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ORIGINAL ARTICLE

AMINOGLYCOSIDE RESISTANCE GENES IN *Pseudomonas aeruginosa* ISOLATES FROM CUMANA, VENEZUELA

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SUMMARY

The enzymatic modification of aminoglycosides by aminoglycoside-acetyltransferases (AAC), aminoglycoside-adenyltransferases (AAD), and aminoglycoside-phosphotransferases (APH), is the most common resistance mechanism in *P. aeruginosa* and these enzymes can be coded on mobile genetic elements that contribute to their dispersion. One hundred and thirty seven *P. aeruginosa* isolates from the University Hospital, Cumana, Venezuela (HUAPA) were evaluated. Antimicrobial susceptibility was determined by the disk diffusion method and the *aac*, *aadB* and *aph* genes were detected by PCR. Most of the *P. aeruginosa* isolates (33/137) were identified from the Intensive Care Unit (ICU), mainly from discharges (96/137). The frequency of resistant *P. aeruginosa* isolates was found to be higher for the aminoglycosides tobramycin and amikacin (30.7 and 29.9%, respectively). Phenotype VI, resistant to these antibiotics, was the most frequent (14/49), followed by phenotype I, resistant to all the aminoglycosides tested (12/49). The *aac(6´)-Ib*, *aphA1* and *aadB* genes were the most frequently detected, and the simultaneous presence of several resistance genes in the same isolate was demonstrated. Aminoglycoside resistance in isolates of *P. aeruginosa* at the HUAPA is partly due to the presence of the *aac(6´)-Ib*, *aphA1* and *aadB* genes, but the high rates of antimicrobial resistance suggest the existence of several mechanisms acting together. This is the first report of aminoglycoside resistance genes in Venezuela and one of the few in Latin America.

KEYWORDS: Aminoglycosides; AME; Resistance; PCR.

INTRODUCTION

The aminoglycosides tobramycin, gentamicin, and amikacin are commonly used to treat hospital-acquired infections caused by *Pseudomonas aeruginosa*. These infections generally require treatment with a combination of antimicrobials in order to achieve a greater bactericidal effect and reduce the levels of resistance[1]. The use of a combination of antimicrobials, however, is associated with resistance mediated by aminoglycoside modifying enzymes (AME). Four of these enzymes, encoded by *aac(6')-I*, *aac(6')-II*, *ant*(2")-I, and *aph(3')-VI*, are of particular significance because they are among the most common modifying enzymes present in *P. aeruginosa*, and their substrates are the most important antipseudomonal aminoglycosides[2]. The spread of these enzymes can occur through genetic elements exchanged between the same or different taxa, and is favored by the selective pressures present in a hospital environment where there is a constant use of antimicrobial compounds. This has led to bacterial resistance which is becoming an increasing threat to public health[3].

In recent years, there has been an increased interest in studying the mechanisms of resistance associated with the different antimicrobial families used in clinical practice. According to epidemiological studies, Latin America is one of the regions with the highest incidence of hospital-associated outbreaks produced by bacteria resistant to several antibiotics[4,5]. Different patterns of resistance to betalactamics, quinolones and aminoglycosides have been reported in Mexico[6], Peru[7], Brazil[8], and Venezuela[9,10,11], and in Latin American surveys[12], showing an increase in resistance in the *P. aeruginosa* strains.

Genes resistant to aminoglycosides in this species have, however, only been reported in Europe, where *aac(6´)-II* and ant(2´´)-I are the most prevalent enzymes, and Korea where the most frequent enzymes are *aph(3′)-VI*, *ant(2´´)-I,* and *aac(6´)-I*, acting either on their own or in combination[1]. In Mexico and Brazil *aac(6´)-31/aadA1*, and *aadA2* have also been reported in Hospital-acquired strains[13,14]. In Venezuela there have been no studies of genes that confer resistance to aminoglycosides in *P. aeruginosa*. However, these genes have been reported in Enterobacteria, with *aadA, aadB,* and *aac(6)-Iq* genes being the most frequently detected in a class I integron, in strains of *Klebsiella pneumoniae* from the University Hospital *Antonio Patricio de Alcala* (HUAPA), in Cumana, Venezuela[15]. Resistance in clinically important gram-negative bacteria is a growing concern which in recent years has broken the within-hospital barrier and is now also affecting outpatients. Bearing this information in mind, we evaluated the *in vitro* antimicrobial resistance of hospital-acquired strains of *P. aeruginosa* isolated from HUAPA and identified their resistant genes to aminoglycosides. We hope

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that this will broaden our understanding of the different aminoglycoside resistance genes, and the mechanisms of resistance in the *P. aeruginosa* strains present in this hospital.

METHODS

We studied 137 different isolates of *Pseudomonas aeruginosa*, from patients with diagnosis of infection, attending different units within the HUAPA, from September 2010 to December 2011. For the identification of bacteria, the isolates were grown in Luria Bertani (LB) agar and taken to the Molecular Genetics Laboratory at the Research Institute of Biomedical and Applied Science "Dra. Susan Tai", *Universidad de Oriente* (IIBCAUDO), where they were kept until their analysis. Isolates were taken only from those patients who had signed a written consent form, after complete disclosure of the aims, risks, and scope of the research project was given. Treatment of patients, analysis of isolates, and information generated was conducted according to the bioethical and biosafety guidelines as stated by the Bioethics and Biosafety Commission of the IIBCAUDO (CoBioBios).

To define the presence of infection, we used the criteria established by the Spanish Society of Infectious Diseases and Clinical Microbiology (http://www.seimc.org/documentoscientificos.php?mn_MP=3&mn_MS=358). In addition, an infection was considered a Healthcare-associated Infection (HAI) if the date of detection of the site-specific infection, as defined by the US National Healthcare Safety Network (NHSN), had taken place after the 3rd day of admission to an inpatient. Otherwise, they were regarded as a community-acquired infection (the infection was already present on the day of admission).

Bacteriological Diagnosis

The viability and purity of the isolates was confirmed using standard culture and biochemical tests, according to the procedures and protocols for the identification of non-fermenting gram-negative bacilli[17]. Microbial susceptibility was determined by the disc diffusion method[18] using the following antimicrobials (BD): amikacin (30 µg), gentamicin (10 µg), netilmicin (30 µg), tobramycin (30 µg), ciprofloxacin (5 µg), cefepime (30 µg), ceftazidime (30 µg), piperacillin (30 µg), piperacillin/tazobactam (100/10 µg), meropenem (10 µg), imipenem (10 µg), and aztreonam (30 µg), according to the guidelines proposed by the Clinical Laboratory Standard Institute for *P. aeruginosa*[19].

Molecular identification of aminoglycoside resistance genes

Bacterial genomic DNA was extracted using the Kit Wizard® Genomic (Promega) according to the manufacturer instructions for gram-negative bacteria. The genes coding for aminoglycoside resistance: *aac(3)-IIa* (*aacC2*-F/R 5´-actgtgatgggatacgcgtc-3´/5´-ctccgtcagcgtttcagcta-3´)[20], *aphA1* (*aphA1*-F/R 5´-atgggctcgcgataatgtc-3´/5´-ctcaccgaggcagttccat-3´) and *aphA2* (*aphA2*-F/R 5´-gaacaagatggattgcacgc-3´/5´-gctcttcagcaatatcacgg-3´)[21], *aac(6´)-Ib* (*aac(6´)-Ib*F/R 5´-taggagtggctaaatcgat-3´/5´-cccgctttctcgtagca-3´)[22] and *aadB* (*aadB*-F/R 5´-cgtcatggaggagttggact-3´/5´-cgcaagacctcaacctttc-3´)[23] were detected using PCR. *Klebsiella pneumoniae* strains Kp01, which contains the *aac(3´)-IIa* gene; Kp28, which contains the *aac(6´)-Ib* and *aph* genes; and KpM7, which contains the *aadB* gene, were used as positive controls[15]. The amplification products were run on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and buffered in TBE 1X, for 30 minutes at 100 V.

RESULTS

From the total number of *P. aeruginosa* isolates obtained from different patients who were admitted to the HUAPA for various treatments, 83.2% (114/137) were identified from patients with hospital-acquired infections. These infections were defined when the clinical and epidemiological information of each individual showed that the patients stayed at least 72 hours after they were admitted. Most isolates were from patients treated in the Medicine A-B unit (n = 46), in the intensive care unit (ICU) (n = 33), and in the nursery (n = 10). The patients treated were hospitalized over a long period (between 55 and 250 days, mean 109 days) and were either immunosuppressed or in a critical condition.

The isolates were mainly from discharges, and were obtained from different parts of the body (96 isolates, 70.0%), followed by catheter tips (15 isolates), sputum (13 isolates), urine (7 isolates), blood (4 isolates), and peritoneal liquid (2 isolates). It should be mentioned that the identification of *P. aeruginosa* from catheter tips was only undertaken when the bacterium was also isolated from a blood sample of the same patient.

The resistance profiles of the *P. aeruginosa* isolates showed that 65.0% (89/137) were resistant to one or more antibiotics: 51.1% (70/137) were resistant to ciprofloxacin, 36.5% (50/137) to meropenem, 35.0% (48/137) to imipenem, 32.8% (45/137) to piperacillin, and 32.1% (44/137) to piperacillin-tazobactam (Fig. 1). In addition, 30.7% (42/137) and 29.9% (41/137) of the isolates showed resistance to the aminoglycosides tobramycin and amikacin, respectively. A total of 9 aminoglycoside resistance profiles (phenotypes) were identified in the isolates (Table 1), of which, the most frequent was the phenotype VI, which showed resistance to tobramycin and amikacin, and type I, which was resistant to all of the aminoglycosides assayed. Furthermore, 95.9% of the isolates with phenotype I also showed multidrug-resistant phenotypes.

The most frequent aminoglycoside resistance gene was *aac(6´)-Ib,* detected in phenotypes I, II, III, IV, VI, and VII (Table 2), followed by *aphA1* (phenotypes I, IX, and VIII) and *aadB* (phenotypes I and V). These genes were also shown to be simultaneously present in all of the phenotype I strains, and in three of the phenotype VI strains.

Nevertheless, the results of the aminoglycoside resistant phenotypes did not always coincide with those of the resistance genes detected. Thus, 10 of the isolates with phenotypic resistance to the aminoglycosides did not yield PCR amplification products for any of the genes evaluated in this study. Six isolates showed the presence of multidrug-resistant phenotypes (Table 3). Only two samples (phenotypes III and VII) came from outpatients (adult observation area). Isolates with phenotypes III and IV and 2 with phenotype VI showed a phenotype that suggests the presence of metallobetalactamases.

DISCUSSION

Pseudomonas aeruginosa has become an important pathogen, constituting 10 to 15% of hospital-acquired infections worldwide,

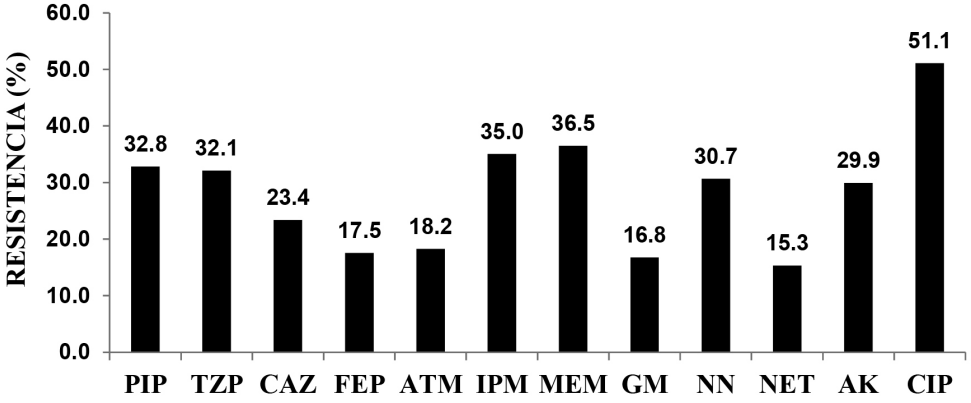

Fig. 1 - Antimicrobial resistance in strains of *P. aeruginosa* isolated from patients at the University Hospital in Cumana, Venezuela. PIP: piperacillin, TZP: piperacillin/tazobactam, CAZ: ceftazidime, FEP: cefepime, ATM: aztreonam, IPM: imipenem, MEM: meropenem, GM: gentamicin, NN: tobramycin, NET: netilmicin, AK: amikacin, CIP: ciprofloxacin.

Table 1
Resistance phenotypes, number of strains (N) and resistance to aminoglycosides in *P. aeruginosa* strains isolated from patients at the University Hospital in Cumana, Venezuela

Phenotype	N	Resistance to Aminoglycosides
I	12	GM NN NET AK
II	4	GM NN NET
III	7	GM NN AK
IV	4	NN NET AK
V	1	GM NN
VI	14	NN AK
VII	1	NN
VIII	1	NET
IX	5	AK
Total	49	

GM: gentamicin; NN: tobramicin; NET: netilmicin; AK: amikacin.

Table 2
Resistance phenotypes and amplified genes in *Pseudomonas aeruginosa* strains collected from patients at the University Hospital in Cumana, Venezuela

Phenotype	N	Amplified Gene				
		aac(3´)-IIa	*aac(6´)-Ib*	*aphA1*	*aphA2*	*aadB*
I	3	-	X	X	-	X
I	1	-	-	X	X	X
I	5	-	-	X	-	X
I	2	-	X	-	-	X
I	1	-	X	-	X	X
II	4	-	X	-	-	-
III	5	-	X	-	-	-
IV	3	-	X	-	-	-
V	1	-	-	-	-	X
VI	1	X	-	-	-	-
VI	3	-	X	-	-	-
VI	3	-	X	-	X	-
VI	2	-	-	-	X	-
VII	1	-	X	-	-	-
VIII	1	-	-	X	-	-
IX	3	-	-	X	-	-
Total	39	1	25	13	7	13

especially in immunocompromised patients in ICUs[24]. These data agree with the findings of our study. Similar results have been reported in Mexico[6] and in Caracas, Venezuela[9]. In addition, we found that 16.8% of community-associated infections were caused by *P. aeruginosa*. This is comparable to a study of patients from India suffering from acquired pneumonia in the community[25], where *P. aeruginosa* was the second most frequent bacteria.

The high frequencies of *P. aeruginosa* isolates resistant to tobramycin and amikacin have also been reported in other health institutions. In Venezuela, in a study of *Pseudomonas* isolates collected in 30 different health institutions throughout the country during the years 1988-1998[26], the resistance shown to gentamicin was 27%, 19% to amikacin and 23% to tobramycin. In a collaborative study, where the resistance pattern of 586 isolates from Argentina, Brazil, Chile, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Panama, Peru, and Venezuela[12] were analyzed, the average resistance found for gentamicin was 32.6%, 24.6% for amikacin and 29.9% for tobramycin. Others studies in Latin American countries have shown a frequency of resistant isolates between 27.8- 42.0% for gentamicin and 16.3- 28.9% to amikacin[27,28,29]. In Spain, resistance frequencies of 24.2% to gentamicin and 16.7% to amikacin have been reported[30].

The inactivation of aminoglycosides by modifying enzymes is the most common resistance mechanism in gram-negative bacilli. This was corroborated in this study performed at HUAPA, where the most frequent genes detected were *aphA1*, *aadB* and *aac(6´)-Ib*, with N-acetylation at the 6' position catalyzed by the last enzyme, being the most usual ways in which aminoglycosides are modified by *P. aeruginosa*[1,31].

Table 3
Resistance phenotypes, hospital areas, sample type and resistance to antibiotics in *P. aeruginosa* strains that did not amplify the aminoglycoside modifying enzyme genes, isolated from patients at the University Hospital in Cumana, Venezuela

Phenotype	N	Hospital Area	Type of Sample	Resistance to Antibiotics
III	1	Medicine B	Discharges	GM NN AK CIP
	1*	Adult Obs.	Discharges	PIP TZP CAZ FEP IPM MEM GM NN AK CIP
IV	1*	Nursery	Blood	PIP TZP CAZ FEP IPM MEM NN NET AK
VI	2*	ICU	Discharges	PIP TZP CAZ FEP IPM MEM NN AK CIP
	1*	Medicine A	Discharges	
	1	ICU	Discharges	PIP IPM MEM NN AK CIP
VII	1	Adult Obs.	Urine	PIP TZP IPM MEM NN CIP
IX	1	Medicine A	Discharges	FEP IPM MEM AK CIP
	1	Medicine B	Discharges	PIP TZP ATM IPM MEM AK CIP

* These isolates showed phenotypes of metallobetalactamases.

This study has also demonstrated the simultaneous presence of several aminoglycoside resistance genes in all the phenotype I isolates, and in three of the phenotype VI strains evaluated. In Korea, the presence of *aph(3')-VI*, *aad(2")-I,* and *aac(6')-I* genes has been reported in 250 isolates of *P. aeruginosa*[32], where the genes were found either alone or combined, with *aac(6')-I* + *aad(2")-I* being the most frequent combination (24/36 isolates). In Iran, *aac(6´)-II* (36%); *aad(2´´)-I* (28%), *aph(3´)-VI* (11%), and *aac(6´)* (7%) genes have been reported in *P. aeruginosa* strains, highlighting the fact that up to four different genes have been found simultaneously in the same isolate[2]. In this sense, integrons have been reported in Brazil[13,33] and Mexico[14], carrying multiple copies of aminoglycoside resistance genes.

Several of the *P. aeruginosa* isolates showing phenotypic resistance to aminoglycosides evaluated in this study did not test positive for the resistance genes evaluated, this suggests that other genes, not tested here, which confer resistance to aminoglycosides, may be present in these isolates, as reported elsewhere[2]. Differences in the distribution of these enzymes could be derived from differences in the selective pressures on the bacterial population with respect to aminoglycoside utilization[34]. There are also other mechanisms that could lead to aminoglycoside resistance, that were not evaluated here, such as: changes in the permeability of the external membrane, active efflux systems, and alterations of the 30S ribosomal subunit conferred by mutations[2,34,35]. Active efflux systems have been demonstrated to be involved in the resistance of *P. aeruginosa* to aminoglycosides in several countries[35,36]. An explanation regarding the presence of resistance genes but not of phenotypic resistance, is the presence of mutations producing non-functional proteins and the lack of promoters in the upstream region where the genes are inserted[37].

The multi-resistance observed in isolates that did not amplify the AME genes evaluated, were mainly obtained from inpatients undergoing long term treatments, and the individuals had received a combination of antimicrobials that could also be partly associated with the indiscriminate use of multiple treatment schemes with broad spectrum antimicrobials, favoring the increase of bacterial resistance caused by multiple mechanisms. However, the high frequency of resistance to different antimicrobials shown by the *P. aeruginosa* isolates from HUAPA suggests the existence of multiple resistance mechanisms acting together.

AUTHOR CONTRIBUTIONS

Salazar E, Guzman M, and De Donato M designed the experiment; Teixeira B collected the samples and epidemiological data. Teixeira B, Carreño N, and Rodulfo H identified the strains and carried out the antimicrobial essays. Teixeira B and Rodulfo H carried out the molecular assays. Rodulfo H, Guzman M, and De Donato M analyzed the data. Teixeira B, Rodulfo H, and De Donato M wrote the manuscript; and Salazar E, Guzman M reviewed the manuscript.

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