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Development of a Multiplex PCR Assay for the Detection of Metallo-Beta-Lactamase Genes in *Pseudomonas aeruginosa*

Desarrollo de un PCR Múltiple para la Detección de Genes Metalo-Beta-Lactamasa en *Pseudomonas aeruginosa*

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SUMMARY: The appearance of *Pseudomonas aeruginosa* strains with multi-resistance to antibiotics is a clinical problem of great relevance. The methods for detecting these resistances are laborious and slow, which is a complication when treating patients promptly. In this work, we developed a simple method for simultaneous detection of several carbapenem resistance genes using a multiplex PCR assay. The PCR assay developed, followed by electrophoretic separation of fragments, allows to simultaneously identify the presence of 6 antibiotic resistance genes: *bla-VIM* (261 bp), *bla-IMP* (587 bp), *bla-SPM* (648 bp), *bla-GIM-1* (753 bp), *bla-NDM-1* (813 bp) and *bla-KPC* (882 bp). We analyzed 7 clinical isolates of *P. aeruginosa* obtained in Chile, finding the resistance genes *bla-VIM, bla-IMP, bla-SPM, bla-GIM*, and *bla-NDM* in 5 of them. We found a perfect correlation between the detection of various resistance genes by PCR and the results obtained by antibiograms. Interestingly, 2 of the strains possessed 3 different resistance genes simultaneously. Finally, in this work, we found the presence of 3 genes never described before in clinical isolates of *P. aeruginosa* in Chile (*bla-IMP, bla-SPM*, and *bla-GIM-1*). We developed a rapid multiplex PCR test for the simultaneous detection of up to 6 antibiotic resistance genes of the metallo-β-lactamase family in *P. aeruginosa*.

KEY WORDS: Antibiotic resistance; *P. aeruginosa*; Multiplex PCR; Carbapenem.

INTRODUCTION

Pseudomonas aeruginosa is a well-known opportunistic pathogen with major clinical relevance (Mohammed *et al*., 2019). This microorganism rarely causes disease in healthy people without a predisposing factor (Faure *et al*., 2018). *P. aeruginosa*, in fact, is commonly found colonizing injured parts of the body (like burns, surgical wounds, or physical injuries in the eyes), and the respiratory tract of people with underlying diseases. From these colonized areas, it can invade the organism and cause necrosis, septicemia, and meningitis (Recio *et al*., 2020; Rodríguez-Lucas *et al*., 2020).

This bacterium is one of the most critical pathogens involved in healthcare-associated infections (Olaechea *et al*., 2010; D´Souza *et al*., 2019), constituting a latent risk to the integrity of the patients (Mohammed *et al*., 2019). The treatment for severe infections caused by *P. aeruginosa* is often difficult since it has a natural resistance to a large variety of antimicrobials available on the market. Hence, the use of carbapenems and broad spectrum β-lactam antibiotics is more frequent (Beyene *et al*., 2019). However, β-lactams ineffectiveness is widespread because of carbapenemases, present in carbapenem-resistant bacteria

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that can cause serious infections. Furthermore, the increasing use of carbapenem has led to a selective pressure, where the resistance mediated by cephalosporinases, such as β-lactamases and seri-β-lactamases, is more frequent, and nowadays it is producing worldwide dissemination of strains with this resistance mechanism.

Considering that carbapenem-resistant *P. aeruginosa* infections are more common, and the conventional methods to identify the antibiotic resistance pattern of different *P. aeruginosa* strains are often slow and inefficient, putting the patient's life at risk. Hence, an early and fast detection technique is highly desirable.

This study reports an alternative method, with a genetic approach for the analysis of carbapenem-resistant *P. aeruginosa*. This new system is based on the identification of several metallo-β-lactamase coding genes using a multiplex PCR test to detect the resistance for different β-lactamase antibiotics in a single test. Six carbapenem resistance genes have been identified and targeted in this study: *bla-KPC*, *bla-VIM*, *bla-IMP*, *bla-SPM*, *blaGIM-1*, and *blaNDM-1*.

MATERIAL AND METHOD

Bacterial strain: Seven strains of *Pseudomonas aeruginosa* and one strain of *Klebsiella pneumoniae* were used for the experiments, and these belong to the culture collection of the Molecular Microbiology Laboratory of the Institute of Biomedical Sciences at Universidad Autónoma de Chile.

Bacterial culture and antibiogram test: Before the development of the multiplex PCR assay, the antibiotic resistance of the strains was studied by standard microbiological techniques: microbiological cultures were prepared on plates with 20 mL of nutritive agar (Difco Laboratories, USA) and were incubated at 37 °C for 24 h. Then, the *P. aeruginosa* colonies were transferred to 10 mL of nutritive broth (Difco Laboratories, USA) and incubated at 37 °C for 16 h with orbital agitation to 180 rpm. Susceptibility of the *P. aeruginosa* isolates was tested for a large variety of antibiotics using the standard disc agar diffusion method on Müeller-Hinton (Difco Laboratories, USA) agar plates. A strain of *Klebsiella pneumoniae* was used for quality control of the susceptibility studies.

Genomic DNA isolation: Bacterial genomic DNA was extracted from 1.5 mL of a culture of the different *P. aeruginosa* strains. Cultures were centrifuged at 12,000 rpm for 5 min and the pellets were resuspended in 467 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). 30 µL of 10 % SDS and 3 µL proteinase K at 20 mg/µL were added and the samples were incubated for 3 h at 37ºC. Then, 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) (Winkler, Chile) were added, and the samples were centrifuged at 14.000 rpm for 10 min. The aqueous phase was obtained and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Then, the samples were centrifuged at 14,000 rpm for 10 min. The DNA from the aqueous phase was precipitated with 1/10 volume of sodium acetate 3M and 0.6 volume of isopropanol, at -20 ºC for 20 min. DNA pellets were washed with 70 % ethanol (150 µL) and centrifuged at 14,000 rpm for 5 min. The pellets were dried and resuspended in 50 µL of sterile MiliQ water. To evaluate the quality of genomic DNA, an agarose gel electrophoresis was run (1 % agarose and TAE 0.5X, from Winkler) for 60 min at 100 volts.

Multiplex PCR assay for the detection of metallo-beta-lactamase genes: Based on the metallo-β-lactamase *vim, imp, spm, gim, ndm* and *kpc* sequences available in the GenBank database, proper primers were designed (Table I) to amplify these six genes in a single amplification reaction from genomic DNA. Utilizing the primers detailed in Table I, a multiplex PCR was designed. The different β-lactamase genes were first amplified individually and then simultaneously, in a Mastercycler gradient thermocycler (Eppendorf, USA).

Table I. Primers used in PCR assays.

Primer	Sequence	Tm (ºC)	DNA Fragment (bp)
Vim A-f	5`-AGT-GGT-GAG-TAT-CCG-ACA-G 3`	54	261
Vim A-r	5`-ATG-AAA-GTG-CGT-GGA-GAC-3`	54	
Imp A-f	5`-CTA-CCG-CAG-CAG-AGT-CTT-TG-3`	62	587
Imp A-r	5`-AAC-CAG-TTT-TGC-CTT-ACC-AT-3`	62	
Spm A-f	5`-CCT-ACA-ATC-TAA-CGG-CGA-CC-3`	62	648
Spm A-r	5`-TCG-CCG-TGT-CCA-GGT-ATA-AC-3`	62	
Ndm_1 A-f	5`-GCA-ACC-GCG-CCC-AAC-TTT-GG-3`	66	252
Ndm_1 A-r	5`-GCT-ATC-GGG-GGC-GGA-ATG-GC-3`	66	
Ndm_1 B-f	5`-ATG-GAA-TTG-CCC-AAT-ATT-ATG-CAC -3`	66	813
Ndm_1 B-r	5`-TCA-GCG-CAG-CTT-GTC-GGC-CAT -3`	68	
Gim_1-f	5`-ACA-ACC-TTG-ACC-GAA-CGC-AG-3`	62	753
Gim_1-r	5`-ACT-CAT-GAC-TCC-TCA-CGA-GG-3`	62	
Kpc B-f	5`-ATG-TCA-CTG-TAT-CGC-CGT-CTA-3`	62	882
Kpc B-r	5`-TTA-CTG-CCC-GTT-GAC-GCC-CAA -3`	66	

-f = forward primer; -r = reverse primer

The simultaneous amplification -or multiplex PCR assay- was optimized standardizing the DNA concentration of all the samples to 20 μg/μL. Then, the PCR was optimized for variables like temperature, $MgCl_2$ concentration, and cycle number, until the simultaneous amplification of all the metallo-β-lactamase genes was successful. At this stage, DNA from the *P. aeruginosa* strains was mixed with *K. pneumoniae* DNA and all the primers. The amplification reaction contained 10 μg/mL of genomic DNA, 2.5 μL of buffer 10X, 2 μL of 25 mM $MgCl_2$, 2 μL of dNTPs at 10 mM each, 1 μL of multiple-F and multiple-R primers to 25 μM each, 0.5 μL Taq DNA Polymerase (5 U/μL) from Fermentas Inc. (USA) and nuclease-free water to adjust the final volume of the reaction to 25 μL. Standard conditions for the multiplex PCR were: 5 min to 94 ºC, 36 cycles of 30 sec at 94 ºC, 50 sec at 60.6 ºC, 1 min at 72 ºC, and a final elongation step of 7 min at 72 ºC. The PCR products were analyzed by agarose gel electrophoresis (2 % agarose, 0.5X TAE).

RESULTS

The antibiogram results showed that all *P. aeruginosa* strains were resistant to β-lactam antibiotics and some none β-lactam antibiotics, such as quinolones and aminoglycosides. As a summary, the resistance detected to β-lactam antibiotics is described as following: CD29 strain was resistant to imipenem, aztreonam, and meropenem; CD30 strain showed resistance to imipenem and meropenem; HSJD2 strain was resistant to imipenem, meropenem and piperacillin; HSJD4 strain has resistance to meropenem, imipenem, and aztreonam; HSJD6 strain evidenced resistance to aztreonam, imipenem, and meropenem.

For development to multiplex PCR assay for the detection of metallo-beta-lactamase genes, first individual amplification using only one pair of primers at a time, with the template DNA from all strains mixed, showed amplicons for *bla-VIM* (261 bp), *bla-IMP* (587 bp), *bla-SPM* (648 bp), *bla-GIM-1* (753 bp), *bla-NDM-1* (813 bp) and *bla-KPC* (882 bp) (Fig. 1A). *P. aeruginosa* genomic DNA was utilized for amplification of *vim, imp, spm, gim*, and *ndm* sequences. For *kpc* gene, DNA from *K. pneumoniae* was used. As can be seen in Figure 1B, multiplex PCR amplification produced the same bands that had been amplified using the pairs of primers individually (Fig. 1A).

Next, the DNA of each strain of *P. aeruginosa* was analyzed by multiplex PCR with all pairs of primers. The PCR products were analyzed by agarose gel electrophoresis (2 % agarose, 0.5X TAE). Figure 2 shows the PCR analysis on CD29, CD30, CD32, CD34 HSJD2, HSJD4, and HSJD6 *P. aeruginosa* strains and *K. pneumoniae*. From all the strains studied, CD32 and CD34 had no carbapenemases genes (not shown in Fig. 2). On the other hand, strains CD29 and HSJD6 had more than one class of carbapenemases gene.

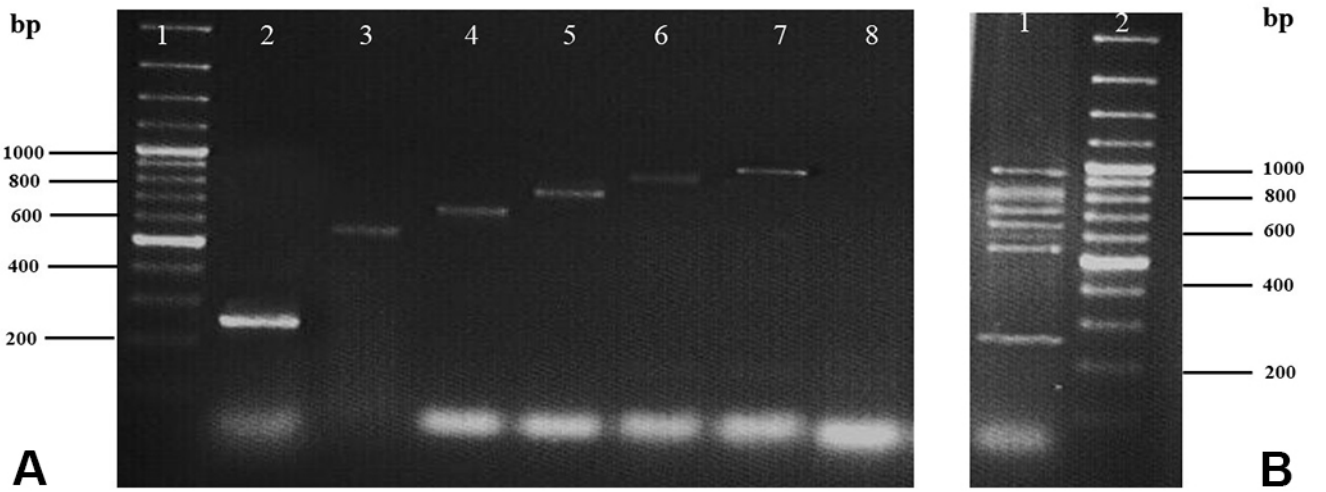

Fig. 1. Individual and multiplex PCR amplification of metallo-β-lactamase genes. (A) Individual PCR amplification. Lane 1: 100 bp molecular size standard. Lane 2: *bla-VIM* amplicon (261bp). Lane 3: bla-IMP amplicon (587bp). Lane 4: *bla-SPM* amplicon (648 bp). Lane 5: *blaGIM-1* amplicon (753 bp). Lane 6: *bla-NDM-1* amplicon (813bp). Lane 7: *bla-KPC* amplicon (882 bp). Lane 8: genomic DNA from *P. aeruginosa* without metallo-b-lactamase gene amplification. (B) Multiplex PCR amplification. Lane 1 shows amplicons for *bla-VIM* (261 bp), bla-IMP (587 bp), *bla-SPM* (648 bp), *bla-GIM-1* (753 bp), *bla-NDM-1* (813 bp) and *bla-KPC* (882 bp). Lane 2 corresponds to a 100 bp molecular size ladder DNA standard.

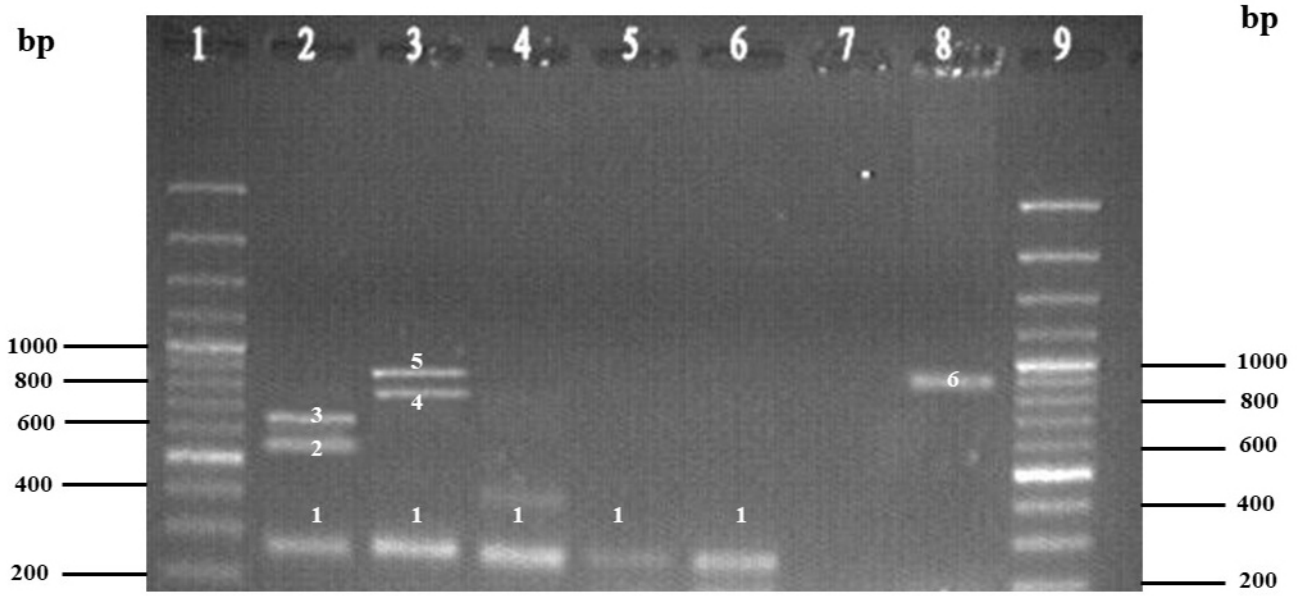

Fig. 2. Multiplex PCR amplification of different metallo-b-lactamase genes in the *P. aeruginosa* strains. Lanes 1 and 9: 100 bp molecular size standard. Lane 2: *P. aeruginosa* strain CD29. Lane 3: *P. aeruginosa* strain HSJD6. Lane 4: *P. aeruginosa* strain HSJD4. Lane 5: *P. aeruginosa* strain HSJD2. Lane 6: *P. aeruginosa* strain CD30. The lane 7 is empty. Lane 8: *K. pneumoniae*. The numbers over amplified DNA band identified different gene fragment or amplicons (1: *bla-VIM* of 261 bp; 2: *bla-IMP* of 587 bp; 3: *bla-SPM* of 648 bp; 4: *bla-GIM* of 753 bp; 5: *bla-NDM* of 813 bp; 6: *bla-KPC* of 882 bp).

DISCUSSION

The detection of *bla-KPC* gene was consistent with what has been reported so far since the presence of this gene has been frequently described in *Enterobacteriaceae* family bacteria as *K. pneumoniae* (Nordmann *et al*., 2009), but it is rarely found on *P. aeruginosa* (Ge *et al*., 2011). The *bla-KPC* gene has been reported in bacterial isolates in the USA, Greece, Israel, Brazil, Argentina, Colombia, and China (Hong *et al*., 2015; Lee *et al*., 2016).

Identification of the *bla-VIM* gene was consequent with the data reported in Chile in 2008 (Pérez *et al*., 2008), where the presence of metallo-β-lactamase was mostly due to *bla-VIM* type genes. The emergence of this gene was described in 2004 in a *Pseudomonas fluorescence* strain (Mendes *et al*., 2004).

Analysis of the *P. aeruginosa* strains for the *bla-IMP* gene only detected the presence of a 587 bp amplicon in the CD29 strain. This is a novel result since, in previous studies of imipenem-resistant *P. aeruginosa*, no *bla-IMP*-positive strains were found in Chile (Hong *et al*., 2015).

Detection of the *bla-SPM* gene was positive only for CD29 *P. aeruginosa* strain. There were no records of a *bla-SPM*-positive strain in Chile; however, they are endemic of countries like Brasil and Switzerland (Hong *et al*., 2015).

P. aeruginosa HSJD6 was the only strain that tested positive for *bla-GIM-1*, and there is no previous report of its presence in Chile (Hong *et al*., 2015). It was also positive for the *NDM-1* gene (an 831 bp amplified fragment, Fig. 2).

The antibiograms indicated resistance to several types of antibiotics in the strains tested. These results were corroborated by the PCR assays, proving a perfect match between beta-lactam antibiotic resistance and the carbapenemase-coding genes for each *P. aeruginosa* strain. Every strain studied evidenced beta-lactam resistance (Imipenem, Meropenem, Aztreonam) and presented one or more resistance genes to these antibiotics as can be seen in Figure 2. This data correlates with the emergence and propagation of multidrug-resistant bacteria worldwide (Potron *et al*., 2015).

Overall, a fast and efficient multiplex PCR assay was created for the simultaneous detection of several metallo-β-lactamase genes, that can also detect more than one resistance gene in each strain. This would help to diminish the detection time, to take the prompt necessary measures to treat these bacterial infections. Additionally, it reduces analysis costs since the detection of these genes was generally done one by one, resulting in high-cost procedures for those requesting the study.

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RESUMEN: La aparición de cepas de *Pseudomonas aeruginosa* con resistencias a diversos antibióticos es un problema clínico de gran relevancia. Los métodos de detección de dichas resistencias son laboriosos y lentos, lo que genera una complicación al momento de tratar a los pacientes oportunamente. En este trabajo desarrollamos un método simple de detección simultánea de varios genes de resistencia a carbapenem, mediante un sistema de PCR múltiple. El ensayo de PCR desarrollado, seguido de una separación electroforética de los amplicones, permite distinguir simultáneamente la presencia de 6 genes de resistencia a antibióticos: *bla-VIM* (261 pb), *bla-IMP* (587 pb), *bla-SPM* (648 pb), *bla-GIM-1* (753 pb), *bla-NDM-1* (813 pb) y *bla-KPC* (882 pb). Analizamos 7 aislados clínicos obtenidos en Chile, encontrando en 5 de ellos los genes de resistencia *bla-VIM*, *bla-IMP*, *bla-SPM*, *bla-GIM* y *bla-NDM*. Encontramos una perfecta correlación entre la detección de diversos genes de resistencia y los resultados obtenidos mediante antibiogramas. Interesantemente, 2 de las cepas mostraron poseer simultáneamente 3 genes de resistencia distintos. Por último, en este trabajo encontramos la presencia de 3 genes nunca antes descritos en aislados clínicos de *P. aeruginosa* en Chile (*bla-IMP*, *bla-SPM* y *bla-GIM-1*). Hemos desarrollado un test rápido de PCR múltiple, para la detección simultánea de hasta 6 genes de resistencia a antibióticos de la familia.a de las metallo-b-lactamases en *P. aeruginosa*.

PALABRAS CLAVE: Resistencia a antibióticos; *P. aeruginosa*; PCR múltiple; Carbapenem.

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