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Identification of carbapenem resistance genes in *Pseudomonas aeruginosa.*

Identificación de genes de resistencia a carbapenémicos en *Pseudomonas aeruginosa.*

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing a series of nosocomial infections, mainly in immunocompromised patients. Currently, it has been reported that the bacteria present intrinsic and acquired resistance to antibiotics, due to the low permeability of its outer membrane, the overexpression of efflux pumps, and the production of enzymes that degrade the antibiotic. The latter is a resistance mechanism presented by bacteria through the acquisition of a transmissible gene that produces a specific enzyme for the hydrolysis of carbapenems, called Metallo-β-lactamases. The objective of the present study was to identify carbapenem resistance genes (VIM and IMP metallo-β-lactamases) in 50 isolates of *P. aeruginosa*. The identification and antimicrobial susceptibility were performed using the VITEK-2 automated system. Subsequently, the *rpoB*, $bla_{VIM,}$ and bla_{IMP} genes were searched by polymerase chain reaction; for the phenotypic determination of Metallo-β-lactamases, the double disk synergy test was performed. The results obtained corroborate that 100 % of the analyzed isolates correspond to *P. aeruginosa*. The sensitivity profile revealed that 82 % of the isolates were resistant to imipenem, while 46 % were sensitive to amikacin. The presence of the bla_{IMP} gene was evident in 90 % of the isolates, being the most frequent, followed by bla_{VIM} in 32% of the cases. Phenotypically, a positive result was obtained in 88 % of the *P. aeruginosa* isolates.

KEY WORDS: *Pseudomonas aeruginosa*, rpoB, bla_{VIM}, bla_{IMP}, antibiotic resistance.

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RESUMEN

Pseudomonas aeruginosa es un patógeno oportunista capaz de ocasionar diferentes infecciones nosocomiales. Se ha reportado que la bacteria presenta resistencia intrínseca y adquirida a antibióticos, debido a la baja permeabilidad de su membrana externa, la sobreexpresión de bombas de eflujo y la producción de enzimas que degradan el antibiótico. El objetivo del presente estudio fue identificar genes de resistencia a carbapenémicos (Metalo-β-lactamasas VIM e IMP) en 50 aislados de *P. aeruginosa*. La identificación y susceptibilidad antimicrobiana se realizó mediante el sistema automatizado VITEK-2. Posteriormente se realizó la búsqueda de los genes *rpoB*, bla_{VIM} y bla_{IMP} por reacción en cadena de la polimerasa; la determinación fenotípica de Metalo-β-lactamasas se llevó a cabo por la prueba de sinergia de doble disco. Los resultados obtenidos corroboran que el 100% de los aislados analizadas corresponden a *P. aeruginosa*. El perfil de sensibilidad mostró que 82 % de los aislados presentaron resistencia al imipenem, mientras que el 46 % mostraron sensibilidad a la amikacina; se evidenció la presencia del gen bla_{IMP} en 90 % de los aislados siendo el más frecuente, seguido de bla_{VIM} con el 32 % de los casos. Fenotípicamente, se obtuvo un resultado positivo en el 88 % de los aislados de *P. aeruginosa*.

PALABRAS CLAVE: *Pseudomonas aeruginosa*, rpoB, bla_{VIM}, bla_{IMP}, resistencia a antibióticos.

Introduction

Pseudomonas aeruginosa is a Gram-negative, facultative aerobic bacillus that does not ferment lactose or glucose and can use many organic molecules as an energy source (Diggle & Whiteley, 2020). It produces a series of soluble pigments, such as pyocyanin, pyoverdine, and pyomelanin, and it has a particular aroma, like grapes or corn tortillas (Martínez-Zavaleta *et al*., 2023). *P. aeruginosa* is considered a ubiquitous pathogen, because it can adapt and resist humid environments, both in water and soil, with minimal nutritional requirements and tolerating different conditions, since it can grow at temperatures from 4 °C to 42 °C, standing out from other *Pseudomonas* for its ability to develop at high temperatures (Solórzano & Parrales, 2021; Liao *et al*., 2022).

P. aeruginosa is an opportunistic pathogen that is generating a global impact, since it can cause a series of nosocomial infections mainly in immunocompromised patients, and it is rare that it can cause infections in healthy people (Diggle and Whiteley, 2020; Solórzano and Parrales, 2021). Infections caused by this bacteria can affect the skin, subcutaneous tissues, bones, ears,

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eyes, urinary tract, lungs, heart, the central nervous system, even the bloodstream; however, the affected site varies depending on the access route and the immunological deficiencies of the person (Bolívar-Vargas *et al*., 2021; Radhika *et al*., 2022).

Recently, *P. aeruginosa* has acquired high resistance to antibiotics. In 2017, the World Health Organization (WHO) reported *P. aeruginosa* as one of the critical priority multi-resistant pathogens, which has shown increased resistance to carbapenems (bactericidal beta-lactam antibiotics that have an extremely broad spectrum of activity), with high mortality and, therefore, affects the health of the population (Tacconelli *et al*., 2017; Fernández-Billón *et al*., 2023). The production of metallo-β-lactamases (MBLs) is one of the main resistance mechanisms due to their horizontal transfer capacity.

MBLs belong to class B of the Ambler classification, and within the Bush-Jacoby-Medieros classification system, they are in group 3, require zinc ions for their activity, and are capable of hydrolyzing a wide range of β-lactam antibiotics, not only penicillins and cephalosporins, but also carbapenems (Radhika *et al*., 2022). This ability to hydrolyze virtually all β-lactams makes infections caused by MBLs-producing *P. aeruginosa* strains particularly difficult to treat. The presence of several types of MBLs has been described in *Pseudomonas*, including metallo-β-lactamase encoded by the Verona integron (VIM), imipenemase (IMP), Seoul imipenemase (SIM), German imipenemase (GIM), São Paulo metallo-β-lactamase (SPM), and New Delhi metallo-β-lactamase (NDM). Of these, VIM and IMP are the most prevalent and reported types worldwide, associated with different outbreaks (Nicolau and Oliver, 2010; Wang & Wang, 2020). Therefore, the objective of the present work was to isolate *P. aeruginosa* strains from clinical samples and identify the presence of carbapenem resistance genes (VIM and IMP metallo-β-lactamases).

Material and Methods

Biological material

Fifty strains with morphological characteristics of *P. aeruginosa* were isolated from patients with healthcare-associated infections, present in different services and referred to the Microbiology Department of the General Hospital “Dr. Aquiles Calles Ramírez” in Tepic city, Nayarit, during the period from December 2022 to August 2023. The isolates came mainly from urine cultures, secretions, and bronchial aspiration. Samples of ulcers, peritoneal fluid, pleural fluid, endometrial tube, dialysis fluid, and cerebrospinal fluid were also obtained. The strains were kept in cryopreservation in LB broth with 20 % glycerol, at -20 °C.

Phenotypic identification and antibiotic susceptibility testing

Culture characteristics were studied by initial diagnosis of bacterial colonies on blood-base, cetrimide, and MacConkey agar. Subsequently, the identification and antimicrobial susceptibility of the isolated strains were performed using the VITEK 2 automated system (BioMerieux, France), which is an automated system for bacterial identification and antimicrobial sensitivity

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testing. Bacterial identification was performed by inoculating a suspension of microorganisms on cards with colorimetric reagents, with certain panels of biochemical reactions. Antimicrobial susceptibility was determined similarly, using cards containing standardized dilutions of different antibiotics corresponding to the sensitivity breakpoints established by the National Committee for Clinical Laboratory Standards (NCCLS, 2018). The following antibiotics were tested: ceftazidime, cefepime, meropenem, piperacillin-tazobactam, imipenem, ciprofloxacin, levofloxacin, amikacin, gentamicin, and tobramycin.

DNA extraction

For molecular identification of *P. aeruginosa* species, DNA was first extracted using the protocol described by Varela-Rodríguez *et al*. (2023). The quality and integrity of the DNA were assessed through observation in a 1.2 % agarose gel, stained with ethidium bromide, and the concentration was determined by spectrophotometry.

Molecular identification of *P. aeruginosa*

For this procedure, the polymerase chain reaction (PCR) was carried out using the methodology proposed by Benie *et al*. (2017). To identify *P. aeruginosa*, the presence of the *rpoB* gene (759 bp) was sought. In addition, a chromosomal region corresponding to the 16S rRNA (956 bp) was amplified, which was used as an internal control to improve the reliability of the technique (Spilker *et al*., 2004). A negative control PCR reaction was carried out simultaneously using *E. coli* DNA. The PCR mixture used was for a final volume of 25 µL, containing 10 mM of Tris-HCl buffer, 2 mM of $MgCl_2$, 0.15 mM of deoxynucleotide triphosphate (dNTP), 15 µM of each primer, 1 U of Taq DNA polymerase, and 2.0 µL of the DNA extraction product (50 ng). The specific oligonucleotides used are found in Table 1. The amplification was carried out in a thermocycler with the following conditions: initial denaturation cycle of 94 °C for 3 min, 35 amplification cycles (94 °C/1 min, 58 °C/1 min, 72 °C/2 min), and final extension of 72 °C/7 min. The amplified PCR products were confirmed through electrophoresis on a 1.5 % agarose gel stained with ethidium bromide (0.9 µL/30 mL agarose), subjected to 100 volts for 50 min. Subsequently, the gel was visualized on a desktop UV transilluminator. Amplicon size was verified based on the 100 bp DNA Ladder molecular weight marker.

Identification of bla_{IMP} and bla_{VIM} genes

To determine the presence of these genes, the conditions described by Poirel *et al*. (2011), were used. The PCR mixture used contained 10 mM of Tris-HCl buffer, 2 mM of $MgCl_2$, 0.15 mM of dNTPs, 15 µM of each primer, 1 U of Taq DNA polymerase, and 2.0 µL of the DNA extraction product (50 ng). The specific oligonucleotides used are listed in Table 1. Amplification was performed in a thermocycler with the following conditions: initial denaturation cycle of 94 °C for 10 min, 36 amplification cycles (94 °C for 30 s, 52 °C for 45 s, 72 °C for 50 s), and final extension of 72 °C for 5 min. Amplicons were verified through electrophoresis on a 1.5 % agarose gel stained with ethidium bromide, subjected to 100 volts for 50 min. Then, the gel was visualized

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on a desktop UV transilluminator. Amplicon size was verified based on the 100 bp DNA Ladder molecular weight marker.

Phenotypic Identification of Metallo-β-Lactamases (MBLs)

The double-disc synergy test described by Arakawa *et al*. (2000) was used for phenotyping. This test is based on the inhibition of MBLs by metal-chelating agents such as EDTA. The plates were inoculated according to NCCLS recommendations, and the EDTA, imipenem (IMP), and meropenem (MEM) discs were placed at 15 mm apart, center-to-center, between the discs containing the antibiotic and the inhibitor. The plates were incubated at 37°C for 24 h. Later, the test was read and interpreted. A positive test for MBLs production was considered when the inhibition zone of the carbapenems (IMP or MEM) containing the EDTA solution was at least 5 mm greater than the zone of inhibition of the carbapenems without the EDTA solution.

Table 1. Oligonucleotides used in PCR protocols.

Target gene	Amplicon size (bp)	Sequence (5´ to 3´)	Reference
16S rRNA	956	Fw- GGGGGATCTTCGGACCTCA Rv- TCCTTAGAGTGCCCACCCG	Spilker *et al*., 2004.
rpoB	759	Fw- CAGTTCATGGACCAGAACAACCCG Rv- ACGCTGGTTGATGCAGGTGTTC	Benie *et al*., 2017.
***bla*$_{IMP}$**	232	Fw- GGAATAGAGTGGCTTAAYTCTC Rv- GGTTTAAYAAAACAACCACC	Poirel *et al*. 2011.
***bla*$_{VIM}$**	390	Fw- GATGGTGTTTGGTCGCATA Rv- CGAATGCGCAGCACCAG	Poirel *et al*., 2011.

Results and Discussion

Isolation of *P. aeruginosa*

During the period from December 2022 to August 2023, 50 isolates of *P. aeruginosa* were collected, isolated from hospitalized and outpatients with infections at the “Dr. Aquiles Calles Ramírez” General Hospital in Tepic, Nayarit; the isolates come mostly from urine cultures 17 (34 %), secretions 11 (22 %) and bronchial aspiration 12 (24 %). Samples were also obtained from ulcers 4 (8 %), peritoneal fluid 1 (2 %), endotracheal tube 2 (4 %), pleural fluid 1 (2 %), dialysis fluid 1 (2 %), and cerebrospinal fluid 1 (2 %) (Figure 1).

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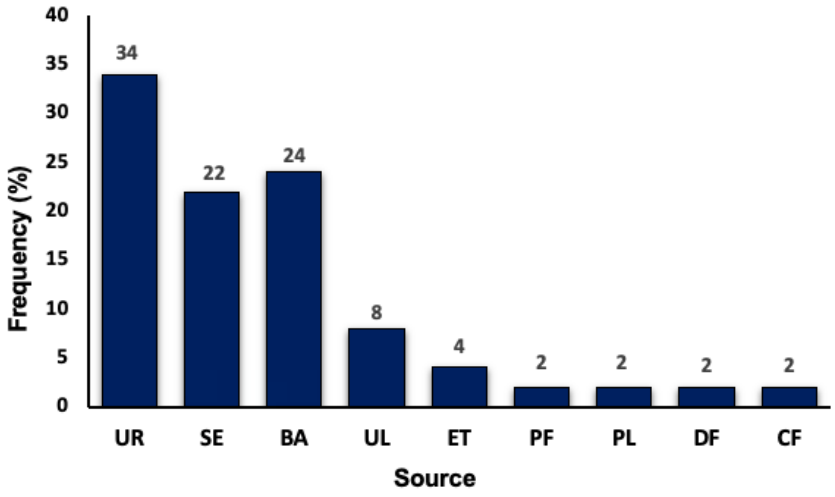

Figure 1. Origin of *P. aeruginosa* strains.

UR, urine culture; ES, secretion; BA, bronchial aspirate; UL, ulcer; ET, endotracheal tube; PF, peritoneal fluid; PL, pleural fluid; DF, dialysis fluid; CF, cerebrospinal fluid.

The isolates were grown on cetrimide, MacConkey, and blood agar at 37 °C under aerobic conditions for a period of 24 h. Irregular, flat and colorless colonies were observed on MacConkey agar, due to this bacteria cannot ferment lactose, some even presented green pigmentation; they also had a very characteristic smell of corn tortilla and when observed under the microscope they were visualized in the form of red bacilli (Gram negative). On the other hand, gray colonies with β-hemolysis were observed on blood agar, due to the ability to lyse erythrocytes, while on cetrimide agar, the colonies presented an intense green pigmentation (Figure 2). The results coincide with those described by Tapia and Jaramillo (2023), who reported that are some tests to identify these bacteria; Similarly, Radhika *et al*. (2022) also included these tests in the identification of their isolates and the characteristics obtained are the same as the results of the present study.

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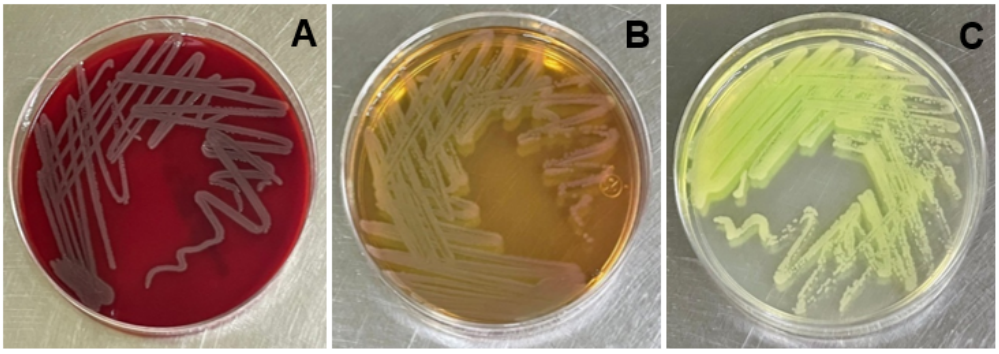

Figure 2. Morphological characteristics of *P. aeruginosa* on A. blood agar, B. MacConkey agar, and C. cetrimide agar.

Phenotypic identification and antibiotic resistance

Bacterial identification and antimicrobial susceptibility of clinical isolates were carried out using the automated VITEK 2 system, which is a system that uses panels with biochemical tests for the identification of bacteria where different metabolic processes such as acidification, alkalinization, enzymatic hydrolysis and development, in the presence of inhibitory substances are evaluated; as well as different antibiotics to determine antimicrobial susceptibility. The system indicated that all isolates belong to *P. aeruginosa*. These results coincide with those reported by Varela-Rodríguez *et al*. (2023) who used this system for the identification of these bacteria in 61 isolates.

The resistance and sensitivity profiles were then analyzed. It was identified that the largest number of isolates were resistant to imipenem with 82 %, while gentamicin with 48 %, was the antibiotic with the lowest number of resistant isolates. On the other hand, the highest prevalence of isolates with sensitivity was to amikacin with 46 % (Table 2). In the case of imipenem, resistance was identified in 82 % of the isolates, this result is like that of Rada *et al*. (2021), who obtained a resistance percentage of 84.8 %. Similar results were also reported by Estepa *et al*. (2015) with 88 %, Martínez-Zavaleta *et al*. (2023) with 89.1 % and Ali *et al*. (2023) with 100 %; In contrast, other studies report a lower resistance to imipenem than that obtained, such as that of Raouf *et al*. (2018), who reported a resistance of 28.57 % and Radhika *et al*. (2022) of 20 %. On the other hand, amikacin presented a sensitivity percentage of 46%; Martínez-Zavaleta *et al*. (2023) reported a percentage of 44.3 %, so the results are very similar, unlike Ali *et al*. (2023), who obtained a lower percentage, since only 18.2 % were sensitive to this antibiotic. It is worth mentioning that the variation in the results reported in antimicrobial resistance is probably due to

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the bacteria have been in contact with others of the same or different genus, but with very similar characteristics, through horizontal genetic transfer to acquire resistance (Arbizú *et al*., 2019; Rada, *et al*., 2021).

Table 2. Resistance of *P. aeruginosa* to the antimicrobial agents tested.

Antibiotic	Number of resistant isolates	%
Ceftazidime	39	78
Cefepime	38	76
Meropenem	32	64
Amikacin	26	52
Gentamicin	24	48
Ciprofloxacin	34	68
Levofloxacin	34	68
Imipenem	41	82
Piperacillin-Tazobactam	36	72
Tobramycin	27	54

The PCR technique was performed to corroborate that these isolates correspond to *P. aeruginosa*. The *rpoB* gene, which encodes the β subunit of RNA polymerase, was amplified. It is a highly conserved gene, and a copy is present in all bacteria due to its essential role in cellular metabolism (Ait Tayeb *et al*., 2005). The *rpoB* gene was amplified in all 50 isolates, confirming that 100 % of the strains correspond to *P. aeruginosa*. The sizes of the amplification products obtained by PCR were identical to those predicted from the *rpoB* primers, approximately 759 bp (Figure 3). The results obtained coincide with those reported by Ali & Abdulrahman (2020), who used the *rpoB* gene in their study and confirmed that all their isolates corresponded to *P. aeruginosa*. This gene can be used to reliably identify this bacterium at the species level (Benie *et al*., 2017; Gaballa *et al*., 2021).

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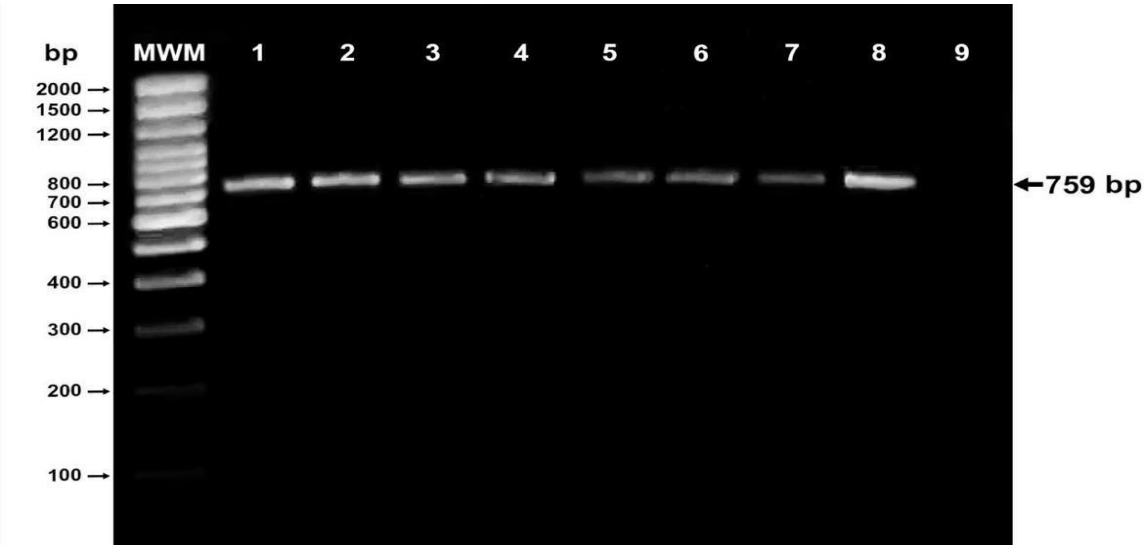

Figure 3. Molecular identification of the *rpoB* gene in clinical isolates of *P. aeruginosa*.

Lanes 1 to 8 correspond to representative isolates, and amplicons of approximately 759 bp are observed in a 1.5 % agarose gel. Lane 9, negative control *Escherichia coli*. MWM, Molecular Weight Marker.

Once the identity of the strains was confirmed, the PCR technique was performed to search for the bla_{VIM} gene, which amplifies a 390 bp product, and the bla_{IMP} gene of 232 bp; these genes encode the transferable MBLs IMP and VIM. In the case of VIM, it was found that 32 % (16/50) of the *P. aeruginosa* strains contain the bla_{VIM} gene of Verona Integron-encoded Metallo-β-lactamase type enzymes, which was evidenced by the presence of amplification products of approximately 390 bp (Figure 4).

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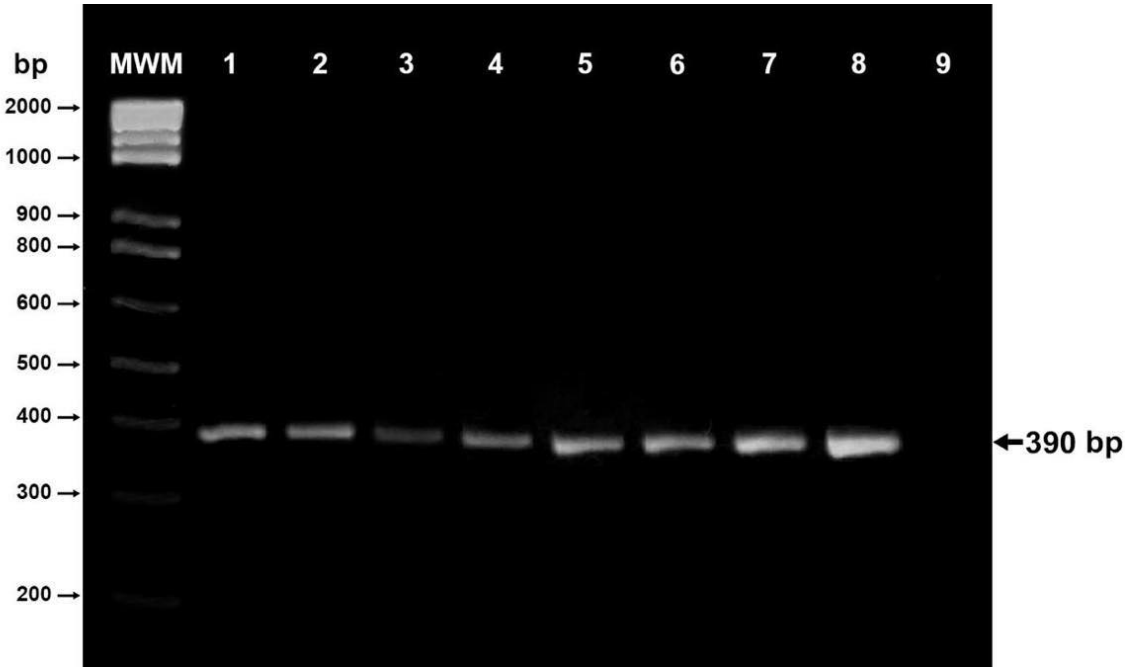

Figure 4. PCR amplification of the *bla*$_{VIM}$ gene in clinical isolates of *P. aeruginosa*.

Lanes 1 to 8 correspond to representative isolates, and amplicons of approximately 390 bp are observed in a 2 % agarose gel. Lane 9, negative control (sterile water). MWM, Molecular Weight Marker 100 bp DNA Ladder.

The results obtained coincide with those reported by Guerra-Sarmiento *et al*. (2021), who conducted research in Colombian hospitals from 2012 to 2017 and reported that the *bla*$_{VIM}$ gene was present in 32.8 % of the isolates. They are also very similar to the results obtained in Egypt, where they reported the presence of this gene in 34 % of the isolates (Raouf *et al*., 2018). In contrast, the results obtained differ from those reported by Martínez-Zavaleta *et al*. (2023) in a study carried out in a hospital in Mexico City in the period from 2011 to 2018, where they obtained that the *bla*$_{VIM}$ gene was found in 59.6 % of the strains and was the gene most frequently found in class B MBLs. In addition, in other countries, a higher prevalence has been reported compared to our results, in Chile 47.5 % (Costa *et al*., 2021), in Nicaragua 68 % (Arbizú *et al*., 2019), and in Colombia 60 % in one study (Saavedra *et al*., 2014) and 47.8 % in another study (Rada *et al*., 2021). It is important to highlight that the difference in results may be due to different factors, including the distribution of the different MBLs by country, even between regions and hospitals in the same country, as well as the evolution they have presented over time (Melgarejo-Touchet *et al*., 2021), since new variants and genes combined with other classes of carbapenemases have been reported; all this since MBL genes are carried in mobile genetic elements such as integrons, transposons or plasmids, which facilitates their spread (Arbizú *et al*., 2019; Costa *et al*., 2021;

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Harris *et al*., 2023; Kazmierczak *et al*., 2015; Rada *et al*., 2021; Remolina *et al*., 2021; Sawa *et al*., 2020).

In the case of IMP, it was found that 90 % (45/50) of the isolates contain the bla_{IMP} gene of imipenemase-type enzymes, because the presence of amplification products of approximately 232 bp was evident. On the contrary, in 10 % (5/50) of the isolates, the presence of this gene was not observed (Figure 5). The results obtained are very similar to those reported by Salvador-Luján *et al*. (2018), who conducted a study in Peru in 2016, which reported that the bla_{IMP} gene was found in 95.8 % of the isolates. On the other hand, Guajardo-Lara *et al*. (2021) investigated in Mexico, in the period from 2017 to 2018, where they found that 16 % of the isolates were IMP type; Likewise, Cho *et al*., (2015) reported in Korea that the bla_{IMP} gene was found in 36.1 % of the isolates and although there is a difference in the percentages in both studies with respect to the results obtained, it was also the gene that predominated in the MBLs. In comparison, Martínez-Zavaleta *et al*. (2023) reported that the bla_{IMP} gene was found in 14.9 % of strains, and although a considerable difference can also be seen in the percentages obtained, they reported that it was the second most prevalent gene after VIM.

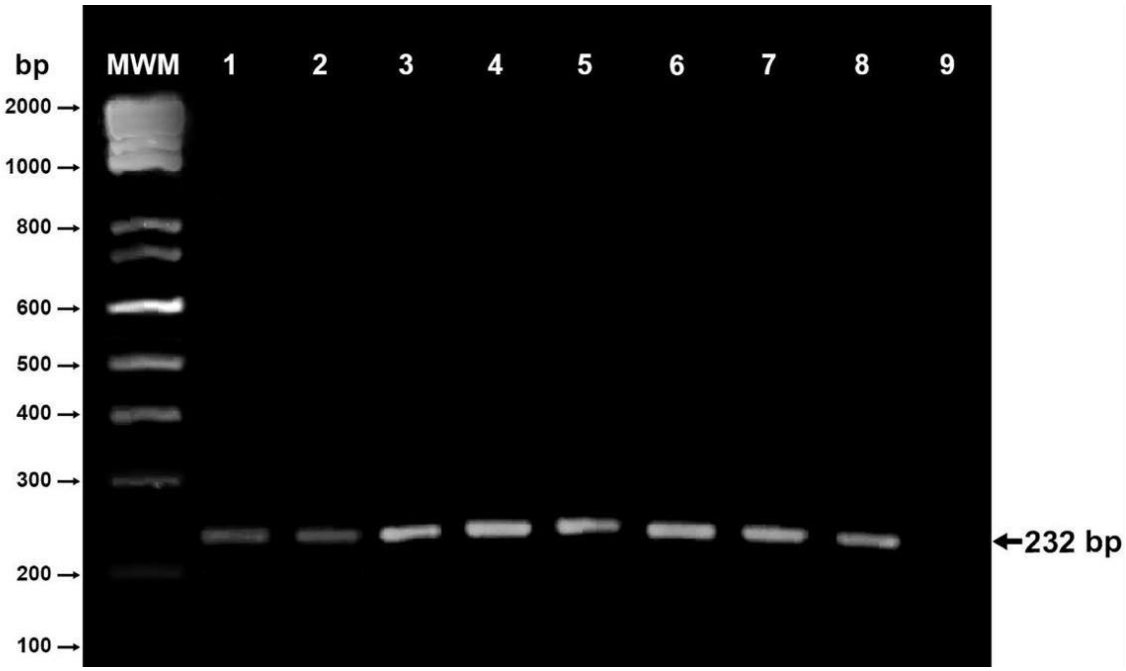

Figure 5. PCR amplification of the bla_{IMP} gene in clinical isolates of *P. aeruginosa*.

Lanes 1 to 8 correspond to representative isolates, and amplicons of approximately 232 bp are observed in a 2 % agarose gel. Lane 9, negative control (sterile water). MWM, Molecular Weight Marker.

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Subsequently, the phenotypic test was performed to detect the presence of MBLs-type enzymes in *P. aeruginosa*; in this research, the double-disk synergy test was used, which is based on the fact that MBLs require zinc++ ions as a cofactor that are trapped by the chelating agent EDTA and consequently inhibit the enzymatic action (Perozo *et al*., 2013; Salvador-Luján *et al*., 2018). The phenotypic test was performed on the 50 strains of *P. aeruginosa*, a positive result was obtained in 44 isolates, since they presented synergy with EDTA, the carbapenems meropenem, imipenem, or both. The presence of MBLs was observed in 88 % of the strains, while in 12 % (6/50), the synergy was not evident (Figure 6). It should be noted that the results obtained show a higher incidence of MBLs using the double-disk synergy method compared to what was reported in other articles, where 13.8 %, 12.9 %, 41 % and 31.6 % were reported, respectively (Julca-García *et al*., 2020; Ophelie and Molin, 2016; Radhika *et al*., 2022; Salvador-Luján *et al*., 2018).

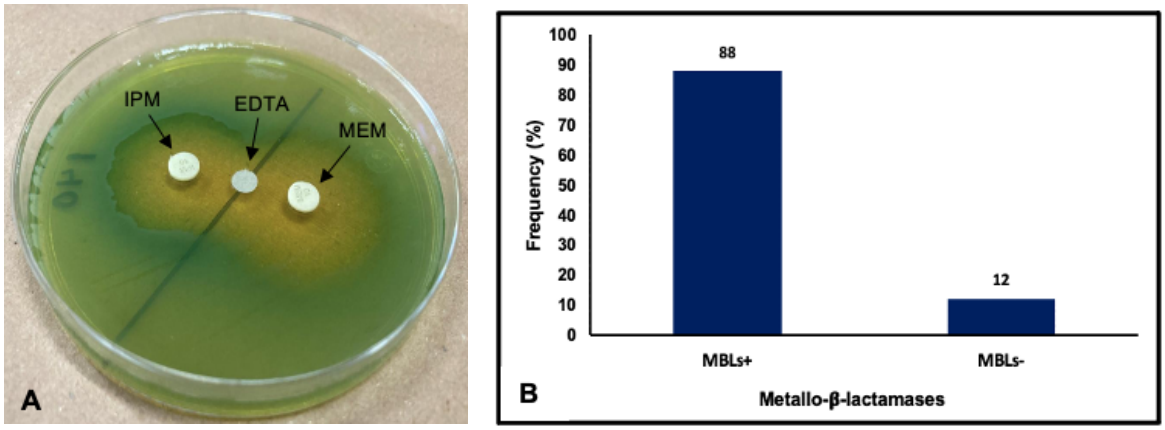

Figure 6. Phenotypic identification of MBLs.

A) Double-disk synergy test; synergy of both MEM and IMP towards EDTA is observed, confirming the presence of MBLs enzymes. B) Percentage of isolates presenting MBLs by the double-disk synergy test.

When analyzing the results, it was found that MBLs enzymes are present in *P. aeruginosa* isolates, with the most frequent being the IMP type, followed by VIM. In addition, it is essential to mention that 22 % (11/50) of the clinical isolates presented both bla_{VIM} and bla_{IMP} genes. On the other hand, 6 % (3/50) of the isolates do not contain any gene under study, a negative result was obtained in the genotypic test through PCR, but they were positive by the phenotypic method. As previously mentioned, other genes code for other MBL-type enzymes in these bacteria, which are distributed both in Mexico and other countries in Latin America and the world (Guajardo-Lara *et al*., 2021; Kazmierczak *et al*., 2015; Salvador-Luján *et al*., 2018; Tapia and Jaramillo, 2023; Tenover *et al*., 2022). It is important to continue testing to determine the presence of these enzymes in these bacteria, both phenotypically and genotypically. Due to the high resistance to different antibiotics that *P. aeruginosa* strains are acquiring, the development of new treatment

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alternatives is necessary, as well as the implementation of epidemiological surveillance programs that regulate the use of broad-spectrum antimicrobials.

Conclusions

The results indicate that there is a wide variety of clinical isolates of *P. aeruginosa* carrying MBLs of the bla_{IMP}, bla_{VIM}, or both types, the most common being bla_{IMP}. Early diagnosis of MBL-producing *P. aeruginosa* is crucial because it is resistant to many antibiotics, and selecting the appropriate treatment is essential to improve patient prognosis. Furthermore, early detection of these resistant strains (both phenotypically and genotypically) helps to implement more targeted measures, such as the implementation of hospital infection control policies, which is key to preventing nosocomial outbreaks and further spread of this resistant pathogen.

Authors contribution

Conceptualization, ANRO, JLAG, CGR; methodology development, PERR, CGR; software management, PERR, LCRR; experimental validation, PERR, ANRO, CGR; analysis of results, JLAG, LCRR, ANRO, CGR; data management, PERR, LCRR, JLAG, CGR; manuscript preparation and writing, PERR, ANRO, CGR; drafting, revision, and edition, ANRO, JLAG, CGR; project administrator, CGR; funds acquisition, CGR.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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