Bol Med Hosp Infant Mex 2013;70(2):133-144

RESEARCH ARTICLE

Pathogenic characteristics of *Pseudomonas aeruginosa* strains resistant to carbapenems associated with biofilm formation

Sara A. Ochoa,[1] Fernanda López-Montiel,[1]* Gerardo Escalona,[1] Ariadnna Cruz-Córdova,[1] Leticia B. Dávila,[1] Briseida López-Martínez,[2] Yolanda Jiménez-Tapia,[2] Silvia Giono,[3] Carlos Eslava,[4] Rigoberto Hernández-Castro,[5] Juan Xicohtencatl-Cortes[1]

ABSTRACT

Background. In recent years the worldwide emergence of multidrug-resistant strains of *Pseudomonas aeruginosa* has been observed. This opportunistic pathogen produces mechanisms of resistance to several antibiotics. The resistance to carbapenems in *P. aeruginosa* strains has been associated with bacterial biofilm formation favored by the presence of exopolysaccharides (EPS) embedded in an extracellular matrix and to the production of type IV pili (T4P). We undertook this study to assess biofilm formation in clinical strains of *P. aeruginosa* resistant to carbapenems isolated at the Hospital Infantil de México Federico Gómez (HIMFG) through quantification of total-reducing EPS and its association with the phenotypic expression of T4P.

Methods. Antibiotic susceptibility tests were performed using the Kirby-Bauer method in 92 clinical isolates of *P. aeruginosa*; likewise, the minimum inhibitory concentration (MIC) was determined for imipenem (IMP) and meropenem (MEM) using the serial dilution method in agar plates with a Steers replicator. Production of metallo-β-lactamase (MBL) was determined by the disk diffusion method and synergism. Biofilm formation was performed in clinical isolates of *P. aeruginosa* resistant to carbapenems through the quantification of crystal violet, total sugar (anthrone), and reducing sugar (DNS), in addition to the phenotypic expression of T4P activity of twitching motility. The genetic diversity of biofilm-forming strains and producers of reducing sugars was evaluated by pulsed-field gel electrophoresis (PFGE).

Results. There were 30.4% (28/92) of *P. aeruginosa* strains of pediatric origin and 50% (46/92) of urine samples that were recovered from the pediatric surgical ward. The results using the Kirby-Bauer method showed that >50% of *P. aeruginosa* strains were resistant to 12 different antibiotics. The MIC to carbapenems was 64 μg/mL, with 43.1% (25/58) for MEM and 56.8% (33/58) for IMP. Likewise, MBL production was observed in 43% (25/58) for MEM, 2% (1/58) for IMP, and 12% (7/58) for both. Qualitative and quantitative analysis showed that 82% (48/58) of *P. aeruginosa* strains resistant to carbapenems were high biofilm formers using the crystal violet method. From the high biofilm forming strains, 46.5% (27/58) showed concentrations of total EPS between 2000 and 6000 μg/mL and 27.5% (16/58) showed concentrations of reducing EPS between 316 and 1108 μg/mL. In addition, 75% (44/58) of these strains showed phenotypic activity of twitching motility.

Conclusions. Detection of total sugars, reducing sugars, and the phenomenon of twitching motility are factors that promote the development of biofilms in clinical strains of *P. aeruginosa* resistant to carbepenems, which are also MBL producers. Our data suggest that these factors are involved in biofilm formation, which confer bacterium with the ability to survive, persist, and colonize its host.

Key words: *Pseudomonas aeruginosa*, clinical isolates, antibiotic resistance, biofilm, pili.

1 Laboratorio de Bacteriología Intestinal, Hospital Infantil de México Federico Gómez
2 Laboratorio Clínico Central, Hospital Infantil de México Federico Gómez
3 Laboratorio de Bacteriología Médica, Departamento de Microbiología, Instituto Politécnico Nacional
4 Departamento de Salud Pública, Facultad de Medicina, Universidad Nacional Autónoma de México
5 Departamento de Ecología de Agentes Patógenos, Hospital General Dr. Manuel Gea González
* Becario PROBEI

México, D.F., México

Received for publication: 2-5-13
Accepted for publication: 3-5-13

INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative bacillus considered to be an opportunistic pathogen. It is a highly versatile microorganism able to tolerate low oxygen conditions. It can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C.[1] These characteristics allow it to attach itself and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immunocompromised patients.[1,2] *P. aeruginosa* can cause pneumonias, urinary tract infections and bacteremias as well as causing high morbidity and mortality in patients with cystic fibrosis due to chronic infections that eventually cause pulmonary damage and respiratory insufficiency. Infections due to *P. aeruginosa* are difficult to eradicate because of their elevated intrinsic resistance as well as their capacity to acquire resistance to different antibiotics.[3]

P. aeruginosa produces various mechanisms of resistance to antibiotics such as broad-spectrum β-lactamases, metallo-β-lactamases (MBL), alteration of protein binders of penicillin (PBP), porin mutations, plasmid enzymatic modification, DNA-gyrase mutation and active expulsion pumps.[4,5] Carbapenemics (imipenem and meropenem) are broad-spectrum antibiotics used for the treatment of nosocomial infections caused by *P. aeruginosa*. Specific resistance to carbapenemics is attributed to the lack of porin permeability (OprD), an increase in the expression of the active expulsion pumps (MexAB-OprD) and to production of metalloenzymes.[5-7]

Carbapenem-resistant *P. aeruginosa* is associated with the production of MBL and has the ability to hydrolyze all β-lactam antibiotics except aztreonam. It is responsible for nosocomial outbreaks in tertiary care centers.[6,8-10] Three groups of MBL have been identified: class A (serine dependent and partially inhibited by clavulanic acid are inducible and nontransferable), class B (zinc dependent, inhibited by EDTA, inducible or associated with conjugative plasmids) and class C (oxacillinase).[6,7]

Resistance to various antibiotics and substances with antimicrobial activity has been associated with bacterial biofilm formation and phagocytosis by components of the adaptive immune system[11] as well as various nosocomial infections caused by *P. aeruginosa*. Biofilms are embedded in an extracellular matrix consisting of an outer membrane protein, pili, exopolysaccharide (EPS) and nucleic acids.[12] The components of the EPS involved in the formation of *P. aeruginosa* biofilm are encoded mainly by different genes located in three independent operons: *algU*, *psl*, and *pel*.[12,13]

Type IV pili (T4P) produced by *P. aeruginosa* have an independent movement of the flagellum through a solid surface due to the action of contraction and relaxation and referred to as twitching motility. These have been associated with biofilm formation, an essential event in host colonization.[14-16] These filamentous structures located at one pole of the bacteria are involved in various mechanisms such as adherence to human cells, formation of microcolonies, bacterial aggregation, phage receptor, evasion of the immune response and cellular signaling.[16-18]

Globally, in recent decades the emergence of *P. aeruginosa* strains resistant to carbapenems commonly used in the treatment of infections associated with this pathogen has been observed.[9] The aim of this study was to evaluate biofilm formation in clinical strains of *P. aeruginosa* resistant to carbapenems isolated at the Hospital Infantil de México Federico Gómez (HIMFG) through quantification of total and reductor exopolysaccharides (EPS) and their association with the phenotypic expression of the T4P.

MATERIALS AND METHODS

Bacterial strains

A total of 92 strains of *P. aeruginosa* were selected and isolated from clinical samples of pediatric patients at the HIMFG from February 2008 to January 2009. Phenotypic identification of these strains was performed at the Central Clinical Laboratory using the Vitek automated system (bioMérieux, France) and by conventional biochemical tests at the HIMFG Intestinal Bacteriology Laboratory. Biochemical identification was based on the production of catalase, oxidase, the presence of pigments (pyocyanin and pyoverdin), sodium citrate growth, growth at 42°C, nitrate reduction and arginine hydrolysis. Strains were grown on BHI agar (Brain Heart Infusion) (Becton Dickinson, France) and stored in skim milk at -70°C.

Antibiotic susceptibility testing

Antibiotic susceptibility tests were performed using the Kirby-Bauer disk-diffusion method according to the 2012 CLSI (Clinical Laboratory Standards Institute). Five colo-

nies of each strain were grown in Mueller Hinton (MH) broth (Oxoid, Basingstoke, Hampshire, England) at 37°C with constant stirring for 2-5 h until reaching an optical density equivalent to 0.5 on the McFarland scale (NMF). A massive seeding was done from the bacterial suspension on MH agar plates using a sterile swab. Discs with the antibiotics were immediately placed on the inoculated plates and incubated at 37°C for 24 h.

For susceptibility testing a total of 12 antibiotics were assessed: piperacillin-tazobactan (100/10 ug), ticarcillin-clavulanate (75/10 mg), cefotaxime (30 mg), ceftazidime (30 mg), ceftriaxone (30 mg), cefepime (30 mg), aztreonam (30 mg), gentamicin (10 mg), ciprofloxacin (5 mg), levofloxacin (5 μg), meropenem (10 mg) and imipenem (10 g) (Oxoid). Inhibition zones were determined and compared with the reference tables according to the CLSI-2012. The sensitivity or resistance for each strain was reported based on the criteria established by the CLSI-2012. *Escherichia coli* strains ATCC 25922 and *P. aeruginosa* ATCC 27853 (American Type Culture Collection) were used as quality controls.

Determination of the MIC for carbapenems

Minimum inhibitory concentration (MIC) was determined for *P. aeruginosa* strains resistant to imipenem (IMP) and meropenem (MEM) by the serial dilution method on MH agar plates using a Steers replicator in accordance with the CLSI 2012. Serial dilutions in triplicate of the antibiotics were used (IMP and MEM) at a range of 0.031–512 μg/ml. A bacterial suspension adjusted to a concentration of 1.5 x 10^8 bacteria/ml was placed in Steers replicated wells in MH agar plates with antibiotic dilutions.

Identification of strains of *P. aeruginosa* producers of metallo-β-lactamases

Disk-diffusion and synergism techniques were used to evaluate the presence of MBL. Two disks of IMP (10 μg) and two of MEM (10 μg) were used; one disk with each antibiotic was impregnated with 10 μl of EDTA (0.5 M). As a reagent control a disk with EDTA and without antibiotic were used. The disks were placed on a MH agar plate containing a strain of a bacterial suspension adjusted to a concentration of 1.5 x 10^8 bacteria/ml. *P. aeruginosa* strains with halos of 7 mm in diameter of difference between the disks of IMP + EDTA and IMP-EDTA were considered as MBL producers.[10]

In the test of synergism, disks of IMP and MEM were placed at a distance of 1.5 cm with respect to a second blank disk impregnated with 10 μl of EDTA 0.5 M and incubated at 37°C for 24 h. An increase in the inhibition zone toward the EDTA disk was considered to be a positive test. *P. aeruginosa* ATCC 27853 was used as a negative control and a clinical strain producer of MBL (IMP and MEM) as positive control.[10]

Biofilm formation in clinical strains of *P. aeruginosa*

Qualitative and quantitative analyzes of the biofilms produced were performed of clinical isolates of *P. aeruginosa* according to the protocol described by Xicohtencatl-Cortes et al.[14] Clinical strains of *P. aeruginosa* were incubated for growth in trypticase-soy broth (TSB) at 37°C for 24 h. For biofilm formation, 24-well plates containing 1 ml of TSB were inoculated with 50 μl (1.5 x 10^8 bacteria/ml) of a bacterial suspension of each of the *P. aeruginosa* strains and incubated at 37°C for 24 h. Biofilms were washed with phosphate buffer solution (PBS) (pH 7.4) and fixed with 2% formalin at 4°C overnight. Subsequently, the fixative solution was removed with PBS and the films were stained with 1 ml of 1% crystal violet for 15 min. Excess crystal violet was removed and 1 ml of methanol at 70% was added for quantification of the biofilm to an optical density of 600 nm (OD_{600nm}). Likewise, biofilms contained in glass coverslips 12 mm in diameter were mounted on slides and viewed with a light microscope at 100X. Assays were performed in triplicate at three different times. *P. aeruginosa* ATCC 27853 and *E. coli* K-12 HB101 were used as positive and negative controls, respectively.

Determination of total carbohydrates in *P. aeruginosa* Biofilms

Production of total carbohydrates in biofilms of clinical strains of *P. aeruginosa* resistant to carbapenems was determined. These were carried out in triplicate in 24-well plates as previously described. A 0.5 ml bacterial suspension of biofilms of each clinical strain of *P. aeruginosa* was resuspended in 1.5 ml of cold anthrone solution at 2% sulfuric acid. The reaction mixture was incubated at the boiling point for 10 min and read with a spectrophotometer at 600 nm. To determine the concentration of total sugars, data obtained were extrapolated into a previously standardized glucose curve of 0-10,000 μg/ml.[19]

Determination of reducing carbohydrates in *P. aeruginosa* biofilm

Reducing carbohydrates were determined in clinical strains of carbapenem-resistant *P. aeruginosa* using the DNS method (3,5-dinitrosalicylic acid). Assays were performed in triplicate. The reaction mixture was prepared with 5 g of DNS and 150 g of double sodium-potassium tartrate dissolved in 250 ml of distilled hot water. Subsequently, 100 ml of 2N sodium hydroxide was added and gauged to a volume of 500 ml with distilled water. A 1.5-ml suspension of the biofilms formed by clinical strains was mixed with 2 ml of NaOH 2N and incubated in a water bath for 15 min to obtain alkaline lysis. An aliquot of 0.5 ml of the lysis was subsequently mixed with 0.5 ml of DNS solution. The mixture was incubated for 10 min in a water bath and read with a spectrophotometer at 600 nm. The results were extrapolated into a previously standardized glucose curve of 0-2000 μg/ml.[19]

Trials of twitching motility in the formation of biofilms

The procedure to determine the phenomenon of twitching motility in clinical isolates of carbapenem-resistant *P. aeruginosa* was performed according to the protocol described by Xicohtencatl-Cortes et al.[14] Previously, strains were grown in TSA agar at 37°C for 24 h. A colony of each strain was vertically inoculated into each well of PPLO media (pleuropneumonia-like organism broth) in 1% bacteriological agar. The plates were incubated at 37°C for 24 h and the halos observed were measured to indicate the production of twitching motility. *P. aeruginosa* strains ATCC 27853 and *E. coli* K-12 HB101 were used as the positive and negative controls, respectively.

Pulsed field gel electrophoresis (PFGE)

To determine the profiles of PFGE, 16 clinical strains of *P. aeruginosa* resistant to carbapenems with a high production of reducing EPS associated with their ability to produce biofilms were selected. DNA extraction was done in agarose blocks according to the protocol described by Morales-Espinosa et al.[20] Then, 150 μl of a bacterial suspension was mixed with 150 μl of low point fusion agarose at 1.8%. The fusion block generated was incubated with 1.5 ml of an EC lysis solution [Tris-HCl 1 M (pH 8.0), NaCl 1 M, EDTA 0.5 M (pH 8.0), sodium deoxycholate 0.5%, N-lauryl sarcosine 12.5%, RNAase 5 mg/ml and lysozyme 10 mg/ml] at 37°C for 24 h.

The blocks were then incubated in an EPS solution [Tris HCl 10 mM (pH 7.4), EDTA 1 mM, N-lauryl-sarcosine 0.25% and proteinase K 0.1 mg/ml]. Finally, the blocks were washed seven times with a cold TE solution [Tris-HCl 10 mM (pH 8.0), EDTA 1 mM (pH 8.0)] and were kept in the same solution at 4°C. The blocks were treated with 3 μl restriction Spel enzyme (30 U) and were incubated at 37°C overnight.[20] Digestion fragments were separated by CHEF Mapper (Bio-Rad) using 1% agarose gel stained with 1.0 μg/ml ethidium bromide. The runs were carried out with 0.5% TBE (Tris-borate-EDTA) at 10°C and 2- to 50-sec pulses for 20 h at 6 V/cm.[20] Obtained fragment sizes were estimated using a molecular weight Lambda Ladder PFGE marker (Biolabs). Analysis of the PFGE patterns was performed using the NTSYS-pc program (Numerical Taxonomy and Multivariate Analysis System, v.2.0). To express similarity among strains using the dendrogram, we estimated the Jaccard similarity coefficient and clustering by the UPGMA method (Unweighted Pair-Group Method Using Arithmetic Average). PFGE patterns generated were interpreted based on the guidelines of Tenover et al.[21]

RESULTS

P. aeruginosa is found widely distributed in the environment and is considered an opportunistic pathogen. It is mainly characterized for colonizing immunocompromized patients. In this study we selected 92 clinical *P. aeruginosa* strains isolated from HIMFG pediatric patients from February 2008 to January 2009. Using the Vitek system (bioMérieux) and conventional biochemical tests, *P. aeruginosa* strains isolated from the pediatric patients in different departments were identified: 30.4% (28/92) from the operating room, 16.3% (15/92) from the pediatric intensive care unit, 10.8% (10/92) from the pediatric ward, 9.7% (9/92) emergency department, 7.6% (7/92) surgery, 7.6% (7/92) from nephrology, 4.3% (4/92) from oncology, 3.3% (3/92) surgical therapy, 3.3% (3/92) from neurosurgery, 3.3% (3/92) intermediate care and 3.3% (3/92) from the neonatal intensive care unit (Figure 1A). Likewise, *P. aeruginosa* strains were recovered from different clinical samples: 50% (46/92) from urine, 31.5% (29/92) venous blood, 6.5%

(6/92) catheter, 3.2% (3/92) from bronchial aspirate and 2.1% (2/92) from surgical wound culture (Figure 1B).

The results obtained by the Kirby-Bauer method in 92 clinical *P. aeruginosa* strains showed >50% resistant to 12 antibiotics tested: aztreonam (63%), gentamicin (64.1%), ciprofloxacin (64.1%), levofloxacin (66.3%), ceftazidime (64.1%), ceftriaxone (66.1%), cefotaxime (75%), cefepime (63%), ticarcillin-clavulanic acid (73.9%) and piperacilin-tazobactam (63%) (Figure 2A); 63% of the strains were identified as multiresistant because they showed resistance to at least three different groups of antibiotics (Figure 2A). Also, there was 63% resistance (58/92) to IMP, 60.8% (56/92) to MEM and intermediate resistance to 2.17% MEM. The MIC for MEM and IMP showed that 63% (58/92) of the clinical isolates for *P. aeruginosa* were resistant to carbapenems, confirming the results obtained by the Kirby-Bauer resistance profiles (Figure 2).

MIC observed in clinical *P. aeruginosa* isolates was 43.1% (25/58) to MEM and 56.8% (33/58) to IMP, with a concentration of 64 μg/ml of the antibiotic. Additionally, 8.6% (5/58) of the strains showed a MIC ≥256 μg/ml to MEM (Figure 2B). Production of MBL in *P. aeruginosa* strains resistant to carbapenems *P. aeruginosa* with the disk-diffusion method (with and without EDTA) and synergism with dual disc showed similar results: 43% (25/58) of the strains were negative for both MBL production, 43% (25/58) were producers of MBL for MEM, 2% (1/58) of MBL for IMP and 12% (7/58) to both MBL (Figure 2C).

Qualitative and quantitative analyses of the biofilms in clinical *P. aeruginosa* strains were carried out only for the 58 strains resistant to carbapenems; 82.7% (48/58) of clinical *P. aeruginosa* strains showed absorbance values 8.8-212 de OD_{600nm}. These were considered as high biofilm formers (Table 1). Furthermore, 15.5% (9/58) of the biofilms with absorbance values of 4.4-8.7 de OD_{600nm} were considered as average biofilm formers and 1.7% (1/58) with absorbance values 1.0-4.3 de OD_{600nm} were considered as low biofilm producers (Table 1).

It was of interest that 55% (32/58) of the high biofilm formers of *P. aeruginosa* strains were mainly isolated from urine samples: 10.3% (6/58) from probes, 6.8% (4/58) from blood, 3.4% (2/58) from catheters, 3.4% (2/58) from bronchial aspirate and 3.4% (2/58) from surgical wound sites (Figure 3A). One low biofilm former strain (1/58) was identified in blood. Average biofilm forming strains were found in different percentages: 8.6% (5/58) in urine samples, 1.7% (1/58) in blood, 3.4% (2/58) in catheters and 1.7% (1/58) in bronchial aspirate (Figure 3A). Qualitative analysis of low, medium and high clinical *P. aeruginosa* strains showed different levels of biofilm formation when observed by light microscopy at 100X (Figure 3B). Laboratory strain K-12 HB101, a non-biofilm producer, was used as a negative control (data not shown).

The results obtained from the quantification of the total EPS of the biofilms with the anthrone method was extrapolated into a standard glucose curve of 0-10,000 μg/ml with a previously standardized linear correlation coefficient (r =

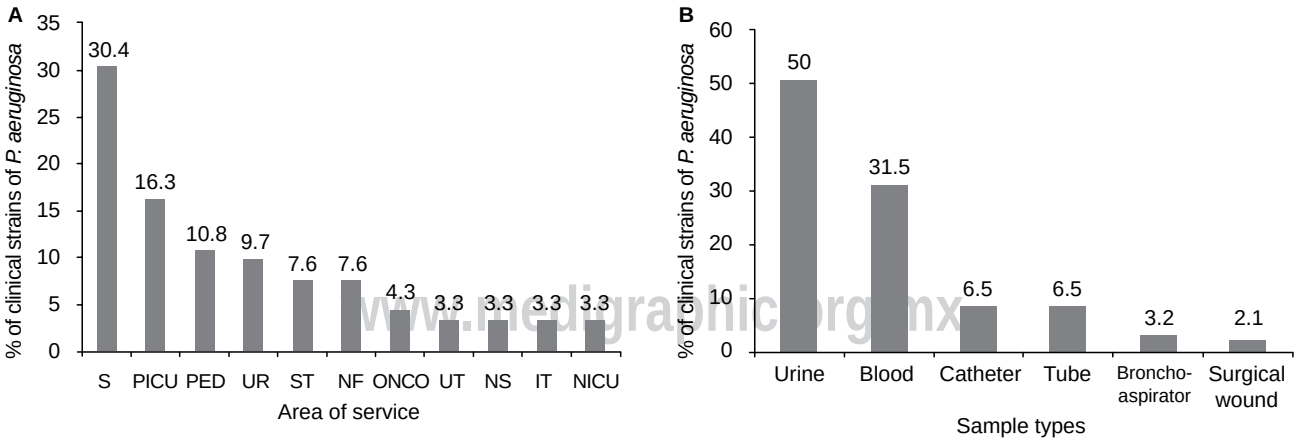

Figure 1. Identification of resistant and sensitive clinical strains of *P. aeruginosa* in different service areas **(A)** and types of sample **(B)**. S, surgery; PICU, pediatric intensive care unit; PED, pediatrics; UR, urgent care, ST, surgical therapy; NF, nephrology; ONCO, oncology, UT, urgent therapy; NS, neurosurgery; IT, intermediate therapy; NICU, neonatal intensive care unit.

0.9924) (data not shown). According to the analysis of the biofilms, the clinical *P. aeruginosa* strains resistant to carbapenems were classified according to the amount of EPS detected in three groups: 18.9% (11/58) as low producers of EPS with a concentration between 40 and 577 μg/ml, 34.4% (20/58), as average producers of EPS with a concentration between 578 and 2000 μg/ml and 46.5% (27/58) as high producers of EPS with a concentration between 2001 and 6000 μg/ml (Table 1). Furthermore, quantification of the reducing sugars was determined by the DNS method using a glucose curve from 0-2000 μg/ml with linear correlation coefficient (r = 0.9631); 27.5% (16/58) of the biofilms were considered as high producers of EPS reducers with values from 316-1108 μg/ml; 27.5% (16/58) as average producers of EPS reducers with values from 207-315 μg/ml; and 44.8% (26/58) low producers of EPS reducers with values from 83-206 μg/ml (Table 1).

The formation of twitching motility was determined in 58 clinical strains of carbapenem-resistant *P. aeruginosa*. The results showed that 75.8% (44/58) of the strains produced halos of different diameters, indicating the presence of the T4P (Figure 4). Interestingly, 53.4% (31/58) of the strains isolated from urine showed twitching motility activity, maintaining a high correlation with biofilm formation (55%) by crystal violet (Figure 3). In addition, this same phenomenon was observed in low percentag-

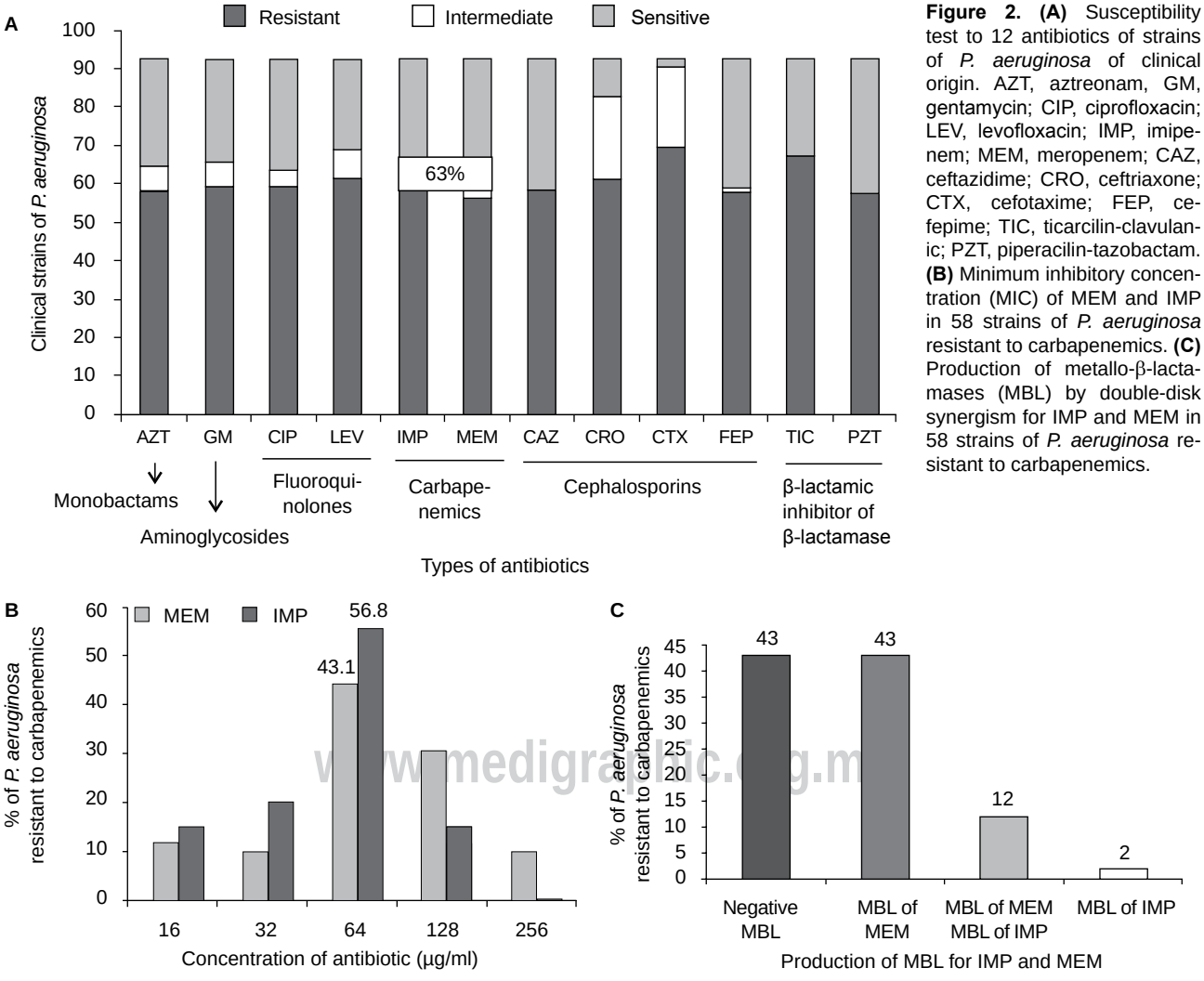

Figure 2. (A) Susceptibility test to 12 antibiotics of strains of *P. aeruginosa* of clinical origin. AZT, aztreonam, GM, gentamycin; CIP, ciprofloxacin; LEV, levofloxacin; IMP, imipenem; MEM, meropenem; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; TIC, ticarcilin-clavulanic; PZT, piperacilin-tazobactam. **(B)** Minimum inhibitory concentration (MIC) of MEM and IMP in 58 strains of *P. aeruginosa* resistant to carbapenemics. **(C)** Production of metallo-β-lactamases (MBL) by double-disk synergism for IMP and MEM in 58 strains of *P. aeruginosa* resistant to carbapenemics.

es in strains isolated from catheter, surgical wound and blood, whereas in strains from bronchial aspirate isolates no twitching motility activity was seen (Figure 4).

PFGE assays were performed in 16 *P. aeruginosa* strains resistant to carbapenems and high producers of EPS reducers during biofilm formation. Dendrogram analysis showed the presence of four patterns (A, B, C and D) with restriction digests of Spe I enzyme, generating profiles of between 16 and 20 bands with molecular weights >436.5 kb and <48.5 kb kb (Figure 5). Pattern A grouped the greatest number of strains with 68.7% (11/16), pattern B with 18.7% (3/16), pattern C with 6.2% (1/16) and pattern D with 6.2% (1/16) (Figure 5).

DISCUSSION

P. aeruginosa is an opportunistic nosocomial pathogen of great importance due to its resistance to multiple antibi-

Table 1. Biofilm quantification of the 58 strains of *P. aeruginosa* resistant to carbapenemics using diverse methods

	Quantification method		
Biofilms	*Crystal violet* *# of strains (%)*	*Anthrone* *# of strains (%)*	*DNS* *# of strains (%)*
Low	1 (1.7%) OD_{600nm} 1.0-4.3	11 (18.9%) CV= 40-577 μg/ml	26 (44.8%) CV= 83-206 μg/ml
Average	9 (15.5%) OD_{600nm} 4.4-8.7	20 (34.4%) CV= 578-2000 μg/ml	16 (27.5%) CV= 207-315 μg/ml
High	48 (82.7%) OD_{600nm} 8.8-212	27 (46.5%) CV= 2001-6000 μg/ml	16 (27.5%) CV= 316-1108 μg/ml
Total	58	58	58

CV, cutoff value; OD_{600nm}, optical density at 600 nm; DNS, 3,5-dinitrosalicylic acid.

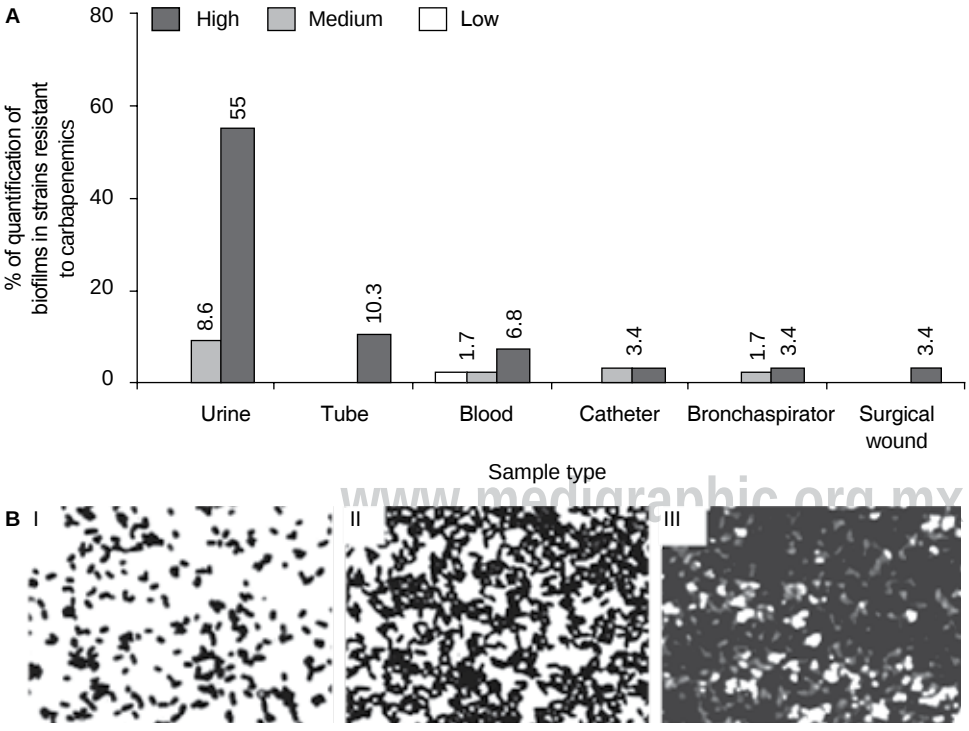

Figure 3. (A) Qualitative analysis of the formation percentage of biofilms in clinical strains of *P. aeruginosa* resistant to carbapenemics. **(B)** Quantitative analysis of the formation of biofilms using crystal violet of clinical strains of *P. aeruginosa* resistant to carbapenemics. Images were processed using a light microscope (100X). i) Low biofilm formers (2.31 OD_{600nm}); ii) average biofilm formers (4.92 OD_{600nm}); and iii) high biofilm formers (212 OD_{600nm}).

otics, making patient treatment difficult. Its versatility to remain in the environment and in substrates (such as disinfecting solutions, soaps, surgical equipment and in common usage in hospitals) make it a bacterium of interest in nosocomial infections.[1,22] An important characteristic of these bacteria is its natural resistance to various antibiotics and their capacity to horizontally acquire genetic material that promotes genetic exchange among intrahospital species, as observed in the transference of extended spectrum b-lactamase genes and MBL among intrahospital pathogens such as extended-spectrum MBL among nosocomial pathogens such as *E. coli* and *K. pneumoniae*.[23]

Carbapenems are antibiotics used as the treatment of choice for infections caused by *P. aeruginosa*. In recent years, in several countries (Africa, Europe, México, Central and South America)[8,24,25] an increase in *P. aeruginosa* strains resistant to carbapenems has been observed, which has generated a health problem of great interest for therapeutic treatments.[26-28] Data obtained in this study showed a range of resistance >50% for different groups of antibiotics, and 63% of the strains were identified as multiresistant. These data correlate with what has been reported in other Latin American countries.[25,26] Gomes et al. discussed the importance of multiresistant *P. aeruginosa* strains related to mortality of patients hospitalized with AIDS and the need for multidisciplinary intervention in the prevention and management of these infections.[29]

The strains studied were recovered from hospitalized children in different departments of the HIMFG, considered a tertiary hospital. The operating rooms, pediatric intensive care unit and pediatric departments were the areas that showed a higher percentage of isolation of this nosocomial pathogen, with 30.4% (28/92), 16.3% (15/92) and 10.8% (10/92), respectively. Moreover, *P. aeruginosa* strains resistant to carbapenems were isolated mainly in urine samples with a percentage of 50% isolation (46/92). These data correlated directly with the presence of *P. aeruginosa* as one of the main etiological agents of urinary tract infection, which is the site where it disseminates to cause systemic infections.[30] Hammami et al.[31] and Vitkauskienė et al.[32] described the presence of clinical strains of *P. aeruginosa* resistant to carbapenems in various hospital intensive care units.

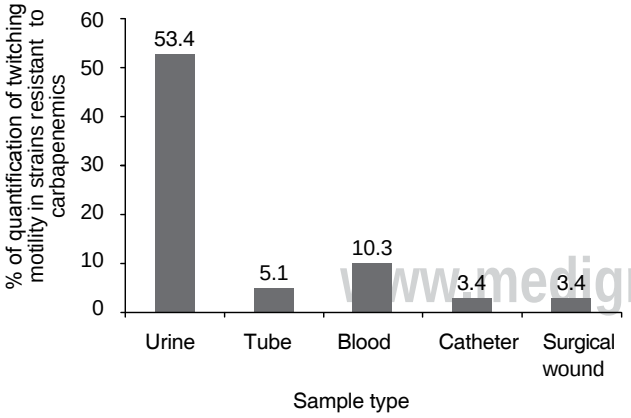

Figure 4. Qualitative analysis of the activity of twitching motility in *P. aeruginosa* strains resistant to carbapenemics of different origins.

Treatment of nosocomial infections caused by strains of *P. aeruginosa* due to overuse of carbapenems (meropenem and imipenem) has facilitated the emergence of an elevated resistance to these antibiotics. In the studies by Kumar et al. in nosocomial *P. aeruginosa* isolates in India, it was concluded that the high prevalence of *P. aeruginosa* strains resistant to carbapenems and MBL producers was due to the excessive use of carbapenems in hospitals when treating nosocomial infections.[33] The results in *P. aeruginosa* strains of clinical origin of the HIMFG showed a profile of high resistance to meropenem and imipenem: 63.4% (58/92) and 60.8% (56/92), respectively. In this work it was considered important to identify *P. aeruginosa* strains producers of MBL as a mechanism of resistance to these antibiotics.[27] The results showed that 43% (25/58) of the *P. aeruginosa* strains resistant to carbapenem of clinical origin were producers of MBL for MEM, 2% (1/58) producers of MBL for IMP, 12% (7/58) for both MBL and 43% (25/58) negative for the production of MBL. According to these data, clinical strains of *P. aeruginosa* showed a high frequency of MBL cases for meropenem, a carbapenem widely used for the treatment of nosocomial infections caused by this microorganism.[9,27]

MBL production in imipenem was found in 2% as compared to the production of 43% of MBL for meropenem. Imipenem is an antimicrobial used in combination with cilastatin to promote absorption and bioavailability of the antibiotic. Due to reported side effects, limitations exist for its use in pediatric patients.[4,28] Twelve percent of *P. aeruginosa* strains were producers of both MBL, which indicated a rapid spread of bacterial resistance. MBL encoded in mobile elements are easy to move in hospitals

among bacteria of the same genus and other pathogens circulating in the hospital environment.[10] Recently, a prevalence of 26.9% of multiresistant hospital *P. aeruginosa* strains was reported in India with a mortality of 34.2% of infected patients.[33]

Additionally, production of MBL as a known and causative factor of resistance in nosocomial strains, the production of biofilms by *P. aeruginosa* makes intrahospital infections difficult to treat due to its highly organized structure, which functions as a barrier for antimicrobial action.[34] Bacteria within the biofilms are more resistant to physical and chemical changes by different chemotherapeutic agents than bacteria in their platonic growth phase.[34,35] In this study we evaluated the formation of biofilms in *P. aeruginosa* clinical strains that were resistant to carbopenem with three methods: crystal violet, anthrone, and DNS. The qualitative and quantitative analysis of crystal violet assays showed that 82.7% (48/58) of *P. aeruginosa* strains resistant to carbapenems were high biofilm formers, whereas 15.5% (9/58) of the strains were considered average biofilm formers and 1.7% (1/58) were low biofilm formers. With this method it was determined that of the strains isolated from urine, 55% (32/58) were categorized as strains that were high biofilm formers. Subramanian et al. conducted biofilm studies on bacteria isolated from urine samples, identifying *P. aeruginosa* as a

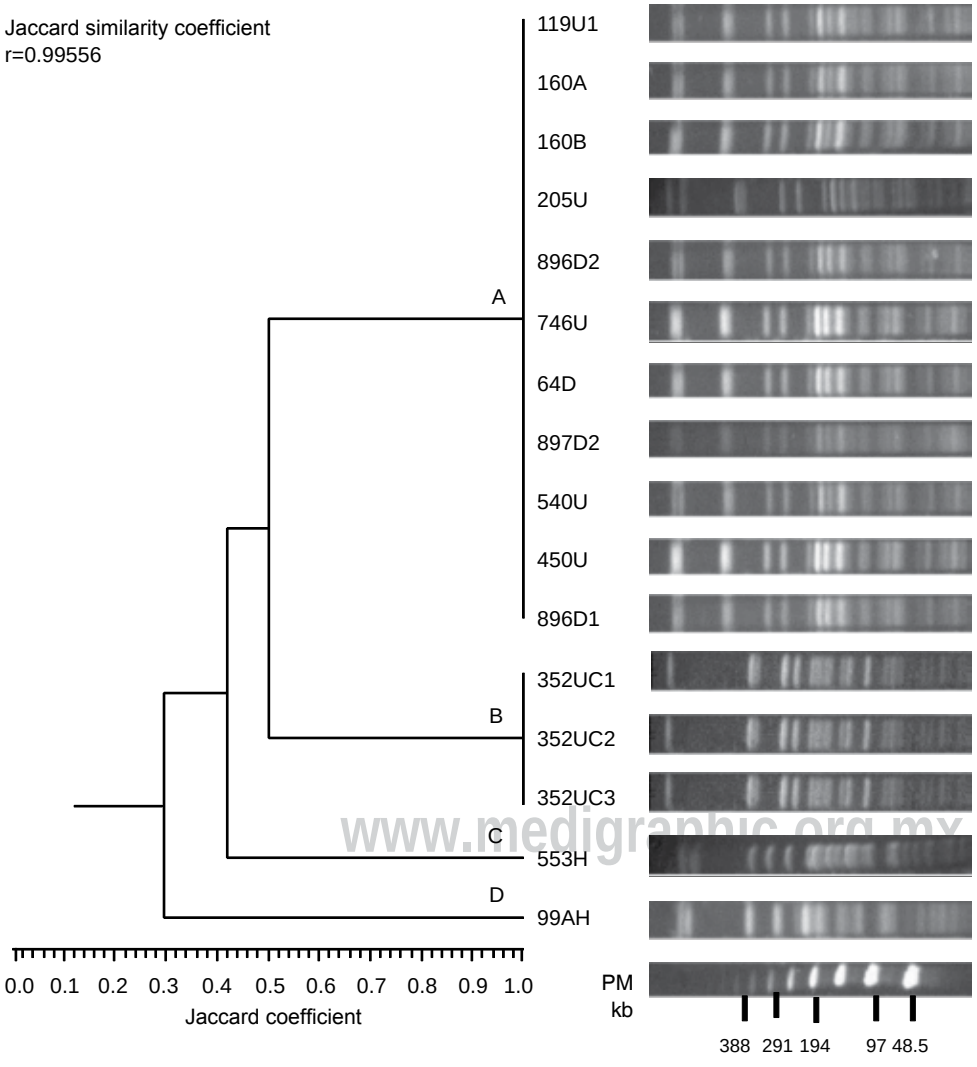

Figure 5.

Dendrogram of the 16 products using pulsed field gel electrophoresis (PFGE) in strains of *P. aeruginosa* resistant to high biofilm forming carbapenemics according to the DNS method (3,5-dinitrosalicylic acid).

pathogen involved in biofilm formation with the ability to maintain a high resistance to various antibiotics.[36,37]

Total EPS presence was determined by the anthrone method in biofilms of strains of *P. aeruginosa* resistant to carbapenems. Furthermore, quantification of total sugars showed heterogeneity in the production of EPS with values from 40-6000 μg/ml in the 58 strains of *P. aeruginosa*; 18.9% (11/58) of the strains showed EPS values between 40 and 577 μg/ml and were classified as low biofilm formers, 34.4% (20/58) showed values from 578-2000 μg/ml and were classified as average biofilm formers, and 46.5% (27/58) showed values between 2001 and 6000 μg/ml and were classified as high formers of biofilms. Taking the same criteria into consideration, we determined the levels of sugar reducers involved in biofilm formation in strains of *P. aeruginosa* strains resistant to carbapenems according to the DNS method; 44.8% (26/58) of the strains of *P. aeruginosa* were classified as low biofilm-forming with values between 83 and 206 μg/ml, 27.5% (16/58) were classified as average biofilm formers with values between 207 and 315 μg/ml and 27.5% (16/58) were classified as high biofilm formers with values between 316 and 1108 μg/ml. Regardless of the detection method used to quantitatively determine formation of biofilm in strains of *P. aeruginosa* resistant to carbapenems, there was a high percentage of strains classified as high producers of biofilms. Likewise, with a high content of total-reducers of sugars, structural elements are required for bacterial host colonization. Irie et al. carried out biofilm models in *P. aeruginosa* and showed the presence of intracellular secondary messenger with diguanylate cyclase activity, which acts by stimulating the production of polysaccharide matrix components of biofilm and activity of the *psl* operon for alginate production.[38]

Twitching motility activity in *P. aeruginosa* is generated by the presence of the T4P involved in biofilm formation on biotic and abiotic surfaces.[39,40] The results obtained in this study showed that 75.8% of *P. aeruginosa* strains resistant to carbapenems produced halos of different diameters, indicating the phenotypic expression of the T4P. Furthermore, 53.4% of clinical strains with twitching motility activity were observed in urine samples. This correlates with the high percentages (55%) of the samples of biofilm formers quantified with crystal violet.

PFGE analysis in the 11 strains grouped in pattern A showed a highly related profile with a similarity of 100%, indicating the presence of the same clone according to the criteria of Tenover et al.[23] The B pattern demonstrated similarity with pattern A. On the other hand, patterns C and D demonstrated a similarity of 46.2 and 59.2% with pattern A, respectively. These similar values were considered to be unrelated.[41,42] Diverse typing methods such as PFGE have been used in epidemiological studies with *P. aeruginosa,* with the goal of understanding the clonal relationship of the strains and their clinical profile.[42] Yousefi et al. genotypically characterized a collection of multiresistant *P. aeruginosa* clones isolated from a burn unit in Iran and identified, by PFGE, the presence of 12 different genotypes with a similarity of >80%.[43] Recently, 14 Mexican strains of *P. aeruginosa* producers of MBL from a hospital source demonstrated four clonal patterns.[44]

A high percentage of children admitted to the HIMFG are immunocompromised patients. This status favors colonization and infection by carbapenem-resistant strains of *P. aeruginosa*. Detection of total sugars, reducing sugars and twitching motility activity are factors that are involved in the development of biofilms and the resistance to carbapenems and production of MBL in *P. aeruginosa* strains. These factors may facilitate colonization and infection by this opportunistic pathogen in immunocompromised patients.

Correspondence: Dr. Juan Xicohtencatl Cortes
Laboratorio de Bacteriología Intestinal
Hospital Infantil de México Federico Gómez,
México, D.F., México
E-mail: juanxico@yahoo.com

REFERENCES

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:959-964.
2. Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr Opin Microbiol 2009;12:61-66.
3. Breidenstein EBM, De la Fuente-Nuñez C, Hancock REW. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol 2011;19:419-426.
4. Garza-Ramos U, Silva-Sánchez J, Martínez-Romero E. Genetics and genomics for the study of bacterial resistance. Salud Publica Mex 2009;51(suppl 3):S439-S446.
5. Tan TT. "Future" threat of gram-negative resistance in Singapore. Ann Acad Med Singapore 2008;37:884-890.

6. Sánchez A, Salso S, Culebras E, Picazo JJ. Resistencia a carbapenemes por metaloenzimas en aislamientos clínicos de *Pseudomonas aeruginosa*. Rev Esp Quimioter 2004;17:336-340.
7. Xavier DE, Picão RC, Girardello R, Fehlberg LC, Gales AC. Efflux pumps expression and its association with porin downregulation and b-lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in Brazil. BMC Microbiol 2010;10:217.
8. Pitout JD, Revathi G, Chow BL, Kabera B, Kariuki B, Nordmann P, et al. Metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolated from a large tertiary centre in Kenya. Clin Microbiol Infect 2008;14:755-759.
9. Samuelsen O, Toleman MA, Sundsfjord A, Rydberg J, Leegaard TM, Walder M, et al. Molecular epidemiology of metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolates from Norway and Sweden shows import of international clones and local clonal expansion. Antimicrob Agents Chemother 2010;54:346-352.
10. Pitout JD, Gregson DB, Poirel L, McClure JA, Le P, Church DL. Detection of *Pseudomonas aeruginosa* producing metallo-beta-lactamases in a large centralized laboratory. J Clin Microbiol 2005;43:3129-3135.
11. Høiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PO, et al. The clinical impact of bacterial biofilms. Int J Oral Sci 2011;3:55-65.
12. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. Curr Opin Microbiol 2007;10:644-648.
13. Kipnis E, Sawa T, Wiwnwr-Kronish J. Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Med Mal Infect 2006;36:78-91.
14. Xicohtencatl-Cortes J, Monteiro-Neto V, Saldaña Z, Ledesma MA, Puente JL, Girón JA. The type 4 pili of enterohemorrhagic *Escherichia coli* O157:H7 are multipurpose structures with pathogenic attributes. J Bacteriol 2009;191:411-421.
15. Hahn HP. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*. Gene 1997;192:99-108.
16. Bertrand JJ, West JT, Engel JN. Genetic analysis of the regulation of type IV pilus function by the Chp chemosensory system of *Pseudomonas aeruginosa*. J Bacteriol 2010;192:994-1010.
17. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 1998;30:295-304.
18. Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. Sensors 2012;12:2519-2538.
19. Tapia-Jiménez AM, Castro-Escarpulli G, Enriques-Nieto C. Cuantificación de Exopolisacárido Producido en la Biopelícula de *Aeromonas* spp. Tesis. Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional, México D.F. 2010.
20. Morales-Espinosa R, Soberón-Chávez G, Delgado-Sapién G, Sandner-Miranda L, Méndez JL, González-Valencia G, et al. Genetic and phenotypic characterization of a *Pseudomonas aeruginosa* population with high frequency of genomic islands. PLoS ONE 2012;7:e37459.
21. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233-2239.
22. Vaz-Moreira I, Nunes OC, Manaia CM. Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water. Sci Total Environ 2012;426:366-374.
23. Warnes SL, Highmore CJ, Keevil CW. Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. mBio 2012;3:e00489-12.
24. Rodríguez MC, Ruiz del Castillo B, Rodríguez-Mirones C, Romo M, Monteagudo I, Martínez-Martínez L. Molecular characterization of *Pseudomonas aeruginosa* isolates in Cantabria, Spain, producing VIM-2 metallo-beta-lactamase. Enferm Infec Microbiol Clin 2010;28:99-103.
25. Wirth FW, Picoli SU, Cantarelli V, Gonçalves AL, Brust FR, Santos LM, et al. Metallo-β-lactamase-producing *Pseudomonas aeruginosa* in two hospitals from southern Brazil. Braz J Infect Dis 2009;13:170-172.
26. Tsakris A, Poulou A, Kristo I, Pittaras T, Spanakis N, Pournaras S, et al. Large dissemination of VIM-2-metallo-β-lactamase-producing *Pseudomonas aeruginosa* strains causing health care-associated community-onset infections. J Clin Microbiol 2009;47:3524-3529.
27. Rojas LF, Newton-Sánchez O, Martínez AG, Salinas DY, Ramírez RI, Tinoco FJC, et al. Identificación de genes responsables de resistencia a carbapenémicos en cepas nosocomiales de *Pseudomonas aeruginosa* aisladas de algunos hospitales de México. Enferm Infec Microbiol 2009;29(suppl 1):S58.
28. Romano-Mazzotti L, Gayosso C, Alcántar CMD, Santos-Preciado JI, Alpuche-Aranda CM. Transmisión cruzada: un factor primordial en bacteremias por *Pseudomonas aeruginosa* resistente a carbapenemes en un hospital pediátrico de tercer nivel. Enferm Infec Microbiol 2008;28(suppl 1):S83.
29. Gomes MZ, Machado CR, da Conceição Mde S, Ortega JA, Neves SM, Lourenco MC, et al. Outbreaks, persistence, and high mortality rates of multiresistant *Pseudomonas aeruginosa* infections in a hospital with AIDS-predominant admissions. Braz J Infect Dis 2011;15:312-322.
30. Gales AC, Sader HS, Jones RN; SENTRY Participants Group (Latin America). Urinary tract infection trends in Latin American hospitals: report from the SENTRY antimicrobial surveillance program (1997-2000). Diagn Microbiol Infect Dis 2002;44:289-299.
31. Hammami S, Boutiba-Ben Boubaker I, Ghozzi R, Saidani M, Amine S, Ben Redjeb S. Nosocomial outbreak of imipenem-resistant *Pseudomonas aeruginosa* producing VIM-2 metallo-β-lactamase in a kidney transplantation unit. Diagn Pathol 2011;6:106.
32. Vitkauskiené A, Skrodenienė E, Dambrauskiené A, Baksyté G, Macas A, Sakalauskas R. Characteristics of carbapenem-resistant *Pseudomonas aeruginosa* strains in patients with ventilator-associated pneumonia in intensive care units. Medicina (Kaunas) 2011;47:652-656.
33. Kumar SH, De AS, Baveja SM, Gore MA. Prevalence and risk factors of metallo β-lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter* species in burns and surgical wards in a tertiary care hospital. J Lab Physicians 2012;4:39-42.
34. Sommerfeld RS, Fiegel J. Nutrient dispersion enhances conventional antibiotic activity against *Pseudomonas aeruginosa* biofilms. Int J Antimicrob Agents 2012;40:177-181.
35. Lopes SP, Ceri H, Azevedo NF, Pereira MO. Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection. Int J Antimicrob Agents 2012;40:260-263.

36. Subramanian P, Shanmugam N, Sivaraman U, Kumar S, Selvaraj S. Antiobiotic resistance pattern of biofilm-forming uropathogens isolated from catheterised patients in Pondicherry, India. Australas Med J 2012;5:344-348.
37. De la Fuente-Nuñéz C, Korolik V, Bains M, Nguyen U, Breidenstein EB, Horsman S, et al. Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. Antimicrob Agents Chemother 2012;56:2696-2704.
38. Irie Y, Borlee BR, O'Connor JR, Hill PJ, Harwood CS, Wozniak DJ. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 2012;109:20632-20636.
39. Burrows LL. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. Annu Rev Microbiol 2012;66:493-520.
40. Jain R, Behrens AJ, Kaever V, Kazmierczak BI. Type IV pilus assembly in *Pseudomonas aeruginosa* over a broad range of cyclic di-GMP concentrations. J Bacteriol 2012;194:4285-4294.
41. Hall AJ, Fothergill JL, Kaye SB, Neal TJ, McNamara PS, Southern KW, Winstanley C. Intraclonal genetic diversity amongst cystic fibrosis and keratitis isolates of *Pseudomonas aeruginosa*. J Med Microbiol 2013;62(Pt 2):208-216.
42. Waters V, Zlosnik JE, Yau YC, Speert DP, Aaron SD, Guttman DS. Comparison of three typing methods for *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis 2012;31:3341-3350.
43. Yousefi S, Nahaei MR, Farajnia S, Aghazadeh M, Iversen A, Edquist P, et al. A multiresistant clone of *Pseudomonas aeruginosa* sequence type 773 spreading in a burn unit in Orumieh, Iran. APMIS 2013;121:146-152.
44. Castillo-Vera J, Ribas-Aparicio RM, Nicolau CJ, Oliver A, Osorio-Carranza L, Aparico-Ozores G. Unusual diversity of acquired β-lactamases in multidrug-resistant *Pseudomonas aeruginosa* isolates in a Mexican hospital. Microb Drug Resist 2012;18:471-478.