Sys Rev Pharm 2020;11(10):1105-1110
A multifaceted review journal in the field of pharmacy

The Role of Efflux Pump *adeJ* Gene in Levofloxacin Resistance among *A.baumannii*

Ali M Abdelaal, Suhad S Mahmood

Department of Biotechnology, college of sciences, University of Baghdad, Iraq.

ABSTRACT

The rapid development of Acinetobacter baumannii resistant to almost all antibiotics even Colistin was making these bacteria a significant public health problem. This study aimed to determine the role of *adeJ* gene of RND-efflux pump, and it is a correlation with levofloxacin resistance in *Acinetobacter baumannii*. A total of 130 bacterial isolates were collected from patients admitted to Baghdad hospitals. Isolates were identified biochemically and conformed with Vitec 2 compact system, and only 70 isolates were confirmed as Acinetobacter baumannii. The Disc diffusion method for 8 types of antibiotics was used in this study to determine the antibiotics susceptibility for all isolate of *A.baumannii*. The results of antibiotics susceptibility test showed that the bacterium was resistance to Amikacin in percentage (61.42%). While it is resistant to Trimethoprim was (88.57%) followed by Piperacillin, Cefepime (82.85%), (71.42%) respectively. On the other hand, the resistance to Tetracycline, Ampicillin-sulbactam, Imipenem and levofloxacin was (47.14%), (61.42%), (54.28%), (51.42%) respectively. In addition, only 15 isolates were selected to determine the minimum inhibitory concentration of levofloxacin, and these 15 isolates were chosen because they were resistant to most of the antibiotics tested and it is MIC to Levofloxacin was ranging between 64 µg/100 µl to 128 µg/100 µl. Real-time PCR was used to determine the relative expression of *adeJ* gene of RND-*adeIJK* and its correlation with levofloxacin resistance in *A.baumannii*. The result shows the relative mean of *adeJ* was 3.1 for resistance group before exposing to levofloxacin and was 7.20 for resistance *A.baumannii* after exposing to levofloxacin and show significant difference (P=0.039). This study indicates that *AdeJ* has a role in providing resistance to levofloxacin .In addition; the overexpression of *adeJ* may be one of the reasons contributing to Resistance to a wide variety of antibiotics.

Keywords: A.baumannii, RND-efflux pump, levofloxacin, Real-time PCR, MIC

Correspondence:
Ali M Abdelaal
Department of Biotechnology, college of sciences, University of Baghdad, Iraq.

INTRODUCTION

Acinetobacter spp. is gram-negative, as well as it is non-fastidious, purely aerobic bacteria, coccobacilli, non-fermenting typically occur in diploid-formation or different-length chains. They are non-motile, oxidase-negative and catalase-positive (1). *Acinetobacter spp*. appears as a nosocomial pathogen with the capability to cause diseases such as Clinical infections, particularly wound infections, pneumonia and catheter-related bacteremia. *Acinetobacter baumannii* is the most prevalent of the different species that cause infection medically and epidemiologically, and it is the main pathogen with mortality rate reaches 60% in patients (2), (3).

The resistance against multiple antibiotics needs to be understood, as well as successful controlling dependent on them is necessary. Research has displayed that efflux pumps perform a significant role in the evolution of drug resistance (4). *A.baumannii*, possess various antibiotic resistance mechanisms among of which the antibiotics outflow pump, which is a transport located in the cytoplasmic membrane of bacteria. It is a mechanism responsible for removing antibiotics from the bacterial cell by using his body. In the case of overexpressed of the antibiotics effluent pump, the antibiotics in bacteria decreases, resulting in drug resistance (3).

At least five families of efflux pumps have been classified in *A.baumannii* based on their phylogeny. These efflux pumps can generally assist in the evolution of antibiotic-resistant strains by reducing the antibiotic concentration inside the bacterial cell (5).Resistance nodulation cell division is one of the efflux pumps found in Acinetobacter baumannii and play an essential role in assisting bacteria in fighting antibiotics. Three RND systems have been overexpressed with multidrug resistance *Acinetobacter baumannii* (6). The first system is *AdeABC*; provide resistance to chloramphenicol, tetracyclines, trimethoprim, fluoroquinolones, and aminoglycosides such as gentamicin (7). The second RND pump system is *AdeFGH* genes, with resistance to lincosamides such as clindamycin, tetracyclines, and chloramphenicol fluoroquinolones such as ciprofloxacin, trimethoprim (6).

While *AdeIJK*, pump express resistance to a number of antibiotics such as trimethoprim, erythromycin, tetracyclines, β-lactams such as Imipenem, fluoroquinolones, chloramphenicol (8).

METHODS

The ethical committee of Collage of Science University of Baghdad approved the study (CSEC/0121/0010) in which About 130 bacterial isolates were collected from patients who admitted to the educational laboratory of the medical city, the burn center of al Yarmouk hospital and Adnan Hospital in the City of Medicine from a period started from April 2020 to November 2020. All bacterial isolates were identified by using a biochemical test, and the results of identification were confirmed by using the Vitek 2 system as well as by selective synthetic media (CHROMagar).

The antibiotic susceptibility of Acinetobacter baumannii:

This test was performed by Disk diffusion method according to the guideline of CLSI (2020). This was conducted on eight antibiotics, including one antibiotic from each group (Levofloxacin, Trimethoprim, Imipenem, Amikacin, Piperacillin, Ampicillin-sulbactam, Cefepime and Tetracycline). the results were interpreted depending on guideline of CLSI (2020).

Minimum Inhibitory Concentration (MIC):
MIC (Minimum Inhibitory Concentration) was used to determine the minimum concentration of levofloxacin that able to stop the growth of bacteria. MIC was determined by using broth Microdilution method according to guidelines of CLSI (2020), with different concentration of levofloxacin (2µg/100 µl, 4 µg/100 µl, 8 µg/100 µl, 16 µg/100 µl, 32 µg/100 µl, 64 µg/100 µl, 128 µg/100 µl and 256 µg/100 µl). The results were interpreted after 18 hours of incubation at $37C^0$ depending on guideline of CLSI (2020).

GenomicDNA extraction:
WizPrep gDNA Mini Kit was used to extract DNA from *Acinetobacter baumannii*. Silica membrane technology was used by this kit to complete the tedious steps and provide an easy, reliable way to efficiently extract DNA with high precision and less time.

Detection of RND efflux pumps genes by PCR:
One primer was used to detect the presence of *adeJ* gene of *adeIJK*-RND pump in Acinetobacter baumannii Table (1.1). The primer was designed by using (prime3 https://primer3.ut.ee/) and manufactured by alphaDNA Company in (Canada).

Table 1.1. The Primer sequence used in this study.

Gene name		Primer sequence 5'→3'	Product size	References
AdeJ	**F**	**GTGATGCCATGAAAGCAATG**	**237 bp**	**This study**
	R	**TTGCACCAATGACACCAAGT**		
	R2	**TTGAGCACCAGACTCACGTT**	**130 bp**	
gyrA	**F**	**AAATCTGCCCGTGTCGTTGGT**	**344bP**	**(Hujer, Kristine M et al. 2006)**
	R	**GCCATACCTACGGCGATACC**		

Amplification of *AdeJ* gene:
The Detection was performed in DNA template extracted from A. baumannii by amplifying *adeJ* gene, by using a reaction mixture with a volume of 25 µl, The PCR reaction was carried out using 12µl of Go Tag Green Master Mix and mixed with 1µl from forward primer and 1µl from revers primer, then 3µl of DNA was added to the mixture, and the mixture was completed to 25µl by addition 6.5 µl of nuclease-free water. The PCR reaction was carried out in MyGenie 96/384 Gradient Thermal Block (Bioneer/Korea). The amplification of *adeJ* gene was started with one cycle of denaturation step at 95C for 5 minutes in order to activate the enzyme. Followed by 35 cycles of denaturation at 95 C for 30 seconds for each step, after denaturation, the reaction mixture was entered to 35 cycles of annealing step with the annealing temperature 58C for *adeJ* gene for 30 seconds, and this step was followed with 35 cycles of elongation step at 72C for a minute, and finally, the reaction ended with one cycle of final extension at 72°C for 5 min. PCR products were electrophoresed in 2 grams of agarose dissolved in 100ml of 1X TBE buffer. Gel electrophoresis was performed to ensure the primers set were successfully amplifying the target genes.

Total RNA extraction with TRIzol:
TRIzol manufactured by (TransGen Biotech) was used to extract total RNA. The extraction was carried out by using the protocol provided by the manufactured company.

CDNA synthesis from mRNA:
mRNA is rapidly and easily degraded during use; it is important to convert mRNA to complementary DNA. It is also difficult to handle mRNA in the laboratory. The EasyScript® One-Step gDNA Elimination and CDNA Synthesis SuperMix was used to reverse the mRNA to complementary DNA. This was performed by mixing 10µl of EasyScript RT/RI enzyme, 1µl of Genomic cDNA remover, OligdT (1µl), Random primers (1µl) and 5µl of total RNA finally the volume was completed to 20µl by addition 2µl of nuclease free water. After the mixture was prepared was converted into cDNA by using congenital PCR.

Quantitative Real-Time PCR (qRT–PCR):
The level of expression of the RND-*AdeJ* genes suspected to be associated with levofloxacin resistance in *A.baumannii* was calculated using RT-PCR. QPCR software was used to perform the determination of genes expression by using SYBR Green as fluorescence dye and this was done with using Corbett Real-time PCR System. All reactions with RT-PCR were achieved in duplicate and included a negative amplification control (NAC), negative DNA control (NTC) and negative primer control (NPC) as negative controls. The sequence of the primers for *adeJ* gene were designed by using (https://primer3.ut.ee/) and synthesized by AlphaDNA Ltd (Canada) and stored lyophilized at (-20°C). In addition, the housekeeping gene was obtained from the article of Kristine (9) and used as an internal control for determining Delta CT (Table 1.2) as well as sensitive strain of A. baumannii was used as a calibrator for determining Delta Delta CT. The gene expression levels were calculated by measuring the threshold cycle (Ct).

RESULTS

Using a biochemical examination, all bacterial isolates were identified, and the findings revealed that only 70 bacterial isolates were identified as Acinetobacter baumannii out of 130 bacterial isolates, based on the biochemical test results showing negative results for indol and oxidase. While Simmon citrate was positive at the same time, the results were confirmed by the Vitek 2 device and the results were also confirmed using selective synthetic media (CHROMagar).

The antibiotic susceptibility of Acinetobacter baumannii:
The rapid evolution of Acinetobacter baumannii to become extensively drug-resistant and multidrug-resistant is thought to be a critical curative challenge that can be explained by many theories; one of them is the inappropriate use of antibiotics, in addition to the widespread use of antibiotics, and this make these bacteria were, therefore, the object of the researchers' attention. Disk diffusion method was used to determine the susceptibility test of Acinetobacter baumannii. In this experiment, the Acinetobacter baumannii susceptibility test for eight antibiotics was conducted, and the *A.baumannii* revealed that these bacteria were resistant to Amikacin in percentage (61.42%). While Trimethoprim resistant was (88.57%) followed by Cefepime, Piperacillin is (82.85%) (71.42%) respectively. In addition, it is resistance to Ampicillin-sulbactam, Levofloxacin, and Imipenem was (61.42%), (51.42%), (54.28%) respectively. Whereas Tetracycline resistance was (47.14 %). The results of antibiotic resistance were shown in figure (3.7).

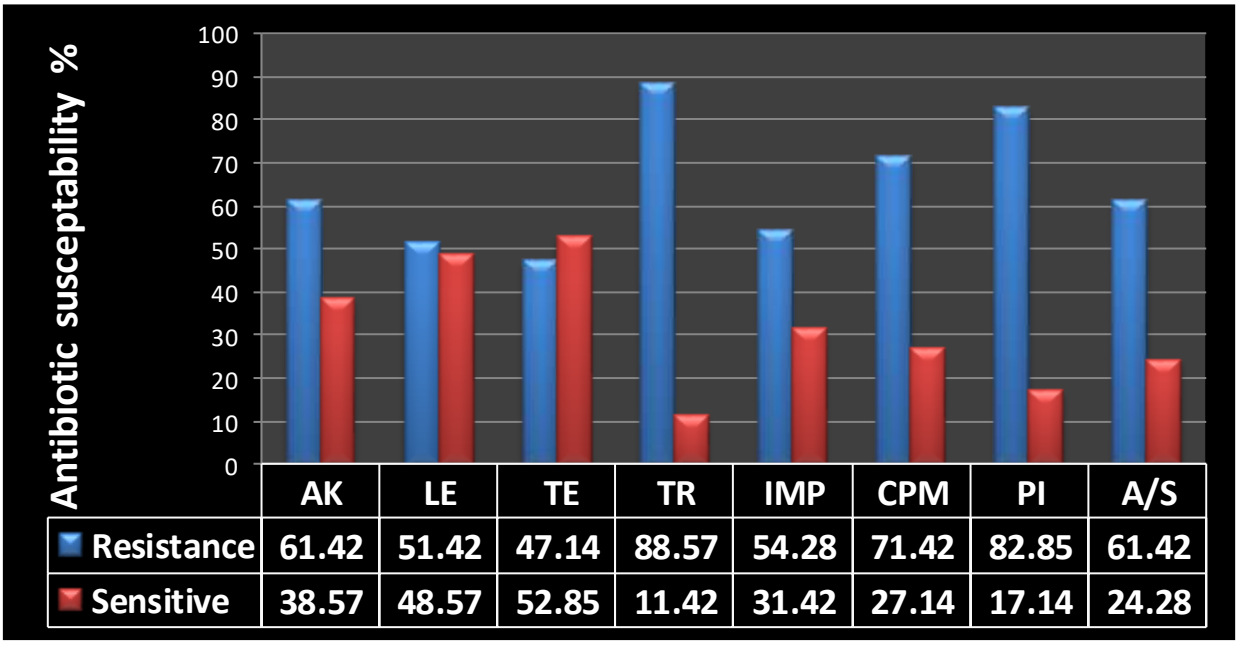

Figure 3. 1 Antibiotic susceptibility of A. baumannii. Amikacin (AK), Piperacillin (PI), Imipenem (IPM), Tetracycline (TE), Ampicillin-Sulbactam (A/S), Cefepime (CPM), Trimethoprim-Sulfamethoxazole (TR) and Levofloxacin (LE).

Molecular detection of RND-Efflux pump:
Molecular detection was conducted on 70 isolates of A. baumannii by using a conventional PCR technique. The result obtained revealed the existence of *AdeJ* in all 70 isolates of A. baumannii. After amplification, the gel electrophoresis was used to detect the PCR product and screened with UV-light. The pictures taken after UV-Light screening showed that the product size was for *AdeJ* and (222). Figure (1.5) shows the presence of *AdeJ* gene. The obtained result about the presence of the adeJ in all strain of A.baumannii was agree with the work of Bily (10).

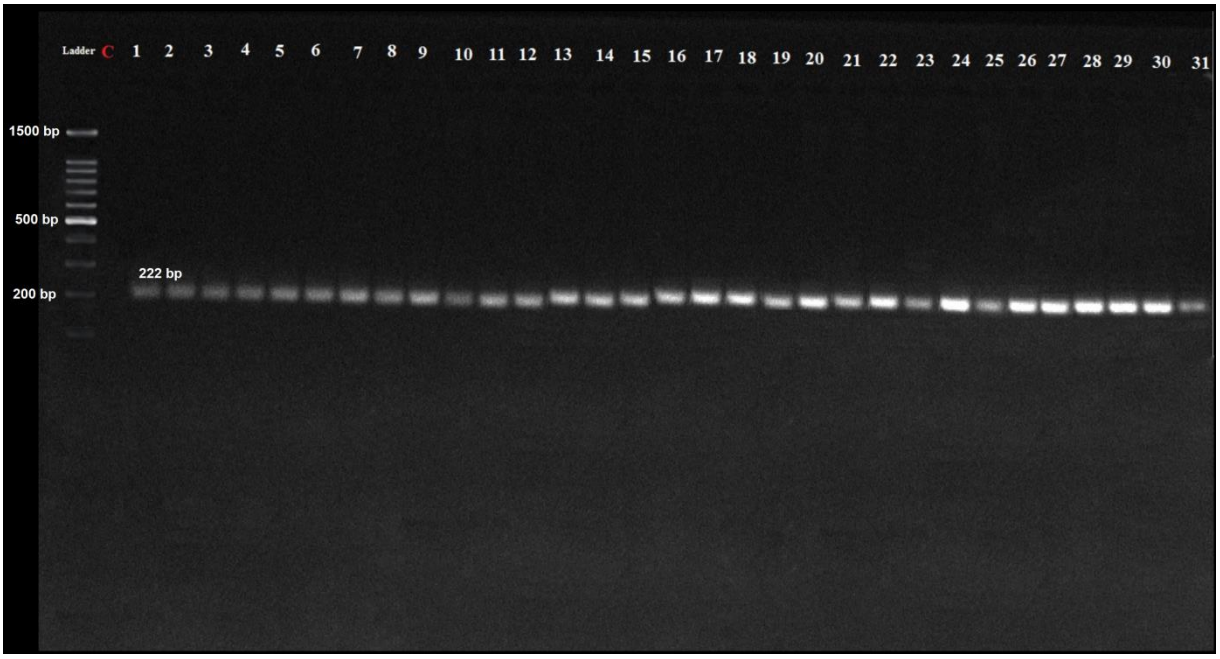

Figure 1.3 Detection the presence of *adeJ* in *A.baumannii* by using gel electrophoresis after amplification the genes with conventional PCR program.

Determination of MIC:

The purpose of determining the MIC of Levofloxacin is to be exposing 15 bacterial isolates under the pressure of Levofloxacin to analyze the correlation between the tolerance of bacterial isolates to Levofloxacin and *AdeJ* through the use of Real-Time Quantitative PCR by determining the gene expression of this gene. Five sensitive bacterial isolates and five resistance bacterial isolates were exposed to different concentrations of Levofloxacin (256/100µl, 128/100µl, 64/100µl, 32/100µl, 16/100µl, 8/100µl, 4/100µl and 2/100µl) and result showed that all five sensitive bacterial isolates were died after exposure to (4/100µl) concentration of Levofloxacin. While some resistance *A.baumannii* was resistance to the Levofloxacin with a concentration of (128/100µl) while other with concentration (64/100µl) as shown in figure (1.6), and Vitek 2 system was used to ensure the correct determination of MIC.

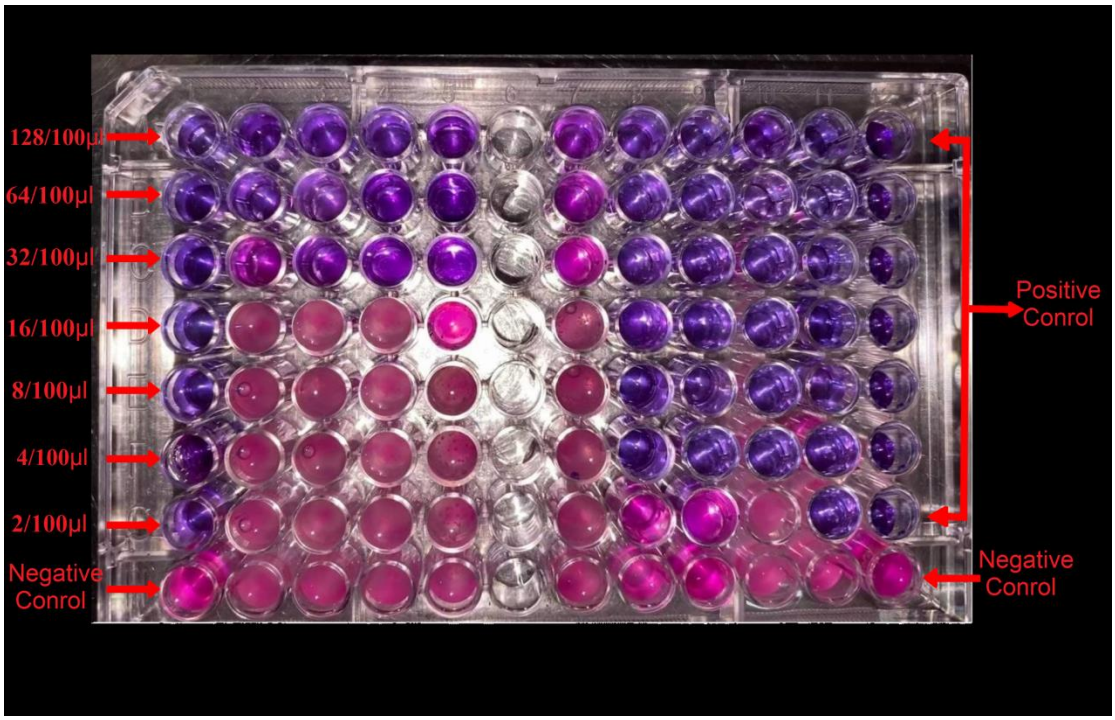

Figure 1.3 Microdilution plate show the growth of some *A.baumannii* was inhibited at concentration 128/100µl, 64/100µl and 32/100µl while sensitive *A.baumannii* was inhibited at concentration 2/100µl.

Determination of gene expression for *AdeIJK* genes by using real-time PCR:

The *adeJ* expression level of the study was measured by real-time RT-PCR and sensitive A. baumannii was used as a calibrator. As a result, the expression level of resistance *A. baumannii* before exposure to levofloxacin was 3.1, and for resistance *A.baumannii* after exposure to levofloxacin was 7.20. There was a statistically significant difference

(P=0.03) in the mean gene expression of *adeJ* between the resistance A. baumannii before exposure and resistance *A.baumannii* after exposure to levofloxacin. As shown in figure (1.3).

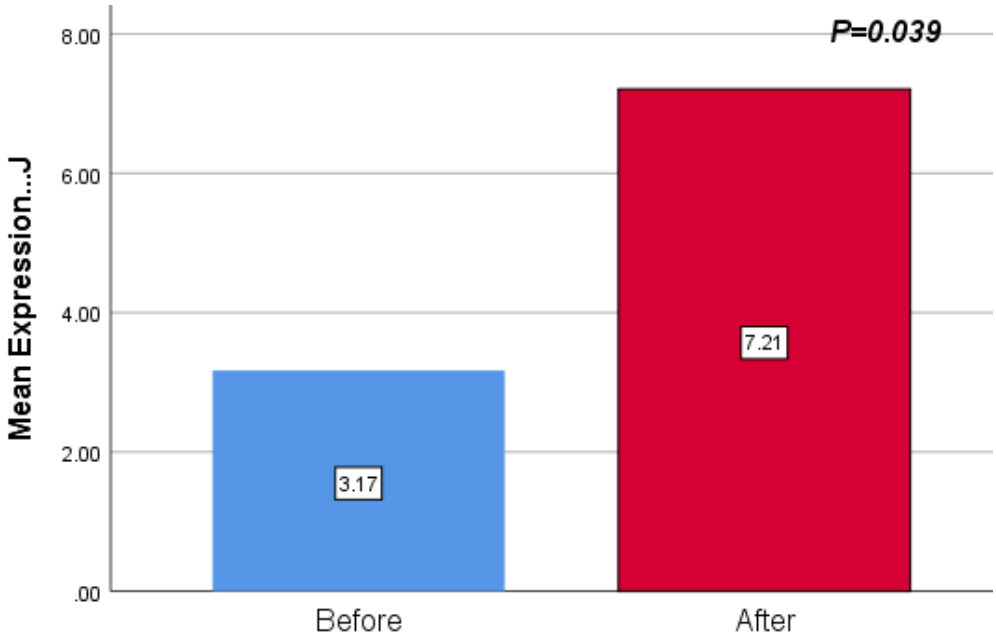

Figure 1. 3 The relative expression level for *adeJ* was determined by real time PCR for resistance strain before exposer to the levofloxacin and after exposure to the levofloxacin. Sensitive strain was used as calibrator.

DISCUSSION

The rapid development of this bacterium to resist most antibiotics made it the focus of researchers' attention, which prompted us to study one of the efflux pump genes (*AdeJ* of *adeIJK*- RND pump) and try to find out the role of this gene in the formation of resistance to levofloxacin. Because there is no article study the role of this gene with levofloxacin which prompted us to figure out what is the relation of this gene with levofloxacin resistance. In addition, 130 isolates were collected from hospitalized patients, as well as from burns and wound surfaces, and they were diagnosed Biochemically and were confirmed with Vitec II system and chrome agar. That 20 bacteria were resistant to all tested antibiotics, including levofloxacin, which prompted us to calculate the minimum inhibitory concentration for levofloxacin, and the results showed that the bacteria were resistant to a concentration ranging between 64/100ul to 128/100ul. A real-time PCR was used to measure the gene expression for this gene. The results showed that the bacteria resistant to levofloxacin before exposure to this antibiotic were 3.17, but after exposure to this antibiotic, the expression level increased to 7.21 and the p-value was 0.039, and this indicates that the gene has a role in providing resistance to levofloxacin.

REFERENCES

1. Wang, T., Costa, V., Jenkins, S. G., Hartman, B. J., & Westblade, L. F. (2019). Acinetobacter radioresistens infection with bacteremia and pneumonia. IDCases, 15, e00495. https://doi.org/10.1016/j.idcr.2019.e00495
2. Dissemination of carbapenemases producing Gram negative bacteria in the Middle East.Zahedi Bialvaei A, Samadi Kafil H, Ebrahimzadeh Leylabadlo H, Asgharzadeh M, Aghazadeh MIran J Microbiol. 2015 Oct; 7(5):226-46.
3. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Perichon B. 2010. Overexpression of resistance-nodulation-cell division pump *AdeFGH* confers multidrug resistance in Acinetobacter baumannii. Antimicrob. Agents Chemother. 54:4389–4393.
4. Attia NM, ElBaradei A. Fluoroquinolone resistance conferred by gyrA, parC mutations, and AbaQ efflux pump among Acinetobacter baumannii clinical isolates causing ventilator-associated pneumonia. Acta Microbiol Immunol Hung. 2019 Dec 13:1-5. doi: 10.1556/030.66.2019.040. Epub ahead of print. PMID: 31833383.
5. Reza A, Sutton JM, Rahman KM. Effectiveness of efflux pump inhibitors as biofilm disruptors and resistance breakers in Gram-negative (ESKAPEE) bacteria. Antibiotics. 2019;8(4):E229.
6. Coyne, S., Courvalin, P., & Périchon, B. (2011). Efflux-mediated antibiotic resistance in Acinetobacter spp. Antimicrobial agents and chemotherapy, 55(3), 947–953. https://doi.org/10.1128/AAC.01388-10
7. Peleg, A. Y., Adams, J., & Paterson, D. L. (2007). Tigecycline Efflux as a Mechanism for Nonsusceptibility in Acinetobacter baumannii. Antimicrobial agents and chemotherapy, 51(6), 2065–2069. https://doi.org/10.1128/AAC.01198-06
8. Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T., & Courvalin, P. (2008). *AdeIJK*, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. Antimicrobial agents and chemotherapy, 52(2), 557–562. https://doi.org/10.1128/AAC.00732-07
9. Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., ... & Bonomo, R. A. (2006). Analysis of antibiotic resistance genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrobial agents and chemotherapy, 50(12), 4114-4123.

10. Xu, C., Bilya, S. R., & Xu, W. (2019). *adeABC* efflux gene in Acinetobacter baumannii. New microbes and new infections, 30, 100549. https://doi.org/10.1016/j.nmni.2019.100549