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Role of some proteins in resistance of clinical Acinetobacter baumannii isolates to imipenem

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Abstract*---Acinetobacter baumannii*** is one of the ESKAPE pathogens which are the leading cause of nosocomial infections throughout the world. The aim of this study is to detect the role of Some Proteins in Resistance of *Acinetobacter baumannii* to imipenem. The research included the collection of 100 different clinical specimens of(urine, burns, and wounds) isolated from patients in Al-Diwaniyah Teaching Hospital for the period from September to December 2021. 20 isolates out of 100 isolates belonging to *A.baumannii* were obtained. The samples were collected from different clinical specimens distributed as follows:7(35%) Swabs of burns,8(40%) swabs of wounds, and 5(25%)from urine, an examination was conducted for (8)Antibiotics by (Antibiotic Susceptibility Test-AST) on 20 isolates. The results showed that all isolates are resistant to antibiotics except for imipenem showed a sensitivity of 20% and resistance of 80% to imipenem. The results of the Minimum Inhibitory Concentration level of imipenem that were conducted for five isolates showed that all isolates are resistant to imipenem at concentrations of (128 mg/ml and 256 mg/ml), while 2(40%) isolates out of 5 isolates were resistant to imipenem in the concentration of 64 mg /ml. As for the results of genetic analysis of the 20 isolates through the PCR technique to detect outer membrane proteins (*Caro, OprD*)that encode enzymes Carbapenemase, they were 9(45%) isolates out of 20 isolates carrying the gene Caro and 8(40%) isolates carrying the *OprD* gene. While *16sRNA*found in 20(100%) isolates, As for the gene bla_{OXA-51}, it was found in 19(95%) out of 20 isolates. The conclusions of this study were that bacteria are Resistance to most antibiotics , which has been proven by many studies, as well as its high resistance to imipenem. The gene bla_{OXA-51} was considered a diagnostic gene and a marker for

the diagnosis of this bacteria (*A. baumannii*). In conclusion, must lead to the development of new drugs or innovative treatment techniques for dealing with the difficulties of carbapenem-resistant *A. baumannii* (CRAB) and limiting the future spread of the infection.

***Keywords*---**CRAB, ESKAPE, carO, OprD.CDC., AST, OMP, MDR.

Introduction

The emergence of *A.baumannii* astonished the world as one of the many pathogens and its unique ability to acquire genes that are resistant to a number of treatments, enabling it to cause mutations in the resistance. In Iraq, the bacteria entered through the wounded and burns of soldiers in 2003 because it is extensive in patients whose immunity is reduced, That's why it was called Iraq's bacteria(Mea *et al.*,2021). *Acinetobacter baumannii* is considered a tool that threatens global health, and this was announced and confirmed by the American Infectious Diseases Association, the World Health Organization, and the Center for Disease Control and Prevention (CDC), where it was found that it has resistance to many treatments, which enables it to this danger (Rello *et al.*, 2018).One of the ESKAPE pathogens that causes lung infections, pneumonia, and urinary tract infections is *Acinetobacter baumannii.* Gram-negative, catalase-positive, oxidase-negative, non-motile, non-fermenting coccobacilli is an aerobic, pleomorphic, and non-motile bacillus. Acinetobacter genus organisms may be found in practically all soil and surface water samples, indicating that they are ubiquitous in nature. *A. baumannii* is a pathogen that prefers to infect wet tissues like mucous membranes and exposed skin(Howard *et al.*,2012).Carbapenems are antimicrobial agents used to treat hospital infections due to their comprehensive spectrum of activity, in addition to their effectiveness against positive and negative bacteria. Therefore, they are used to treat patients with many diseases. *A. baumannii* are associated with infections acquired from hospital infections, and they are one of the pathogens of aerobic bacteria that cause inflammation of the skin, bloodstream, and urinary tract. and soft tissues, but in recent years bacteria have gained resistance to these treatments, which has become a threat to humanity (Bartal *et al.*,2022).Outer membrane protein(OMP),It has been addressed in many studies due to its functional importance, its distribution and its role in antibiotic resistance(Nie *et al.*,2020).OMP: Mono- or triangular-shaped porins allow the molecules to diffuse into the space surrounding the bacteria.The membrane contains dozens of them, *Caro, OprD.* In many functions, bacteria confer the ability to withstand environmental conditions and resist limiting treatments(Uppalapati *et al* .,2020). *CarO* is an 8-stranded beta barrel-shaped outer membrane channel protein that does not have a continuous channel (Zahn *et al.*, 2015), but it does regulate the inflow of beta-lactams into *A. baumannii* (Mussi *et al.*, 2005). CarO is divided into two sub-groups: *CarOa and CarOb; CarOb* has a two-fold higher specificity for IMP than *CarOa* (Catel-Ferreira *et al.*, 2011). Antibiotic-resistant strains lacking the Caro gene are sometimes studied, demonstrating the variety of resistance mechanisms in *A. baumannii* (Li *et al.*, 2015). In contrast to studies that relate carbapenem resistance to *CarO* loss(Vahhabi *et al.*,2021). *OprD* It is an orthologous protein to a porin involved in the basic amino acid and imipenem

transport. The amino acid conservation at structural domains *A. baumannii OprD* porins indicates its putative function in *A. baumannii.* However, sequence and homology analysis of *A. baumannii OprD* showed that it belongs to a protein involved in resisting low-iron or magnesium and low oxygen stresses (Catel-Ferreira *et al.*, 2012). Recombinant *A. baumannii OprD* did not conduct antibiotics but was partially bound to Fe2+ and Mg2+ cations. *OprD* have been frequently identified in MDR *A. baumannii* signifying its role in resistance(Lai *et al.*, 2018) *OprD* was renamed to OccAB1 by (Zahn *et al.* ,2016), while solving its crystal structure. In their work, Zahn *et al.*, resolved structures of four carboxylate channels OccAB1, 2, 3, and 4, and showed that OccAB1 has the largest channel size with correspondingly high rates of the small-molecule shuttle, including amino acids, sugars, and antibiotics, contrary to previous observations(Evans *et al.*,2021). *16sRNA* has been used to study the different strains and classification, and it is more common because it is available in almost all types of bacteria, and its function has not changed over time and has not been subjected to mutations, as well as its large enough size for information purpose(Karstens *et al.*,2019). The most clinically predominant enzymes in this respect are Class D carbapenemases, commonly known as OXA-type enzymes or oxacillinases. The majority of these enzymes, including the class D-lactamase, have been discovered in diverse Acinetobacter clinical isolates. OXA-51 is a carbapenemase that has emerged in multi-resistant *A. baumannii* strains(Szczypta et al.,2021).

The Aim of the Research

The aim of the study is to characterize the imipenem resistance mechanisms in resistant *A. baumannii* isolates recovered from patients with infections in hospitals through detection of *Caro ,OprD* genes in *A.baumannii.*

Materials and Methods

The research dealt with 20 specimens of *A.baumannii* out of 100 clinical specimens collected from swabs of burns, wounds, and urine and percentages (35%, 40%,25%), respectively. An antibiotic sensitivity test was conducted for 8 Antibiotics as (Amikacin 30mg ,Piperacillin/Tazobactam 10mg ,Cefepime 30mg ,Cefotataxime 30 ,Ciprofloxacin 5mg ,Colistin 25mg ,I mipenem 10mg and Tetracycline 30mg)for 20 isolates and results recorded depended on CLSI,2021. MIC for imipenem by agar dilution methods on 5 isolates in concentration (0.125 mg/ml-256 mg/ml) for imipenem and according CLCI,2021 read the results , finally detected the presence of genes *16sRNA, Caro, OprD* and bla_{OXA-51} in *A. baumannii* isolates by PCR techniques.

Isolation and Identification of Acinetobacter baumannii

Isolates were identified depending on the morphological properties and biochemical tests . Atotal of 20 specimens were collected from urine, and full depth burns and wounds from patients at different hospitals in Diwaniya city, to isolate *A. baumannii.* Clinical relevance were determined by the clinical microbiologists and attending physicians. The specimens were inoculated initially on MacConkey agar and chromagar then incubated for 24hr at 37 °c . The bacterial isolates identified according to(Maccfadin,2000).

Antibiotic Susceptibility Test

The susceptibility of 20 isolates of *A. baumannii* towards 8 type of antibiotics were evaluated by using Kirby –Bauer method (DDT). Antibiotic susceptibility by disk diffusion method on Muller Hinton Agar according to the clinical and laboratory standard institute (CLSI, 2021). The resulting zones of inhibition were measured and compared with the breakpoints standard value of Clinical Laboratory Standards Institute CLSI (2021).

Determination of Minimum Inhibitory Concentration for Imipenem

The minimum inhibitory concentration (MIC) using agar dilution method was used in current study,for *imipnem.* To determine the lowest concentration that inhibits bacterial growth, by dissolving the imipenem with the mollar- Hinton-Agar, culturing the isolates on the medium, and then incubating for 24 hours at 37 °C. The results are recorded based on the readings (CLSI,2021).

Molecular detection of imipenem resistance *A. baumannii* by PCR

The genomic DNA of the bacterium was extracted according to the genomic DNA purification kits (G-SPIN), Supplemented by the manufacturing company (Bioneer,Korea) ,the reaction of PCR (25 µl reaction mixture).Including(0.5 µl Forward Primer ,0.5 µl reverse Primer,1 µl Template DNA ,11.5 µl OneTaq Quick-Load 2X Master Mix with , 11.5 µl Nuclease-free water).The PCR product of 4 genes (*16sRNA ,Caro ,OprD ,blaOXA-51*),the primer sequences and thermal cycler condition listed in Table(1).The amplification products were separated in 1% agarose.DNA ladder(100-1500pb).After electrophoresis, the gel was photographed under UV light.

Table (1) Primers sequences and amplicon results in this study

Primer Name	Sequence(5'_3')	Product size (bp)	References
16SrRNA	F5'-CAGCTCGTGCGTGAGATGT -3'	150	(Ghaima,2016) (Zhu,2019)
	R5'-CGTAAGGGCATGATGACTT -3'		
Bla$_{OXA-51}$	F5'-TAATGCTTTGATCGGCCTTG -3'	353	
	R5'-TGGATGCACTTCATCTTGG -3'		
carO	F5'-ATGAAAGTATTACGTGTTTTAGTGACAAC 3'	729	
	R5'-TTACCAGTAGAATTCTACACCAACT -3'		
oprD	F5'-ATGCTAAAAGCACAAAAACTTACATTAGC A-3'	1320	
	R5'-TTAGAATAATTTCACAGGAATATCTAAGA A -3'		

Results and Discussion

Isolation and Identification of *Acinetobacter baumannii* isolates

In the current study, 100 clinical specimens were taken from patients suffering from different infections. Out of 100 specimens, 20 isolates were recovered as *A.*

baumannii (20%). (The specimens were 8 wounds, 7 burns, 5urine). However,69 isolates out of 100(69%) were identified as other bacterial spp. While no growth represents (11%). Compered with another study by (Ribeiro et al .,2020)found the rate of *A. baumannii* was 55.6% while another study by (Al-Hasnawy *et al* .,2020) showed 13% *A. baumannii* . The difference in isolation rates is due to environmental factors or genetic mutations that enable bacteria to dominate and overcome conditions and treatments.

20(20%) isolates were obtained belonged to *A. baumannii* collected from different clinical specimens , distributed in burn swabs7 (35%), wound swabs 8(40%) , urine 5 (25%). The results of current study were highest from studies by (Raheem, 2020; Rahi, 2021) ,established that isolation rate of *A.baumannii* in AL-Hillah (3.33%) isolates. However, Al-Masoudi, (2014) found that the isolation rate(15%), the current study were similar to studies by (Al-Warid, 2014; Al-Harmoosh, 2015; Al-Kadmy *et al.*,2019; Al-Baroody and AL-*Ghnimi*,2020), that found the isolution rate of *A.baumannii* (21.5%) isolate,. while studies by Hamza and Hadi, (2020) found that the isolation rate of *A. baumannii* was (20%) and study by Al-Zubaibi, (2020) found that the isolation rate of *A. baumannii* was (9.23%).

Antibiotic Susceptibility Profile of *A .baumannii* isolates:

The susceptibility of 20 isolates of *A. baumannii* towards 8 type of antibiotics were evaluated by using Kirby –Bauer method (DDT). Antibiotic susceptibility by disk diffusion method on Muller Hinton Agar according to the clinical and laboratory standard institute (CLSI, 2021). The resulting zones of inhibition were measured and compared with the breakpoints standard value of Clinical Laboratory Standards Institute CLSI (2021). showed that highest level of resistance in *A. baumannii* isolates to all antibiotics used in current study. All isolates were resistant to Piperacillin/Tazobactam, that similar to study by Rahi, (2021), and local study in Babylon province by Al-Warid, (2014), Carbapenems group Imipenem) showed resistance rate in (100%)15 isolate (75%), which varied from different hospitals in Thailand Thirapanmethee *et al.*, (2020), found that *A.baumannii* isolates were resistance, and different from the study of Rahi, (2021) who found (100%) resistance rate t*o* imipenem, while other study by (Mshachal *et al* .,2017), showed resistance rate (50%) for imipenem.

Determination of Minimum Inhibitory Concentration for Imipenem

The Minimum Inhibitory Concentration (MIC) using agar dilution method was used in current study,for imiepneme. The results showed that 5 isolates exhibited imipenem resistance (100%) in three concentrations (64,128,256)mg/ml) , all 5 isolates showed resistant in concentrations (128, 256) mg/ml while only two isolates(20%) showed resistance in concentration (64) mg/ml, with comparable to another study done by (Fonseca,2013) ,for 84 isolates and all isolates showed resistance for imipenem at concentrations ranging from(0,125-0,50)mg/ml according to the CLSI (2021), according to the (CLSI, 2021), However, in a study concluded in Oxford university by (S.pournaras,2021) ,revealed that the MIC for imipenem resistance at concentration (64mg/ml),

Molecular Characterization of Imipenem resistance *A . baumanii*

The Caro gene was detected by PCR with a specific primer with a product of (729pb) shown in figure(1).In this study, conventional PCR was used to detect genes for resistance to imipenem(*Caro, OprD*)in *A .baummannii* isolates. The results showed 9(45%)isolates of *A . baumannii* carrying *Caro* genes while another study by (Pajand *et al* .,2013)showed 72(94%)carrying Caro out of 76 *A .baumanii* isolates. And a study by (He *et al.*,2011)found 5(1.8%) carrying these genes out of 272 isolates also study by(Zhu *et al* .,2019)conducted 100% isolates carrying *Caro* genes. while *OprD* gene 8 (40%) out of 20 isolates showed in figure (2) with specific primers and products of (1320pb) product for PCR reaction and study by (Al-Ouqaili *et al.*,2018)showed 100% isolates carrying OprDgene out of 44 *A . baumannii* isolates and another study by(Lin *et al.,*2010) conducted 40(49.3%) carrying *OprD*gene out of 81 isolates and another gene *blaOXA-51* present in 19(95%) out of 20 isolates while the study by (Rahi,2021)showed 17(85%) carrying *blOXA-51* gene out of 20 isolates and study by(Mekkey *et al* .,2020) found 33(66%) carrying the gene out of 50 *A. baumannii* isolates. Figure (3) showed the results of this study for this gene finally,1*6sRNA* found in 20(100%) out of 20 isolates in this study . Study by (Beikmohammadi *et al.,*2022)found 60(30%) isolates carrying this gene out of 200 *A.baumannii* isolate.And another study by (Karstens *et al.,*2019) show 100% isolates carrying this gene. With these results, the dominance of gene 16sRNA was shown in the first place, then next *blaOXA-51,* after it *Caro*, and *OprD* finally. These genes are essential for the identification of Imipeneme-resistant *A.baumannii.*

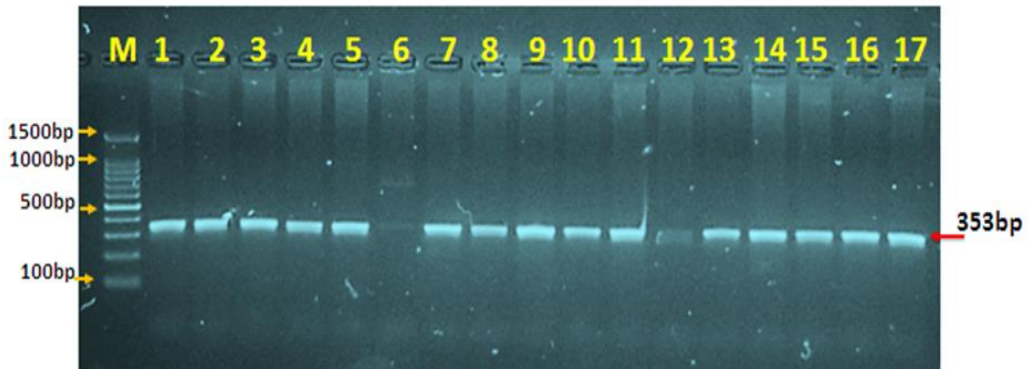


Figure (1): Agarose gel electrophoresis image that showed the PCR product analysis of bla-OXA51 gene in Acinetobacter baumannii isolates. Where, Lane (M) Marker ladder (1500-100bp), lane (1-17): showed positive bla-OXA51 gene in Acinetobacter baumannii isolates at (353bp) PCR product size.

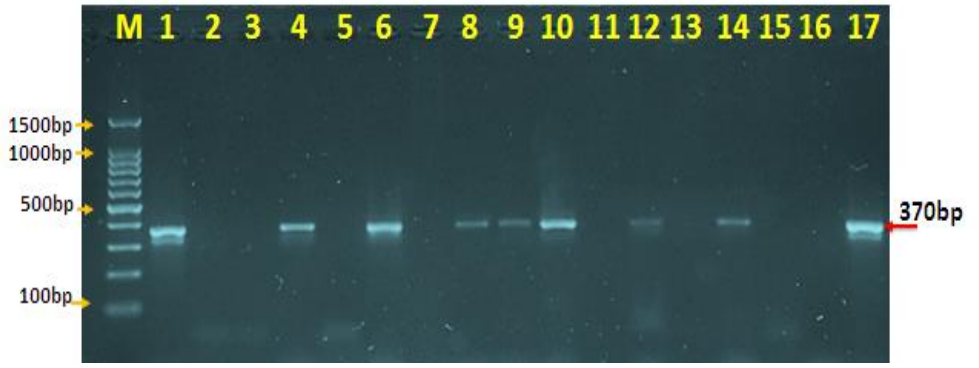


Figure (2): Agarose gel electrophoresis image that showed the PCR product analysis of Caro gene in Acinetobacter baumannii isolates. Where, Lane (M) Marker ladder (1500-100bp), lane (1-17): showed positive Caro gene in Acinetobacter baumannii isolates at (370bp) PCR product size.

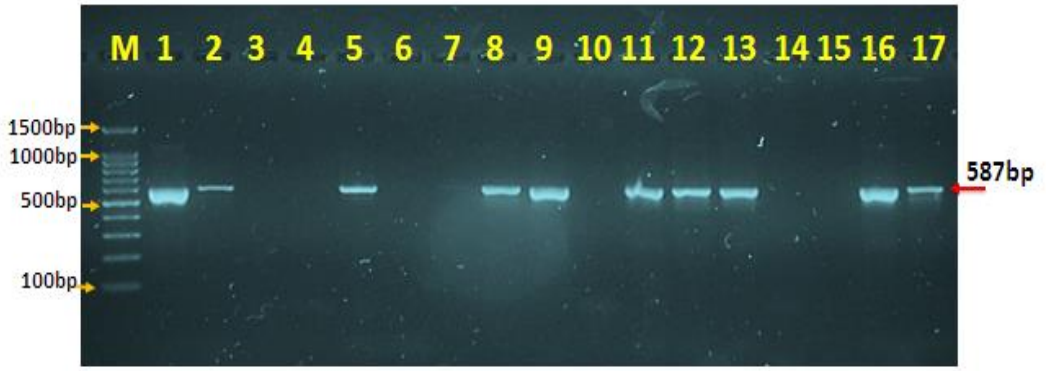
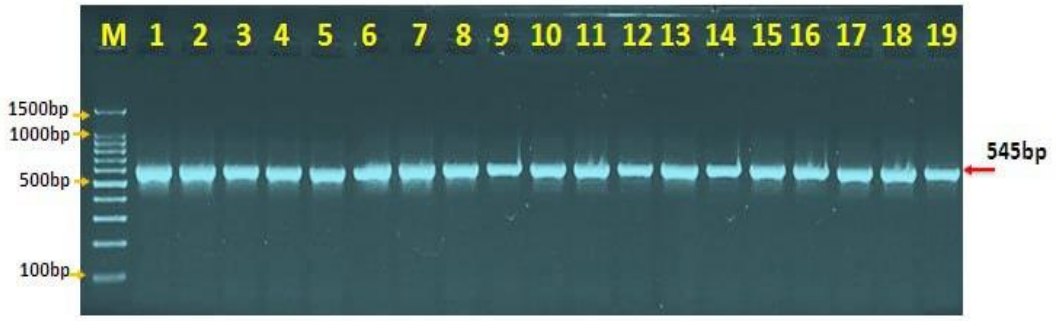


Figure (3): Agarose gel electrophoresis image that showed the PCR product analysis of OprD gene in Acinetobacter baumannii isolates. Where, Lane (M) Marker ladder (1500-100bp), lane (1-17): showed some positive OprD gene in Acinetobacter baumannii isolates at (587bp) PCR product size.



Figure(4). PCR product analysis of 16sRNA gene in Acinetobacter baumannii isolates

Conclusion

Imipeneme resistance -*A.baumannii* poses a great danger to humanity because imipeneme is considered one of the wide-ranging treatments and has an impact on dangerous diseases. Therefore, we must focus on this treatment because it is the best solution in severe pathological cases, and study the genes that cause this resistance to extract and enable bacteria to overcome the treatment. The bla_{OXA-51} gene is also a diagnostic gene for *A.baumannii* and is an indicator of this bacteria.As for the 16sRNA gene, it is present in most types of bacteria, and its advantage is the possibility of studying it and the difficulty of obtaining mutation in it, in addition to its large size that enables it to absorb much information.

Ethical approval

All ethical consents were obtained from patients and informed of their rights to accept or refuse to participate in this research.

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Interests in conflict

The writers' views may not always reflect the official positions of the scientific organizations to which they belong. There are no conflicts of interest declared by the authors.

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