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Major Article

Detection of multidrug-resistant *Pseudomonas aeruginosa* harboring bla_{GES-1} and bla_{GES-11} in Recife, Brazil

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Abstract

Introduction: *Pseudomonas aeruginosa*, an important pathogen globally, presents several resistance mechanisms. This study aimed to investigate the presence of bla_{GES} in clinical isolates of *Pseudomonas aeruginosa* obtained from various clinical specimens from patients admitted to three different hospitals in Recife, Brazil. The Guiana extended spectrum beta-lactamase (GES) enzymes are responsible for conferring broad spectrum resistance to beta-lactam drugs, including the carbapenems. **Methods:** A total of 100 carbapenem-resistant *P. aeruginosa* isolates underwent polymerase chain reaction (PCR) testing to identify bla_{GES}, bla_{KPC}, bla_{SPM-1}, bla_{IMP}, and bla_{VIM}. Additionally, PCR products positive for bla_{GES} were sequenced. The clonal profiles of these same isolates were then determined by means of enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis. **Results:** PCR analysis revealed that four isolates harbored bla_{GES}; DNA sequencing showed that two harbored bla_{GES-1} and two bla_{GES-11}. Beta-lactamase genes bla_{SPM-1}, bla_{IMP}, bla_{VIM}, and bla_{KPC} were investigated; none of these genes was detected. Automated susceptibility testing methods (Vitek®2, bioMérieux) showed that the bla_{GES-1}-positive isolates were only susceptible to polymyxin B. The patterns obtained with ERIC-PCR methods showed clonal relationship between the two isolates that harbored bla_{GES-11}, whereas different clonal profiles were found in the isolates harboring bla_{GES-1}. **Conclusions:** We detected the presence of bacterial isolates positive for two different variants of the enzyme GES in three different hospitals from Recife, Brazil. These enzymes have a great capacity for dissemination among Gram-negative bacteria and confer broad-spectrum resistance to beta-lactam antibiotics and to the carbapenems.

Keywords: *Pseudomonas aeruginosa*. Guiana extended spectrum beta-lactamase. Polymerase chain reaction. Antimicrobial resistance.

INTRODUCTION

Pseudomonas aeruginosa, a pathogen associated with opportunistic infections, has been isolated from patients in every part of the world[1,2]. The versatility of *P. aeruginosa* in terms of acquiring resistance and its capacity to adapt to the environment make it capable of causing a wide range of healthcare-related diseases[3,4]. Resistance mechanisms, both intrinsic and acquired, are among the commonest causes of treatment failure related to infections caused by this pathogen.

Among the intrinsic mechanisms, multi-drug efflux pump systems, AmpC chromosomal beta-lactamases, and reduced expression of porin are of particular importance[5,6]. Acquired beta-lactamase enzymes in *P. aeruginosa* include metallo-beta-lactamases (MBL), *Klebsiella pneumoniae* carbapenemases (KPC), and extended-spectrum beta-lactamases (ESBL)[7,8]. The Guiana extended spectrum beta-lactamase (GES) enzyme was first described in France, from a *K. pneumoniae* isolate obtained from a female patient who had previously been hospitalized in French Guiana. According to Bush and Jacoby, this enzyme belongs to the 2f group, and currently has 26 variants (http://www.lahey.org/studies/other.asp#table1)[9-11]. Even though initial studies showed that GES-1 has a low affinity towards imipenem, evolution of its *in vitro* hydrolytic activity has been reported, including to the carbapenems[12].

In Brazil, the first reported case of an isolate carrying an ESBL codifying gene was the bla_{GES-1} variant, detected in a biologic specimen from a patient who underwent a hysterectomy in São Paulo in 2002[8]. It is important to search for this enzyme in *P. aeruginosa* isolates, as it confers broad spectrum resistance to beta-lactams, monobactams, and carbapenems, which are important in the increasing occurrence of multidrug-resistant phenotypes. The aim of the current study was to identify whether

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bla$_{GES}$ is present in clinical isolates of *P. aeruginosa* obtained from patients admitted to three different hospitals in the City of Recife, Brazil.

METHODS

Bacterial isolates

This study included a total of 100 *P. aeruginosa* isolates with multidrug resistance (resistance to three or more antibiotic drug classes, including imipenem and/or meropenem) obtained from various clinical specimens from 100 patients admitted to various departments in three hospitals — one university hospital, one general hospital, and one cancer hospital — in Recife, Pernambuco, Brazil. The clinical isolates, obtained from the microbiology laboratories of the hospitals, were collected between 2011 and 2013; they were identified by the BD Phoenix™ (Becton Dicknson, United States) and VITEK® (bioMérieux, France) automated systems.

Ethical considerations

This study was approved by the Human Research Ethics Committees of the Cancer Hospital Pernambuco/ Brazil (CAAE 05554812.7000.5205) and of the Federal University of Pernambuco/Brazil – Center for Health Sciences (CAAE 0490.01.17200-11).

DNA extraction of bacterial isolates

All 100 isolates were incubated at 37°C for 18-24h in tubes containing Luria-Bertani broth. After being cultured, total deoxyribonucleic acid (DNA) extraction was performed using Brazol (LGC – Biotecnologia, Brazil) according to the manufacturer's instructions. The extracted DNA was re-suspended in Tris-ethylenediaminetetraacetic acid (EDTA) solution and appropriately quantified by spectrophotometry (NanoDrop 2000) at a wavelength of 260nm.

Polymerase chain reaction for *bla*$_{GES}$ gene research

The *bla*$_{GES}$ genes were identified using specific primers: GES1A 5'-ATGCGCTTCATTCACGCAC-3' e GES-1B and 5'-CTATTTGTCCGTGCTCAGG-3'[13]. Amplification reactions were prepared in a total volume of 25µl per tube, comprising 10 ng of genomic DNA, 10pmol of each primer, 1 × buffer, 200µM of deoxyribonucleotide triphosphate (Ludwig Biotec), 1.5mM $MgCl_2$, and 2U Taq DNA polymerase (Promega). The cycle parameters were as follows: 93°C for 3 min, followed by 40 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 1 min; extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Polymerase chain reaction (PCR) products were placed in 1% agarose gel for electrophoresis. A 100pb marker (Amresco) was used as the standard molecular weight. After electrophoresis, the fragment corresponding to the gene was visualized under ultraviolet light and was properly photodocumented.

PCR for beta-lactamases *bla*$_{SPM-1}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{KPC}$

Beta-lactamase genes *bla*$_{SPM-1}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{KPC}$ were investigated using specific primers[14-16]. For each gene, the amplification reactions were performed in a total volume of 25µL per tube: For the *bla*$_{SPM-1}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{KPC}$ genes, 25ng of genomic DNA, 10pmol of primer, 1 × buffer, 100µM of deoxyribonucleotide triphosphate (Ludwig Biotec), 1.5mM of $MgCl_2$, and 1.0U Taq DNA polymerase (Promega) were used[15,16]. PCR amplification was performed in a thermocycler (Biosystems) as follows: Initial denaturation at 95°C for 5 min followed by 30 cycles (*bla*$_{SPM-1}$, *bla*$_{IMP}$, *bla*$_{VIM}$) or 35 cycles (*bla*$_{KPC}$) of denaturation at 95°C for 1 min, annealing according to the temperature previously described for 1 min, and extension at 72°C for 1 min, and then a final extension of 10 min at 72°C[14-16].

The PCR products were stained with blue dye (Jena Bioscience), subjected to electrophoresis on 1% agarose gel in a Tris/Borate/EDTA buffer, visualized under ultraviolet light, and photodocumented. A negative control was included, substituting bacterial DNA for H_2O MilliQ. Positive control strains for the genes *bla*$_{SPM-1}$ (*P. aeruginosa* PSA319), *bla*$_{IMP}$ (*P. aeruginosa* 48-1997A), and *bla*$_{VIM}$ (*P. aeruginosa* VIM-1) were supplied by the *Laboratório Alerta, Universidade Federal de São Paulo*. A positive control strain for the gene *bla*$_{KPC}$ (*P. aeruginosa* P22A) was obtained from the culture collection of the *Laboratório de Bacteriologia e Biologia Molecular, Departamento de Medicina Tropical, Universidade Federal de Pernambuco*.

Molecular typing by enterobacterial repetitive intergenic consensus-PCR

Amplification reactions were prepared in a total volume of 25µl per tube, comprising 100ng of genomic DNA, 10pmol of primers (ERIC-1 [5'-ATGTAAGCTCCTGGGGATTCAC3'] and ERIC-2 [5'AAGTAAGTGACTGGGGTGAGCG-3'])[17], 1 × buffer, 200µM of deoxyribonucleotide triphosphate (Promega), 1.5mM $MgCl_2$, and 1U Taq DNA polymerase (Promega). The amplification parameters used in the enterobacterial repetitive intergenic consensus- polymerase chain reaction (ERIC-PCR) were 95°C for 3 min, followed by 40 cycles of denaturation at 92°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 8 min, followed by a final extension at 72°C for 16 min. PCR products were stained blue-green (LGC – Biotecnologia, Brazil), subjected to electrophoresis on 1.5% agarose gel, visualized under ultraviolet light, and photodocumented for clonal profile analysis.

Sequencing of *bla*$_{GES}$

PCR product was purified using the DNA kit Wizard® SV Gel and PCR Clean-Up System (Promega Corporation) according to the manufacturer's instructions and was subsequently quantified by spectrophotometry. After quantification, the amplicons of the DNA fragments were taken for sequencing. The sequencing results were analyzed using Chromas Lite 2.1.1 software where the authenticity of the sequence was confirmed by comparison with sequences in the online database GenBank (NCBI https://www.ncbi.nlm.nih.gov/genbank/). The corresponding amino acid sequences of the nitrogenous bases of each triplet were obtained using ExPASy software (Expert Protein Analysis System; https://www.expasy.org/). Nucleotide sequence of *bla*$_{GES-1}$ genes and *bla*$_{GES-11}$ have been deposited in GenBank under the following accession numbers: KF679347 (P118UH- *bla*$_{GES-1}$), KT321970 (P8GH- *bla*$_{GES-11}$), KT321971 (P30CH- *bla*$_{GES-1}$).

RESULTS

A total of 100 multidrug-resistant *P. aeruginosa* isolates obtained from patients in three hospitals in Recife, Brazil, were evaluated for the presence of bla_{GES}. Fifty-two were from the university hospital, 33 from the cancer hospital, and 15 from the general hospital. Approximately half (53%) of clinical samples were tracheal aspirates, and most (59%) isolates were from patients admitted to intensive care units. All multidrug-resistant isolates with carbapenem resistance were evaluated for the presence of bla_{GES}. Overall, bla_{GES} was detected in four isolates (**Table 1**).

Of the 53 isolates from the university hospital, one harbored bla_{GES}. This isolate (isolate number P118UH) was obtained from a right foot fragment sample from a male patient admitted to the intensive care unit (**Figure 1**). The patient was treated with polymyxin B and gentamycin but showed no clinical improvement; he subsequently died. After performing sequencing analysis on this isolate, a GES-1 variant was identified.

Of the 33 isolates from the cancer hospital, bla_{GES} was detected in one (isolate number P30CH). Isolate P30CH, obtained from the urine sample of a male patient admitted to the surgical ward, was found to be multidrug-resistant. After performing sequencing analysis, a GES-1 variant was found. ERIC-PCR revealed a different clonal profile from the two clones found in isolates from the general hospital **(Figure 1)**. The patient was discharged, but was rehospitalized two months later; he died from complications of advanced prostate cancer with bone metastasis.

Of the 15 isolates from the general hospital, bla_{GES} was detected in two (isolates number P8GH and P12GH). These isolates were obtained from tracheal aspirate samples from two different patients admitted to the intensive care unit within a short space of time. Both patients had developed hospital-acquired pneumonia and were mechanically ventilated. One was treated with piperacillin/tazobactam and the other with polymyxin B. Sequencing analysis revealed that the gene was the GES-11 variant. The results of the ERIC-PCR analysis showed that isolates number P8GH and P12GH had the same clonal profile; this profile differed from that of isolates number P118UH and P30CH (**Figure 1**). Additionally, all 100 isolates studied were subjected to PCR for bla_{SPM-1}, bla_{IMP}, bla_{VIM}, and bla_{KPC} genes; none of these genes were detected.

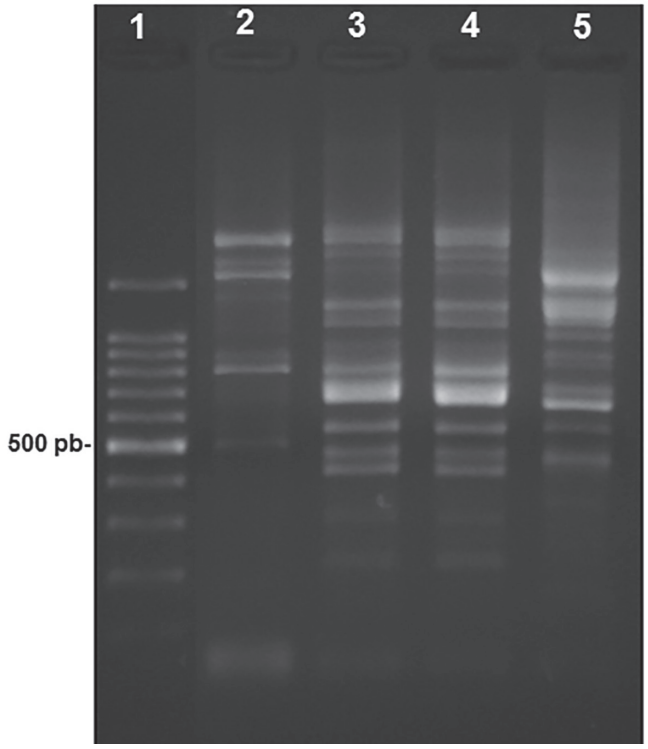

FIGURE 1 - ERIC-PCR results of the four *Pseudomonas aeruginosa* isolates from the University, General, and Cancer Hospitals. **1:** molecular weight marker; **2:** P118UH; **3:** P8GH; **4:** P12GH; **5:** P30CH. **pb:** base pairs; **ERIC-PCR:** enterobacterial repetitive intergenic consensus-polymerase chain reaction; **UH:** University Hospital; **GH:** General Hospital;. **CH:** Cancer Hospital.

DISCUSSION

The emergence of GES-type beta-lactamases, which have been reported in a number of hospitals around the world, is noteworthy as some amino acid substitutions have resulted in

TABLE 1: Antimicrobial resistance profile of the multidrug-resistant *Pseudomonas aeruginosa* isolates harboring bla_{GES} obtained from clinical specimens of four patients in three hospitals in Recife, Pernambuco, Brazil.

Isolate number/bla_{GES} identified	Inpatient department	Clinical sample type	Antimicrobial resistance profile
P118UH/bla_{GES-1}	Internal medicine ward	Right foot fragment	CAZ, CPM, CTX, CRO, IMP, MPM,CIP, NOR (Int.), OFX, ATM, TAC, GEN, AMI
P30CH/bla_{GES-1}	Surgical ward	Urine	CAZ, IMP, MPM, CIP, NOR, GEN, AMI
P8GH/bla_{GES-11}	Intensive care unit	Tracheal aspirate	CAZ, IMP, MPM, ATM, GEN, AMI,CIP, PIT, LEV, TOB
P12GH/bla_{GES-11}	Intensive care unit	Tracheal aspirate	CAZ, IMP, MPM, ATM, GEN, AMI,CIP, PIT, LEV, TOB

***bla*$_{GES}$: Beta lactamase GES ; UH:** University Hospital; **CH:** Cancer Hospital; **GH:** General Hospital; **CAZ:** ceftazidime; **CPM:** cefepime; **CTX:** cefotaxime; **CRO:** ceftriaxone; **IMP:** imipenem; **MPM:** meropenem; **CIP:** ciprofloxacin; **NOR:** norfloxacin; **Int.:** intermediate; **OFX:** ofloxacin; **ATM:** aztreonam; **TAC:** ticarcillin/clavulanate; **GEN:** gentamicin; **AMI:** amikacin; **PIT:** piperacillin/tazobactam; **LEV:** levofloxacin; **TOB:** tobramycin.

increased hydrolytic activity[12]. In Brazil, the first ESBL to be found in *P. aeruginosa* was GES-1, detected in an isolate from a patient in São Paulo who underwent a hysterectomy due to endometrial neoplasia[8]. The GES-1 variant has also been reported in Rio de Janeiro in ceftazidime-resistant *P. aeruginosa* isolated from blood stream infections and, recently, from burn wounds[18,19]. A *P. aeruginosa* clone isolate containing bla_{GES-1}, which was restricted to the south east of Brazil, has subsequently also been detected in the state of Amazonas in the north of the country[20]. The findings of the present study corroborate those of a previous study showing the occurrence of *P. aeruginosa* isolates harboring bla_{GES} in Brazil's Northeastern region[21]. The fact that the GES-1 variant was found in two different hospitals serves as a warning and emphasizes the importance of molecular research related to bacterial resistance genes; such research provides genetic data regarding antimicrobial resistance in hospitals and assists the work of Hospital Infection Control Commissions.

Another GES enzyme variant was found in the Amazon region: In a study involving isolates showing susceptibility to colistin only, the variant found (other than GES-1 which had already been described in the region) was GES-5. This new variant was identical to one found in Korea in *Klebsiella pneumoniae* isolates[19]. It is important to highlight that, as in other studies[22-24], the present study did not detect a high occurrence of bla_{GES}-positive *P. aeruginosa* isolates. Only four were found in a total of a hundred isolates, and two of these were clones, representing a low frequency of the gene.

The other GES variant found in present study was GES-11, an enzyme variant with a total of 287 amino acids that differs from GES-1 by a glycine-to-alanine substitution at Ambler position 243. Such a gly243 substitution has been previously reported in GES-9, but that variant had a glycine-to-serine substitution[25,26]. The isolates showed different characteristics from those of the GES-1-producing cloned isolates, as both clones carrying bla_{GES-11} were obtained from separate patients. This serves as proof of the importance of the antiseptic process and the need to use individual protection equipment when handling hospitalized patients, especially those in intensive care units. This finding is extremely significant for the molecular epidemiology of bla_{GES}, as it is the first reported case in *P. aeruginosa*. Moreover, the enzyme dissemination that accompanied its evolution is notable, as it has only recently been described[12].

GES enzyme variants GES-1 and GES-11 can promote broad-spectrum resistance to beta-lactams and carbapenems. The isolates present in this study, however, likely presented with a multidrug-resistant phenotype due to the presence of distinct mechanisms, such as efflux pumps and reduced porin expression[2,5,12]. The results of this study show the importance of searching for resistance genes (such as ESBLs) in *P. aeruginosa*, as enzymes such as GES effectively contribute to changes in multidrug-resistance phenotypes. The presence of bla_{GES} in *P. aeruginosa* is epidemiologically relevant, especially as there few reports of the occurrence of bla_{GES}-type genes in bacteria in Brazil. The findings of the present study confirm the low prevalence of bla_{GES-1} in *P. aeruginosa* in some hospitals in Brazil; however, we hypothesize that bla_{GES-1} could be spreading across Brazil and that this could be determined if more genetic studies were realized. A point mutation that does not change the amino acid formation (threonine), has also been identified; this could be used as an epidemiologic molecular marker. Additionally, clones carrying bla_{GES-11} have been found. This deserves special attention, given that these genes codify enzymes that give bacteria an extended spectrum of resistance to antibiotics, including the carbapenems, making the treatment of infections caused by *P. aeruginosa* more difficult.

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Conflict of interest

The authors declare that there is no conflict of interest.

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