Biotechnological Journal of Environmental Microorganisms "BJEM"

Biotechnological Journal of Environmental Microorganisms(BJEM) 3(10) 2024 500-509

Molecular and Phenotypic Evaluation of Gentamicin Resistance in Clinical ısolates of *Acinetobacter* spp.

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Received:03 August 2024/ Revised 19 August 2024/ Accepted 24 August 2024

Abstract

Acinetobacter is an opportunistic pathogen involved in nosocomial urinary tract infections, bacteremia, wound and burn infections. *Acinetobacter* is responsible for difficult nosocomial infections due to its high resistance to the main antibiotics available. The spread of antibiotic resistance genes such as gentamicin through integron structures has become an important problem in the treatment of infections caused by *Acinetobacter.* The aim of the present study was to determine the phenotypic and molecular resistance to gentamicin in clinical isolates of *Acinetobacter.* In this study, 30 *Acinetobacter* were collected from medical centers in Lahijan and Amlash. The isolates were examined using various biochemical tests. The antibiotic resistance pattern of gentamicin in the isolates was determined by the disk diffusion method based on the CLSI standard. Also, the minimum inhibitory concentration (MIC) of gentamicin was determined on resistant Acinetobacter isolates by the tube dilution method (macrodilution) and according to the CLSI standard. Then, the genomic DNA of the isolates was extracted by boiling method and the frequency of the *aac(3)-IV* gene was determined using the PCR method. According to the results of this study, resistance to gentamicin was determined in 40% of the isolates by the disk diffusion method. Also, resistance to gentamicin was reported in 33.33% of *Acinetobacter* isolates by the macrodilution method, with the highest and lowest MIC values being 125 µg/ml and 7.81, respectively. The frequency of the aac(3)-IV gene was observed to be 30%. These results indicate the essential role of the *aac(3)-IV* gene in the development of resistance to gentamicin. Determining the frequency of gentamicin resistance in native isolates of Guilan using phenotypic and molecular methods is helpful in determining an effective treatment protocol.

Key words: *Acinetobacter,* Gentamicin, Phenotypic, Molecular, *aac (3)-IV* gene

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doi:10.71886/bioem.2024.1217872

Introduction

Acinetobacter is one of the most important causes of hospital-acquired infections. *Acinetobacter* is a gram-negative coccobacillus that can be isolated from many human and environmental sources and is more prevalent in the summer than in other seasons. This bacterium is a gram-negative, non-fermentative, obligate aerobic bacterium that is generally found in soil, water, and wastewater(Mohammadi et al., 2020). *Acinetobacter* is an opportunistic pathogen involved in nosocomial urinary tract infections, bacteremia, wound infections, and burns. *Acinetobacter* is responsible for difficult nosocomial infections due to its high resistance to the main antibiotics available(Bergogne-Berezin, 1994). Aminoglycosides are widely used to treat infections caused by *Acinetobacter baumannii*, although the number of resistant isolates reported has increased in recent years(Mak et al., 2009). Aminoglycosides are natural or semisynthetic antibiotics derived from actinomycetes. They were among the first antibiotics to be introduced for routine clinical use, and several have been approved for use in humans. Aminoglycosides were widely used as first-line antimicrobials in the early days, but were eventually replaced by cephalosporins, carbapenems, and fluoroquinolones in the 1980s(Eliopoulos et al., 2007). Resistance of *Acinetobacter* species to aminoglycosides results primarily from inactivation of the antibiotic by modifying enzymes. Several aminoglycoside modifying enzymes, including acetylases, adenylases, and phosphorylases, have been identified in *Acinetobacter* species. Genes encoding aminoglycoside modifying enzymes may be located on plasmids and transposons, and some of these genes have been found on class 1 integrons in MDR *Acinetobacter* isolates in Europe(Moniri et al., 2010). Aminoglycoside resistance is mediated by the *aacC1* genes (encoding acetyltransferase that confers resistance to gentamicin), the aadA1 gene (encoding adenyltransferase that confers resistance to streptomycin and spectinomycin), the *aadB* gene (encoding adenyltransferase that confers resistance to gentamicin, tobramycin, and kanamycin), and the *aphA6* gene (encoding a phosphotransferase that confers resistance to gentamicin, amikacin, kanamycin, and neomycin).

Resistance to penicillins and cephalosporins is mediated by the beta-lactamase enzyme encoded by the *ADC7* gene or the *OXA*set gene(Hujer et al., 2006). Gentamicin is active against most gram-positive cocci, including penicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. The susceptibility of some types of *Streptococci* is variable, whereas gram-negative cocci are generally resistant. Gram-positive bacilli are also relatively resistant to the concentrations of gentamicin commonly found in plasma and other biological fluids, Gram-negative bacilli, including *Aerobacter*, *Klebsiella*, and *E. coli*, are quite susceptible to low levels of gentamicin, and about 95% of all strains of these organisms are susceptible to its achievable levels. For example, the susceptibility of *Pseudomonas aeruginosa* to clinically achievable levels of gentamicin varies from 91% to 99% in most series. Many isolates of *Mycobacterium tuberculosis* are also inhibited. Such inhibition is comparable to the activity of streptomycin(Wang et al., 2021). The aim of this project is to investigate antibiotic resistance to gentamicin in clinical isolates of *Acinetobacter* bacteria. First, using molecular methods, genes associated with resistance are identified. Then, using phenotypic evaluation, the level of resistance and its impact on bacterial behavior are analyzed. This study helps to better understand the mechanisms of resistance and determine its prevalence. Ultimately, the results can help improve the treatment of infections caused by this bacterium in clinical settings.

Materials and Methods

Sample Collection

In this study, various clinical samples (including urine, blood, wound, tracheal, sputum, and other samples) were collected from patients hospitalized or referred to Shafa Hospital in Lahijan and Hekmat Amlash Laboratory in a cross-sectional and random manner. After collecting 30 clinical samples of *Acinetobacter*, they were transferred to the Microbiology Laboratory of Azad University, Rasht Branch, and re-identified. The confirmed clinical samples were stored in a -20°C freezer with glycerol for further testing.

Identification and confirmation of collected clinical specimens of *Acinetobacter*

For confirmation and purification, these 30 clinical specimens were cultured on MacConkey agar medium. MacConkey agar medium was prepared according to the manufacturer's instructions. After linear culture of clinical specimens of *Acinetobacter,* they were incubated at 37°C for 24 hours.MacConkey agar is a selective medium for the isolation and identification of gram-negative bacteria. Bile salts and crystal violet inhibit the growth of gram-positive bacteria. The purple color of colonies of lactose-positive bacteria is related to the acid reaction resulting from the fermentation of lactose sugar in the presence of bile salts and the absorption of neutral red(Elazhary et al., 1973).

Gram staining

Clinical isolates of *Acinetobacter* were first cultured and fixed onto glass slides. The Gram staining procedure was then performed in sequential steps. Initially, crystal violet was applied to the smear for 1 minute to uniformly stain bacterial cells, followed by gentle rinsing with distilled water to remove excess dye. Subsequently, a few drops of iodine solution were added and left for 1 minute, then the slide was rinsed again with distilled water. For decolorization, the slide was held at a 45-degree angle while a solution of acetone-alcohol was applied for 10 seconds, followed by immediate washing with water. Finally, the counterstain safranin was applied for 30 to 60 seconds and then rinsed off. Slides were air-dried, covered with a coverslip, and examined under a light microscope using an oil immersion lens to assess staining characteristics and bacterial morphology. This method facilitates differentiation between Gram-positive and Gram-negative bacteria(Cappuccino, 2018).

SIM test

According to the manufacturer's instructions, this medium was prepared and distributed in test tubes. Then it was sterilized at 121°C for 20 minutes. After closing the culture medium inside the tubes, a colony was removed from the nutrient agar medium with a needle sterilized with a gas flame and placed inside the tube. Finally, the cultured tubes were placed in a greenhouse for 24 hours at 37°C. This semi-solid medium is used to determine SH2 production, indole, and motility(Elazhary et al., 1973).

TSI test

The Triple Sugar Iron (TSI) agar medium was prepared according to the manufacturer's instructions. The medium was initially dissolved in a flask and sterilized by autoclaving at 121°C for 20 minutes. Once cooled to an appropriate temperature, the sterile medium was aliquoted into test tubes in a slanted position to solidify. Under sterile conditions, bacterial inoculation was performed by stabbing the deep butt of the medium with a sterile needle vertically and streaking the slant surface in a zigzag manner. The inoculated tubes were then incubated at 37°C for 24 hours. The TSI medium contains 1% lactose, 1% sucrose, and 0.1% glucose, with phenol red as a pH indicator to detect acid production resulting from carbohydrate fermentation. This medium is commonly used to differentiate bacterial species based on their ability to ferment glucose, lactose, and sucrose, as well as to detect hydrogen sulfide gas production. The variation in sugar concentrations enables differentiation of metabolic capabilities, while phenol red color changes reflect pH shifts linked to fermentation processes(Elazhary et al., 1973).

Oxidase test

To perform this test, first the oxidase disk was placed on a clean slide. Then a drop of sterile physiological serum was poured on it and then the bacterial colony was picked using a non-metallic tool (such as the end of a wooden swab) and placed on the oxidase paper(Elazhary et al., 1973).

Preparation of 0.5 McFarland Standard

This solution is used to determine the turbidity of the standard bacterial suspension in the antibiogram test. To prepare 0.5 McFarland standard, 0.5 ml of 0.1% M barium chloride was added to 9.5 ml of 1% sulfuric acid. The absorbance of the prepared solution was read at a wavelength of 625 nm. Then, it was poured into a dry and clean tube, completely blocked in the tube, and stored at room temperature in the dark. This solution is equivalent to 1.5×108 cfu/ml of bacterial solution.

To prepare a microbial suspension equivalent to half McFarland, fresh (24-hour) colonies of *Acinetobacter* were inoculated into a tube containing sterile physiological serum, then by comparing its turbidity with the turbidity of the standard half McFarland solution, a microbial suspension equivalent to half McFarland was prepared. The optical absorbance or OD of this suspension at a wavelength of 625 nm should be about 0.08 to 0.13(Elazhary et al., 1973).

Disk Diffusion Method

In the disk diffusion method, a bacterial suspension with a turbidity of 0.5 McFarland was first prepared. For this purpose, nutrient broth medium was prepared according to the manufacturer's instructions. After sterilizing and cooling the medium, a colony was removed using a sterile loop and added to the nutrient broth medium and incubated for 24 hours in a 37°C incubator. After 24 hours, an appropriate amount of bacteria was mixed in physiological serum to reach a turbidity of 0.5 McFarland. After preparing the bacterial suspension, a 24-hour microbial suspension with a turbidity equivalent to half McFarland was cultured on Mueller Hinton Agar medium using sterile swabs. Using sterilized forceps, the antibiotic gentamicin disks were removed and carefully placed on the plate (after 15 minutes of cultivation) under the hood and in its center. After 15 minutes of placing the discs, the plates were incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured with a ruler and interpreted according to the CLSI standard. Based on the diameter of the zone of inhibition, the isolates were divided into 3 groups: susceptible, semi-susceptible, and resistant(Tendencia, 2004).

Determination of antibiotic resistance by the dilution method in the tube (macrodilution)

This test was performed by the macrodilution method in Mueller Hinton Broth medium and according to the CLSI standard instructions.Using physiological serum, 1 ml of successive dilutions of the antibiotic gentamicin was prepared in sterile tubes. Then, 1 cc of the culture of Acinetobacter under study with a turbidity equivalent to 0.5 McFarland tube was added to each dilution and the tubes were placed in a 37 ° C incubator for 24 hours. After the incubation time, the presence of turbidity resulting from bacterial growth in different concentrations of the antibiotic was examined.

Gentamicin (4 µg / ml) was used in this method. To prepare 4 µg/ml gentamicin, first one ml of gentamicin ampoule (80mg/2ml) was dissolved in 9 ml of sterile physiological saline and finally one ml of the obtained solution was dissolved in 9 ml of physiological saline. In this way, a concentration of 400 µg/ml of gentamicin was prepared. Then, from this concentration of the drug, serial dilutions were performed in 10 test tubes in Mueller Hinton Broth culture medium. Then, 100 µl of a 24-hour culture of Acinetobacter with half McFarland turbidity was added to all tubes except the eleventh tube (medium control). The tubes were incubated for 24 hours at 37°C(Shanholtzer et al., 1984).

DNA extraction

DNA extraction using the boiling method was performed as follows: Fresh colonies from 24-hour clinical cultures of *Acinetobacter* isolates were selected for DNA extraction. One to three colonies from each sample were suspended in 1.5 mL microtubes containing 200 µL of sterile distilled water. The suspensions were vortexed until completely homogenized. The microtubes were then placed in a water bath at 100°C for 10 to 15 minutes, ensuring that the water level covered two-thirds of the tube height. After boiling, the samples were centrifuged at 14,000 × g for 5 to 10 minutes. The supernatant, containing the extracted DNA, was carefully transferred to a new sterile microtube for downstream applications such as PCR. This boiling method offers a rapid, cost-effective, and simple approach to obtain bacterial DNA suitable for molecular analyses(Ahmed & Dablool, 2017).

PCR test

For amplification of the *aac(3) IV* gene in Acinetobacter, the primer pair designed by Van de Klundert et al. was utilized. The nucleotide sequences of the primers used in this study are listed in Table 3-5. Primers were synthesized by Bioneer, South Korea, and received in lyophilized form from Takapouziist Company.

According to the manufacturer's instructions, stock solutions of each primer were prepared at a concentration of 100 pmol/µL using sterile deionized distilled water, and stored at -20°C. For PCR reactions, working primer solutions were prepared at 20 pmol/µL by mixing 10 µL of the stock solution with 40 µL of sterile deionized distilled water. This primer set targets a 286 bp fragment of the *aac(3) IV* gene, which encodes an aminoglycoside acetyltransferase involved in gentamicin resistance. This methodology enabled specific and sensitive detection of the resistance gene in clinical *Acinetobacter* isolates(Van De Klundert & Vliegenthart, 1993).

Electrophoresis method

The PCR product was loaded onto a 1% agarose gel and electrophoresed. For this, 5 µl of the PCR product was poured into the wells. 4 µl of the 100 base pair marker (Ladder) was removed and poured into one of the wells (refrigerated materials were placed on ice packs during all stages to prevent material deterioration). Due to the negative charge of DNA, the PCR products were placed on the negative pole of the electrophoresis machine so that it would move from the negative pole to the positive pole during electrophoresis. Sterile distilled water was used as a negative control instead of DNA. Then the tank lid was closed and the wires were connected to the main electrical power supply. Electrophoresis was performed at a voltage of 120 V for 45 minutes. Finally, the gel was photographed under UV light using a transilluminator. Thus, the abundance of the aac (3) IV gene was determined by PCR using specific primers(Bier, 2013).

Results

Confirmation of clinical samples of *Acinetobacter*

Various microbiological and biochemical tests were used to confirm clinical samples. To identify Acinetobacter, Gram staining (gram-negative bacilli), negative oxidase test, lack of motility in SIM test, lack of pigment production in Mueller-Hinton agar culture medium, lack of lactose fermentation in MacConkey agar medium were performed, and as a result, 30 samples were confirmed as *Acinetobacter.*

Results of determining antibiotic susceptibility of *Acinetobacter* to gentamicin by disk diffusion method All 30 clinical samples of *Acinetobacter* were examined for antibiotic susceptibility to gentamicin by disk diffusion method (Figure 1). The resistance rate to gentamicin is given in Table 1.

Results of determining antibiotic susceptibility of *Acinetobacter* to gentamicin in resistant samples using the MIC method

The antibiotic susceptibility of *Acinetobacter* isolates resistant to gentamicin was determined using the MIC macrodilution method (Figure 2). Among the 12 gentamicin-resistant isolates, 1 isolate had an MIC of 125 µg/mL, 5 isolates had an MIC of 62.5 µg/mL, 4 isolates had an MIC of 31.25 µg/mL, and 2 isolates exhibited an MIC ≤15.62 µg/mL.presents the percentage of gentamicin-resistant *Acinetobacter* isolates based on MIC results. According to CLSI standards (2020), 10 isolates (83.33%) out of the 12 resistant isolates were classified as resistant by MIC testing. The results showed that 10 isolates (33.33%) demonstrated the highest inhibitory concentration in the presence of gentamicin. The highest and lowest MIC values recorded for gentamicin were 125 µg/mL and 7.81 µg/mL, respectively.

PCR test to identify the *aac(3)-IV* gene

With a specific primer for the *aac(3)-IV* gene, this gene was examined in all clinical isolates of *Acinetobacter* by PCR. Out of 30 clinical isolates of *Acinetobacter*, 7 samples (23%) carried the *aac(3)-IV* gene. According to the data obtained from the gentamicin antibiogram test by disk diffusion method, among these 7 isolates, 5 samples were resistant and 2 samples were semi-susceptible.Figure 3 shows the electrophoresis view of the PCR product of the gentamicin resistance gene (*aac(3)-IV*) on a 1% agarose gel.

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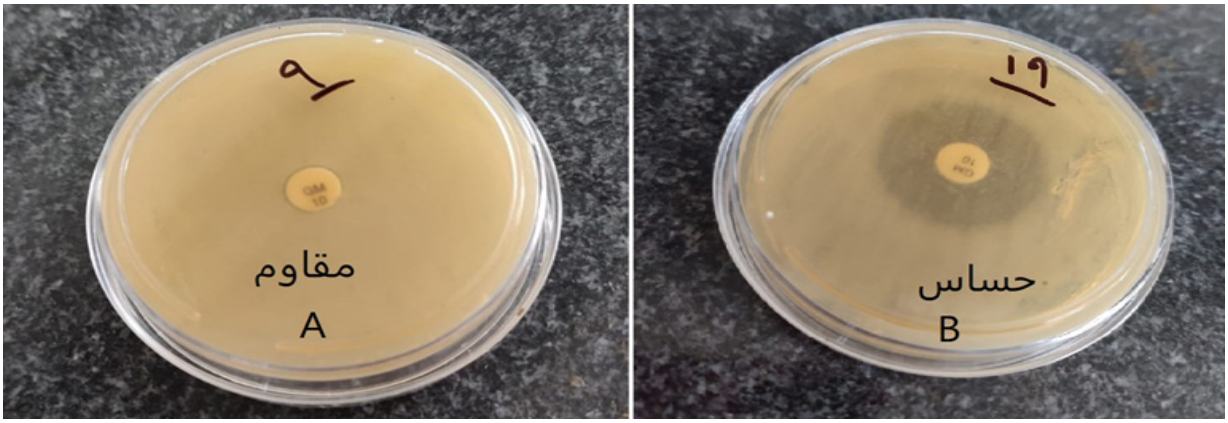

Figure 1: Gentamicin antibiogram test results in *Acinetobacter*. Left (A) results of isolates resistant to the antibiotic gentamicin. Right (B) results of isolates sensitive to the antibiotic gentamicin.

Table 1: presents the antibiogram results for 30 *Acinetobacter* isolates against the antibiotic gentamicin, categorized as sensitive, intermediate, and resistant based on CLSI standards using the disk diffusion method.

Antibiotic	Sensitive (S)	Intermediate (I)	Resistant (R)	Total
Gentamicin	11 (36.33%)	7 (23.33%)	12 (40%)	30 (100%)

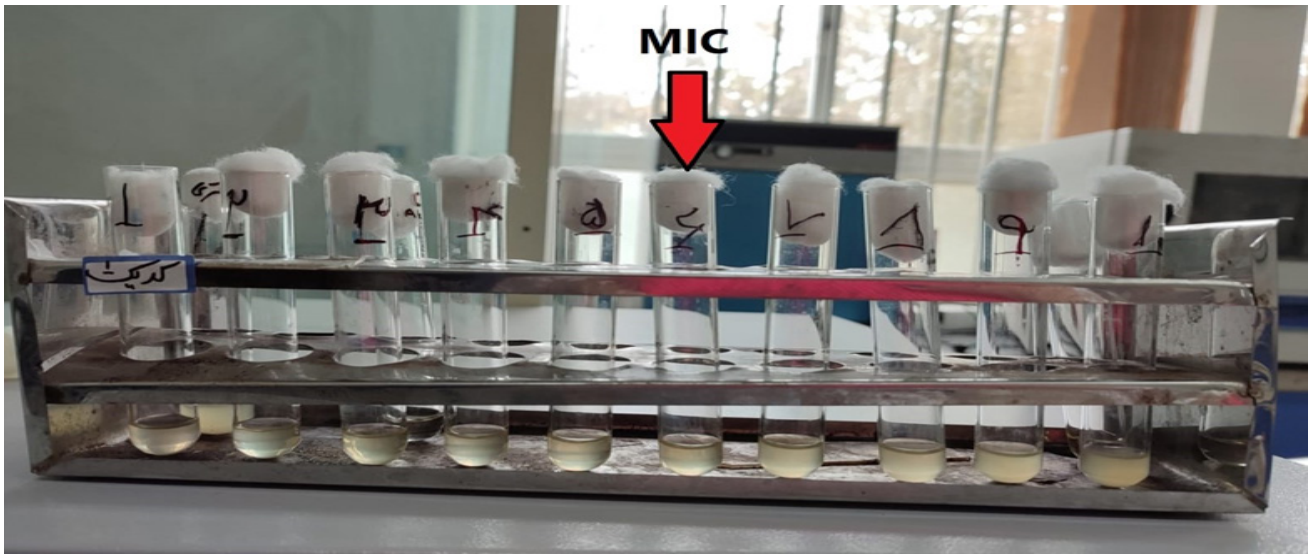

Figure 2:Results of MIC of gentamicin in resistant *Acinetobacter.* Tube number 6 is the MIC. This means the lowest concentration of antibiotic that was able to stop the growth of the bacteria.

Table2: The MIC results obtained by the macro-dilution method for all gentamicin-resistant *Acinetobacter* isolates are presented according to CLSI 2020 standards as follows:

Bacterial Code	Resistance Status	MIC (µg/ml)
1	Resistant (R)	62.5
5	Intermediate (I)	15.62
9	Resistant (R)	125
10	Resistant (R)	31.25
13	Resistant (R)	62.5
15	Resistant (R)	31.25
16	Resistant (R)	62.5
18	Resistant (R)	62.5
26	Susceptible (S)	7.81
27	Resistant (R)	62.5
29	Resistant (R)	31.25
30	Resistant (R)	31.25

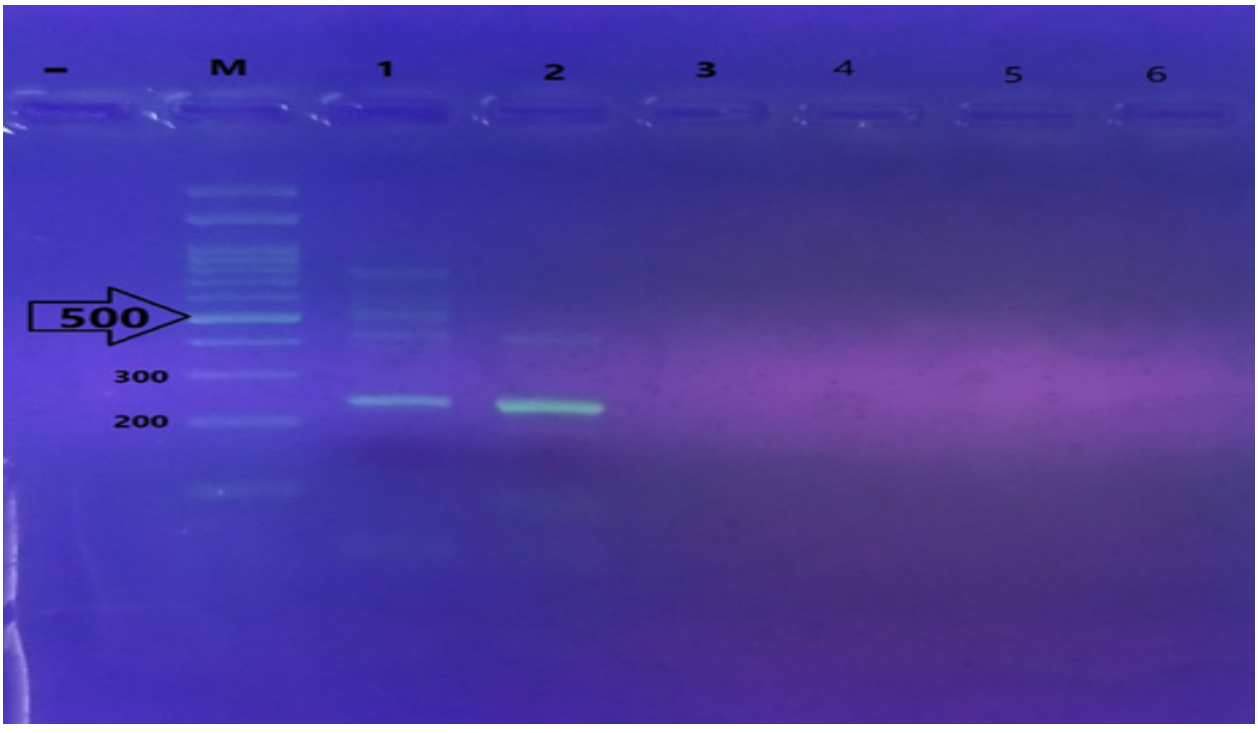

Figure 3 Results of PCR product electrophoresis to identify the gentamicin resistance gene aac(3)-IV on a 1% agarose gel. In the above image, the presence of the specific aac(3)-IV gene (286 base pairs) as a single band is observed in columns 1 and 2 (M:Ladder). The first column is the negative control and columns 3-6 are the negative samples.

Discussion

The present study aimed to identify phenotypic and molecular resistance to gentamicin in clinical isolates of *Acinetobacter* isolated from clinical samples of Lahijan and Amlash medical centers between June 2010 and September 2011.Based on the results of this study, out of 30 *Acinetobacter* samples examined by disk diffusion method, 12 (40%) samples were reported to be resistant to gentamicin. The minimum inhibitory concentration for these 12 gentamicin-resistant isolates was examined by MIC method, thus 10 samples (83.33%) of the 12 isolates were found to be resistant according to CLSI 2020. The highest MIC concentration was 125 µg/mL. Also, 8.33% of the isolates were reported to be semi-susceptible to gentamicin, indicating that these isolates may become resistant in the future, which requires further investigation and research. In the study of Farahani et al. (2009), antibiotic susceptibility was performed on 60 *Acinetobacter* species using the disk diffusion method and based on CLSI criteria. PCR was used to identify and amplify the genes under study (Hussain, 2022). In this study, 40% resistance to gentamicin was reported. Also, in the study of Tawakol et al. 2015, 40.31% resistance to gentamicin was observed (Tavakol & Momtaz, 2015). The results of these studies are similar to the present study. In the study of Ali Akbarzadeh (2014), the antibiotic susceptibility pattern to gentamicin was reported to be 86% (Tavakol & Momtaz, 2015), and similarly, Noormohammadi et al.2014 measured the antibiotic resistance of *Acinetobacter baumannii* isolates isolated from different clinical samples (urine, blood and sputum) to gentamicin using the Kirby-Bauer disk diffusion method and found 85% resistance to gentamicin (Alikhani et al., 2018) . In the study of Hossein and his colleagues 2022, the results of antimicrobial resistance showed that 95% of *Acinetobacter baumannii* isolates were resistant to gentamicin (Hussain, 2022). Also, in the study of Sarafan Sadeghi 2019, 64% resistance to gentamicin was reported using the Kirby-Bauer disk diffusion method (Sarafan Sadeghi et al., 2019). In a 2022 study by Hossain et al., molecular analysis of the aadA1 and *aac(3)-IV* gene showed that the aadA1 gene was detected in 15% of the isolates. However, the *aac(3)-IV* gene was not detected in any of the isolates (Hussain, 2022). Aishwarya et al.2020, studied aminoglycoside-modifying enzymes in Gram-negative bacteria that cause human infections. In this study, 386 clinical isolates were examined, of which 38 were *Acinetobacter baumannii.* The frequency of the *aac(3)-IV* gene in *Acinetobacter baumannii* was reported to be 5% (Aishwarya et al., 2020). In a study by Tavakol et al. 2015, a descriptive study was conducted in two hospitals in Tehran on 121 *Acinetobacter baumannii* isolates isolated from clinical infections, and the frequency of the *aac(3)-IV* gene was observed to be 56.19% (Tavakol & Momtaz, 2015). The results of these studies are inconsistent with the present study, which could be due to the use of a different method for DNA extraction. In this study, the frequency of genes encoding gentamicin-modifying enzymes in clinical isolates of Acinetobacter was determined. With a primer specific for the *aac(3)-IV* gene, this gene was examined in all clinical isolates of *Acinetobacter* by PCR. Of the 30 clinical isolates of Acinetobacter, 7 samples (23%) carried the *aac(3)-IV* gene.

Conclusion

These results indicate the essential role of the *aac(3)-IV* gene in the development of resistance to gentamicin. Determining the frequency of gentamicin resistance in native isolates of Guilan using phenotypic and molecular methods is helpful in determining an effective treatment protocol.

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