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Evaluating the Activity of Ultrasound on Biofilm Formation by *Acinetobacter baumannii* isolated from clinical Specimens

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Abstract:

Acinetobacter baumannii received attention for its multi-drug resistant associated with many severe infections and outbreaks in clinical environment. The aims of the study are to investigate the antibiotic susceptibility profile of clinically isolated *A. baumannii*, biofilm production, and the efficiency of Low Frequency Ultrasound (LFU) and honey to attenuate biofilm production. A total of 100 samples were taken from different sources from Baghdad hospitals. The susceptibility patterns revealed the percentage of pan drug resistant (PDR) isolates were 1.5 %, 72.7 % were extended drug resistant (XDR), 16.7 % were multidrug resistant (MDR), and 9.1 % were non MDR and sensitive to most antibiotics used. The ability to form biofilm was detected by crystal violet staining, and the results showed that 20% were strong biofilm, and 31.8% were moderate biofilm. The biofilm formation percentage was decreased using Low frequency ultrasound LFU and honey. Moreover, PCR results revealed that not all of them harbouring biofilm-related genes or integrons (*bap*, *csuE*, *IntI-1*, *IntI-2*), although, they are strong biofilm producers. These results conclude that low frequency ultrasound and chemical components of honey might be a good choice to restrain *A. baumannii* biofilm formation, and negative correlation between antibiotic resistance and biofilm ability.

Running title: Antimicrobial drug resistance, *Acinetobacter baumannii*, biofilm inhibition**Keywords:** *Acinetobacter baumannii*, Antibiotic resistance, Biofilm production, Clinical isolates, Low frequency ultrasound.

Introduction:

A. baumannii is a Gram-negative pathogen, aerobic non- motile and non-fermentative coccobacillus, it is strictly aerobic and is able to persistent in heterogeneity of the surrounding environment. *A. baumannii* also known as "Iraqibacter", causes a major problematic infection among soldiers through Iraq and Afghanistan war ¹. *A. baumannii* is a major nosocomial microorganism that causes a wide range of diseases in hospital setting, such as meningitis, wound infection, endocarditis, and bacteremia in mostly ICU patients. It also causes a ventilator-associated pneumonia (VAP), catheter-associated bloodstream, and urinary tract infections ².

Nowadays, *A. baumannii* is largely coupled with a significant infection among patients ³, as it exhibits a tremendous resistance against practically

all known antibiotics as well as to a host immune response, which makes it a crucial concern in treatment and public health ⁴ The efficiency of *A. baumannii* to form biofilm and it's persistence in dry and desiccated surrounding conditions generated problems and outbreaks in health-care treatment options, particularly for ill patients in the intensive care units (ICUs) ⁵. Biofilm is the aggregation of bacterial cells embedded in an extracellular polymeric substances (EPS), called matrix, which enable bacterial cells to attach to various abiotic and biotic surfaces and protect their growth from the immune system and antibiotics ⁶. Because biofilm formation is a multistep processes, several factors contribute to the formation and establishment of biofilm, *bap* gene is the biofilm-associated protein which displays an indispensable

role in the formation of biofilm, intercellular adhesion and aggregation of bacteria cells⁷. Furthermore, the attachment and biofilm formation are largely mediated by pili, encoded by a *csu* operon cluster genes, that form a pilus-like pack structure in *A. baumannii*. Among *csu* operon, it is found that the *csuE* gene (Chaperon-Usher pilus) plays an important role in biofilm assemble in *A. baumannii*. Similarly, it was reported that a partial contribution in biofilm formation is mediated by the outer membrane protein A (*OmpA*), which enhanced the ability of *A. baumannii* to adhere to biotic surfaces such as epithelial cells and lung epithelial cells and, may induce apoptosis in epithelial cells of human^{8,9}.

Several recent studies have shown that the clinical isolates of *A. baumannii* have a robust system to persistence on biotic surfaces; this is attributed mostly to the presence and expression of *bla*_{PER-1} genes which enhance and increase the ability of these isolates to invade lung epithelial cells¹⁰. Moreover, antimicrobial sensitivity can be affected by several biofilm-associated genes, and thus may refer to a correlation between biofilm formation and drug resistance¹¹. However, this correlation is still unconfirmed, in recent study, the correlation between antibiotic sensitivity and biofilm formation with the associated genes are significant¹².

Nowadays, we required a new strategy to combat the increasing problems of *A. baumannii* drug resistance. Low Frequency Ultrasound (LFU) is one of the safest and promising physical approaches¹³. Ultrasound has been used in research and diagnosis for decades, with frequency of 20 kHz or higher. The LFU is also known as a high-power ultrasound and has a frequency ranging from 20 to 100 kHz¹⁴. The effect of ultrasound on microorganisms is mediated by mechanical forces, such as shock waves, shear forces, and microjets. The creation and collapse of microbubbles in a liquid medium increase the permeability of biofilm and accelerate the penetration of antibiotics, and this cavitation is assumed to be the major mechanism behind ultrasound's bactericidal effects¹⁵.

The objective of this study is to investigate the potential effect of LFU on biofilm activity of clinical isolated *A. baumannii*, and the correlation between antibiotic resistance and biofilm formation of integron-harboring isolates.

Methods:

Specimen Collection and Identification.

A total of 100 specimens (Urine, blood, sputum, fluids, burns and wounds) were collected from different hospitals in Baghdad, the collected

samples were isolated from sputum, swab, blood, urine, and fluid included cerebrospinal fluid (CSF). A total of sixty six 66 samples were identified as *A. baumannii* using the morphological assays, Vitek and Epi20E. The isolates were routinely cultured on MackonCey agar plates under aerobic conditions at 37 °C. All isolates were stored at - 80 °C for further use. In this study, *A. baumannii* ATCC17904 was used as a standard strains.

Antibiotic Susceptibility Test of *A. baumannii*

Antibiotic susceptibility pattern of *A. baumannii* isolates were detected using disc diffusion methods by Kirby–Bauer and based on CLSI guidelines¹⁶. Eighteen antimicrobial agents from different classes were used to determine the sensitivity test including ampicillin-sulbactam (SAM), amikacin (AK), ceftriaxone (CTX), ceftazidime (CAZ), cefotaxime (CFT), ciprofloxacin (CIP), cefepime (CPM), gentamicin (GM), levofloxacin (LVE), meropenem (IMI), meropenem (MEM), piperacillin (PIR), piperacillin-Tazobactam (PTZ), trimethoprim-sulfamethoxazole (TS), tetracycline (T) Doxycycline (DXT), ticarcillin-K clavulanate (TIM), and tobramycin (TOB). *A. baumannii* isolates (adjusted to 0.5 MacCferland) were spread out on Muller-Hinton agar plates (MHA) (Merck, Germany), and each antibiotic disc was placed on the plates. All plates were incubated at 37 °C for 24 hours. Inhibition zone was measured by calculating the diameter of hollow zones around each disc. According to CLSI guidelines, the antimicrobial efficiency against the strain *in vitro* is expressed as “susceptible” or “intermediate”, and the strain that known as resistant or “non-susceptible” means that it is “not inhibited” *in vitro* by a concentration of the antibiotic disc. MIC was determined by Vitek 2 compact system (AST222 Cards).

A. baumannii Biofilm Formation Assay

In order to examine the ability of *A. baumannii* to form biofilm, microtiter plate assay was used¹⁷. Briefly, an overnight culture of *A. baumannii* was prepared in fresh Luria Bertani (LB) broth (OD₆₀₀ of 0.5) as previously mentioned^{2, 18}, and incubated at 37 °C for 24 h. A 200 µl of diluted bacteria 1:50 was inoculated in 96 wells plate and incubated over night at 37°C for 24 h. The microtitre plates were washed three times with 1 X phosphate buffered saline (PBS). The plates were fixed by methanol 99 %, and 200µl of Crystal violet 1% was added for 30 min, and left to dry at room temperature for 15 min. The microplates were washed with PBS, and all reads were recorded at OD_{c 570 nm} using ELISA reader. The assays were done in triplicate¹⁹. The adhesion ability of isolates was organized into four groups. The mean optical density of the

negative control (contained broth only) was deemed as the cut-off optical density (OD_c). The isolates were grouped as follows:

- If $OD \leq OD_c$, the isolate is non-biofilm producer
- If $OD_c < OD \leq 2 \times OD_c$, the isolate is weakly biofilm producer.
- If $2 \times OD_c < OD \leq 4 \times OD_c$, the isolate is moderately biofilm producer.
- If $4 \times OD_c < OD$, the isolate is strongly biofilm producer.

Low Frequency Ultrasound

To unequivocally determine the efficiency in the use of low frequency ultrasound (LFU) to inhibit the biofilm formation in clinical isolates *A. baumannii*, overnight culture of bacterial isolate was inoculated in 100 ml nutrient broth. The bacterial suspension was transferred to 250 ml conical flask and placed in a shaker incubator (200 rpm) at 37°C for 24 h. The suspension was centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended with sterile distilled water. The bacterial suspension was exposed to different intensities of 20KH 5Amp, 15Amp, 50Amp and several time intervals zero, 5, 10, 15, and 30 min, time zero is considered as OD positive control. To avoid the potential heating resulted from each exposure, temperature was continuously measured and the suspension was cooled in the ice box when it requires²⁰.

To precisely assess the anti-biofilm activity of LFU, the OD₆₀₀ of bacterial suspension from each exposure was measured, and the suspension was adjusted to 1×10^5 . The suspension was further diluted to 1:50 in LB broth, and about 200 μ l of suspension was inoculated in polystyrene microtiter plates, followed by incubation at 37°C for 72 h. The anti-biofilm was measured according to the following equation:

Biofilm inhibition = $\frac{OD(\text{positive control}) - OD(\text{treatment})}{OD(\text{positive control})} \times 100\%$

Anti-biofilm Formation Assay

Anti-biofilm formation assay was performed using the honey as a chemical compound against the clinical isolates of *A. baumannii*. Briefly, a loopfull of overnight bacterial growth of *A. baumannii* was inoculated in fresh LB broth and incubated at 37°C

for 24 h. The culture was then diluted to 1:50 with LB broth and nine different concentrations of honey were added 10, 20, 30, 40, 50, 60, 70, 80, 90%, and incubated at 37°C for 24 h. Biofilm formation assay was performed as previously described in 2.3²⁰.

DNA Extraction and Manipulation

DNA has been extracted according to Genomic kit's instruction procedure; the procedure has been set to isolates from each group of strong and moderate biofilm formation in addition to the standard strain of *A. baumannii* ATCC 17904. To determine the DNA concentration and purity, the Nano drop system has been used, and the purity has been measured depending on the following equation:

$$\text{DNA purity} = \frac{OD_{260}}{OD_{280}}$$

The concentration of DNA was calculated as:

$$\text{DNA concentration } (\mu\text{g ml}^{-1}) = OD_{260} \text{ nm} * 50 * \text{dilution factor}^{21}.$$

PCR for Biofilm related genes and Integrons (*bap*, *csuE*, *Int1*, *Int2*)

The PCR reaction of genes of interest (*bap*, *csuE*, *Int1* and *Int2*) was performed in a 25 μ l reaction mixture containing 12.5 μ l of Green Master Mix, 1 μ l (10 pmol μ l⁻¹) of forward and reverse primers listed in table 1. Genomic DNA (2 μ l) was added, and the volume was completed to a final volume of 25 μ l by nuclease free water. PCR conditions were set up according to annealing temperature of each primer as indicated in table 2. PCR products were resolved on 1% agarose gel, and visualized by using UV transilluminator, and the image was captured by Gel documentation (Witeg Labortechnik GmbH, Germany).

Statistical analyses

Statistical analyses were done using one-way ANOVA and Tukey's post hoc correction. To make of multiple comparisons, all stated P values were modified. Pearson's correlation analysis was used to examine the relationship between the phenotypes. P values less than 0.05 were deemed as significance. The JASP package was used. Values of ≤ 0.05 were considered as significance. All data analyzed in this work were derived from at least three biological replicates.

Table 1. Oligonucleotides used in this study

Primers	Primer sequence 5'-3'	Reference
<i>bap</i> -F	ATGCCTGAGATACAAATTATTGCCAAGGATAATC	Designed in this study
<i>bap</i> -R	AGGTGCTGAAGAATCATCATCATTAC	
<i>csu</i> E-F	TTGTGGGAATCGGGGTGTTCTTTG	
<i>csu</i> sE-R	GAGAGTGAACCAAGCGAGTCTGC	
<i>Int1</i> -F	CAGTGGACATAAGCCTGTTC	
<i>Int1</i> -R	CCCGAGGCATAGACTGTA	
<i>Int2</i> -F	CACGGATATGCGACAAAAAGGT	
<i>Int2</i> -R	GTAGCAAACGAGTGACGAAATG	

Table 2. PCR conditions of biofilm- related genes

Optimization	Temp ^o C	Time m:s	No.of Cycles
Initial denaturation	94 °C	5:00	1
Denaturation	94 °C	00:30	25
Annealing	<i>bap</i>	55°C	
	<i>csu</i> -E	61°C	00:45
	<i>Int1</i>	55°C	
	<i>Int2</i>	55°C	
Extension	72 °C	00:45	
Final extinction	72 °C	5:00	1
Hold	10	10:00	

Results:

Dissemination of Clinically Isolated *A. baumannii*

A total of 100 samples were collected from different hospitals in Baghdad /Iraq, and from different sources, around 66 isolates out of 100 were diagnosed as *A. baumannii*. Most of *A. baumannii* were isolated from swab 22 (33.3%), sputum 19 (28.8%), and blood 15 (22.7%), whereas, *A. baumannii* was less distributed in fluid and urine 6 (9.1%), 4(6.1%) respectively. (S1; supplementary file)

Antimicrobial Sensitivity Profile of Clinical Isolated *A. baumannii*

The antibiotic susceptibility pattern of *A. baumannii* isolates was examined by disc diffusion assay, and the results showed that the majority of clinical isolated *A. baumannii* 95.5 % were resistance to ceftriaxone, ceftazidim, and ticarcillin-K clavulanate, and about 92.4 %, 91 % were resistance to cefepime and ciprofloxacin respectively, whereas, Doxycycline exhibits the lowest activity against isolates 63.6% Fig. 1, S2, S3; supplementary file.

Multidrug-resistant (MDR) pathogens were defined as those were resistance to at least three antibiotic groups, including penicillin, cephalosporin, fluoroquinolones, and

aminoglycosides. MDR isolates that are carbapenem resistance, classified as extensively drug-resistant (XDR) ²² XDR strains that were resistance to colistin, and all other groups of antibiotics, defined as pan drug-resistant (PDR). In sputum, 1 (19) was (PDR), 12 (19) were (XDR), 4 (19) were (MDR), and 2 (19) were sensitive to the most antibiotics (non MDR). In swabs, 17 (22) were (XDR), 4 (22) were (MDR), and 1 (22) was non MDR. In fluids, 5 (6) were (XDR), and 1 (6) was (MDR). Moreover, most isolates 93.9% were resistance to all antibiotics that used. Interestingly, 12.1 % of the total isolates were MDR, 80.3 % were (XDR), 1.50% were (PDR), and only 6.1% were sensitive to the most antibiotics that used Fig. 2.

Notably, *A. baumannii* isolates from fluid and swabs were resistant to most antibiotics (94.22%) and (93.9%) respectively, whereas, isolates in urine, sputum and blood were less resistant 86.12 %, 84.76 %, 81.84 % respectively. The high resistance patterns of *A. baumannii* isolates in fluid and swabs may be due to several resistance mechanisms mediated by related genes, resulted from the extreme using of antimicrobial agents. On the other hand, current study revealed that the increase in resistance of *A. baumannii* clinical isolates to different antimicrobial agents is diverse within the sources.

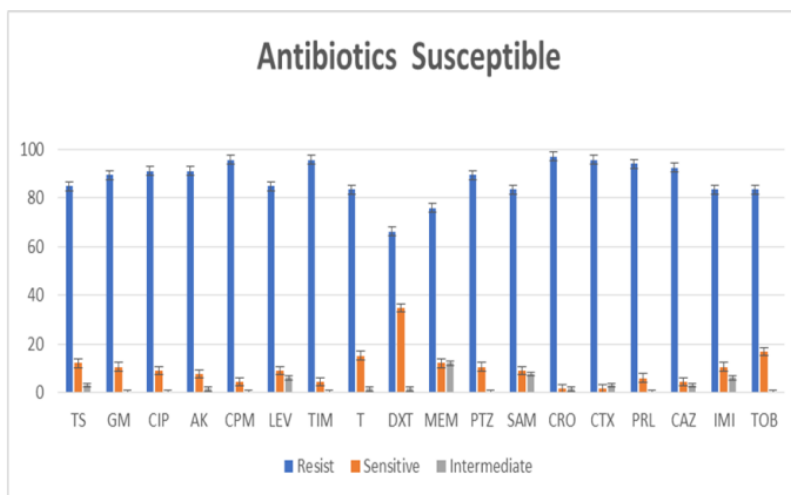


Figure 1. Percentage of Antibiotic sensitivity of *A. baumannii* clinical isolates. TS: Trimethoprim/sulpha, GM: Gentamicin, CIP: Ciprofloxacin, AK: Amikacin, CPM: Cefepime, LEV: Levofloxacin, TIM: Ticarcillin/ clavulanate, T: Tetracycline, DXT: Doxycycline, MEM: Meropenem, PTZ: Piperacillin/ tazobactam, SAM: Ampicillin/sulbactam, CRO: Ceftriaxone, CTX: Cefotaxime. PRL: Piperacillin CAZ: Ceftazidime. IMI: Imipenem. TOB: Tobramycin.

Determination of Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration is defined when the value of antimicrobial agents was equal to or higher than the breakpoints, which means that the bacteria or pathogens were resistant to the antibiotics used. In the current study, the MIC was determined by Vitek 2 compact system (AST222 Cards), and the rates of resistance to the antibiotics are shown in Table 3. The results showed that most *A. baumannii* isolates were sensitive to colistin except for one isolate. The MIC value to imipenem was ($\geq 16 \mu\text{g ml}^{-1}$), whereas, ²³, determine the lowest MIC value to imipenem as ranged from 8 - 64 $\mu\text{g ml}^{-1}$. It is clear that the increase in the initial breakpoint of MIC means that the antibiotic does not affect *A. baumannii* at a concentration lower than 16 $\mu\text{g ml}^{-1}$. Moreover, the reduced susceptibility to imipenem in clinical and

nonclinical isolates to 80.4%-83.3% and the MIC ranged from 8-64 $\mu\text{g ml}^{-1}$ ²⁴.

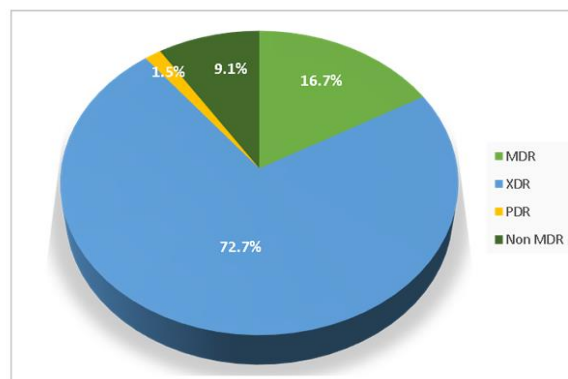


Figure 2. Antimicrobial susceptibility patterns of clinical *A. baumannii* isolates. The susceptible profile ratios, 72.7 % of clinical isolates exhibit XDR, followed by 16.7 % MDR, 1.5 % PDR, and 9.1 % were non MDR.

Table 3. Determination of Minimum Inhibitory Concentration (MIC) in Clinical isolated *A.baumannii*

Antibiotics	S	I	R	Antibiotics	S	I	R
TS	2(≤ 20)		28(≥ 320)	TIM	1(≤ 8)	0	29(≥ 128)
GM	4(≤ 1)	0	26(≥ 16)	MEM	4(≤ 0.25)	4(=8)	22(≥ 16)
CIP	3(≤ 0.25)	0	27(≥ 4)	IMI	3(≤ 0.25)	4(=8)	27(≥ 16)
PIR	2(8)	0	28(≥ 128)	TOB	6(≤ 1)	0	24(≥ 16)
PIT	3(≤ 4)	0	27(≥ 128)	CPM	2(≤ 1)	0	28(≥ 64)
TIM	2(≤ 8)	0	28(≥ 128)	CAZ	1(≤ 1)	1(=16)	28(≥ 64)
MIN	10(≤ 1)	8(=8)	12(≥ 16)	TIC	15(≤ 0.5)	0	15(≥ 128)
Col	29(≤ 0.5)	0	1(=8)	SAM	5(≤ 2)	0	26(≥ 32)

TS: Trimethoprim/ sulpha,GM: Gentamicin, CIP: Ciprofloxacin, AK: Amikacin, CPM: Cefepime, LEV: Levofloxacin,TIM: Ticarcillin/clavulanate, T:Tetracycline, DXT: Doxycycline, MEM: Meropenem,PTZ: Piperacillin/ tazobactam, SAM: Ampicillin/sulbactam, CRO: Ceftriaxone, CTX: Cefotaxime. PRL: Piperacillin CAZ: Ceftazidime. IMI: Imipenem. TOB: Tobramycin, Col: Colistin

Biofilm Formation Capacity

The results of biofilm forming ability revealed that all clinical *A. baumannii* isolates produced strong biofilm on polystyrene plates Fig. 3, S4: supplementary file. Strains isolated from swabs had a biofilm ratio of 38.46%, and the mean values of about 12.821 ± 22.206 , whereas, strains isolated from sputum had the ability to form biofilm with the ratio of 30.77%, and the mean values of 10.256 ± 17.765 . Moreover, strains isolated from fluid had less biofilms ratio of 15.38%, and the mean values of 5.128 ± 8.882 , and the isolates from fluid had similar biofilm ratio 15.38%, and the

mean values of 5.128 ± 8.882 . Comparatively, the strains isolated from blood and urine Foly tip could only formed biofilm at (7.69%) ratio, with the mean values of 2.563 ± 4.440 . On the other hand, *A. baumannii* ATCC17904 forms weak biofilm. From the above results, it is obvious that strains isolated from swab and sputum form strong biofilm than other isolates (blood, fluid, and urine) $P < 0.001$. Most notably, there was a statistical significant difference between antibiotic resistances and biofilm formation including (strong, moderate, weak and non-biofilm) isolates at a statistical level $P < 0.0001$ Fig. 4, S5: Supplemented file.

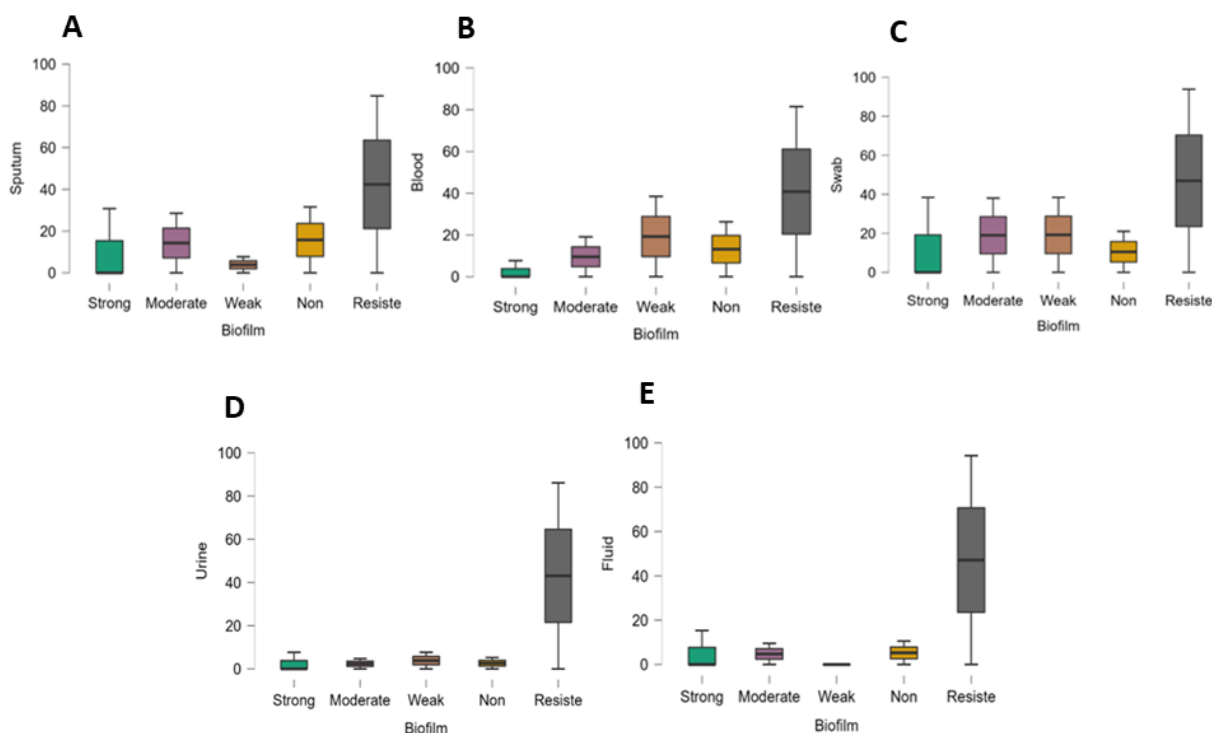


Figure 3. The relation of antibiotic resistance values with biofilm formation pattern of *A. baumannii* isolates. *A. baumannii* isolates from different sources were investigated for biofilm formation ability in correlation with their resistance profile. **A:** biofilm formation of *A. baumannii* in sputum; **B:** in blood; **C:** in Swabs; **D:** in Urine; and **E:** in Fluid. The assays were done in triplicate.

Biofilm Inhibition using of LFU

The potential activity of LFU to reduce the ability of *A. baumannii* isolates to form biofilm was evaluated. The analysis of biofilm inhibition revealed that the biofilm ability was decreased from 44.3% to 50.7% at 5 min exposure and under different amplitudes 5, 15, 50 AMP/ 20KH. Fig. 5, whereas, after 10 min exposure, the inhibition percentage of biofilm is increased remarkably from 51.7 to 57.9 % under similar intensities. However, there is no significant reduction in biofilm production after 15 min 58.7 to 60.7%, and 30 min 58.7% to 62.9%. " S6: supplementary file". On the other hand, Odc_{600 nm} was measured after each exposure to LFU, and the results showed that there

is an obvious decrease in the density of all treated isolates (Data not Shown).

The impact of LFU on antibiotic susceptibility profile of *A. baumannii* was determined. The results indicate that there is no obvious effect on *A. baumannii* susceptibility pattern (Data not Shown).

The probability to inhibit the biofilm formation by clinically isolated *A. baumannii* chemically was also investigated" S7: Supplementary file". The 5-hydroxymethylfurfural; an organic compound presents in the honey; is annotated to be able to inhibit biofilms formation of *A. baumannii*. The mechanism mediated by 5-hydroxymethylfurfural is unknown, but it was reported once that this compound is toxic for human

under high temperature. The results showed that the inhibition percentage is concentration dependent, at 10 % the inhibition rate was 38.38 %, in comparable, at 100 % the inhibition rate was 50.80%.

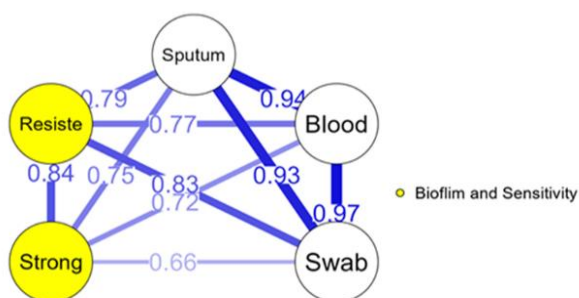


Figure 4. Correlation between biofilm formation and antimicrobial resistance profile of *A. baumannii* isolates. The correlation between strong biofilm ability and antibiotic resistance patterns of *A. baumannii* isolates in blood, sputum, and swab. The correlation value is about 0.84 as indicated in the figure.

Detection of Biofilm -Related genes (*bap*, *csuE*)

One of the objectives of the present study is to detect the presence of common biofilm related genes in all *A. baumannii* isolates. The majority of 15 isolates including: strong, moderate

and non-biofilm producer were subjected to investigate the presence of the main biofilm-related genes (*bap*, *csuE*), and whether the resistance pattern is biofilm dependent. The genes of interest were PCR amplified using primer pairs listed previously in Table 1. The PCR products were electrophoresed and visualized under the UV light. Interestingly, the results showed that the prevalence of the *bap* gene 650 bp was 93.3%, whereas the *bap* gene was absent in one strong biofilm forming isolate 6.7% Fig. 6A. On the other hand, the prevalence of chaperone-usher pili assembly system gene *csuE* 103 bp were 86.6% in *A. baumannii* isolates, and 13.4% of isolates were lack the *csuE* gene Fig. 6 B.

In order to unequivocally determine the correlation between antibiotic resistance and biofilm formation ability of *A. baumannii*, integron genes (*intl-1* and *intl-2*) were detected in 15 selected clinical isolates of *A. baumannii*. The amplified genes were analyzed with 1.5 % agarose gel, and the results showed that the prevalence of the *intl-1* 200 bp and *intl-2* 700 bp was 87.5% and 13.3% respectively, whereas, about 12.5% and 86.6% of selected isolates were lack the *intl-1* and *intl-2* genes respectively Fig. 6 C, D.

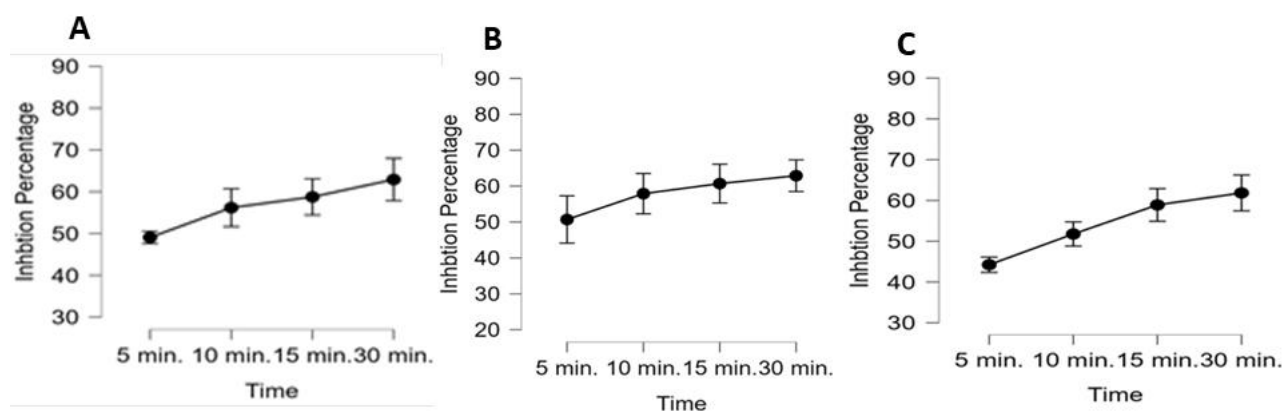


Figure 5. Biofilm Inhibition percentage of Low Frequency Ultrasound against *A. baumannii* clinical isolates. A culture of *A. baumannii* isolates was exposed to different amplitudes of LFU, the isolates were subjected to biofilm assays and the inhibition percentage was calculated. A: represents the inhibition percentage at 5AMP; B: inhibition percentage at 15AMP; C: inhibition percentage at 50AMP. The experiments were done in triplicates.

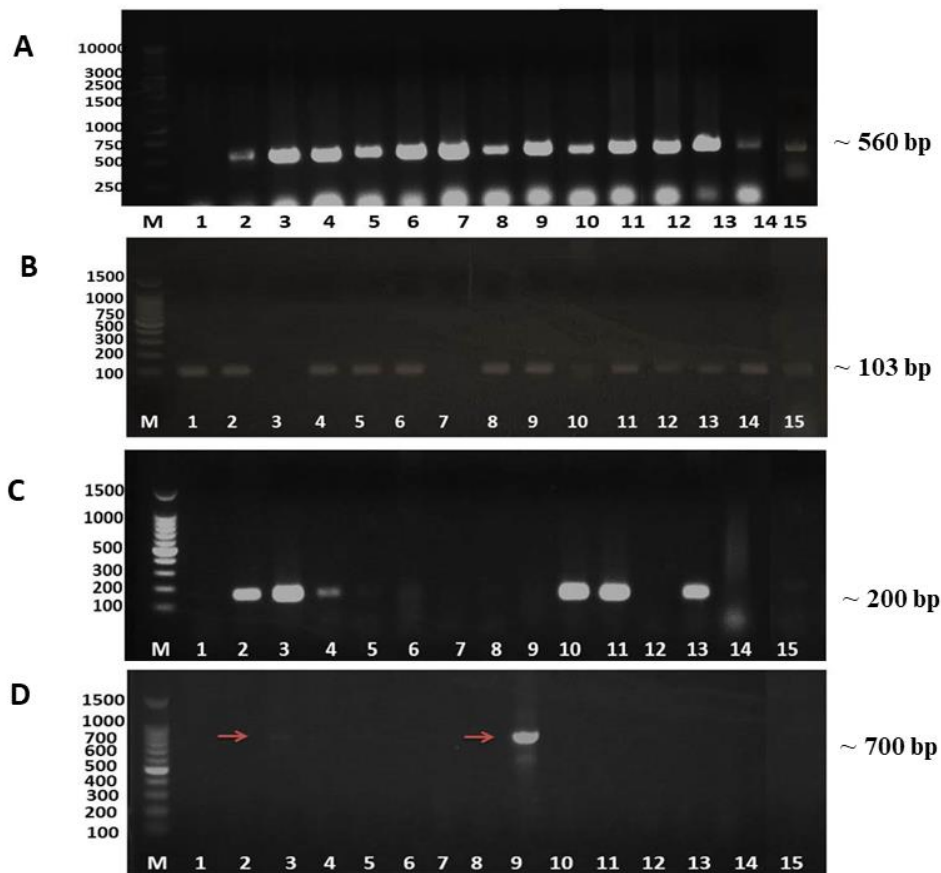


Figure 6. Detection of biofilm-related genes and integrons in *A. baumannii* isolates. PCR amplification of (*bap*, *csuE*, *intI-1*, and *intI-2*) genes were done using specific primer pairs for each gene, and the products were electrophoresed on 1.5 % agarose gel, and visualized by UV-transilluminator. Lane M: Hypper ladder I Molecular marker 1 Kb (biolone); Line: 1,2,3,7,8,9,10,11 and 13: represent the strong biofilm producers, Line: 4, 6, and 14: refer to moderate biofilm producers; Line: 5, and 12 refer to nonbiofilm producer, Line 15 the standard strain of *A. baumannii* as a control. A: *pab* gene ~560 pb, B: *scuE* ~103 pb, C: *intI-1*~200 pb, and D: *intI-2* ~700 pb.

Discussion:

In the recent years, *A. baumannii* have been reported as nosocomial pathogens due to their prevalence in the hospitalized patients. Statistical analyses of *A. baumannii* isolated based on the source of isolation indicate that the prevalence in swabs (burns and wounds) 33.3%, sputum 28.8%, and blood 22.7% was higher than that in fluid 9.1%, and urine 6.1%. Locally, it was reported that the majority of *A. baumannii* were isolated from sputum 61%, wounds 19%, blood 9%, urine 3%, and other specimens 8 % included pus, synovial fluid, and ascetic fluid ²⁵. Furthermore, it was recorded that the majority of *A. baumannii* strains were isolated from sputum, wound, swabs, and urine ²⁶. Our findings were agreed with ²⁷ who reported that *A. baumannii* was isolated from burn skin and wound. *A. baumannii* have several adhesion factors facilitate the colonization and persistence in the respiratory system and mucous cells.

The prevalence of *A. baumannii* in Baghdad hospitals became the main issues and raise a remarkable interest that required serious steps to turning off the rapid spread of this pathogen and decrease the mortality and morbidity rates. In the current research, *A. baumannii* isolates were resistant to mainly all antimicrobial agents used 86.7%. The resistance to ciprofloxacin 90.9 %, imipenem 83.3%, and gentamicin 89.4% is similar to ²⁸, who showed that the resistance to Ciprofloxacin was 95%, meropenem 74.2%, and Gentamicin 35%.

Moreover, it was reported that the majority of *A. baumannii* isolates 92% were resistant to Amikacin, and 83.3% were resistant to tetracycline ²⁹. Moreover, 84.8% of *A. baumannii* isolates were resistant to levofloxacin, however, it was mentioned that 91% of pathogenic isolates were resistant to levofloxacin ³⁰.

In addition, of the 66 clinical *A. baumannii* isolates evaluated, 1.5 % were qualified as PDR, 72.7 % were designed as XDR and 16.7 %, were

qualified as MDR, whereas, 85.6 % of *A. baumannii* isolates were designed as MDR, and 14.4% were qualified as XDR about ³¹. The resistance phenotype of Gram negative bacteria in urine is diverse, among all isolates in urine 35, 62.8 % were qualified as MDR, and 28.5 % were qualified as XDR. However, they all are considered as biofilm producers ³².

The clinical isolates of *A. baumannii* exert different patterns of resistance against antibiotics, 95.5% were resistance towards ceftazidime, ceftriaxone and CTX, and 90.9 % were resistance to ciprofloxacin and piperacillin. It was reported by Mekkey et al., 2020 ³³, that 100% of *A. baumannii* were resistance to ceftazidime, 83 % were resistant to meropenem, and 98 % were resistant to ciprofloxacin. Most notably, all *A. baumannii* strains isolated from different sources display various resistance profiles to most antibiotics, all *A. baumannii* isolates were not susceptible to carbencillin, but 50 % were resistance to ampicillin-sulbactam ³⁴. In addition, the resistance to ampicillin- sulbactam was increased 65%, due to the prevalent use of antimicrobial agents in Baghdad hospitals. The resistance pattern of *A. baumannii* strains was varied in AL- yarmok Teaching Hospital, as 65% were resistance to piperacillin, 100 % were resistance to cefotaxime, 65% were resistance to ceftazidime, 100 % were resistance to cephalixin, 65% were resistance to ceftriaxone, 50% were resistance to imipenem ³⁵. Additionally, the majority of *A. baumannii* 99% were resistant to ciprofloxacin, 98% were resistance to ceftazidime, meropenem, and imipenem, 77% were not sensitive to gentamicin, and 48% were resistance to amikacin ³⁶. Our results showed that most of *A. baumannii* isolates 98.5% were highly susceptible to colistin, as it mainly targets the LPS components, and attenuates the colonization of *A. baumannii* in lung epithelial cells ³⁷.

Based on our results, of the 66 clinical *A. baumannii* isolates; 11, 16.7% were designed as MDR, 48, 72.7% were qualified as XDR, 1.5% was qualified as PDR, and 6, 9.1% were susceptible to the most antibiotics. The prevalence of XDR isolates is attributed to the extensive use of antibiotics in Iraqi hospitals ³⁸.

The ability of *A. baumannii* isolates to form biofilm promotes the persistence in different environments, and increasing the risk of healthcare-associated infections. This study assesses the biofilm-production capacity of the clinical isolates *A. baumannii*, and the potential correlation between biofilm formation and susceptibility to antibiotics. Most notably, there were significant statistical differences between biofilm formation ability and

antibiotic resistance at level $p \leq 0.01$, indicating for a positive correlation between biofilm patterns and antibiotic resistance ³. The current study revealed that most *A. baumannii* isolates 72.7 % were able to form biofilm, but only 20% isolates were able to produce strong biofilm, because the link between biofilm forming capacity and pathogenic resistance to antibiotics is currently unorganized, and the outcome between studies was even contrast ^{39, 40}. However, a positive correlation between bacterial antibiotic resistance and biofilm formation was mentioned ⁴¹ in contrast with finding of our result which, indicating that these pathogens were not depending on antibiotic resistance for survival, as the non-biofilm forming isolates were resistance to antibiotics as well. Our study indicated that the antimicrobial resistance is not correlated with the biofilm forming ability, and antibiotic resistance is one of the main survival mechanisms for *A. baumannii*, in addition to biofilm forming capacity.

Furthermore, most of 75% *A. baumannii* isolates were able to form biofilm, of which 43% isolates were able to form strong biofilm, and 32% isolates were formed moderate biofilm, while, 25% were produced weak and non-biofilm producers. It was notably that the biofilm formation ability decreases the effect of antimicrobial agents, and alternative strategies of treatment are seriously needed, there for, dissecting of the virulence factors in pathogenic bacterial isolates is urgent, and important with the use of most authoritative methods ⁴².

Because the ability of pathogens to form biofilm is not correlated with the resistance to antimicrobial agents, there are significant challenges needed to inhibit the biofilm formation of *A. baumannii* isolates. In this study, among 66 of *A. baumannii* isolates, three isolates evaluated as strong biofilm formers, were exposed to LFU. The results investigate that the sonication of bacterial suspension at continues frequency 20 KHz, stimulates the initial decrease in the number of bacterial cells, and the number of cells was continuously decreased after 15 min exposure. Subsequently, there was a gradual and continuous decrease in the number of bacterial cells after 30 min exposure ⁴³. When a bacterial suspension is exposed to low frequency ultrasound, a large bubble cavitation will form and fall, this will generate a high level of energy, and in contrast, high frequency ultrasound generates shorter time for cavitation bubbles to form with less energy, ultimately, leads to collapse ⁴⁴. This indicates that low frequency ultrasound 20 KHz is might be a good approach to significantly reduce the bacterial suspension. In addition, there are many factors affect the

sensitivity of bacteria to sonication such as; the composition of medium, the movement of sound, distribution of power and viscosity⁴⁵.

However, the using of LFU will temporarily decrease the biofilm formation of *A. baumannii*, there are many factors enhance the efficiency of LFU such as; power intensity, environmental conditions, frequency, time of irradiation, and duty cycle. Low frequency ultrasound is a promising assay to decrease the biofilm formation ability due to many reasons; the probe directivity, and remedation efficiency, both target the deep tissues without damaging the tissue. Furthermore, the low frequency ultrasound approach may release extra oxygen and nutrition into the biofilm, which accelerates the bacterial growth and retrieves the antimicrobial susceptibility to antibiotics⁴⁶.

It was demonstrated that the continuous wave of ultrasound at a frequency of 70 KHz, and power density 10mW/cm² are efficient to improve the bactericidal effect of antibiotics more than low intensity 1mW/cm², and the pulsed wave intensity is effective as a continuous ultrasound with identical density²¹.

On the other hand, the chemical components in honey play potential roles as antimicrobial drugs against *A. baumannii* isolates, the activity is due to several factors; high osmotic effect, non-hydrogen peroxide, phytochemical components that involved in the controlling of bacterial colonization, and subsequently disruption of biofilm⁴⁷. Additionally, it was suggested that the high concentration of sugar and high acidity in honey reduce the ability of pathogens to form biofilm. Our study revealed that there was a significant invert correlation between the high concentration of honey and biofilm inhibition rate^{48, 49}.

Biofilm-related genes detection

The ability of *A. baumannii* isolates to form biofilm is induced by a complex of genes that involved in biofilm maturation and completion. In our study, the *bap* gene was screened out in 15 isolates. It was reported that the frequency of *bap* gene in the isolates were 92%, and there was a strong correlation between the *bap* gene and the biofilm production⁵⁰. The *bap* gene encodes for Bap protein, is one of the major virulence factors and plays a substantial role for adherence to the epithelial cells and maturation of biofilms. In addition, another study⁵¹ found that 89% of MDR *A. baumannii* have *bap* gene and suggested that there was strong correlation between biofilm formation and biofilm gene in the multi –drug resistance

A. baumannii. Furthermore, the prevalence of *bap* gene in 30 clinical *A. baumannii* isolates was 76.6%. The prevalence of *bap* genes in the strong biofilm forming isolates is clearly explained the ability of isolates to form biofilm, and this may be attributed to the influence of biofilm associated protein (Bap) in the biofilm maturation⁵⁰. Our findings showed a significant correlation between *bap* gene and biofilm formation ability. Our findings are similar to 66, who demonstrated a strong correlation between biofilm formation and *bap* genes. Furthermore, *bap* gene was detected in 48.43% clinical *A. baumannii* isolates⁵².

According to our results, of the 15 evaluated *A. baumannii* isolates, the prevalence of *csuE* was 86.6%. Similarly, the prevalence of *csuE* gene in clinical isolates *A. baumannii* was 93.2%^{53, 54}. Moreover, it was recorded that the *csuE* gene was predominance in all evaluated *A. baumannii* isolates 100%²⁵. Based on the above findings, there was a strong correlation between biofilm, antibiotic resistance, and biofilm related genes (*bap*, *csuE*), as the bacterial pathogens in the biofilm community are able to tolerate the antimicrobial agents 100 to 1000 folds more than the plankton community⁵⁵. Furthermore, the formation of pili is fundamental for the initial steps in biofilm formation. The results of the current study revealed the prevalence of (*bap* and *csuE*) genes in strong, moderate, and weak biofilm producers, these results proposed that the expression level of these genes play a key role in the determination of biofilm formation capacity⁵⁶.

Integrations

In our study, the prevalence of integrations (*Intl-1* and *intl-2*) genes in *A. baumannii* isolates were 87.5% and 13.3% respectively. Many mentioned that several factors involved in the distribution of integrations within clinical isolates in particular, for example; the wrong uses of antibiotics for treatment, overexpression of gene cassettes included integrations class -1, integrations capacity to accept a new gene cassette, and disability to build a national surveillance system in the medical centers as a part of the national managements for antimicrobial resistance mechanisms. Because of the *Intl-1* gene is transferred by a common genetic elements such as; conjugative, plasmids, and transposons, suggested to be the main reasons for the significant increase in the emergence of MDR *A. baumannii* in Iraqi hospitals^{57, 58}.

The study of⁵⁹ reported that the prevalence of *intl-1* and *intl-2* genes among the clinically isolates *A. baumannii* were 63.9% and 78.2% respectively. However, many studies have been

conducted to investigate the presence of integron class- I worldwide. Most notably that, the prevalence of integron class-1 is variable, due to the geographical distribution of *A. baumannii*, the source of infection, or the source of isolation ⁶⁰.

Furthermore, the results clearly showed that there was a strong correlation between the prevalence of *intl-1* gene and antimicrobial resistant patterns, and with the biofilm formation capacity. These correlations explain the significant role of integrons in antibiotic resistance and thereby in the epidemic attitude of *A. baumannii* ⁶¹. Other study ⁶² found that the prevalence of integron class-1 and integron class-2 were 70.77 % and 26.15% respectively. Integrons; are transposons (like genetic elements), have a conserved region, and encodes for antibiotic resistance determinants and have extra ability for chromosomal integration in bacteria ⁵⁸.

Many classes of integrons have been identified, and the most common classes are integrons class-1 and class-2 in MDR *A. baumannii* strains ⁶⁶. According to the previous study, integron class-1 is the most important gene in the distribution of MDR *A. baumannii* ⁶².

Our study demonstrated that the integron class-1 was predominant in the (XDR) and (MDR) *A. baumannii* isolates, and there was a correlation between integron class-1 and the resistance gene cassettes.

Conclusion:

The predominance of *A. baumannii* in Baghdad hospitals is particularly in Gazii AL-Hariry Hospital, and burn hospital increasing day by day, and the increase in antibiotics resistance is crucial. *A. baumannii* clinical isolates exhibit high resistance ability to cephalosporins, whereas the isolates were sensitive toward doxycycline, suggesting that doxycycline is effective against *A. baumannii*. In addition, *A. baumannii* is well known for its ability to form biofilm on biotic and abiotic surfaces, so novel approaches are needed to attenuate and decrease biofilm formation ability. The use of green energy of LFU with low frequency ultrasound is a promising technique to inhibit biofilm production, particularly at low energy and short time. In addition, the natural components in honey reduce the biofilm production. Subsequently, they reduce the transfer of pathogen through medical equipment, and decrease the number of patients infected with *A. baumannii*.

Authors' declaration:

- Conflicts of Interest: None.

- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.

- **Ethics Approval:** The form of Ethical consideration Approval is approved by National Centre for Teaching Laboratories of Medical City Hospital, and assigned by all authors.

Authors' contributions statement:

The authors have contributed equally in the lab works, writing up and typing this paper. Sh. A.A. contributed in lab work, methodology and writing up the manuscript, and some data analysis. H. H.A. H. Conceptualization writing of original draft, methodology, review and editing, data analysis. A.M A. Conceptualization, supporting the idea, aim and goal, methodology and reviewing.

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تقييم تأثير الموجات فوق الصوتية على تكوين الغشاء الحيوي لبكتريا *Acinetobacter baumannii* المعزولة سريريا

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الخلاصة:

تلقي بكتريا الراكدة البومانية *Acinetobacter baumannii* اهتمامًا كبيرًا لمقاومتها الواسعة للمضادات الحيوية ولارتباطها بالعديد من الإصابات الشديدة وتفشي الأمراض السريرية. هدفت الدراسة إلى معرفة مدى تأثير المضاد الحيوي ضد بكتريا *A. baumannii* المعزولة سريريا والمنتجة للأغشية الحيوية، وكفاءة الموجات فوق الصوتية منخفضة التردد (LFU) والعسل لتخفيف إنتاج الأغشية الحيوية. تم جمع 100 عينة من مصادر مختلفة من مستشفيات بغداد. أظهرت أنماط الحساسية أن نسبة العزلات المقاومة للأدوية (PDR) كانت 1.5%، 72.7% كانت مقاومة للأدوية ذات الطيف الواسع (XDR)، 16.7% مقاومة للأدوية المتعددة التأثير (MDR)، 9.1% كانت غير مقاومة للمضادات المتعددة التأثير وحساسة لمعظم المضادات الحيوية المستخدمة. تم الكشف عن القدرة على تكوين الغشاء الحيوي عن طريق التلوين الكريستالي البنفسجي، وأظهرت النتائج أن 20% تكون غشاء حيوي قوي، و 31.8% تكون غشاء حيوي متوسط. تم تقليل نسبة تكوين الأغشية الحيوية الرقيقة باستخدام الموجات فوق الصوتية منخفضة التردد LFU والعسل. علاوة على ذلك، كشفت نتائج تفاعل (PCR) أن العزلات لا تحتوي جميعها على جينات أو إنتيجرونات مرتبطة بالأغشية الحيوية (*bap*، *csuE*، *Int1-1*، *Int1-2*)، على الرغم من أنها منتجة قوية للأغشية الحيوية. توصي النتائج إلى أن الموجات فوق الصوتية منخفضة التردد والمكونات الكيميائية للعسل قد تكون خيارًا جيدًا لتقييم تكوين الأغشية الحيوية *A. baumannii*، وان العلاقة بين مقاومة المضادات الحيوية والقدرة على إنتاج الأغشية الحيوية هي علاقة سلبية.

الكلمات المفتاحية: بكتريا الراكدة البومانية، مقاومة المضادات، تكوين الاغشية الحية، العزلات السريرية، الموجات فوق الصوتية منخفضة الشدة.