates were identified [33]. Additionally, Rees *et al*.,reported hypermutable and highly drug-resistant strains in respiratory tract isolates from patients with cystic fibrosis; authors suggest that patients´ lung microenvironment favors a high mutation rate, microenvironment adaptation which offers a selective pressure and the panresistance establishment [34].

In this context, the expression of virulence factors depends on microenvironment of infection site even under treatment with antimicrobial drugs and pathogenicity of *P. aeruginosa* is mediated by virulence factors including toxins, extracellular enzymes, siderophores, and secretion systems that directly inject virulence factors into the eukaryotic host cell [35]. A major virulence factor is biofilm that is a complex matrix that protects bacterium against immune system; another factor is the enzyme elastase (LasB) that supports the infection and colonization process by damaging tissue and degrading immune proteins [36], while pyocyanin is a blue redox active that can directly accept electrons from reducing agents such as NADPH and reduced glutathione [37]; finally alkaline protease cleaves various proteins and impairs host immune response [38]. Moreover, the structural and physiological characteristics of biofilm provides it with an innate protection against antimicrobials, for this reason biofilm-associated bacterium are able to resist antimicrobial agents by different mechanisms, such as an efficient evasion to phagocytosis as well as the host immunological mechanisms. Additionally, it is speculated that biofilm may act as a niche for resistant organisms generation, due to the capacity of certain bacterium for exchanging genetic material by conjugation and thereby contribute to the transmision of possible resistance factors to antimicrobials or to the factors intervening in the adhesion and biofilm development [39].

Additional studied mechanisms for intrinsic resistance include β-lactamases constitutive expression and efflux bombs with low permeability of its external membrane; as well as its high capacity to acquire multiple- drug resistance mechanisms mainly by random gene mutations or by horizontal transfer due to plasmids acquisition, transposons and integrons which leads to acquired resistance [40]. Our results showed the highest virulence factor production for both biofilm and alkaline protease (98.2%), followed by pyocyanin (95.8%) and elastase (93.8%). Also, a high variation in the production of virulence factors according to the sampling anatomical site was observed. From this data, it could be hypothesized that resistance mechanisms to these antibiotics could be mediated by other factors such as acyl-homoserine lactones and even the expression of genes related to quorum sensing signals such as las and rhl systems rather than the production of virulence factors.

In a study reported by Karatuna and Yagci [41], the highest virulence factor reported was biofilm (67%) followed by alkaline protease (44%) and pyocyanin (31%). Previously, it was reported that the production of two proteases with anti-flagellin activity provides a failsafe mechanism for *P. aeruginosa* to ensure the maintenance of protease-dependent immune-modulating, they concluded that alkaline protease (AprA) and elastase (LasB) are capable of degrading exogenous flagellin under calcium-replete conditions and prevents flagellin-mediated immune recognition [42]. Finally, during another study authors analyzed strains isolated from cystic fibrosis patients and found mutations as well as genomic arrays which are in accordance with phenotypic variability among populations, this may explain why some epidemic and transmissible *P. aeruginosa* strains dominate and displace others during infection, authors suggest that pyocyanin production is capable of leading this process and that one possibility is that intraspecies competition is driven by the production of bacteriocins as pyocyanins [43]. All previously described mechanisms may be present in isolated strains in this study since it was found that a high percentage of the strains express

biofilm, alkaline protease, pyocyanins and elastase as virulence factors.

As previously mentioned, *P. aeruginosa* can posses a high rate mutation and great capacity to integrate plasmidic/chromosomic genetic information, these mechanisms explain the high phenotypic and genotypic variability which provides it with the adaptation to multiple conditions; thereby different studies have been focussed on analyzing genetic variability and clonality of strians. In this study, ERIC-PCR results demonstrated a very high clonal diversity (more than 90%) (Figure 4). Similarly, Khosravi *et al*., [44] reported a high heterogeneity among *P. aeruginosa* isolates from burn infections in Iran (85%); also, Stehling *et al.* [45] found 80% similarity among *P. aeruginosa* isolates from cystic fibrosis patients in Brazil. These findings highlight the importance of clonal relationship among isolates causing infections at hospitals regardless the site of infection. Conversely and surprisingly, in our study the same clone was isolated from the three different hospitals studied, suggesting that the personal from all hospitals somehow transported the bacteria causing its dissemination. As seen in Figure 4 the genetic characteristics did not match the antibiotic resistance profiles probably due to the role the micro-environment of each the isolates is performing.

In this regard, the genes responsible for drug resistance in *Pseudomonas spp.*, are generally of plasmidic origin, however, there are reports of some strains in which genes that confer resistance have been found on chromosomal DNA. While the virulence factors biofilm, elastase, pyocyanin and alkaline protease are encoded by chromosomal genes, this chromosomal variability is what could be reflected in the analysis by ERIC-PCR; although no direct relationship was found between the clonality of the strains and the resistance to drugs, both factors are related and expressed according to the microenvironment, such as the origin of the sample and the selective pressure exerted by the administration of drugs.

The present study showed that resistance to antibiotics and the production of β-lactamase is a challenge in the fight against infectious diseases, the production of virulence factors is a problem that is present in most of the isolates and there is a great genetic diversity of *P. aeruginosa* strains in hospitals in northeastern Mexico indicating a complex relation between these variables. The higher prevalence of antibiotic resistance among *P. aeruginosa* isolates underscores the need for more epidemiological studies to determine the mechanisms of antibiotic resistance at all levels. Additional studies involving genes and signaling mechanisms that regulate the quorum sensing that in turn regulates antibiotic resistance in these isolates are suggested.

Conclusion

Nosocomial infections are a public health problem causing high costs and *P. aeruginosa* is frequently responsible of acute and chronic infections. The isolates of *P. aeruginosa* sampled in the hospitals of northeastern Mexico showed antibiotic resistance, virulence factors production and high variability of genotypes, also was observed a complex relation between variables. The increased prevalence of antibiotic resistance among *P. aeruginosa* isolates underlines the necessity for better clinical interventions at all levels.

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Ethical approval

Ethical approval for this study was granted by the Bioethics Committee of Facultad de Ciencias Químicas, Universidad Juárez del Estado de Durango (UJED), Number 123301538X0201 COFEPRIS.

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