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Molecular detection of *arr2* Gene of Rifampcin Resistant *Acinetobacter baumannii* Isolated from Patients Referring Baghdad Hospitals

[1]Safaa H. Hussein, [1]Mayaada S. Mahdi

[1]College of Biotechnology, AL-Nahrain University

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Abstract: The *Arr* enzymes that catalyze ADP-ribosylation of rifamycins to render it inactive Which contributes to increasing bacterial resistance to this antibiotics , that genes encoding predicted *arr* enzymes are widely distributed in the genomes of pathogenic and nonpathogenic bacteria, This study aimed to isolate *A. baumannii* from various clinical specimens and detection of arr2 gene in rifampcin resistant *A.baumannii*. A total of (300) samples were collected from patients suffering from different infections in Medical City Hospital during the period from (1/7/2022) to (30/1/2023). The Vitek 2 system was used to identify bacterial isolates, and the Kirby-Bauer method was used to test antibiotic susceptibility. Among the collected clinical samples 88 (29.3%) were identified as *A. baumannii.* The majority of *A. baumannii* isolates were found in wounds (45.4%), followed by burns (30.6%), urine (14.7%), and sputum (9.0%). Antibiotic susceptibility showed that *A. baumannii* isolates had varying levels of resistance to the antibiotics used. It was high resistant to cefotaxime, Ceftriaxone, Rifampin, cefepime, Amikacin, Ampicillin-sulbactam and Trimethoprim- sulfamethoxazole at a percentage of (96.5, 86.3, 85.2, 84.0, 80.6, 78.4 and 70.4 %) respectively. While exhibit a moderate level of resistance to doxycycline, meropenem and Levofloxacin at a percentage (52.2, 53.4 and 67.0 %) respectively, thirty multidrug-resistant isolates including rifampicin, were subjected to molecular study by detecting the *arr2* gene, it was concluded that 9 isolates out of 30 isolates (30%) have *arr2* gene

Keywords: *A.baumannii*, Rifampin, Antibiotics Resistance.

Corresponding author: (Email: safaaalzuhairi06@gmail.com).

Introduction

Acinetobacter baumannii-related nosocomial infections are becoming more prevalent in Iraqi hospitals. Their ability to contaminate various surfaces in hospitals for extended periods of time has been linked to nosocomial outbreaks (1). This bacterium has developed its ability as it infects the general population and is not limited to hospital patients. The mortality rate in hospitals is 26%, rising to 43% in intensive care units (2). One of the most serious global public health issues is antimicrobial resistance**.** ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, and Enterobacter* spp.) have emerged as globally critical multidrug-resistant (MDR) pathogens that require ongoing monitoring and drug development (3). Additionally, rifampicin-transferable resistance in *A. baumannii* has been

connected to the arr-2 gene. This gene produces a rifampicin ADP-ribosylating transferase, which ribolyzes the drug to render it inactive Covalent modification of antibiotics is one of many methods used by bacteria of different genera, including pathogens, opportunistic pathogens, and non-pathogens, to render antibiotics inactive (4). Multidrug resistance patterns in Gram-positive and -negative bacteria have resulted in difficult-to-treat, if not untreatable, infections with standard antimicrobials. Because many healthcare settings lack early identification of causative microorganisms and their antimicrobial susceptibility patterns in patients with bacteremia and other serious infections, broad spectrum antibiotics are used liberally and often unnecessarily (5). Rifamycins are bactericidal antibiotics that inhibit RNA synthesis by interacting with the bacterial RNA polymerase subunit encoded by the *rpoB* gene. Rifampicin, rifabutin, rifapentine, and rifaximin are examples of rifamycins. Among these, rifampicin is the most commonly used first-line therapy for mycobacterial disease. Rifampin resistance is a growing issue in both developed and developing nations (6). Rifampin resistance is most likely caused by point mutations as well as small insertions and deletions in the *rpoB* gene, which encodes the RNA polymerase beta subunit (7). The rising rates of antibiotic resistance can be attributed to a variety of factors. The emergence of resistant strains is directly related to the magnitude of antibiotic consumption. Resistance mechanisms can be passed down from one bacterium to another either longitudinally (via relatives) or horizontally (via plasmids), the latter may result in resistance transmission between species. Inappropriate prescribing is another determining factor; studies have shown that 30% to 50% of antimicrobial therapies are prescribed incorrectly (8). This study aimed to isolate *A. baumannii* from various clinical specimens and detection of *arr2* gene in rifampcin resistant *A. baumannii*.

Materials and methods

Sample collection

A total of (300) samples were collected from patients suffering from different infections (burns, wound, sputum and urine samples) in Medical City Hospital during the period from (1/7/2022) to (30/1/2023). All samples were collected by sterile cotton swaps and then streaked on MacConkey agar and blood agar incubated for 24hr at 37ºC.

Identification of bacterial isolates

All clinical samples were cultured for 24 hours on CHROMagar, MacConkey agar, and Blood agar at 37°C. Each bacterial isolate was identified using morphological, microscopic, and biochemical tests such as Oxidase, Catalase, Triple sugar iron agar (TSI), Indole production, Urease production, and Citrate utilization. With a 98% confidence level, the Vitek-2 system was used to accurately identify each bacterial isolate.

Antibiotic susceptibility test

Antibiotic susceptibility test was performed using Kirby-Bauer method according to CLSI, (2021) guideline. In this test, 88 *A. baumannii* isolates previously identified by morphological characteristics and the VITEK-2 system were cultured in 5 ml of nutrient broth and incubated at 37°C for 24 h. The bacterial broth was centrifuged at 5000 rpm for 5 min, then the supernatant was removed and the bacterial precipitate was diluted until it reached 0.5 McFarland turbidity standard, which contains approximately $1\text{-}2\times10^{8}$ CFU/ml. Following that, a sterile cotton swab was dipped in a bacterial suspension and any excess liquid was

removed by pressing on the side of the tube before streaking on Muller-Hinton agar medium in various directions. The inoculated plates were left at room temperature for 10 min to allow excess moisture to be absorbed, then each antibiotic disk was gently pressed on the agar surface with a sterile forceps; no more than 5 discs were placed in each plate. The plates were incubated for 24 h at 37°C. Following incubation, the diameter of the inhibition zone, defined as the area with no obvious visible growth around the disk, was measured with a ruler and compared to the standard value of each drug as published by CLSI 2021.

DNA extraction

Using a commercial purification system (Promega/USA), the genomic DNA of rifampcin-resistant *A. baumannii* (30) clinical isolates was extracted, and the purity and concentration of the DNA were determined using a spectrophotometer (NanoDrop) by measuring the optical density (O.D). The extracted DNA was then stored for future use at -20°C.

PCR reaction mixture

The 0.2 ml Eppendorf tube held the master mix, primers, and nuclease-free water. According to Table (1), the PCR mixture's total volume was 25l.

Table (1): Reaction mixture of PCR.

Components	Reaction volume (µl)	Final concentration
Master Mix (2X)	12.5	1X
Forward primer (10 µM)	1	0.4 µM
Reverse primer (10 µM)	1	0.4 µM
DNA template	2	< 250 ng
Nuclease free water	8.5	------
Final volume	25 µl	------

According to the unique program for each primer pair as shown in Tables(2,3) the PCR tubes were put in the thermal cycler to start the amplification reaction.

Table (2): PCR amplification program of *arr2* genes for *A. baumannii*

Steps	Time (min)	Temperature (°C)	No. of cycles
Initial denaturation	5	95	1
Denaturation	2	95	30
Annealing	0.5	60	
Extension for *arr2*	0.5	72	
Final extension	5	72	1

Table (3): Furthermore, the primers used in this study are listed.

Primer	Sequence (5`----3`)	Primer Size (bp)	Tm	GC %	Product size (bp)	References
arr-2-F	tacaagcaggtgcaaggacc	20	65	55	349	Current study
arr-2-R	attcaacaggatgcccctcc	20	65	55		Current study

Results and discussion

The results showed that 103 isolates (34.4 %) gram positive, 110 isolates (36.6 %) gram negative, Only (88) isolates (29.3%) were found to have identical morphological and biochemical characteristics to *A.baumannii*. The majority of *A. baumannii* isolates were found in wounds (45.4%), followed by burns (30.6%), urine (14.7%), and sputum (9.0%). Clinical sample distribution across various clinical sources Which demonstrates that (45.4%) of the samples were taken from wounds, (30.6) % from burns, (14.7) % from urine, and only (9.0) % from sputum samples as shown in Table (4).

Table (4): The number and percentage of *Acinetobacter baumannii* from different sources.

Source of specimens	No. of samples (%)	No. of *A. banmannii* (%)
Wounds	40	45.4
Burns	27	30.6
Urine	13	14.7
Sputum	8	9.0

All of the isolates studied were gram-negative coccobacilli that occasionally organized as diplococci. Diagnosis of *A.baumannii* isolated from patients suffering from different infections are depend upon isolation and laboratory identification of the bacteria. The burns, wounds, sputum and urine swabs were directly streaked on MacConkey agar and blood agar, then incubated for 24 h at 37ºC. Colonies *of A.bauamannii* on MacConkey agar appeared as small, round, pale, lactose non-fermenter colonies and regular edges. MacConkey agar a selective and differentiating medium is also used to test the isolate's capacity to ferment lactose. While the smooth, spherical, white to cream-colored colonies of *A.baumannii* isolates on blood agar had entire edges. Colonies without hemolysis and a fishy odor which indicative the bacteria's inability to manufacture hemolysins, which are responsible for degrading red blood cells by rupturing their cell membrane. For further diagnosis of *A.baumannii* isolates a loopful of colonies from MacConkey agar that were not fermented with lactose were further subcultured on CHROMagar™ and incubated for 24 h at 37˚C, most isolates had showed a good growth on this medium with special characteristics of red colonies as shown in Figure (1). Furthermore, CHROMagar™ *Acinetobacter* selective medium had 100% sensitivity and specificity for *A. baumannii* isolation. It also includes substrates that allow for color-based identification of colonies recovered within 18 to 24 h of incubation. Furthermore the presence of the supplement CR102, which was used selectively for the screening of multi-drug resistant *A. baumannii* (MDRAB). Based on the results of the CHROMagar™ *Acinetobacter* medium and biochemical tests 88 isolates of *A.baumannii* were recovered from all collected bacterial isolates. The only *Acinetobacter* isolates that can grow on CHROMagarTM Acinetobacter medium. Moreover, *Pseudomonas* species with similar physical characteristics can grow in this media, but an oxidase test can distinguish between them easily.

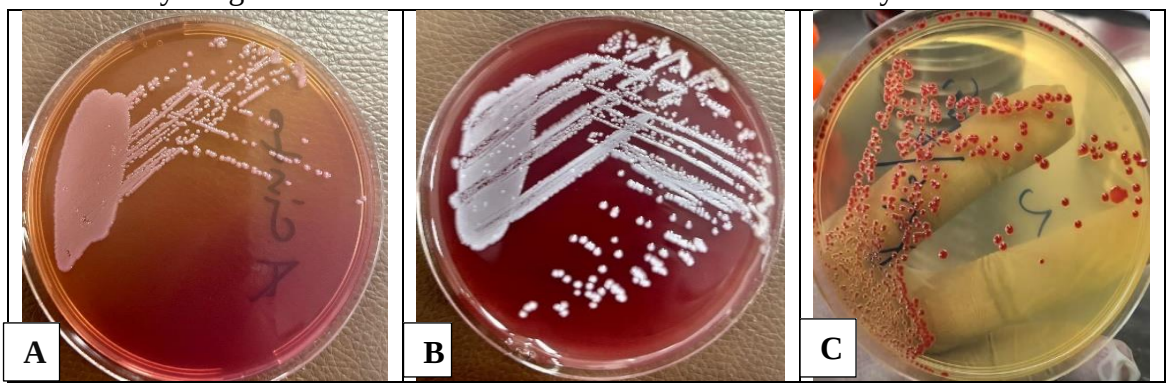

Figure (1): Growth of *Acinetobacter baumannii* on A. MacConkey agar, B Blood Agar, C CHROMagar*TM*

The Kirby-Bauer method was used to detect the antibiotic resistance profile of *A. baumannii* isolates. Eighty eight diagnosed isolates of *A.baumannii* were subjected to this test. The results revealed that *A.baumannii* isolates had varying levels of susceptibility and resistance to these antibiotics. The susceptibility test of *A.baumannii* isolates to 10 different antibiotics as shown in Table (5).

Table (5): Susceptibility test of *Acinetobacter baumannii* isolates to 10 different antibiotics.

Antibiotics	S Isolate No. (%)	I Isolate No. (%)	R Isolate No. (%)
Trimethoprim-sulfamethoxazole	17 (19.3%)	9 (10.2%)	62 (70.4%)
Levofloxacin	23 (26.1%)	6 (6.8%)	59 (67.0%)
Doxycycline	33 (37.5%)	9 (10.2%)	46 (52.2%)
Amikacin	11 (12.5%)	6 (6.8%)	71 (80.6%)
Meropenem	40 (45.4%)	1 (1.1%)	47 (53.4%)
Cefepime	12 (13.6%)	2 (2.2%)	74 (84.0)
Cefotaxime	2 (2.2%)	1 (1.1%)	85 (96.5%)
Ceftriaxone	7 (7.9%)	5 (5.6%)	76 (86.3%)
Ampicillin-sulbactam	15 (17.0%)	4 (4.5%)	69 (78.4%)
Rifampin	12 (13.6%)	1 (1.1%)	75 (85.2%)

S: Sensitive, I: Intermediate, R: Resistance.

Results showed an alarming rise in antimicrobial resistance to most classes of antibiotics, particularly the antibiotics of choice for treating *A.baumannii* infections The current study found that every clinical isolate of *A.baumannii* was highest resistant (96.5%) to cefotaxime. Additionally, a significant resistance to Ceftriaxone (86.3%), Rifampin (85.2%), cefepime (84.0%), Amikacin (80.6%), Ampicillin-sulbactam (78.4%), Trimethoprim- sulfamethoxazole (70.4%), While The doxycycline (52.2%), meropenem (53.4%) and Levofloxacin (67.0%). exhibit moderate level of resistance. The results of antibiotic susceptibility test in this study has been shown that meropenem was the most effective antibiotic against *A.baumannii* isolated from different infections, in which 45.4 % of isolates were sensitive to it. This outcome was closely coordinated with Al Meani *et al*. (2020) in Iraq who showed that 40 % of *A.baumannii* isolates were sensitive to meropenem (8). Which kills bacteria by binding to penicillin-binding proteins in the bacterial cell wall and inhibiting peptidoglycan cross-linking associated with cell wall synthesis, which results in cell death (9). A study in Iran-Tehran Fallah *et al.* (2014) disagree with the findings of our study where it was shown the *A.baumannii* isolates were high resistant to meropenem 91.7%(10). The antibiotics susceptibility test in our study showed the highest resistant of *A.baumannii* isolates to cefotaxime (96.5 %) and this agree with a study by AL-Taati *et al*. (2018) clinical isolates of *A. baumannii* were entirely resistant (100%) to cefotaxime(11). The results also indicated that 80.6 % , 70.4 %, 84.0% of *A.baumannii* isolates were resistant to Amikacin and Trimethoprim- sulfamethoxazole, cefepime respectively, A relatively comparable result reported by Qader, (12) in Iraq- Duhok revealed that amikacin and Trimethoprim-Sulfamethoxazole showed only slight effectiveness against *A.baumannii* isolates, with resistance rates of 88% and 93% respectively (12). Also local study reported by Numan *et al*. (2021)

exhibited a significant percentage of resistant, that *A. baumannii* isolates were resistant to Amikacin 81%, Trimethoprim-Sulfamethoxazole 87%, cefepime 100% (13). The current study exhibit that *A.baumannii* isolates were resistant to levofloxacin and doxycycline 67.0%, 52.2% respectively, a local study in Iraq Duhok by Abdullah and Merza, (2019) also mentioned a moderate level of resistance levofloxacin 51% and doxycycline 39% of *A.baumannii* isolates (14). A local study in Babylon reported by AL-Enawey *et al*. (2020) revealed a close finding to our study whereas *A.baumannii* isolates were resistant to levofloxacin 56% but low level of resistant to Doxycycline 12%. Ceftriaxone, Ampicillin-sulbactam and rifampin revealed high resistance pattern 86.3%, 78.4%, 85.2% respectively in all tested *A.baumannii* isolates (15). This outcome was in strong agreement with those obtained in Iraq – Babylon also showed a high level of resistant by Raheem and Al-Hasnawy, (2021) who recorded that 100% of *A. baumannii* isolates were resistant to ceftriaxone, 100% to Ampicillin-sulbactam and 95% to rifampin (16). A study conducted in Iraqi hospitals showed that More than 90% resistance was seen to ceftriaxone and beta –lactam antibiotics. The quality of the applied health system, the type of antibiotics that are locally available, as well as the timing and methods of using these antibiotics, may all contribute to the global variation in antibiotic resistance rates. Additionally, isolates of the multidrug-resistant *A. baumannii* bacteria may have a genomic structure that enables them to develop resistance genes in response to antimicrobial pressure (17).

Molecular study

According to the findings, the *arr2* gene is present in 9 out of 30 isolates (30%). The arr2-F and arr2 R primers were used to detect the *arr2* gene, and the amplified fragment measured 350 bp. as shown in (Figure 2). Covalent modification of antibiotics is one of many methods used by bacteria of different genera, including pathogens, opportunistic pathogens, and non-pathogens, to render antibiotics inactive These modifications include ribosylation, phosphorylation, acylation, glycosylation, nucleotidylation, and thiol transfer (19). By attaching to conserved amino acids in the active site of bacterial RNA polymerase, rifampicin prevents the start of transcription. Chromosome mutations that lead to changes in the active site's amino acids account for a sizable portion of rifampicin resistance (17). RIF resistance in *Acinetobacter* strains has been linked to the enzymatic modification of rifampin ADP-ribosyltransferase arr-2 (19). Because of the horizontal transfer of mobile genetic elements (MGE), such as bacteriophages, pathogenicity islands (SaPI), plasmids, transposons, and cassette chromosomes between bacterial isolates, this gene is not present in all isolates. MGE can also introduce genetic variation encoding for virulence, antibiotic resistance, and host adaptation (20). All isolates carrying the *arr2* gene were mostly resistant to all antibiotics, except for meropenem, which showed efficiency in most isolates of *A.baumannii* because previously published research has linked carbapenem resistance in *A. baumannii* to PBP changes More than other methods of resistance (21). The rest of the isolates that do not carry the arr2 gene It also showed resistance to rifampicin. This indicates the existence

of other mechanisms of resistance to rifampicin Instead of genes being transferred from other bacteria by mobile genetic elements, mutational changes are the primary cause of drug resistance in bacteria, rifampicin resistance is caused by mutations in the *rpoB* gene, which codes for the RNAP subunit, these changes lessen RNAP's affinity for rifampicin (22).

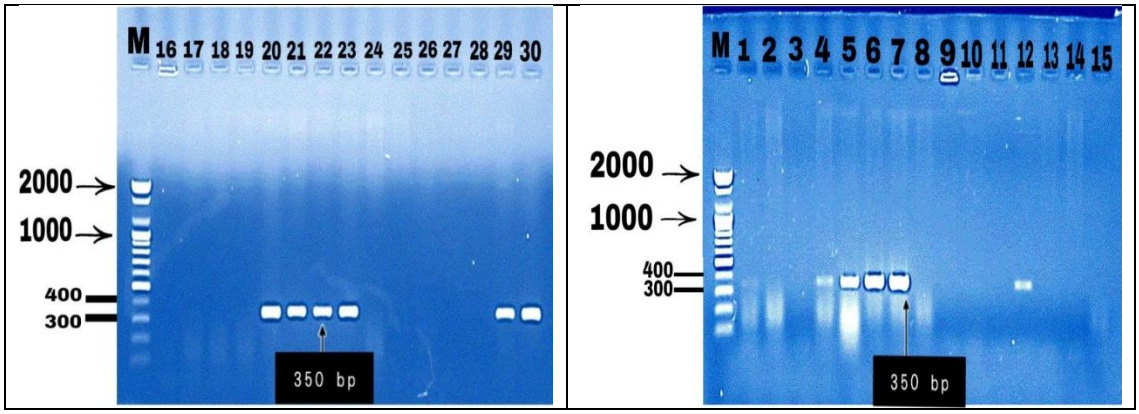

Figure (2): Amplified *arr2* gene of thirty bacterial isolates of *A.baumannii* isolates using specific primers (*arr-2-F and arr-2-R)*. Electrophoresis was done using 1% agarose for 1 h at 70 V. Lane M: 100 bp DNA ladder.

Conclusion

Many of the bacteria found in patient samples with wounds, burns, respiratory and urine infections were *A.baumannii* isolates. Additionally, other pathogenic bacteria like *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* can be isolated from cases of these infections. The majority of *A.baumannii* isolates came from wound infections and The most effective antibiotic against *A.baumannii* isolated from the infections mentioned above was meropenem. *A.baumannii* isolates showed the highest resistance to cefotaxime, ceftriaxone, and rifampcin. 30 percent of *A. baumannii* isolates have *arr-2*. The ability of these rifampicin-resistant determinants to spread through horizontal gene transfer makes it one of the most significant mechanisms of *A. baumannii* resistance to that drug. so the presence of *arr2* gene in *A. baumannii* play a prominent role in rifampicin resistant but it is not the only one mechanism to resistance.

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