RESEARCH ARTICLE

Effects of Meropenem at Sub-inhibitory Doses on the Expression of Biofilm-associated Protein by *Acinetobacter baumannii*

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ABSTRACT

Objective(s): *Acinetobacter baumannii* has a high proclivity for forming biofilm and is a common source of multidrug-resistant (MDR) infections caused by medical devices in hospitals. This research aims to see how sub-inhibitory meropenem doses affect the expression of biofilm-associated proteins.

Methods: Two hundred and fifty samples were collected from various sites in Baghdad, Iraq hospitals and divided into two groups based on their source: clinical 150 and environmental (100) samples, collected from October 2020 to March 2021. Five bacterial isolates were examined for the presence of the bap gene, were treated with sub-inhibitory concentrations of meropenem to evaluate gene expression level.

Results: Eighty-three *A. baumannii* isolates were identified by microscopic examination and biochemical tests. The identification of 83 isolates was verified using a molecular approach based on the rplB gene, an essential gene found in this genus. Antibiotic susceptibility testing was performed on all 83 identified isolates, and the findings revealed that 36 isolates had MDR. This study found that 30 isolates from these 36 isolates carrying the *Bap* gene that confirmed by molecular detection methods, meropenem dose (0.0005 mg/mL) were with very low inhibition activity against the bacterial strains for that they were considered as sub-inhibitory concentrations, *Bap* gene expression showed an almost apparent decrease in all isolates in antibiotic treatment.

Conclusion: There was a clear link between the presence of the bap gene and the prevalence of multidrug-resistant isolates. These findings point to the importance of isolate resistance and the role of the bap gene in biofilm formation by *A. baumannii* strains. Furthermore, these findings point to the necessity for more research. This study demonstrated the role of rplB gene for molecular detection *A. baumannii* at the level of genus and species, respectively. Meropenem at sub-inhibitory concentration has decreased *Bap gene* expression level.

Keywords: Burns, Meropenem MDR, UTI.

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INTRODUCTION

Acinetobacter baumannii is an opportunistic Gram-negative *coccobacilli* extracellular human pathogen that is non-motile.[2] It has emerged as a significant nosocomial pathogen, causing patients' wide range of infections. *A. baumannii* has developed resistance to nearly all antibacterial agents currently available.

Multidrug-resistant strains of *A. baumannii* are infamous for spreading among hospitalized patients and causing outbreaks, which have been documented globally.[8]

Several studies have shown that *A. baumannii* has a higher intrinsic human virulence capacity than other *Acinetobacter* spp. Many conventional antibiotics have been Previously, it was used to treat infections caused by *A. baumannii*, but has recently developed opposition to large groups of antibiotics such as tetracycline, fluoroquinolone, carbapenem, chloramphenicol, penicillin, cephalosporin, and aminoglycosides. In addition to its increased prevalence and the growth of resistance at a significant rate. *A. baumannii* contagion strains are notable for both inherent antibiotic resistance and the ability to acquire genes encoding resistance determinants. The approach to treating this bacterial infection is limited due to its high susceptibility to clinically effective antibiotics.[5,10,13]

Biofilm formation is another way bacteria, particularly *A. baumannii*, which causes biofilm-related medical device contamination, can thrive in the presence of antibiotics.[11]

In recent years, the separation of bacterial species from biofilms and the finding of genetic variables that govern this complicated process have been hot topics. Biofilms are highly organized heterogeneous bacterial colonies encased inside a polymeric conglomerate of polysaccharides and proteins with

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integrated metabolic activities that yield sessile phenotypes different from their planktonic cousins.[12] Biofilms of bacteria have been found on the surfaces of plastics, glass, a range of surgical tools, and many other hospital surfaces.[7]

METHODS

Bacterial Isolation and Identification

Through the period extending from September to March 2020, (250) clinical (UTI, burns, respiratory tract infection, wound infection, and blood) regardless of age and gender, the selection of samples varies depending on the clinical symptoms; (i.e., inflammation, fever, abscesses, pain, rapid breathing, irritation at site of infection). And environmental specimens (beds, tables, sinks, floors and instruments) were collected as swabs and transported in sterilized transport medium containers. Blood agar and MacConkey agar were streaked on the samples, then incubated at 37°C for 24 hours. *A. baumannii* was identified using manual biochemical testing such as the catalase and oxidase tests. Biochemical assays for final validation are included in the VITEK2 small system.

Antibiotic Susceptibility Test

These tests were carried out by the VITEK 2 Compact Instrument utilizing antibiotic sensitivity test number (AST-N222) cards, as directed by the manufacturer. They contained the following antimicrobial agents: Amikacin (AK), Aztreonam (AZT), Cefpime (CPM), Ceftazidime (CAZ), Ciprofloxacin (CIP), Gentamicin (GM), Imipenem (IMI), Meropenem (MEM), Piperacillin (PRL), Ticarcillin/clavulanic acid (TIM), Tobramycin (TM), Colistin (cs), Pefloxacin (PEF).

Identification of *A. baumannii* and *Bap* Virulence Gene by Molecular Method

DNA Extraction

The Wizard genomic DNA purification kit separated genomic DNA from bacterial growth. This kit was developed to isolate DNA from a variety of biological materials. This kit used a bacterial technique to extract DNA (for gram negative bacteria).

Conventional Polymerase Chain Reaction for *Bap* and *rblB* Genes

Concerning the conventional PCR reaction, designed primers were used to detect each gene. As suggested by the provider, lyophilized and dissolved in sterile deionized distilled water to achieve a final concentration of 100 Pico mole/l. The Bap gene's specific primers (Bap-F, 5`-ATG CCT GAG ATA CAA ATT AT-3`and *Bap*-R, 5`-GTC AAT CGT AAA GGT AAC G-3`) and the *rplB* gene (*rplB* -F, 5`-GTA GAG CGT ATT GAA TAC GAT CCA AAC C-3` and *rplB* -R, 5`-CAC CAC CAC CGT GCG GGT GAT C-3`) were used. Table 1. Using 20 µL of PCR reaction volume protocol, 3 µL DNA template (100 ng/µL) was amplified using 10 µL of Go *Taq*® green master mix 2X (Promega, USA) and 1 µL of each primer (10 pmol/ µL) for each specific gene, up to the final volume 20 µL with nucleases free water. At 4°C, the extracted DNA, primers, and PCR premix were thawed, vortexed, and quickly centrifuged to bring the contents to the bottom of the tubes. The negative control had everything but DNA, replaced with DW instead of template DNA. The bap gene was amplified under the following conditions using Thermal cycler gradient PCR (Thermo Fisher/ USA) programs: 95°C for five minutes, then 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute, and 72°C for seven minutes. In addition, the following were the PCR conditions for the rplB gene: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for one minute. In a 1.5% agarose gel, the PCR products were separated.

Preparation of Antibiotics for Sub-inhibitory Concentration

Meropenem test antibiotic solution was diluted in brain heart infusion broth, and serial of dilution was prepared as follows: 50, 5, 0.5, 0.05, 0.005, 0.0005, and 0.00005 mg/mL and quantitative M.I.C was done.[14]

Study of *Bap* gene Expression using RT-PCR Technique

To examine whether the Antibiotics at sub-inhibitory concentrations affect the biofilm. gene expression, 50 **µ**L of bacterial suspension were cultured in 850µl of nutrient broth and directly mixed with 100 **µ**L of appropriate antibiotic in 1.5 mL Eppendorf Tubes. The RNA was extracted after 24 hours incubation at 37°C.

RNA Extraction

RNA was extracted from samples using the qiagen TRIzol Reagent (Total RNA Isolation Reagent) technique. TRIzol Reagent (Total RNA Isolation Reagent) is a ready-to-use reagent for obtaining total RNA from cells and tissues for use in PCR analysis.

One step Quantitative Real-time PCR assay protocol *Bap,* and *rplB* Genes

The precision, sensitivity, and speed of reverse transcription-quantitative PCR (RT-qPCR) separate it from other gene expression approaches. This method has become the gold standard in gene expression analysis. It's vital to remember that in a relative quantification research, the studies are generally

Table 1: One-step quantitative RT-PCR reaction mix.

Components	*Stock/Unit*	*Final/Unit*	*Volume per 1 Sample/uL*
qPCR Master Mix	2x	1 X	5
RT mix	50 x	1 x	0.25
MgCl2			0.25
Forward primer	10 µM	1 µM	0.5
Reverse primer	10 µM	1 µM	0.5
Nuclease Free Water			2.5
RNA	ng/µL	ng/µL	1
Total volume			10
Aliquot per single rxn	9 µL of Master mix per tube and add 1 µL of template		

focused on comparing the amount of expression of a certain gene across several samples (Table 1).[6]

The samples were examined in triplicate and compared to the expression of the rplB gene. The relative variations in mRNA expression levels between antibiotic-exposed and antibiotic-unexposed *A. baumannii* were assessed using the comparative threshold cycle (CT) approach (2-Ct).

Statistical Analysis

To determine the influence of different components in research parameters, the statistical analysis system-[SAS (9)] program was used. The least significant difference (LSD) test (ANOVA) was performed to make a meaningful comparison between means. In this study, the Chi-square test was utilized to compare percentages (0.05 and 0.01 likelihood).

RESULTS

Identification of *A. baumannii*

Using the VITEK 2 system's Gram-negative strain identification card, 83 clinical isolates were identified as *A. baumannii*. This method has been used in many previous studies and has produced satisfactory results in biochemical test diagnosis and validation. This automated device could determine the antibiograms of *A. baumannii* isolates.[1]

Table 2: Isolates of *A. baumannii* in clinical and environmental samples.

Source	*No. of samples*	*Positive samples*	*%*
Wound infection	50 (20%)	27 (10.8%)	32.53
Burn infection	35 (14%)	18 (7.2%)	21.68
Respiratory tract infection (sputum)	25 (10%)	12(4.8%)	14.45
Urine	25 (10%)	10 (4%)	12
Blood	15 (6%)	7(2.8%)	8.43
Environment	100 (40%)	9 (3.6%)	10.84
Total	250 (100%)	83 (33.2%)	100

Table 3: Antimicrobial susceptibility test of 83 *A. baumannii* Isolates to 14 Antimicrobial agents.

Antibiotic	*Resistant*	*Intermediate*	*Sensitive*
Piperacillin/ tazobactam (PTZ)	36 (43.37%)	0 (0.0%)	47 (56.62%)
Colistin(cs)	4 (4.81%)	12 (14.45%)	67 (80.72%)
Trimethoprim/ Sulfamethoxazo le (TMP/SMX)	12 (14.45%)	0 (0.0%)	71 (85.54)
Tobramycin(TM)	20 (24.09%)	12 (14.45%)	51 (61.44%)
Piperacillin (PRL)	36 (43.37%)	0 (0.0%)	47 (56.62%)
Meropenem(MEM)	28 (33.73%)	12 (14.45%)	43 (51.80%)
Minocycline (MNO)	4 (4.81%)	4 (4.81%)	75 (90.36%)
Imipenem (IMI)	16 (19.27%)	20 (24.09%)	47 (56.62%)
Cefepime(CPM)	48 (57.83%)	0 (0.0%)	35 (42.16%)
Ceftazidime(CAZ)	83 (100%)	0 (0.0%)	0 (%)
Ciprofloxacin(CIP)	36 (43.37%)	1(1.20%)	46(55.42%)
Gentamicin(GM)	32 (38.55%)	0 (%)	51 (61.44%)
Ticarcillin	24 (28.91%)	16 (19.27%)	43 (51.80%)
Ticarcillin / Clavulanate(TIM)	24 (28.91%)	12 (14.45%)	47 (56.62%)

Distribution of *A. baumannii* according to Sample Type

According to Table 2, *A. baumannii* was included in 83 (33.2%) of the 250 clinical and environmental samples tested. The samples were taken from various environmental and medicinal sources, including wounds and burns, sputum, and in patients' body fluids (peritoneal fluid) (Table 2).

Susceptibility to Antibiotics Test

All 83 *A. baumannii* isolates were tested for antibiotic susceptibility using the automated VITEK 2 Compact equipment. Each isolate was given a McFarland 0.5 standard suspension in 0.45 percent sodium chloride, and all samples were grown on MacCkonkey agar plates. A liquid suspension of all isolates was fed into the VITEK machine and left overnight to acquire the findings. A total of 14 different antibiotics were tested using the Gram-negative susceptibility card included in the VITEK 2 Compact device. Table 3

Presence of *bap* and *rplB* Genes

PCR analysis confirmed the existence of the *rplB* gene in all 36 (100%) *A. baumannii* isolates, confirming the conventional diagnosis of culture, biochemical tests, and Vitek-2 tests. In the electrophoresis procedure, the formed amplicons of this gene appeared clearly at 475 bp on an agarose,[4] as shown in Figure 1.

PCR assay was used to determine the virulence gene in all 36 MDR *A.baumannii* isolates. PCR assay was used to detect a single gene using particular primers. This analysis showed that 30 isolates (83.33%) carried the biofilm-associated protein (*Bap* gene), as shown in Figure 2.

Subinhibitory Concentration of Meropenem in Different Concentration against *A. baumannii.*

Meropenem is an antibiotic that inhibits microorganisms that are gram-positive and gram-negative. The concentrations (50, 5, 0.5, 0.05, and 0.005 mg/mL) showed highly inhibition power against the bacterial strains. In contrast, the concentration (0.0005, 0.00005 mg/mL) showed a low inhibition power (0.0005 mg/mL) with very low inhibition activity against the

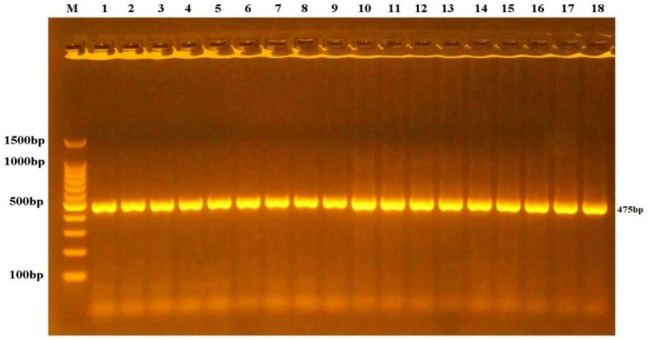

Figure 1: Gel Electrophoresis for PCR Amplification of *A. baumannii rplB* Gene on 1.5% agarose gel, 100V for 75 minutes wells 1-18 positive results, of amplification at 475 bp compared to DNA ladder (100 bp).

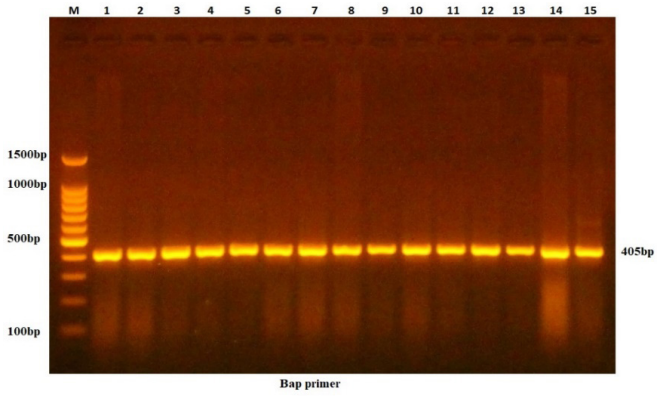

Figure 2: Gel electrophoresis for PCR product of *A. baumannii* Bap gene on (1.5%) agarose gel, 100V. for 75 min,Positive amplicons were detected at 405 bp. Compared to DNA ladder (100 bp).

bacterial strains for that they were considered as sub-inhibitory concentrations.

One step quantitative Real-time PCR Assay for *Bap,* and *rplB genes*

A total of the thirty-six (36) *A. baumannii* isolates were used in the quantitative RT-PCR procedure; the different sources of these isolates were allocated as follows (4 wounds, 1 burn). Antibiotic therapy reduced the expression of the Bap gene in virtually all isolates (meropenem). *Bap* Gene Expression in *A. baumannii* clinical isolates.

DISCUSSION

Acinetobacter baumannii has a greater inherent pathogenicity capability in humans than other Acinetobacter species. In the past, tetracycline, fluoroquinolone, carbapenem, chloramphenicol, penicillin, cephalosporin, and aminoglycosides were used to treat infections caused by A. baumannii, but it has recently developed resistance to large groups of antibiotics, including tetracycline, fluoroquinolone, carbapenem, chloramphenicol, penicillin, cephalosporin, and aminoglycoside In addition to a rise in prevalence and a large increase in resistance. Contagion strains of *A. baumannii* are notable for their ability to acquire genes encoding resistance determinants and their inherent antibiotic resistance. Due to its great resistance to clinically effective medicines, the treatment options for this bacterial infection are restricted.[5,10,13] The current investigation discovered 36 *A. baumannii* isolates from various sources that were highly resistant to several antibiotic classes., According to Table 3.

Many local and international studies in this field have shown that *A. baumannii* isolates are resistant to a wide range of antibiotics, supporting the current findings. For example, Hussein (2017) reported that scientifically identified *A. baumannii* isolates had mild resistance to Imipenem and Meropenem (58.26 %). She also discovered that the highest resistance was to most antibiotics used, including cephalosporins, and they were 100% resistant to Amoxicillin-clavulanic acid, Cefepime, and Cefotaxime. Also, according to Almaghrabi *et al*.,[1] isolated and established *A. baumannii* strain that is multidrug-resistant and widely resistant have been discovered in Saudi Arabia's southern area. According to the analysis, multidrug-resistant pathogens account for 74% of these isolates. About half of these MDR bacteria are extremely drug-resistant isolates sensitive to colistin but resistant to all other medicines of choice. This should be cause for alarm regarding the pathogen's potential danger. Colistin is the most effective antibiotic for treating *A. baumannii*, with a 60% success rate, followed by trimethoprim and sulfamethoxazole (46%). The carbapenem medications imipenem and meropenem were extremely resistant against *A. baumannii* isolates. Among the strains tested, only 5 (0.05%) and 4 (0.04%) isolates were sensitive to imipenem and meropenem, respectively. These isolates were more dangerous than previously identified MDR strains, which were sensitive to both medicines in more than 90% of instances.[1]

The *rplB* gene is specific to this species, and its detection provides a quick and easy way to identify *A. baumannii* and is more accurate than biochemical identification, which is currently used. Because Acinetobacter baumannii is by far the most clinically relevant member of the genus, the ability to distinguish it from other members of the genus will be tremendously beneficial. This particular gene proved to be particularly selective for *A. baumannii*, allowing for species-level recognition of these bacteria, which was confirmed by PCR amplification with specific primers.

Presence genotypic characterization approaches should be used to validate the presence of genes linked to biofilm development. Bap was first discovered in *Staphylococcus aureus*, but it has now been found in various gram-negative and gram-positive pathogenic bacteria. The findings of this study showed that 30 isolates (83.33%) carried the biofilm-associated protein (*Bap* gene). A study carried out for molecular characterization of *A. baumannii* isolated from Iran hospitals environment showed that the distribution of the *Bap* gene among 100 *A. baumannii* isolated strains was ninety-two (92%) isolates possessed the *bap* gene and 36 (36%).[3]

According to Navidifar (2019), Meropenem biofilm inducibility at sub-MIC levels was strain genotype-dependent. Except for adeJ, where the genes involved in biofilm formation, pili assembly, and quorum sensing after exposure to meropenem and tigecycline at their sub-MICs after exposure to meropenem and tigecycline at their sub-MICs showed a significant positive correlation between biofilm formation capacity and the mRNA levels of genes encoding efflux pumps.

CONCLUSION

By reference to the results of the current study, the below conclusions could be elucidated:

A. baumannii is an emerging member of multi-resistant pathogen increasingly known to cause nosocomial infections in Iraq hospitals, especially in burns and wounds infections.

There was a substantial link between the presence of the bap gene and the presence of multidrug-resistant isolates. These findings point to the relevance of isolate resistance and

the role of the bap gene in biofilm formation by *A. baumannii* strains. Furthermore, these findings point to the necessity for more research.

For rapid detection of *A.baumannii,* vitek2 compact system gave credible, accurate and clear results with accurate and clear results.

This study demonstrated the role of *rplB* gene for molecular detection *A.baumannii* at the level of genus and species, respectively.

The meropenem at sub-inhibitory concentration has decreased *Bap* gene expression level.

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