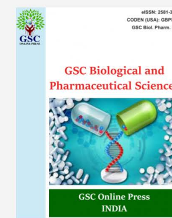
GSC Biological and Pharmaceutical Sciences

eISSN: 2581-3250 CODEN (USA): GBPSC2

Cross Ref DOI: 10.30574/gscbps

Journal homepage: https://gsconlinepress.com/journals/gscbps/



(Research Article)

Molecular identification of *Pseudomonas aeruginosa* and antibiotic resistance testing

Enoc Nicolás Varela-Rodríguez [1], Jackeline Lizzeta Arvizu-Gómez [2], Abril Bernardette Martínez-Rizo [3] and Christian González-Reyes [1,*]

[1] *Academic Unit of Biological and Pharmaceutical Chemical Sciences, Autonomous University of Nayarit, Tepic 63000, Mexico.*

[2] *Research and Postgraduate Secretary, Center for Innovation and Technology Transfer (CENITT), Autonomous University of Nayarit, Tepic 63000, Mexico.*

[3] *Academic Unit of Medicine, Autonomous University of Nayarit, Tepic 63000, Mexico.*

GSC Biological and Pharmaceutical Sciences, 2023, 24(02), 308–316

Publication history: Received on 08 July 2023; revised on 19 August 2023; accepted on 22 August 2023

Article DOI: https://doi.org/10.30574/gscbps.2023.24.2.0335

Abstract

Pseudomonas aeruginosa is a Gram-negative bacillus considered an opportunistic pathogen, usually associated with nosocomial infections in immunocompromised patients. Among the infections caused, the most frequent are urinary tract infections, wound infections, pneumonia, and bacteremia. It has 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. In this study, 61 samples of *P. aeruginosa* collected from the General Hospital "Dr. Aquiles Calles Ramírez" from the city of Tepic, which were phenotypically identified using the Vitek2 automated system. Subsequently, they were molecularly identified with the polymerase chain reaction technique, targeting the *gyrB* gene that codes for the B subunit of DNA gyrase. In 60 of the samples, amplification of the *gyrB* gene was obtained and only one, no amplification was observed. The results showed that infections caused by *P. aeruginosa* are more frequent in male patients, in age range of 41-60 years. Urine culture being the origin of the sample that most predominated. The sensitivity-resistance analysis to antibiotics showed that most of the strains are resistant to β-lactams, except for meropenem, which presented a high sensitivity in most of the strains, as well as in the case of ciprofloxacin, amikacin, and gentamicin.

Keywords: *Pseudomonas aeruginosa*; *gyrB* gene; Antibiotic; Resistance

1. Introduction

P. aeruginosa is a Gram negative, opportunistic pathogenic bacterium, it has a rod shape with dimensions of 0.5 - 1 µm in diameter and 1.0 - 5 µm in length [1]. It can grow in a temperature range from 20 °C to 45 °C and produces pigments such as pyoverdine, this pigment gives a green-yellow or brown-yellow color, also produce pyocyanin that gives a blue color, in addition, some colonies produce pigments called pyorubin and pyomelanin, they give a red or black color, respectively. It is incapable of fermenting lactose, can use acetate and ammonia as carbon and nitrogen sources, and lives on few nutrients. It is a bacterium that is very resistant to environmental conditions; it can be found in any type of soil and water on the planet [2].

P. aeruginosa is one of the nosocomial pathogens that can cause severe disease in immunocompromised and immunocompetent patients. *P. aeruginosa* infections have a mortality rate of approximately 34%, a characteristic that classifies it as high mortality, due to the promotion of the spontaneous generation of multi-drug resistant mutants against any treatment with antibiotics. Most infections caused by *P. aeruginosa* occur in pediatric hospitals, neonatal

* Corresponding author: Christian González-Reyes

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intensive care units, burn units, and oncology. Despite being an etiology for cystic fibrosis, it can also cause bacteremia, bone, central nervous system, urinary, skin, soft tissue, and burned tissue infections [3].

Sometimes, *P. aeruginosa* grows in populations covered by an extracellular polymeric matrix called biofilm, which gives it a high resistance to physical and antimicrobial factors. During the last years, *P. aeruginosa* has acquired a high resistance to antibiotics, even to the latest generation [4].

2. Material and methods

2.1. Bacterial isolates

A total of 61 strains with morphological characteristics of *P. aeruginosa* were collected, isolated from patients with infections associated with health care, present in different services and referred to the Microbiology department of the General Hospital "Dr. Aquiles Calles Ramírez" from the city of Tepic in Nayarit, Mexico, during the period December 2021 to August 2022. The isolated strains come mostly from urine, secretions, and bronchial aspirates cultures. Ulcer, peritoneal fluid, pleural fluid, endotracheal tube, dialysis fluid, and cerebrospinal fluid (CSF) samples were also obtained. The strains were kept in cryopreservation in LB broth (DIBICO) with 20% glycerol, at -20 °C. They were cultured on MacConkey agar (DIBICO) and incubated overnight at 37 °C, under aerobic conditions for their subsequent analysis.

2.2. Phenotypic identification

The culture characteristics were studied through the initial diagnosis of the bacterial colonies on cetrimide and MacConkey agar, where the morphological characteristics of the colonies, color, shape, and texture were noted, as well as their size, height, odor, and the shape of the edges. Subsequently, a microscopic examination of the bacterial isolates under study was carried out, taking the bacterial colony, and placing it on a glass slide, stained with Gram stain to observe the shape of the cells and the method of its aggregation.

The identification of the isolated strains was carried out using the VITEK 2 automated system (BioMerieux), which is an automated system for bacterial identification and antimicrobial susceptibility study. The process was carried out with the inoculation of a suspension of microorganisms in cards with colorimetric reagents with certain panels of biochemical reactions.

2.3. DNA extraction

The extraction of the DNA from bacteria was performed by the thermal shock method described by Donatien-Benie, *et al.* [5]. Briefly, the bacterial isolates were inoculated in nutrient broth culture was inoculated and incubated overnight at 37 °C and 110 rpm in a shaker incubator. Subsequently, 100 µL were taken and placed at boiling temperature (95 °C) in Thermoblock (Labnet) for 10 min. At the end of this period, the tube with the sample was subjected to -20 °C for 10 min in freezer (Norlake Scientific); the last two steps were repeated and later they were centrifuged at 3500 rpm for 10 min. Integrity of the DNA samples was checked by electrophoresis on 1% agarose gel (Invitrogen) and purity was determined by ratio of A260/A280 using a spectrophotometer Multiskan Go (Thermo Sicentific). Finally, the extracted DNA was stored at -20 °C until use.

2.4. Molecular identification of *P. aeruginosa*

A reliable polymerase chain reaction (PCR) target for the detection of *P. aeruginosa* is the *gyrB* gene. In addition, a chromosomal region corresponding to 16S rDNA (956 bp product) was amplified, which was used as an internal control to improve the reliability of the technique [6]. The *Escherichia coli* strain was used as a negative control. The PCR mixture used was for a final volume of 25 µL, which contained the following: 14.8 µL of sterile water, 2.5 µL of Tris-HCl buffer, 2.0 µL of $MgCl_2$, 0.5 µL of deoxynucleotide triphosphate (dNTP) (Invitrogen), 1 µL of each forward and reverse primer (IBT-UNAM), 0.2 µL of Taq polymerase (Invitrogen) and 3.0 µL of the DNA extraction product. A negative control PCR reaction was carried out simultaneously, using *E. coli* DNA. The amplification was optimized in thermocycler (Labnet) with the following conditions: an initial denaturation cycle of 94 °C for 5 min, 35 amplification cycles (94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min), final extension of 72°C for 7 min [7]. The oligonucleotides used in all the tests carried out are described in Table 1. The amplified PCR products were corroborated by agarose gel electrophoresis in a 2% agarose gel stained with ethidium bromide (Promega) subjected to 100 volts for 50 min. Subsequently, the gel was visualized in a desktop UV transilluminator (Benchtop). Amplicon size was verified based on the 100 bp DNA Ladder (Invitrogen) molecular weight marker.

Table 1 Primers used for the identification of *P. aeruginosa*.

Target gene	Sequence (5´to 3´)	Amplicon size (bp)	Reference
16S rDNA	Fw- GGGGGATCTTCGGACCTCA Rv- TCCTTAGAGTGCCCACCCG	956	[6]
gyrB	Fw- CCTGACCATCCGTCGCCACAAC Rv- CGCAGCAGGATGCCGACGCC	222	[7]

2.5. Antibiotic susceptibility tests

Antimicrobial sensitivity was carried out using the VITEK 2 system, through cards containing standardized dilutions of different antibiotics corresponding to the sensitivity cut-off points established by the National Committee for Clinical Laboratory Standards [8]. The procedure used was as follows, a fresh culture batch was taken and resuspended in 1.8 mL of sterile saline solution (0.45%) to obtain a concentration corresponding to tube 0.5 on the McFarland standard, for this a nephelometer was used. The wells were hydrated with 100 µL of the microorganism suspension equivalent to $1.5X10^8$ CFU/mL, incubating them at 37 °C.

The following antibiotics were tested, ceftazidime, cefepime, piperacillin-tazobactam, imipenem, meropenem, ciprofloxacin, levofloxacin, amikacin, gentamicin, and tobramycin.

3. Results and discussion

3.1. Isolation of *P. aeruginosa*

61 samples of *P. aeruginosa* were collected in "Dr. Aquiles Calles Ramirez" General Hospital during the period December 2021 to August 2022. The isolated strains come mainly from urine cultures, secretions, and bronchial aspirates. Ulcer, peritoneal fluid, pleural fluid, endotracheal tube, dialysis fluid, and cerebrospinal fluid samples were also obtained. They were cultivated in LB broth, incubated at 37 °C with shaking for 24 h. Subsequently, they were cultivated on cetrimide, MacConkey and blood agar plates where it was shown that the 61 strains can grow in these media. Colonies of *P. aeruginosa* on MacConkey agar are colorless due to the lack of lactose fermentation, they are large and flattened, round and mucoid. It has a smell like corn tortilla or fruity like grapes and some strains produce bluish (pyocyanin) and green (pyoverdine) pigments that color the culture medium [9]. The bacteria were cultivated on solid blood agar to determine the ability of the bacteria to lyse red cells and determine the type of decomposition, and it was found that 55 isolates were β-hemolytic, which was evidenced by the presence of a transparent halo around the colony (figure 1).

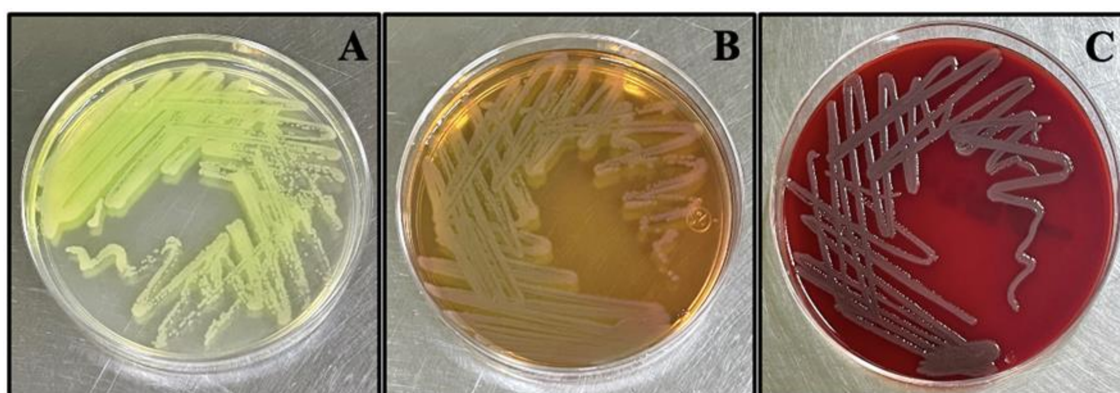

Figure 1 Morphological characteristics of *P. aeruginosa* on A. cetrimide agar, B. MacConkey agar and C. solid blood agar.

Next, the bacteria were cultivated at 4 °C and 42 °C, the growth was monitored since the bacteria were cultivated in the solid nutrient media for each isolate, the first one was incubated at 4 °C and the other at 42 °C. The results showed the ability of all the isolates to grow at 42 °C, which is an important diagnostic characteristic for *P. aeruginosa* over the rest of the species of the genus *Pseudomonas*. However, not all isolates grew at 4 °C, and this agrees with [10].

3.2. Microscope observation

On microscopic examination of Gram-stained bacterial specimens, *P. aeruginosa* bacteria were observed as single rods or short chains and appeared negative for Gram-stained (red color), as shown in the figure 2.

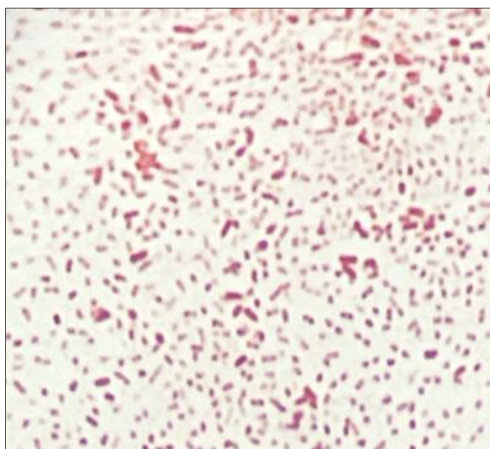

Figure 2 Gram stain of *P. aeruginosa* under magnification (100X) after 24 hours of growth on MacConkey agar, which shows Gram-negative bacilli.

3.3. Identification of *P. aeruginosa*

Pseudomonas strains were conventionally identified with the Vitek 2 system, which uses various substrates that measure various metabolic activities such as acidification, alkalinization, enzymatic hydrolysis, and growth in the presence of inhibitory substances. Once the samples were processed, the system indicated that the 61 clinical isolates obtained correspond to *P. aeruginosa*.

Table 2 Reference tests used in the diagnosis of *P. aeruginosa* isolated from different pathological conditions.

Biochemical test	Outcome
Gram stain	Gram-negative bacillus
Catalase	Positive (bubble formation)
Oxidase	Positive (development of purple color)
Growth at 42ºC	Growth
Growth at 4ºC	Negative
Growth on Cetrimide agar	Round, smooth colonies with regular edges, production of green pigment (pyoverdine)
Growth on MacConkey agar	Round, colorless colonies due to non-use of lactose
Hemolysin	Positive (greenish transparent halo around the colonies
Kligler	K/K (red throughout the tube)
KIA	K/K
Indole	Negative
H_2S production	Negative
Urease	Positive (development of pink color)
Hugh-Leifson glucose medium	Acid production by oxidation of glucose
Motility	Growth around the inoculation site

Identification of *P. aeruginosa* is based primarily on its distinctive colony morphology, pigment and odor production, and its ability to grow at 42 °C. The biochemical tests that were used to identify the *P. aeruginosa* bacterium isolated from different samples are shown in table 2.

Subsequently, the DNA was extracted from the 61 strains that presented phenotypic and biochemical characteristics of *P. aeruginosa*; the DNA was used as a template in the PCR assay, for the search and amplification of the *gyrB* gene, which is a gene present in all bacteria that encodes the β fraction of DNA gyrase, an essential enzyme to carry out DNA replication [11]. Amplified PCR products were detected by agarose gel electrophoresis of each amplification mixture in 2% agarose gels, using ethidium bromide and UV light. The *gyrB* gene has been amplified in 60 isolates, which indicates that it is *P. aeruginosa*. The sizes of the amplification products obtained by the PCR were identical to those predicted from the target *gyrB* primers, approximately 222 pb (figure 3); the presence of the gene was not found in one strain. That strain complies the phenotypic and biochemical characteristics, but not the genotypic ones; this sample may not be a strain of *P. aeruginosa*.

While *P. aeruginosa* rarely causes disease in healthy persons, hospitalized patients are a high-risk group for infection, because the organism is often found in hospitals. It is frequently multi drug resistant, which contributes to the high morbidity and mortality of patients in intensive care units, burn units and surgery wards. The organism may be involved in nosocomial pneumonia, urinary tract, surgical wound, and bloodstream infections, as well as causing other serious infections such as meningitis, external otitis, endophthalmitis, and endocarditis. The most susceptible to *P. aeruginosa* infections are immunocompromised cancer patients, burn patients, cystic fibrosis patients, and bone marrow transplant recipients [12].

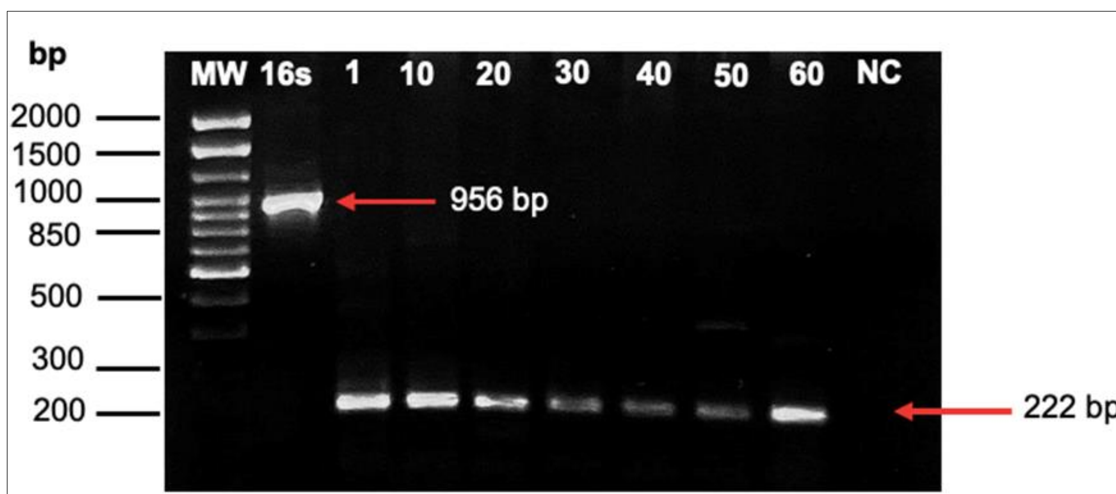

Figure 3 PCR amplification of *gyrB* gene in *P. aeruginosa* with the expected product length of 222 bp in 2% agarose gel. strains 1, 10, 20, 30, 40, 50 and 60. 1Kb DNA ladder was used as a molecular weight marker (MW). 16S rDNA, internal control. NC, negative control (*E. coli*).

Of the 60 strains confirmed by the presence of the *gyrB* gene, 37 (62%) were found to come from male patients and 23 (38%) from female patients. The results obtained indicate that bacterial infection is more common in male than female patients.

The age of the patients ranges between 2 months to 88 years, mean of 45.3 years, values that coincide with those reported by Astal [13], which report an age range of 1 to 80 years. According to the grouping of the data, it shows that the most prevalent is the range of 41 - 60 with 18 cases (30%), followed by 61-80 with 16 cases (27%), 21-40 with 11 cases (18%), 0-20 with 9 cases (15%) and 6 cases (10%) in the range of 81-100 years. The values obtained differ from what was reported by Folic, *et al.* [14], where they report that the age range with the highest presence of the bacteria is 61-80 with 49% followed by 41-60 with 26 %.

Bacterial isolates come from both hospitalized and outpatients. Of the 60 isolates, 16 (26%) come from urine cultures, 13 (23%) from secretions, and 12 (20%) from bronchial aspirates. In addition, 8 (13%) ulcer samples, 3 (5%) peritoneal fluid, 2 (3%) pleural fluid, 2 (3%) endotracheal tube, 2 (3%) expectoration, 1 (2%) dialysis fluid, and 1 (2%) CSF (figure 4). The results obtained agree with those reported by Yetkin, *et al*, and Folic, *et al,* [14-15] who indicate that the isolated *P. aeruginosa* strains come mainly from urine cultures, secretions, and bronchial aspirates; something interesting in

these investigations was the isolation of the bacteria in blood samples (hemoculture), in our investigation, it was not reported in this type of sample.

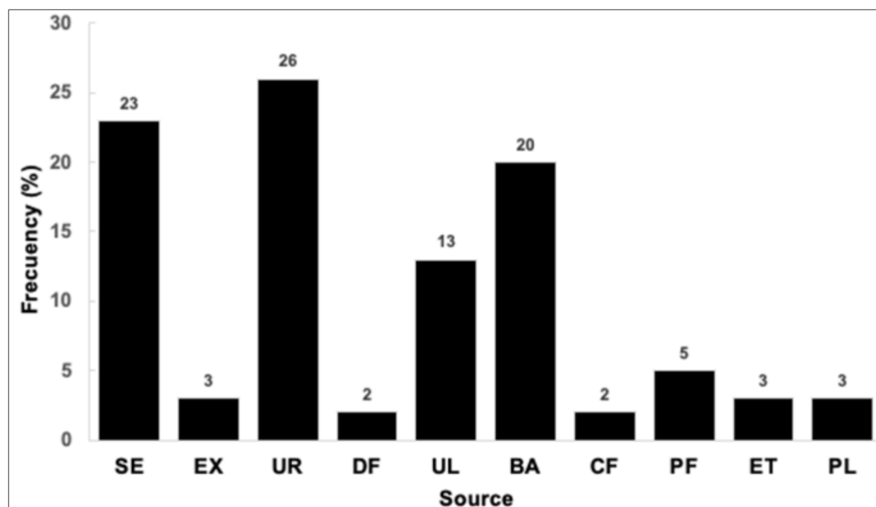

Figure 4 Origin of the sample with the presence of *P. aeruginosa*. SE, Secretion; EX, Expectoration; UR, Urine; DF, Dialysis Fluid; UL, Ulcer; BA, Bronchial Aspirates; CF, Cerebrospinal Fluid; PF, Peritoneal Fluid; ET, Endotracheal Tube; PL, Pleural Fluid.

3.4. Antibiotic resistance of strains

The data analysis of susceptibility-resistance of 60 *P. aeruginosa* isolates to 10 antimicrobial agents, ciprofloxacin, gentamicin, amikacin, meropenem, cefepime, ceftazidime, imipenem, levofloxacin, tobramycin, piperacillin-tazobactam is summarized in table 3. The results of the current study showed that most of the *P. aeruginosa* isolates are resistant to piperacillin-tazobactam (73%), followed by ceftazidime (68%), cefepime (67%) and imipenem (50%). On the other hand, the lowest resistance was to meropenem (27%), amikacin (25%) and finally, gentamicin (22%).

Table 3 Resistance of *P. aeruginosa* to the antimicrobial agents tested.

Antibiotic	Number of resistant isolates	%
Ciprofloxacin	21	35
Gentamicin	13	22
Amikacin	15	25
Meropenem	16	27
Cefepime	40	67
Ceftazidime	41	68
Imipenem	32	53
Levofloxacin	24	40
Tobramycin	19	32
Piperacillin-Tazobactam	44	73

The results obtained in the resistance tests show a high percentage of resistance to the β-lactam antibiotics, piperacillin-tazobactam, ceftazidime, cefepime and imipenem, with 73%, 68%, 67% and 50%, respectively. These results agree with the study carried out by Heidari, *et al.* [16], who reported a resistance rate for these antibiotics between 50 and 60%. Similarly, in another investigation, resistance to imipenem was reported; as well as piperacillin-tazobactam, ceftazidime and cefepime with 61%, 60% and 80% respectively [17-18]. On the other hand, in a study carried out on isolated of patients from a university hospital, it was reported that most of the isolated present a low level of resistance to amikacin (11%), gentamicin (40%), ciprofloxacin (34%), tobramycin (31%) and meropenem (33%). These values coincide with

those obtained in this work, which indicates that these strains do not present a high rate of horizontal genetic transfer. In the case of the tested cephalosporins, the reported results differ from those of this work, because they report a low level of resistance, 27% to ceftazidime and 35% to cefepime [15].

Both, broad intrinsic and acquired resistance to many antibiotics used in medical practice generally contribute to the pathogenicity of *P. aeruginosa*, as bacteria of this genus can show high rates of resistance to most antimicrobial agents commonly used in the hospital environment. Therapeutic options include aminoglycosides, fluoroquinolones, broad-spectrum penicillin's, monobactams, third- and fourth generation cephalosporins, and carbapenems. One of the recommendations for antibiotic therapy in the treatment of *P. aeruginosa* infections is the use of an association that includes a beta-lactam and another antibiotic, normally an aminoglycoside or a quinolone, with beta-lactams being the basis of the chosen association [19].

However, resistance of *P. aeruginosa* to beta-lactam antibiotics can occur due to enzymatic mechanisms, for example, the production of beta-lactamases through the activation of outer membrane waterproofing systems, which are related to porins, and through the expulsion of antimicrobial agents from the bacterial cell, called an efflux pump [20]. Eventually, more than one mechanism may be present, resulting in a broad pattern of resistance to all beta-lactams, with beta-lactamase production being the most studied mechanism in recent decades.

Currently, an increase in strains of *P. aeruginosa* resistant to carbapenems is reported, the main mechanisms are mutations or loss of OprD porin, overexpression of efflux pumps, hyperproduction of chromosomal AmpC beta-lactamase and production of carbapenemases enzymes [20-21]. A variety of class A, B (metallo-β-lactamases) and D (oxacillinases) carbapenemases have been described in *P. aeruginosa*, with metallo-β-lactamases (MBL) being the most prevalent worldwide. One of the characteristics of MBL is that they do not hydrolyze aztreonam, therefore, they do not develop resistance to this antibiotic, which may be indicative of the presence of these enzymes [22].

Due to the high resistance that some strains of *P. aeruginosa* present to various groups of antibiotics, the increase in resistance opens the way for the search for new treatment alternatives, for example, using of phages therapy, antibody, microbiota, vaccines, antibiofilm compounds and nanoparticles combined with other chemical materials [3, 23-25].

4. Conclusion

In this work, 60 strains of *P. aeruginosa* were identified by the presence of the *gyrB* gene. The presence of the bacteria is mostly in male patients, in age ranges between 41-60 years, in samples of urine, secretions and bronchial aspirates cultures. The results of the antimicrobial tests show that many isolates present resistance to piperacillin-tazobactam, ceftazidime, cefepime and imipenem.

Compliance with ethical standards

Acknowledgments

Authors would like to thank Marcela Acosta from the Microbiology department of the General Hospital "Dr. Aquiles Calles Ramírez" for providing the facilities to obtain clinical samples for this research work.

This research was carried out within the framework of a research project with the support of UAN Foundation 2019, of the University Autonomous of Nayarit, México.

Disclosure of conflict of interest

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

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