Journal of Applied and Natural Science
16(2), 470 - 477 (2024)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Prevalence of β-lactamase enzymes and molecular detection of the *AmpC* gene in *Pseudomonas aeruginosa* isolated from various clinical infections

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Article Info
https://doi.org/10.31018/jans.v16i2.5434
Received: January 24, 2024
Revised: March 22, 2024
Accepted: March 27, 2024

How to Cite
Saleh, M.K. *et al.* (2024). Prevalence of β-lactamase enzymes and molecular detection of the *AmpC* gene in *Pseudomonas aeruginosa* isolated from various clinical infections. *Journal of Applied and Natural Science*, 16(2), 470 - 477. https://doi.org/10.31018/jans.v16i2.5434

Abstract
Most species of Gram-negative bacteria produce the enzyme β-lactamase, which can inactivate the structure of β-lactam antibiotics. *Pseudomonas aeruginosa* has revealed countless resistances to β-lactam antibiotics and others over the last few decades. Therefore, the present study aimed to determine the prevalence of ampicillin β-lactamase (AmpC) enzymes, conduct a molecular investigation of the *AmpC* gene expressing the enzymes and assess their impact on the widespread occurrence of antibiotic resistance in *P. aeruginosa*. One hundred and fourteen *P. aeruginosa* isolates were collected from wounds, UTIs, burns, ears, and sputum and identified using various culture media, tests, and the VITEK 2 compact system. The isolates were identified, exhibiting high resistance rates of 100% to Ampicillin and 95.4% to Nalidixic acid. They also demonstrated resistance rates of 18.2% to Chloramphenicol and 22.7% to Ciprofloxacin. The Iodometric method test revealed that 63.1% produced β-lactamase enzymes, while the remaining 36.8% did not produce β-lactamase enzymes. The finding of the CHROM agar and Extended-Spectrum β-Lactamases) ESBLs) tests showed that 100% of isolates produced broad-spectrum β-lactamase. The double disk synergy test (DDST) for detecting AmpC enzymes showed that 19.2% tested positive, while 80.7% were unable to produce these enzymes. Molecular detection of the *AmpC* gene revealed that 81.8% of the isolates possessed this gene, while 18.1% did not. Out of the 22 isolates of *P. aeruginosa* studied, 18 exhibited a high percentage of the *AmpC* gene. This gene produces penicillinase enzymes, contributing to resistance against most studied antibiotics.

Keywords: Ampicillin β-lactamase enzymes, *AmpC* gene, Double Disk Synergy Test, Extended-Spectrum β-Lactamases, *Psuedomonas eruginosa*

INTRODUCTION

The antibiotics that contain β-lactam in their structure are considered more important in medicine in the 21st century (Bush and Bradford, 2016). Penicillins, cephalosporins, carbapenems, and monobactams are the four major chemical classes that contain β-lactams. Because these chemicals are so valuable, the healthcare community is alarmed by the global proliferation of drug resistance (Brüssow, 2017). Enzymes that inactivate β-lactams are responsible for much of the resistance to these compounds (Bush, 2018). The Centers for Disease Control and Prevention (CDC 2013) and the World Health Organization (WHO 2017) have identified β-lactamase-producing Gram-negative bacteria as one of the world's most serious threats. Pharmaceutical efforts to subvert these enzymes by developing novel molecules with broader clinical applications have yielded scientific and some clinical results (Bush and Bradford, 2019). β-lactamase enzymes are one of the most common means of resistance of bacteria to β-lactam antibiotics (Jagad and Vakanee, 2017). They are expressed in most species of Gram-negative bacteria, such as *Pseudomonas aeruginosa*. The production of enzymes ceases as soon as the effectiveness of the antibiotic ceases, and the genes encoding for the production of the enzyme are carried either by plasmid, chromosomally, or on transposon genes

(Castanheira *et al*., 2021). They play the main role in developing resistance to penicillin's, cephalosporins, and carbapenems (Boyd *et al*., 2020).

Every β-lactamase gene is found genetic elements that encode genes encoding antibiotic resistance determinants, sharing multidrug resistance with *P. aeruginosa*. Furthermore, these genetic components are transferable to other Gram-negative bacteria, increasing the number of cases of antibiotic resistance and complicating the treatment of infected patients. The therapeutic usage of these antibiotics is significantly impacted by the hydrolysis of broad-spectrum β-lactams by the enzyme groups ESBLs and carbapenemases, which are dependent on these antibiotics to treat severe infections (Bush, 2013). Therefore, several Gram-negative pathogen infections can now be treated with only a few, if any, antibiotics. Based on the number of organisms that produce these enzymes, Class C cephalosporinases are among the most abundant β-lactamases. These cephalosporinases, known as species-specific AmpC enzymes, are typically found as chromosomal enzymes (*AmpC* gene on the chromosome that encodes the AmpC enzyme) in most Gram-negative bacteria.

P. aeruginosa is an opportunistic bacterium that causes sepsis, pneumonia, Urinary Tract Infections (UTIs), and soft-tissue infections. It also contributes to nosocomial infections, particularly in immunocompromised individuals, because of solid-organ transplantation and immunosuppressive medication (Hong *et al*., 2015). The worldwide emergence of multidrug-resistant bacterial isolates in hospitals continues to be a problem of scientific concern, especially infections caused by *Pseudomonas* species, particularly *P. aeruginosa* , due to their opportunistic character and inherent resistance to many antibiotics. Until recently, the majority of *P. aeruginosa* β-lactam resistance was attributed to the loss of the OprD porin protein associated with high-level synthesis of the naturally occurring class C AmpC, commonly in isolates with elevated efflux systems (Quale *et al*., 2006). The *P. aeroginosa AmpC* gene, encoded by chromosomal genes, may lead to the overproduction of AmpC β-lactamases, causing resistance to cephalosporins, cephamycin, and carbapenems. The β-lactamases AmpC enzymes are among the most important β-lactamases that can break down a large group of antibiotics (Hosu *et al*., 2021). Antibiotic inactivation of enzymes is the most common mechanism in *P. aeruginosa* and is characterized by its ability to produce AmpC β-lactamases and hydrolyze β-lactams rather than inhibiting them (Meletis and Bagkeri, 2013). Moreover, the increased production of AmpC enzymes in *P. aeruginosa* can cause resistance to virtually all β-lactams. Therefore, the present study aimed to reveal the prevalence of β-lactamase enzymes and the molecular investigation of *AmpC* gene expressing AmpC β-lactamases and their role in multiple antibiotic resistance in *P. aeruginosa* in some areas of Iraq.

MATERIALS AND METHODS

Isolates collection

One hundred and fourteen of *P. aeruginosa* were obtained from Shirqat General Hospital and distributed to different clinical infections, including wounds, burns, UTIs, ear infections, and sputum. The isolates were collected from August 1, 2020, to February 1, 2021. Then, it was kept for a short period in a sterilized peptone water tube until a diagnosis was made.

Ethical approval

The study protocols were approved by the Ethics Committee of Scientific Research (ECSR) in the Faculty of Pure Sciences - University of Tikrit – Iraq (No. 2024.1- date. 5. 03.2024), with written consent obtained from all participants.

Identification of the bacterial isolates

The samples were grown in nutrient broth, diluted using the serial dilution method on Nutrient, MacConkey, and Blood agar, and purified on Cetrimide agar. The phenotypic characteristics were diagnosed according to Levinson (2016) and the microscopically diagnosed characteristics were studied according to Betsy and Keogh, (2005). Biochemical tests were performed, which included oxidase, catalase, IMViC (IMViC, a group of individual tests, includes I for indole, M for methyl red, V for Voges-Proskauer, and C for citrate utilization) H_2S gas formation, motility, gelatin decomposition, and urease tests. The tests were carried out as stipulated by (Procop *et al*. 2017), and finally, the confirmatory diagnosis was made using the VITEK-2 compact system.

Antibiotics susceptibility

The assay was conducted according to Syal *et al*. (2017), and 12 antibiotics (supplied by Bioanalyse_Turkey) were used, namely Ampicillin (25 µg), Aztreonam (30 µg), Cefotaxime (30 µg), Chloramphincol(30 µg), Ciprofloxacin (10 µg), Gentamycin (10 µg), Impienem (10 µg), Nalidixic acid (30 µg), Rifampin(5 µg), Tetracyline(10 µg), Trimethoprim(10 µg) and Vancomycin (30 µg). The inhibition zone diameters were measured according to the Clinical and Laboratory Standards Institute (CLSI, 2021).

β-lactamase production

The Iodometric standard method (AL-Rodhan 2008) was used to investigate the ability of bacterial isolates to produce β-lactamase enzymes. The isolates of *P. aeruginosa* at 24 hours of age were prepared. A sterile loop was used to inoculate the Eppendorf tubes containing 100 ml of Penicillin G solution into the bacterial

culture, and the tubes were incubated at 37 °C for 30 minutes. 50 µl of starch solution was mixed with the contents of the tube. Finally, 20 µl of iodine solution was added to the solution. The result was positive for the presence of β-lactamase enzymes when a color change from white to dark blue occurred within 10 minutes of adding the iodine reagent due to the reaction of iodine with starch.

Detection of Extended-spectrum β-lactamases (ESBLs) production

CHROM agar ESBLs

The medium for the CHROM agar test for detection of ESBLs was prepared according to (Uyanga *et al*. 2019). The steps include dissolving 33 g of CHROM agar in 1000 ml of distilled water. Then 10 ml of ESBLs supplement ES372 was added. The medium was sterilized with an autoclave at 121 °C, 1.5 bar, for 15 minutes, left to cool to 45 °C, then poured into a sterilized Petri dish, left to solidify, and stored in dark conditions in the refrigerator. Sterilized medium was cultured and incubated at 37 °C for 24 hours; a positive result was returned when the bacterial isolates could grow.

Double disc synergy test (DDST)

The DDST assay was performed as described by Gazin *et al*. (2012) and was used to detect the production of AmpC enzymes. The isolates to be tested were cultured on a Muller-Hinton agar plate and left to dry for 10 minutes. An amoxicillin-clavulinic acid (Augmentin) disc was placed in the center of the plate, and a Cefotaxime and Ceftazidime discs were placed 20-30 mm from the center of the medial disc. The plates were incubated at 37 ºC for 16–18 hours. A widening of the inhibition zone was observed toward either side discs, which is evidence of the absence of AmpC enzymes. Extension is the result of inhibiting the growth of bacteria. This means that the bacteria possess the AmpC enzymes that disrupted the clavulanic acid activity in the Augmentin tablet and continued to grow without inhibition.

Molecular investigation of the *AmpC* gene

DNA extraction was carried out for the isolates according to Chen and Kuo (1993), and they were electrophoresed according to (de Lipthay *et al*. 2004). The designed gene used in the study has a size of 610 base pairs (bp) and its sequence (F_TACCAGATTCCCCTGCCTGT, R_GAACAATTG CTGCTCCATGA), while the PCR reaction program for the gene was performed using specific thermo-cycler, with a final volume of 25 µL that consisted of 1 µL of each specific primer (10 pmol/L) plus 12.5 µL Master Mix PCR (Promega®) [Taq DNA polymerase (dNTPs, MgCl2, PCR buffer (pH 8.5))], 1.5 µL of nuclease-free water, and 3 µL of the bacterial lysate containing the DNA.

The amplification reactions were executed under the following conditions: 95 ºC for 5 minutes (initial denaturation) followed by 35 cycles of 95 ºC for 30 seconds; annealing for 30 seconds at temperatures 59 ºC; extension at 72 ºC for 30 seconds; and final extension at 72 ºC for 5 minutes.

Gene amplification was determined by electrophoresis by applying 8 µL of each PCR product on agarose gels (1.5% (w/v) in Tris-acetate-EDTA buffer (TAE: 40 mM Tris-acetate and 1 mM EDTA)). A 100 bp DNA ladder (Promega Corporation, Madison, USA) was included in each run. After electrophoresis, the agarose gels were stained with a safe stain (0.5 µg/mL) and photographed under ultraviolet light (UV) at 260 nm.

RESULTS AND DISCUSSION

Susceptibility of bacteria to tested antibiotics

Psuedomonas aeruginosa isolates showed high resistance to Ampicillin, Nalidixic acid, Trimethoprim, Vancomycin, Tetracycline, and Rifampin with 100%,

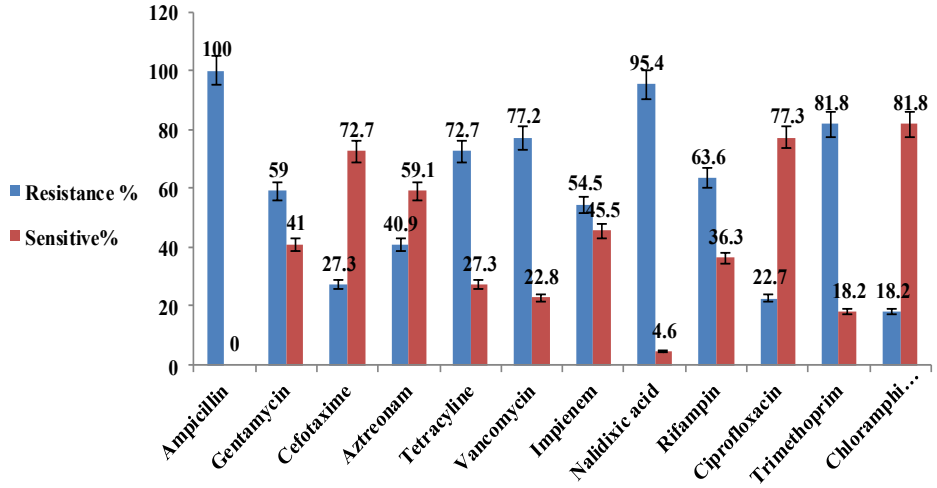

Fig. 1. *Percentage of resistance and sensitivity of Psuedomonas aeruginosa isolates to antibiotics*

95.4%, 81.8%, 77.2%, 72.7%, and 63.6%, respectively. However, moderate resistance appeared to be to Gentamycin, Imipenem, and Aztreonam, with 59%, 54.5% and 40.9%, respectively, whereas less resistance appeared to be to Chloramphenicol, Ciprofloxacin and Cefotaxime at 18.2%, 22.7%, and 27.3%, respectively, as shown in Fig. 1.

Rada *et al*. (2022) indicated that the group of β-lactam antibiotics, especially ampicillin, was of little efficacy in treating infections caused by *P. aeruginosa* by inhibiting the synthesis of peptidoglycan in the bacterium's cell wall. Due to its ability to produce β-lactams enzymes (Penicillinase), which break down the β-lactam ring of antibiotics, modify the antibiotics' structure, and nullify its effect (Bush and Bradford, 2016). In addition, it possesses many mechanisms that enable it to resist different types of antibiotics, such as increasing the efflux of antibiotics out of cells using efflux pumps, modifying the structure of outer membranes, and resistance genes (Tran *et al*., 2021).

β-lactamase production test

The finding of BLs appeared of the 114 isolates, 42 were distributed as follows: 14 wounds, 11 UTIs, 9 burns, 5 ears, and 3 sputum that were not BLs producers. In contrast, 72 isolates were positive for producing the β-lactamase, distributed as wounds, burns, UTIs, ears, and sputum as follows: 25, 22, 12, 7, and 6. The statistical analysis results showed no significant differences between the abilities of *P. aeruginosa*. The production of β-lactamase enzymes and the source of the isolates are shown in Fig. 2 and 3.

The result was almost the same as that of Hong *et al*. (2015), who showed that the high rate of *P. aeruginosa* produced β-lactamase is due to the presence of a permeability barrier, making changes in the target site, or possessing efflux pumps (Hong *et al*. 2016 ; Codjoe and Donkor 2018).

Detection of ESBLs Production

CHROM agar and ESBLs assay

CHROM agar and ESBLs medium were used to detect the ability of *P. aeruginosa* isolates to produce ESBLs. The test results showed that all isolates were 100% productive of ESBLs, as shown in Fig. 4.

The result was not as in the findings of Shaikh *et al*. (2015), who obtained 25.1% of his isolates were producing ESBLs. The difference in the results may be due to the geographical location, where the prevalence of ESBLs varies from one region to another (Uyanga *et al*., 2019). Rajaee Behbahani *et al.* (2019) mentioned that *P. aeruginosa* can produce ESBLs and their genes are carried on plasmids, which helped their spread among different kinds of bacteria, and this led to an increase in bacterial resistance to β-lactam antibiotics during the last 20 years, as indicated by (Winokur *et al*., 2001), that enzymes are encoded by large plasmids ranging from 80 to 300 kbp and are transmitted between different bacterial species, as well as carrying genes that encode resistance to other β-lactam antibiotics. The method is characterized by being one of the easiest, fastest, and most accurate methods for detecting ESBLs (Vázquez-Ucha *et al*., 2021), and the chromogenic medium contains the basic substance chromogen as a screening system. The chromogen leads to discoloration of the medium, indicating the enzyme's presence (Tran *et al*., 2021).

Double Disk Synergy Test (DDST)

The results of the test showed the presence of 22 isolates with a percentage of 19.2% that produced AmpC β-lactamase enzymes, whereas 92 isolates from different sources with a percentage of 80.7% showed their inability to produce AmpC β-lactamase enzymes. There were significant differences ($P < 0.05$) among the isolates producing AmpC enzymes isolated from five clinical sources. The non-producers isolates were distribut-

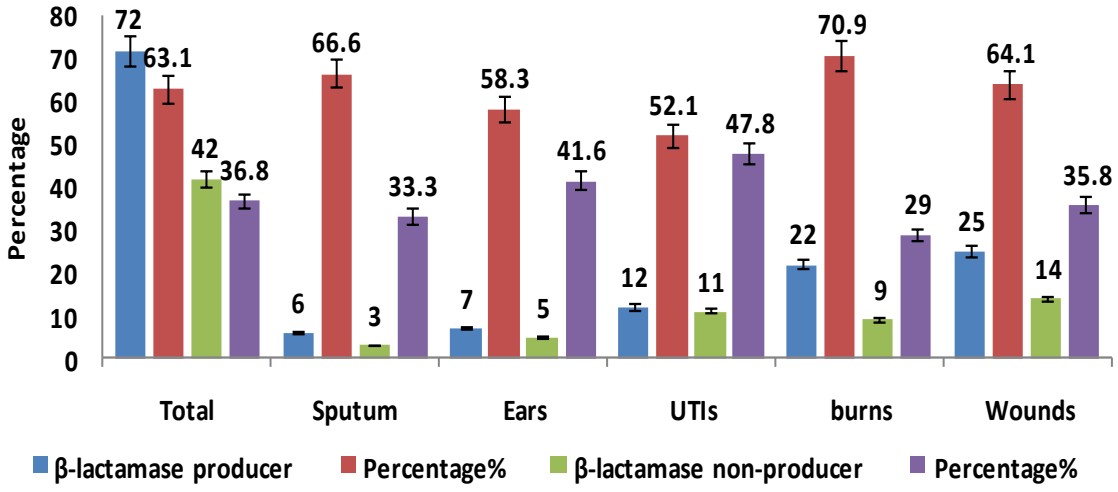

Fig. 2. *β-lactamase production assay for Psuedomonas aeruginosa isolates*

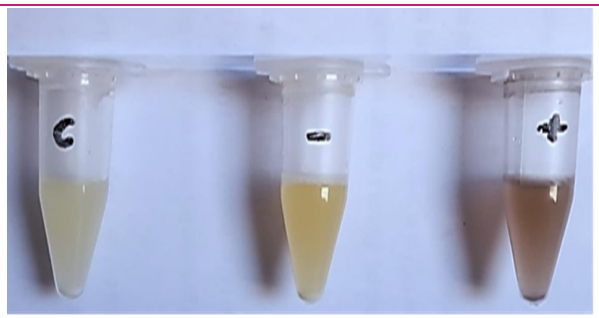

Fig. 3. *β-lactamase assay for Psuedomonas aeruginosa isolates. (C) Control sample, (+) β-lactamase producing, (-) non β-lactamase producing.*

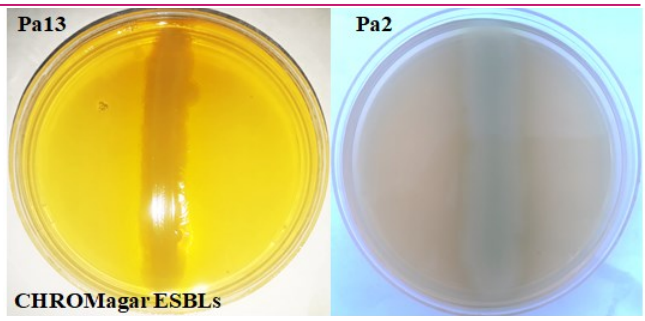

Fig. 4. *CHROM agar test to detect the production of ESBLs in isolates of Psuedomonas aeruginosa, Pa13 negative and Pa2 positive for ESBLs.*

ed between the sources of isolates (Fig. 5. (A)).

The results did not agree with the findings of Abd El-Baky *et al*. (2013), who obtained 72.4% of *P. aeruginosa* isolates that produced AmpC enzymes using the contiguous tablets method. Sridhar (2015) indicated that AmpC enzymes confer high resistance against Ceftazidime and Cefotaxime antagonists, and its genes are carried on plasmids, while Bush (2013) mentioned that AmpC enzymes, which are a subgroup of β-lactamase enzymes, are resistant to penicillins, cephalosporins, and monobactams. AmpC between bacterial species and from one place to another, the reason behind may be due to several reasons that led to the emergence of discrepancy in the rates of their isolation, such as antibiotics and how to use them, as the irregular use of these antibiotics encourages the spread of resistance genes, where AmpC enzymes are among the most antibiotics it is used in the treatment of many inflammatory conditions, due to its high efficacy and low cost these antibiotics and ensuring the sensitivity of the bacteria that cause infection to them is important, to limit the spread of resistant strains with an emphasis on conducting screening tests for ESBLs (Carcione *et al*., 2021). The findings in Fig. 5 (B) are the results of isolates numbered pa19 and pa11 that used specific antibiotics to induce the isolates to produce the β-lactamase enzymes.

Molecular investigation of *AmpC* gene

The entire isolates were ampicillin resistant, while 72 isolates were detected to have β-lactamase, and the results of ESBLs showed that all isolates had it. On the other hand DDST revealed 22 isolates that may have the *AmpC* gene phenotypically. Isolates that may possess the *AmpC* gene have been selected. The results of the investigation for the *AmpC* gene revealed that 18 isolates (81.8%) possess the gene, while 4 isolates (18.1%) do not possess the gene, and most isolates possessing the gene were isolated from wounds, UTIs, and sputum. The non-possessing isolates were isolated from ear infections of two isolates possessing the gene, and the statistical analysis results showed significant differences between the sources of isolates at the level of significance (P<0.01) as in Fig. 6.

The genes for β-lactamase and ESBLs increase the risk of the emergence of more resistant strains to multi-drug and lead to treatment failure. Its large production may cause resistance to Cephamycin antibiotics, greatly harming public health. Berrazeg *et al*. (2015) confirmed that the bacterial resistance in *P. aeruginosa* is due to its possession of chromosomally encoded AmpC enzymes.

P. aeruginosa isolates that possess chromosomal AmpC enzymes are distinguished by their resistance to cephalosporins. The genes of these chromosomal en-

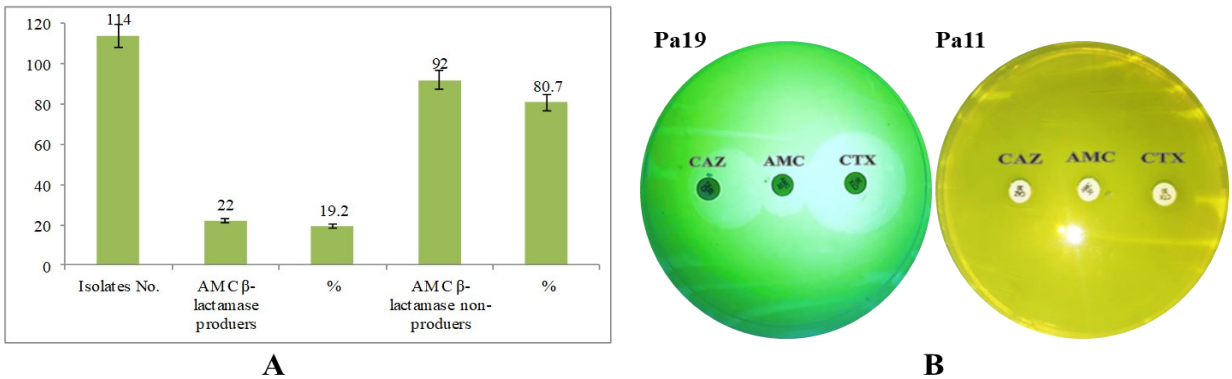

Fig. 5. *(A): DDST for ampicillin β-lactamase (AmpC) production by Psuedomonas aeruginosa isolates.) B): DDST to detect the ability of P. aeruginosa to produce AmpC enzymes, Cefazidime (CAZ), Amoxicillin clavulanic (AMC) and Cefotaxime (CTX). Pa19 AmpC negative, pa11 AmpC positive*

zymes, when transferred to plasmids produce β-lactamase on plasmids that help spread to other highly threatening bacterial species as they are resistant to cephalosporins commonly used in different parts of the world, which leads to the occurrence of epidemic cases (Moore *et al*., 2016). The resistance of bacterial isolates to cephalosporins provides preliminary evidence that they possess AmpC enzymes, which is insufficient for diagnosis. It had *AmpC* genes carried on the chromosome or the plasmid; therefore, molecular methods were used to confirm and determine their location (Barceló *et al*., 2022).

Four isolates showed that they do not possess the *AmpC* gene, as shown in Fig. 7. This may be attributed to Aghazadeh *et al*. (2014), who mentioned that other types of β-lactamase genes play a role in antibiotic resistance. These include *OprD*, *OprA*, and *OprM*. In addition, Bae *et al*. (2011) also indicated that the PER and VEB genes degrade Aztreonam antagonists, Penicillins, Oxyiminos, and Cephalosporins. The *AmpC* gene, which encodes for the production of AmpC enzymes molecularly, was investigated using PCR technology to determine the prevalence of the species producing these enzymes to control them and prevent or reduce their spread among other bacterial species, as phenotypic methods may give negative results in the case of one bacterial isolate possessing more than enzymatic type of AmpC β-lactamase enzymes and ESBLs. In this case, the bacterial isolate was not sensitive to clavulanic acid due to the presence of AmpC enzymes, and thus appears that it does not possess ESBLs enzymes, and some phenotypic tests may fail to detect some strains producing these enzymes. The investigation of these enzymes is important due to their ability to analyze β-lactam antagonists and their resistance to clavulanic acid and other inhibitors affecting ESBLs (Bottalico *et al*., 2022).

Conclusion

The present results indicated a broad spectrum of antibiotic resistance, underscoring the imminent threat posed by isolates of *P. aeruginosa*. The tests revealed the presence of various β-lactamases and ESBLs, explaining their antibiotic resistance and the challenges in treating different infections such as wounds, UTIs, burns, ears, and sputum. Genetic detection of the *AmpC* gene showed a high percentage of isolates possessing this gene, which encodes penicillinase enzymes resistant to penicillins. Consequently, new antibiotics and innovative approaches are imperative to address this bacterial menace. Targeting genes responsible for β-lactamases is essential to reduce

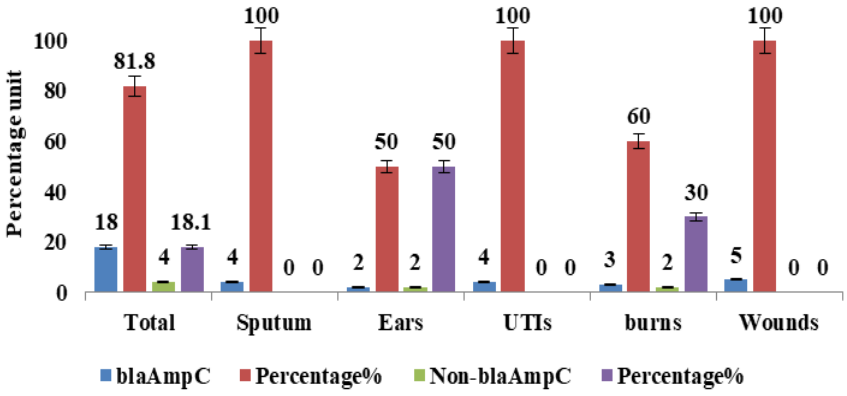

Fig. 6. *Detection of the AmpC gene in Psuedomonas aeruginosa isolates*

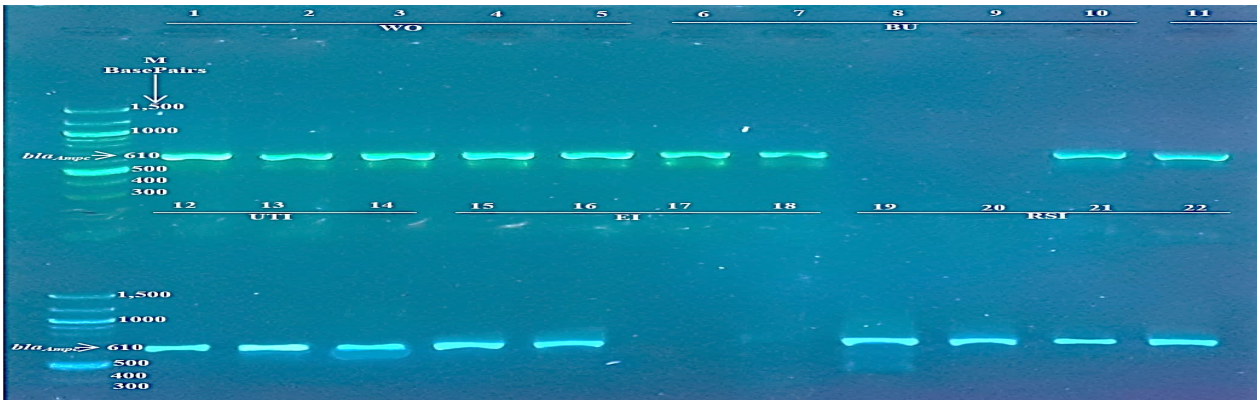

Fig. 7. *Electrophoresis of the of the DNA PCR using AmpC on agarose gel at a concentration of 1.5% M: 100 bp; WO: isolates of wounds (1-5), BU: isolates of burns (6-10), UTIs: isolates of urinary tract infections (11-14), EI: isolates of ear infections (15-18), RSI: isolates of respiratory infections (19-22)*

resistance to commonly used penicillin antibiotics. In response to the resistance of *P. aeruginosa*, it is imperative to explore alternative solutions, such as using safe nanoparticles, to overcome this challenge effectively.

Conflict of interests

The authors declare that they have no conflict of interest.

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