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DETECTION OF BIOFILM FORMATION AND ITS RELATED WITH AMINOGLYCOSIDE RESISTANCE IN *ACINETOBACTER BAUMANNII* ISOLATES, ISOLATED FROM SOME BAGHDAD CITY HOSPITALS

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ABSTRACT
In the last decade the health care sections has face a new strong threaten by new nosocomial as multi-drug resistant known as *Acinetobacter baumannii*, this organisms has the ability to reinforce it colonization by the producing of biofilm result in resistant to many classes of the antibiotics. 125 samples has been collected from Baghdadian hospitals from patients with skin infections (burns, bedsores, diabetic foot ulcers, and wounds), it first examine by microscope, and after the biochemical tests and identification by the mechanical VITEK2, an18(14.4%) isolates was known as *Acinetobacter baumannii*, the ability of the isolates to produce the biofilm has been studied by three methods Congo Red Agar (CRA) Tube Method (TM), and Tissue Culter Plate (TCP), all the isolates were show the ability to produce the biofilm at less in one way, with several degrees ,the sensitivity to biofilm production was in rate0%, 100%, 100%, specifity 0%, when the false positive 0% and the false negative 0%and the positive predictive value was 100% and the negative predictive value was 0% for the three methods, the susceptibility to the Aminoglycoside antibiotics has been study to five antibiotics (Gentamicin GM, Tobramycin TOB, Netlimicin NET ,Amikacin AK ,and Streptomycin S),the isolates show resist to the Gentamicin and Tobramycin in 100 percent , followed by the Streptomycin 77.7%, Amikacin 72%, and the Netlimicin 50%, the correlation between the resistance to the antibiotics and biofilm production has been study. There was a correlation between the ability of production of biofilm and the resistance to the Aminoglycoside antibiotics production but there was no connection between how much the biofilm is strong and the resistant to the aminoglycoside production.

KEYWORDS: Acinetobacter, baumannii, skin infections, Biofilm, Antibiotics resistance, Aminoglycosides, Baghdad hospitals, Vitek 2 compact system.

INTRODUCTION
Genus *Acinetobacter* include gram negative, non-motile, coccibacilli bacteria[1] obligate aerobic, this bacteria known as a comensal, but in the last decades it has been an opportunistic pathogen.[2]

The *Acinetobacter baumannii* is a nosocomial which is causative many disease like: septicemia, pneumonia, skin and soft tissue infection, blood infection and death.[3,4] the main cause of the mortality and morbidity in *Acinetobacter baumannii* may be belong to the high resistance to the known antibiotics.[5], the biofilm is one of the virulence factors which is acquire the bacteria survive in the hospitals and cause devise-related infections[1] biofilm known as the arrangement in which the cells are morphologically, metabolically, and physiologically different from the planktonic counterpart[6] the microorganism which grown in a biofilm, always associated with human chronic infections, and resist to the antibiotics[7], it exhibit a phenotypic characteristics, some of it is the antibiotic resistance and increase the resistant to the host immune defenses[8], biofilm mainly regulated by quorum sensing and two other component regulatory systems, the poly-β-(1,6)-N-acytelglucose amine is extra poly sacchride that's thought to function as intracellular adhesion for the bacteria within the biofilm.[9]

At less there are three methods to detect the ability of biofilm production in bacteria (Tissue Culter Plate) TCP, (Tube Method) TM, and (Congo Red Agar Method) CRA.[7]

increasing use of antibiotics, has led to higher resistance, and one of these is the aminoglycoside antibiotics[10] in general there are two main reasons for aminoglycoside resistance, the first is, involve in the entering of the aminoglycoside antibiotics, and the second is the

methylation enzymes[11] that are controlled by twelve combination different genes.[12]

The main target of this research is to isolate *Acinetobacter baumannii* from skin infections, study the production of biofilm in this isolates, recognize the relationship between biofilm stamina and the kind of the skin infection and the relationship between the biofilm and its ability to resistance for Aminoglycoside antibiotics.

MATERIALS AND METHODS

A hundred twenty five samples were collected in the period between 23/11/2015 to 8/3/2016 from seven hospitals in Baghdad city(Ak-Kindy hospital, Al-Kadhimyah medical city, Baghdad Hospital for surgicals/ city of medicine, Martyr Ghazi al Hariri specialist/city of medicine, burn specialist hospital /city of medicine and teaching labs/city of medicine, Al-Yarmouk teaching hospital), for skin infections: bed sores samples, burns samples (Second and third degrees), diabetic foot ulcers, wounds samples (most of the samples were collected from soldiers injuries in the last Iraqi military operations, gun shouts and surgical wounds), first it examine by microscope and has been identified by classical ways (cultural) on MacConkey Agar(SALUCEA/Netherland), Leeds Acinetobacter Agar (Casien 15g, soya pepton 5g, sodium chloride 5g, fructose 5g, sucrose 5g, manitol 5g, phenyl alanin 1g, ferric ammonium citrate 400mg, phenol red 20mg, Agar 12g) as mention in[13] and biochemical tests which include: Catalase(Schuchardt/Germany), Oxidase(Schuchardt/Germany), IMVIC (LAB/UK), KIA (Kligler Iron Agar) (SALUCEA/Netherland), and O/F with (glucose, arabinose, Milibioes). And final identification has been done with Vitek 2 compact system (Biomérieux, France).

Detection of biofilm

Qualitative method

The qualitative method for detection of biofilm has done with CRA as mention below

Congo Red Agar

The CRA had prepared by dissolve 18.25g brain heart infusion broth, 25g sucrose, 5g Agar in 490ml of deionized water and sterile by autoclave, then 4g of Congo red Stain was mix with 10 ml deionized water vigorously, and then sterile in autoclave, after cooling the dye solution and the medium to 45˚C, it mix and pour in Petri dish, left to solidify and use for detect biofilm formation, by inoculated single colony of the *A.baumannii* on the CRA plate by streaking as mention in[14], the black dry colonies refer to the biofilm production, red or move color of colony is refer to a negative biofilm.[14]

Quantitative methods

The quantitative method for detection has done with Tube Method (TM), and Tissue Culter Plate (TCP) method.

Tube Method

The quantitative method has done with polystyrene tubes as mention in[14], briefly the *A.baumannii* has growth in 5 ml of trypticase soy broth with 1 % Glucose in plastic tubes for 24 hours.

After 24 hours of incubation the growth was discharge and the tubes washed with phosphate buffer PH 7.3, then left to dry, after drying it's stained with crystal violet in concentration 1% for five minutes and washed with tap water, inverted to dry, after drying it compared with the negative control (only the broth without bacterial inoculation) and the result record as a scores:

− if the isolate non-biofilm production.

+ if the isolate has a weak biofilm production.

+ + if the isolate has a moderate biofilm.

+ + + if the isolate has a strong biofilm.

Tissue Culter plate method

The *A.baumannii* had been grown in brain heart infusion broth for 18-24 hour, after incubation period ;1 ml of the bacterial growth add to 10ml of fresh brain heart infusion broth supplemented with 1% glucose and make it equal to McFarland with concentration 0.5 and 250 microliter of the suspension were move to the wells in the microtiter plate in triplicate, control negative was the medium only, incubate for 24 hour more.

After incubation the growth has been remove and wash three times with phosphate buffer(PH7.4) (300 microliter per well) with shaking to remove the non adhere bacteria.

Then the remaining bacteria were fix with 96% ethanol,(250 microliter per well) for 15 minute, then it drain and left to dry, after fixation the wells were stained with crystal violet 2% (200 microliter to each well) for 5 minute, then plate were washed with tap water and left to dry, in this step the biofilm will appear as a visible ring in the wells.

The quantitative biofilm analysis has done by add (glacial acetic acid 33% v/v), 200 microliter to each well, and then measure the optical density on 490 nanometer by the ELISA reader(Bioteck/USA).[15]

The result record as:

The value of the OD	
Cut off	0.064
Negative	0
Weak	0 - 0.128
0.128 – 0.512	Moderate
< 0.512	Strong

Cut off OD=(3 standard deviation + mean of negative control)
Positive control = mean of the triplicate
Weak biofilm= 0 cut off -2 cut off OD value
Moderate biofilm = 2 cut off value – 4 cut off OD value
Strong biofilm = more than 4 cut off OD value

Antibiotic susceptibility

The detect of antibiotic susceptibility was done by Kirby Bauer method as mention in CLSI (2014) with five Aminoglycoside antibiotics: Gentamicin(GM) (10mcg) MAST/UK, Tobramycin(TOB) (10mcg) Bioanalyse/Turkey, Netlimicin(NET) (30 mcg) from MAST/UK, Amikacin (AK) (30 mcg) MAST/UK, and Streptomycin(S) (10 mcg) Bioanalyse/Turkey.

A50 microliter of bacterial suspension (equal to 0.5 McFarland) was spread on Muller Hinton Agar by using of glass spreader, waiting to dry for 10 minute and then the antibiotic discs was put on the bacterial growth and incubate for 18-24 hour in 37˚C, the inhibition zone was measure by Veirner clipper and compare with the standard in CLSI (2014)(16).

RESULTS

A125 samples were collected from Baghdad city hospitals for skin infections include (bed sore, burn, diabetic foot ulcer and wounds) and an18 (14.4%) were identified as (*A.baumannii*) while an 95 (85.6%) were identified as another species of microorgansims as seen in table 1.

Table1: isolation of *A.baumannii*

Sample Kind	Negative	Positive	Total
Bed sore	10(10.52%)	2(11.11%)	12 (9.6%)
Burn	41(43.15%)	5(27.77%)	46 (32%)
Diabetic foot ulcers	9(9.47%)	1(5.55%)	10 (8%)
Wounds	47(49.47%)	10(55.55%)	57 (50.4%)
Total	107(85.6%)	18(14.4%)	125 (100%)

A.baumannii, is appear as a negative coccibacilli in microscopic examine (Fig 1), purple on MacConkey agar, (pink, mucoied and diffused a purple color to the agar) on Leeds Agar Acinetobacter as seen in Fig2 (2,1), in the biochemical test has been appear as Catalase positive, Oxidase negative, Alkaline slant /no change butt on KIA, negative to the Indol, methyl red, Vogas proskauer and Positive to Simmon citrate Fig2(2) and growth in O/F aerobically with the three sugars and this result record according to the change of color of the tube to yellow and recognized as oxidative/non fremantive.

In the final identification by Vitek 2 compact, all the 18 isolates has been identified as *A. baumannii*.

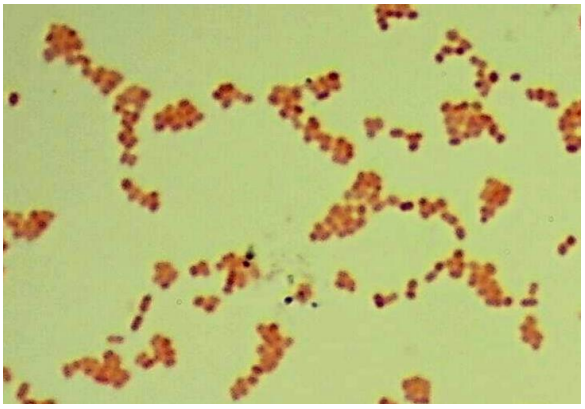

Fig1: Gram stain for *A.baumannii*

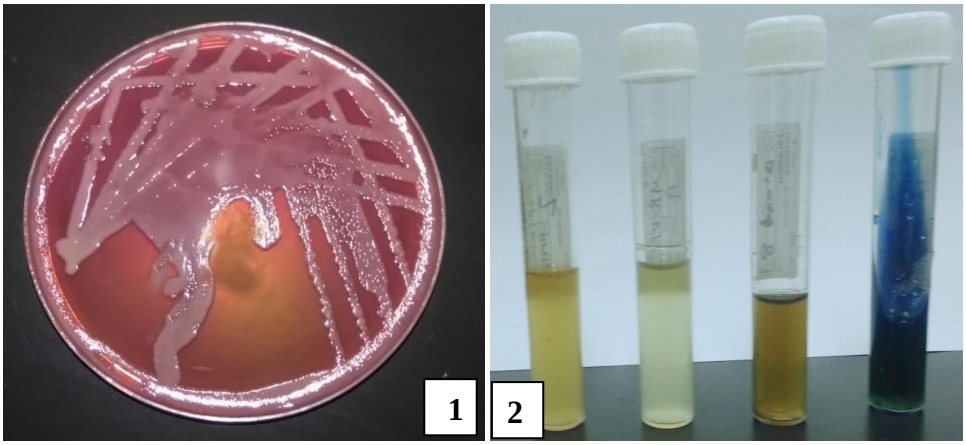

Fig 2: Growing of *A. baumannii* on(1) Leeds Agar Acinetobacter and(2) IMVIC results of *A.baumannii*

In the detection of biofilm on (CRA), all the isolates were grown on the media (Fig 3), Non of the isolates in this method were product biofilm or was product weak biofilm.

And on polystyrene tubes, it appear that all the isolates was biofilm production when it compare with the negative control Fig4, with several degrees.

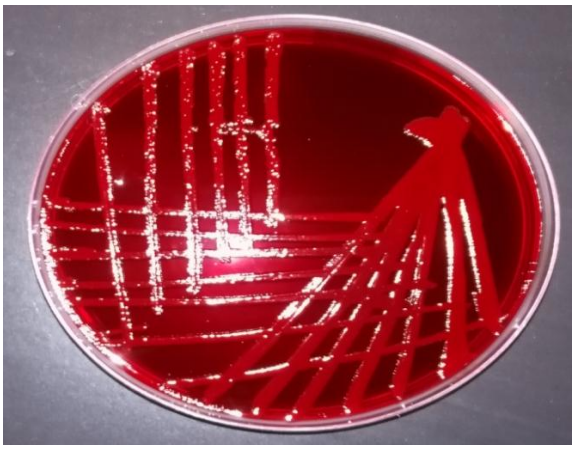

Fig3: growth of *A.baumannii* on Congo Red Agar

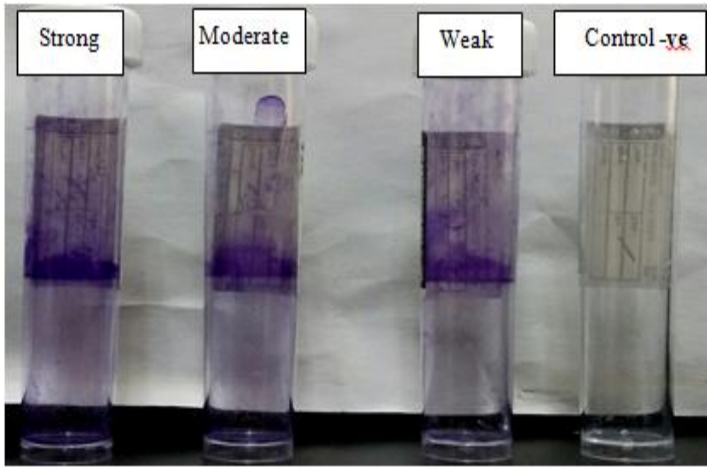

Fig4: Biofilm of *A.baumannii* in Tube Method

On the Tissue Culter Plate method, 1 isolate was identified as weak biofilm production , 2 isolates were strong , and 15 were moderate as in Fig 5.

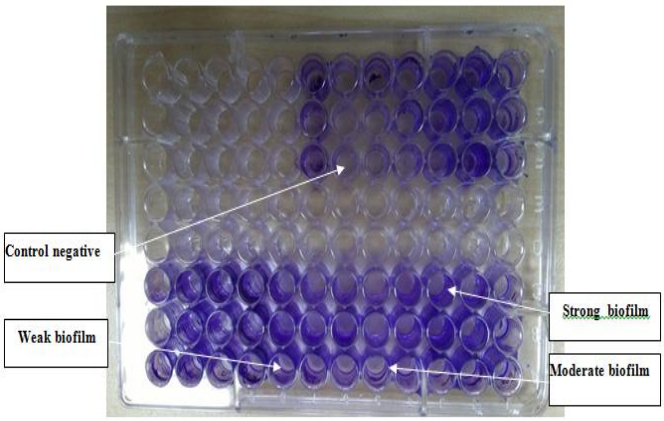

Fig5: Biofilm of *A.baumannii* on Tissue Culter Plate

In the comparing between the three methods we can notice that all the isolates was produce the biofilm with several degrees in the Tube Method and Tissue Culter Plate while it was negative or non produce to the biofilm in Congo Red Agar as we can see in table 2.

Table (2): the ability of *A.baumannii* to produce the biofilm by the three methods

	The formation of biofilm	strong	moderate	weak	non
Isolates 18	CRA	-	-	-	18
	TM	6	5	7	-
	TCP	2	15	1	-

Sensitivity and specificity

The sensitivity and the specificity has been calculated to the isolates for production of biofilm had been record for the tests like bellow (table 3):

Table (3): the sensitivity and specificity of the isolates

	CRA	TM	TCP
Sensitivity	0	100	100
Specificity	0	0	0
False positive	0	0	0
False negative	0	0	0
positive predictive value	100	100	100
Negative predictive value	0	0	0

In the detection of sensitivity and specificity and the false negative and false positive to the biofilm production isolates the sensitivity was0% in CRA, in TB 100% and in TCP 100%, specificity 0% for all the methods, False positive 0%for all isolates, and the false negative was 0%, positive predictive value 100%(CRA), 100%(TM), 100%(TCP) and finally the negative predictive value 0%(CRA), 0%(TM) ,0%(TCP).

In the antibiotics susceptibility to the Aminoglycoside antibiotics we found that all the isolates were resist to Gentamicin(GM) 100% and Tobramycin(TOB) 100%, flowed by Amikacin(AK) 72%, the intermediate were 16% and the sensitive 12%, while the resistance to Netlimicin(NET) was 50% and the sensitivity was 50%, and for streptomycin(S) the resistance was 77.7% and the intermediate 5% while the sensitive 16.6% in percent. As seen in the table(4).

Table (4): the biotyping to *A. baumannii for aminoglycoside antibiotics* susceptibility to the isolates

Biotybing of *Acinetobacter baumannii*	Resistant to Aminoglycoside
Ab1,Ab5,Ab7,Ab9	GM,S,TOB
Ab2,Ab3 ,Ab10,Ab11,Ab12, Ab14,Ab15,Ab17	AK,GM,NET,S,TOB
Ab4	AK,GM,NET,TOB
Ab6	GM,TOB
Ab8 ,Ab16	AK,GM,S,TOB
Ab13,Ab18	AK,GM,TOB

Ab –*Acinetobacter baumannii*

DISCUSSION

A. baumannii had emerged as a one of the health care settings pathogens, partly due to its ability to survive for a long period of time under wide bad environment conditions[18], the source of the infections in *A.baumannii* either exogenous or endogenous ,the exogenous infection is acquired from hospital environment and the endogenous is from the skin of the patient himself[19], in this study the rate of collection to *A.baumannii* was18.36%, locally in 2008[20] has isolate (5.6%), in 2011[21] had a rate of isolation to *A.baumannii* (6.05%), in 2014[22] had been isolate 23of *A.baumannii* (22.3%), we can notice increasing in the rate of infection with *A.baumannii* after different periods of time, and this result can translate to many reasons belong to the ability of *A.baumannii* to resist the known antibiotics, which is make it had morbidity and survive[22] but this results is differ in the rate from our study and this perhaps can belong to the same reason above, here is another reason can explain the variation in the increasing rate of infection in *A. baumannii* is the existence of the dormant cell[23] one more reliable reason is the biofilm formation ,the biofilm mode can explain the prevalence of *A.baumannii* on hospitals settings ,and this can be main role of how is it transmit to the another patients.[24]

To characterize the ability of *A.baumannii* isolates to produce the biofilm we use three methods CRA, TM, and TCB, in CRA method all the isolates were appear as non-biofilm producer, while the ability to form a weak biofilm 38.8%, 5.5% respectively on TM and TCP, the moderate was 27.7%, 83.3%, and the strong biofilm was 33.3%, 11.15%, this results agree with the result of.[25],[26]

We notice that the isolates of *A.baumannii* has several abilities in the formation of biofilm with several materials, the hydrophobicity of the *A.baumannii* may serve as one reason of this differences, the detect method and the additives can be another reason, like the addition of sugar to the medium while using of TCP method can enhancement the producing of biofilm[14] ,[26] had found that the isolates which isolated from wound has ability to form biofilm more than which isolated from UTI, in our result the wound isolates was the strongest biofilm and this result is agree with the results above.[7] suggest that the method of detect the biofilm by TM is better than the CRA in detection, while many researchers had conducted the TCP method is reliable and quantitative method for detect of biofilm, more than the TM and CRA[7,14]

The CRA method that use for detection of biofilm in fact is use to detect the ability of the bacterial strain to secrete the exopolysaccharide (one of the virulence factors share in the biofilm production) and the negative result can refer to not express of the operon which is responsible to produce the exopolysaccharide and fallow a path-way for adhere.[27]

The differences in the forming of biofilm in several degrees between the isolates in the same method which is use to assay by; can belong to the bacterial strain itself and its ability to produce the virulence factor which enhance the ability of biofilm forming, for instance;[12] had suppose the amount of accumulation of outer membrane protein (OMP) is connected with the ability of the bacterial biofilm production, while[24] has mention that "Biofilm can Re-organization the metabolism of fatty acids, aminoacids, active transport, motility, many researches had been found correlation between biofilm production and resistant to the known antibiotics[28] and this perhaps lead to the resistance to the antibiotics which is used.

In the screening for the other antibiotic from the Aminoglycoside antibiotics group we find out the isolates was resist to Streptomycin 77.7%, Amikacin in 72%, and Netlimicin 50%, Tobramycin 100%, and Gentamicin 100% .

In 2008-2010[29] had been found resistance from *A.baumannii* to the Aminoglycoside antibiotics (Amikacin, Tobramycin, Netlimicin, Gentamicin) with the rate 90.3%, 80%, 90.2%, 85.8% respectively, in (2010)Koo and colleagues[30] find all the isolates was high-resist to Streptomycin ,Gentamicin and the most efficacious was Amikacin between 9 antibiotics has been tested, when[10] find the Tobramycin was the more active antibiotic against *A.baumannii* isolates,[31] had find out resistance to Tobramycin 81.5% on clinical isolates from Chinese hospitals,[32] find out resistant to Gentamicin by clinical isolates from Iranian hospital was 83.33%, We can notice a globally high resistance to the Aminoglycoside antibiotics by *A.baumannii*, many reasons can clear the reason behind this ability to fight this type of drugs.

mainly this resistance can explain by the ability of the isolates to destroy the antibiotics by secretion of the Aminoglycoside modification enzymes, and by rRNA methylase the bacterial 16s can decreased the affinity to the drug[11] many researcher had demonstrate a correlation between the biofilm production and the ability to resist the known antibiotics,[33] had been found a connection between the biofilm and resist to the antibiotics in *A.baumannii*, While[26] found a direct correlation between the resistance to the Imipenem and Ciprofloxacin antibiotics with the increasing in the formation of the biofilm ,another reason to resistance is lost of the ability of the antibiotic to reach to the target site; because it cannot penetrate the biofilm stamina.[34]

in our study if we want to check any correlation between the ability of biofilm production with the antibiotic resist, we see the strongest biofilm production on microtiter plate was isolates number 8and 9, in the time were all the isolates were resist to the GM, TOB, and the isolate no.9

was sensitive to AK while the isolate no. 8 Was resist with the rest of isolates , and further, in case of the NET and S we notice that both the isolates no.8and 9 was sensitive to NET and the both of them was resist to S, and in that way we can hypothecate that there is no correlation between the strongest biofilm production isolates and the resist to the antibiotics.

CONCLUSION

In conclusion we notice the ability of *A.baumannii* to the biofilm and the Tissue Culter Plate (TCP) is the more reliable way to detect the ability of biofilm production, the *A.baumannii* has the ability to resist most kind of the Aminoglycoside antibiotics with a high rate, and there is no connection between biofilm production and the resist to the antibiotics.

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