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Evaluation of Biofilm Formation Capacity of *Acinetobacter baumannii* Isolated from Clinical Samples in Baghdad Hospitals using Phenotypic Methods

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Abstract: During the period from November/2016 to March/2017, a total of 475 specimens from various clinical sources (wounds, burns, urine, sputum, blood and throat) were collected from patients suffering different infections from a number of hospitals in Baghadad city (Karkh and Resafa), as follows: (Al-Yarmuk, Al-Karama, Al-Karkh, Al- Kadmia, Martyr Gazi Al-Hariry, the Medical city, Al-Kindi Teaching, Al-Imam Ali, Ibn-Al Balady and Baghdad Teaching Hospitals). *Acinetobacter baumannii* isolates in selective (CHROMagar) and specific differential medium, microscopic features, biochemical tests and API20 NE and VITEK-2 system at probability 98%. Furthermore the identification of isolates depending of molecular method by using polymerase chain reaction (PCR) technique which is used to amplify specific gene by using special primers, to genus level was confirmed finally by detection of *16SrRNA* gene while identification of *A.baumannii* isolates to species level was done by *blaOXA-51*-like gene, gaving ratio 100%. The results showed that the 83 *A.baumannii* isolates were obtained from clinical specimens (17.47%), distiributed according to the sources from highest to lowest percentage as follow in 31(25.20%) wound, 21(19.81%) burn, while 10(12.82%) urin, 8(11.94%) sputum, 7(12.72%) blood and low percentage 6(13.04%) from throat. This study focused on the phenotypic to determine methods the ability of biofilm production using three methods includes: Congo red agar (CRA), Tube method (TM) and microtiter plates (MTP). The results showed a significant differences between all 83 *A.baumannii* isolates. A seventy four isolates (89.15%) have the ability to adherence and produce slim layer with significant differences in thickness degrees (strong, moderate and weak). While 9 isolates which represented (10.84%) from isolates have no ability to adherence and produce slim layer. When comparing between these methods to detect strong biofilm production isolates, the results showed that MTP assay was excelled on CRA and TM at 58(69.87%), 48(57.83%) and 45(54.21%), respectively measured by optical densities values at 630 nm. **Conclusion:** The study concludes that there a positive correlation between biofilm formation and multi drug resistance *A. baumannii*. Each of the three phenotypic methods used for detection of biofilm formation has its advantages and disadvantages. But MTP method is most widely used and was considered as standard test for detection of biofilm formation.

Keywords: *Acinetobacter baumannii,* CHROMOagar acinetobacter, Biofilm Formation, phenotypic methods.

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Introduction:

Acinetobacter baumannii considered as a typical opportunistic pathogen that almost exclusively affects predisposed individuals, and classified as a nosocomial pathogen involved in infections that mainly affect critically ill patients. On the other hand, in immune compromised patients several species,

in particular *A.baumannii* can cause severe, life threatening infections (1).

Another major risk factor for *A.baumannii* infections is the widespread use of broad-spectrum antibiotics (2).

One of the distinctiveness of *A.baumanii* which contributes to its extensive survival in the environment is its ability to bioflim formation, or communities of intercommunicate bacterial cells associated with a surface and encase in an extracellular matrix of carbohydrates, nucleic acids, proteins, and other macromolecules (3).

A.baumannii is can adhere and colonizer to different abiotic surfaces in the hospital environment, such as glass and plastic, keyboards, pillows, gloves, beds and medical equipment. Prolonged periods survival on dry surfaces and high levels of resistance contribute to persistence of *A.baumannii* and complicates eradication during outbreaks(4).

Hypothesized that biofilm development mechanisms in *A.baumannii* from three important factors which contribute to the perseverance of *A.baumannii* in the hospital environment specifically, resistance to major antimicrobial drugs, resistance to desiccation, resistance to disinfectants and disease generation is closely associated with its ability to form biofilm and colonize on biotic and abiotic surfaces. (1).

Biofilm formation has also been correlated with MDR in clinical *A.baumannii* isolates it's to increased antimicrobial resistance. Formation of a biofilm is commonly a characteristic of *A.baumannii* clinical isolates and the ability to form a biofilm has been linked to pathogenesis in this organism (5).

The present study aims at studying the capacity of *Acinetobacter baumannii* isolates for Biofilm Formation.

Materials and methods:

Clinical specimens collection:

During the period between from November 2016 to March 2017, a total of 475 clinical specimens were collected from patients hospitalized in Baghdad city (Karkh and Resafa).

Different sources collected from (Burns swab, Wounds swab, Sputum swab, Urine, Blood and Throat swab). All specimens were collected by using clean sterile containers and transport swabs damped with normal saline, and transported to the laboratory immediately. The time between samples collection and bacteriological exam never exceed 1-2 hrs.

Isolation and Identification of *A.baumannii* isolates:

Each specimen was streaked using direct method of inoculation on a selective media to investigated for the presence of *A.baumannii*. Typical *Acinetobacter* CHROMagarTM *Acinetobacter*/ MDR medium, MacConkey agar and blood agar and then incubated under aerobic conditions for 24 hrs at 37°C (6). Then confirmatory identification was reconfirmed of *A.baumannii* according to Macroscopic and Microscopic examination**,** biochemical tests, API20

NE kit, Vitek-2 system and genotyping by PCR were studies.

Genotypic Techniques detection:

Extraction of Genomic DNA:

Genomic DNA of *A.baumannii* clinical isolates was extracted by using Genomic DNA extraction kit a commercial purification system (Genomic DNA Minni Kit Geneaid, Thailand), and quantification of purity and concentration of genomic DNA using a spectrophotometrically (Nano-drop) instrument to measure the optical density (O.D), and stored at -20°C.

Primers selection and Identification used PCR technique:

In this study, monoplex PCR assay was performed as a confirmative test is used to amplify specific gene by using special primers, to genus level was confirmed finally by detection of *16SrRNA* gene while identification of *Acinetobacter baumannii* isolates to species level was done by *blaOXA-51*-like gene. This assay was done according to method described by (7), as in Table (1) and the programs for each gene that mention in Table (2). All PCR assays were accomplished in institute of genetic engineering and biotechnology.

Table (1): Primers used in this study.

Primer Name	Primer Sequence Oligo sequence F (5'….3') Forward	Primer Sequence Oligo sequence R (5'…3') Reverse	Product size (bp)	Reference
Identification of *A.baumannii* to genus level				
16SrRNA	5′- CAG CTC GTG TCG TGA GAT GT -3	5′- CGT AAG GGC CAT GAT GAC TT -3′	150	(Higgins *et al.*, 2004)
Identification of *A.baumannii* to species level				
blaOXA-51	5′- TAA TGC TTT GAT CGG CCT TG -3′	5′- TGG ATT GCA CTT CAT CTT GG -3′	353	(Woodford *et al.*, 2006)

R: Reverse primer and F: Forward primer

Table (2): Programs of PCR Thermocycling Conditions.

Gene	Temperature (ºC) / Time					Cycle Number
	Initial denaturation	Cycling condition			Final extention	
		Denaturation	Annealing	Extension		
16S rRNA	94 ºC for 4 min.	94 ºC for 35 sec.	55 ºC for 35 sec.	72 ºC for 40 sec.	72 ºC for 4 min.	30
bla OXA-51	95 ºC for 5 min.	94 ºC for 45 sec.	52 ºC for 40 sec.	72 ºC for 45 sec.	72 ºC for 6 min.	30

Agarose gel electrophoresis:

To provides a simple and efficient method for purification of genomic DNA and the amplified PCR products. The DNA fragments and PCR products were separated and visualized by horizontal agarose-gel electrophoresis (8).

Biofilm Formation measurement:

A variety of different assays were used to evaluate production of biofilm

formation to all *A.baumannii* isolates was studied in different medium, on various materials with some modifications:

Culturing on Congo red agar plates (CRA) (qualitative assay):

The morphology of the colonies and their phenotypic changes were studied using CRA medium as previously described by (9) with some modifications. The CRA medium is composed of Brain heart infusion broth (BHIB) (37 g/L), sucrose (50 g/L), agar agar (15 g/L) dissolve in 900 ml of deionized water Congo red stain was prepared (0.8 g/L) was mixed with 100 ml of deionized water vigorously, thus the concentrated aqueous solution was sterilized by autoclaving separately from other medium constituents, after cooling the dye solution and medium to 55°C, it mixed and pour in Petri-dishes, left to solidify and use to detect biofilm formation.

The colonies were streaked on Congo red agar plates and incubated aerobically for 24 hours at 37°C to obtain single bacterial colonies.

Tube Method (TM) (quantitative assay):

A.baumannii isolates were tested for biofilm formation was investigated by the glass test tubes adherence test proposed by (10, 1) with some modifications. 10 ml Trypticase soya broth (TSB) with 1% glucose was inoculated with single colonies of the test bacterial strains on nutrient agar individually and the cultures were incubated statically for 48 hrs at 37ºC, The experiment was performed in duplicates. After incubation, the contents were decanted and washed with phosphate buffer saline PBS (pH 7.3) and dried. The tubes were stained with crystal violet or safranin (0.1%) for 7 min, each tube was then gently rotated to ensure uniform staining of any adherent material on the inner surface and the contents gently decanted. Excess stain was removed and washed with D.W. Tubes were dried in inverted position and observed for biofilm formation.

The scoring for tube method was done according to the results of the control strains.

Microtitre culture plate method (MTP) (quantitative assay):

This analyze determined by microtitre plate method and considered by (10) is performed by a spectrophotometric method, which measures the total biofilm biomass (bacterial cells and extracellular matrix), Biofilm formation assays and the standard test for detection of biofilm formation as follow (11) with some modifications. Briefly, *A.baumannii* isolates were grown overnight in in 10 ml of trypticase soy broth (TSB) with 1% glucose for 24 hrs at 37°C. The culture was diluted fresh medium in a ratio of 1:90 in TSB-1 % glucose, then 200 µl of diluted cell suspension was filled in each well of a sterile 96 well polystyrene flat-bottom microtitre plates, and 200 µl aliquots of only TSB + 1% glucose were dispensed into microtiter plate to serve up as a control, followed by the plates were covered and incubated for 24 hrs at 37ºC.

After three carefully washes with phosphate-buffered saline (PBS) (pH 7.2), then the attached bacteria were

fixed with 200 mL of 99% methanol per well for 15 minutes. Any remaining biofilm was stained with crystal violet (1 %v⁄v) for 30 min and wells were washed again with PBS. The dye bound to the adherent cells was resolubilized with 200 µl of 95% ethanol for 20 min, and were fixed the attached bacteria, and the optical density (OD) was quantified at 630 nm using an ELISA reader. Each assay was performed in triplicate and the average optical density was considered.

According to their optical densities, the adherence capability of each bacterial cell was classified into the following four categories as follows: if OD ≤ ODc, the bacteria were non-biofilm (−); if ODc < OD ≤ 2 × ODc, the bacteria were weakly biofilm producer (+); if 2 × ODc < OD ≤ 4 × ODc, the bacteria were moderately biofilm producer (++); if 4 × ODc < OD, the bacteria were strongly biofilm producer (+++) (12).

Statistical Analysis:

The results data were analyzed by using Statistical analysis system by using computer program (SPSS), analysis of variance (ANOVA) were used, the level of probability at P values below of ≤ 0.05 that used to identify a significant difference (13).

Results and Discussion:

Distribution of *A.baumannii* according to infection:

The results exposed that a total number of 83(17.47%) isolates of *A.baumannii* were obtained from 475 clinical specimens which initially were diagnosed in hospitals from different source. The distributions of the isolates according to specimens types are shown in Figure (1).

The results of current study is correlated with the local studies in Iraq conducted by (14) they diagnosed and identified that isolation in different percentage rates of nosocomial infections caused by *A.baumannii* from same sources and that agreement with present study. Forthermore these results were paralleled with the other comparable studies in world such: (15, 16) in Iran they isolated *A.baumannii* bacteria from different sources of clinical isolates.

The reasons for the variation in rate and percentage of isolation of *A.baumannii* between studies may be due to several factors such as place of collection (unit of hospital), geographic site and the difference in time of collection as well as the variation in the number of the studied samples (17).

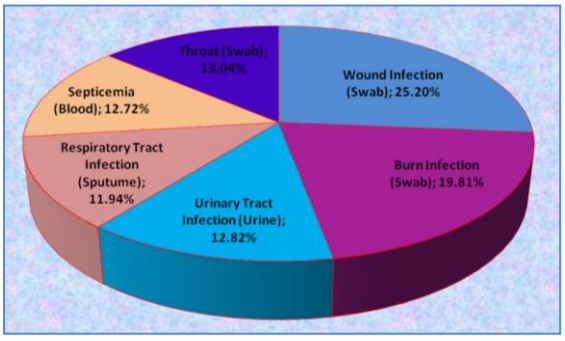

Figure (1): Percentages of *A. baumannii* isolates among different clinical samples.

Bacterial identification:

The phenotypic identification of the *A.baumannii* isolates using bacteriological methods, Grams staining, colonial morphology and microscopically characteristics Figure (2). Then characteristics of isolates were subjected identified by biochemical test Table (3), and confirmatory identification by Api 20 NE and Vitek-2 system. Accurate identification and typing of bacterial isolates are essential, particularly when determining strains involved in hospital outbreaks (18). Results of current study shown to be agreement with all different other studies in this aspect like studies of (19).

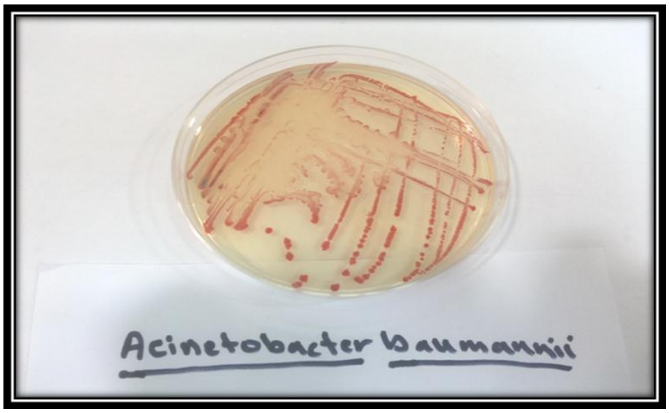

Figure (2): Colonies of *A.baumannii* isolates on selective medium CHROMagar *Acinetobacter*

Table (3): Morphological and Biochemical identification results of *A.baumannii.*

No.	Biochemical Test	Result
1.	Gram stain	(-)
2.	Microscopic shape	Coccobacilli
3.	Growth at 44°C	(+)
4.	Lactose fermentation	(-)
5.	Hemolysin production	(-) γ hemolysis
6.	Oxidase test	(-)
7.	Catalase production test	(+)
8.	Methyle red	(+)
9.	Citrate utilization	(+)
10.	Kliglar iron agar	Alkaline slant /No change bottom, No gas, No H_2S
11.	Urease production	variable
12.	Voges- Proskauer	(-)
13.	Motility test	(-)
14.	Indoe production	(-)

(+) : positive, (-): negative

Genotyping Identification:

Genomic DNA Extraction:

Genomic DNA was successfully extracted from all *A.baumannii* isolates using a commercial Genomic DNA purification Kit (Geneaid) according to instructions of the company. Extraction results were good and the DNA quantification (concentration and

purity) were directly performed by Nanodrop spectrophotometer at 260/280 nm. Extracted DNA concentration from all the isolates ranged between (58 to142) ng/µl and purity was evaluated ranged between (1.8 to 2). Extracted DNA were confirmed and analyzed by gel electrophoresis in 1% agarose for 30 min at 75 volts and exposed to U.V light in which the DNA appears as compact bands shown in figure (3).

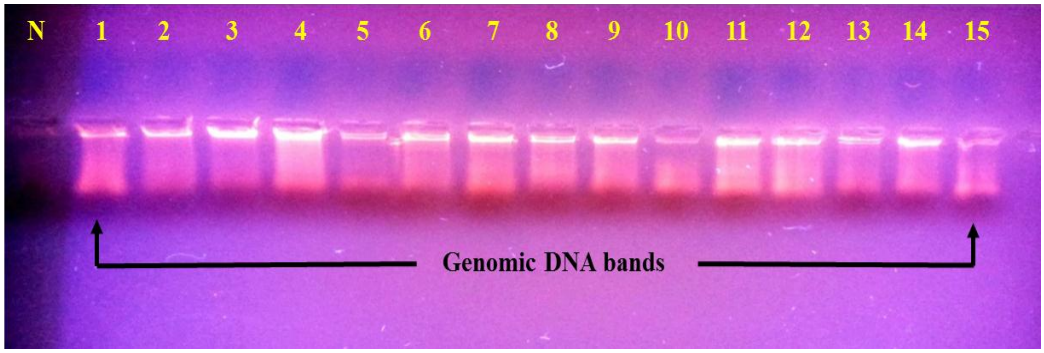

Figure (3): Genomic DNA bands extracted from *A.baumannii* isolates on (1% agarose, 75V, for 30 min stained with ethidium bromide). Lane 1-15: DNA of Lane N: Negative control (water).

Molecular detection of *Acinetobacter* species by detection *16SrRNA* gene:

In this study identification of the 83 isolates was done used molecular identification *A.baumannii*. A specific primer for gene responsible for determined the *Acinetobacter* genous (*16SrRNA*) would identify all the isolates correctly and can be used to perform a single step Conventional PCR technique. Results exhibited that 83(100%) isolates were *A.baumannii* using gene *16SrRNA* with amplified size of 150bp that present in Table (4) and according to the statistical analysis that show high significant at P<0.01. All isolates gives positive results that amplified fragments (150 bp) bands were separated by electrophoresis on a agarose gel, stained with ethidium bromide, photographed under UV light that showed in Figure (4).

The results of current study were agreement with studies in local area in Iraq by (14) they using the gene *16SrRNA* for molecular identification of *Acinetobacter* clinical isolate with high rate of genomic identification. Moreover agreement with other studies which mentioned that detection and sequencing of this gene is an effective means for the identification of clinical isolates of *Acinetobacter* by (3).

16SrRNA gene is one of the structural RNA components of the ribosome. Prokaryotes have 5S and 23S species in large subunit and a *16SrRNA* species in the small subunit (20). Postulated the importance of 16S-23S internal transcribed spacer to detect some bacterial identification at species level. As reported from sequence analysis studies, *16SrRNA* could be used to identify *Acinetobacter* to the genus level (21).

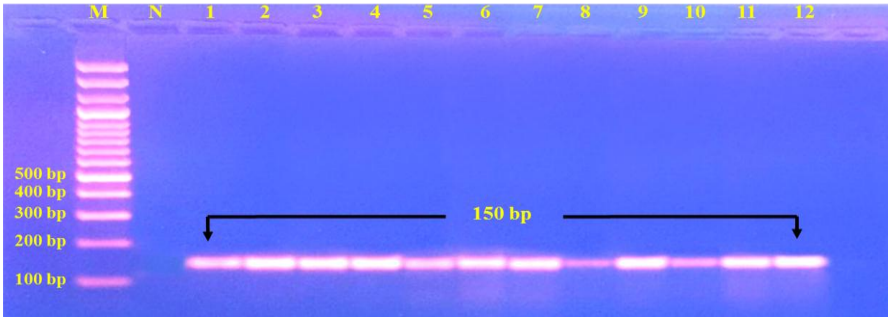

Figure (4): Agarose gel electrophoresis of PCR products (1% agarose,75 volt for 90 min) for *16SrRNA* gene product using DNA template of *A. baumannii* isolates. Lane (M): (100 bp DNA Ladder). lane N: Negative control. All lanes (1-12) of *A. baumannii* isolates show positive results (amplified size 150bp).

Molecular identification of *A.baumannii* by detection *blaOXA-51*-like gene:

The results of the PCR products have been confirmed by comparing their molecular weight with 100 bp DNA Ladder by analysis of the bands on gel electrophoresis, to *blaOXA-51*-like gene is present in all 83(100%) of DNA *A.baumannii* isolates with a PCR product size single amplicon 353bp shown in Figure (5) and that present in Table (4) which indicated that all clinical isolates identified as *A.baumannii.*

The current results is parallel with most of the previous studies which evidence the occurrence of *blaOXA-51*-like gene in all clinical isolates of *A.baumannii* and not detected in the other species of *Acinetobacter spp*. (22). This result was in agreement with other researchers in Iran (15, 16) identified of the isolates *A.baumannii* phenotypically, all confirmed as by using *blaOXA-51*-like primers and observing ~ 353 bp band in the agarose gel.

The purpose of *blaOXA-51*-like gene identification of *Acinetobacter* species has several benefits over phenotypic identification. Even though the detection of the *blaOXA-51*-like gene has been considered a simple, rapid and reliable method for *A.baumannii* genomic species identification as these are naturally occurring carbapenemases genes to *A.baumannii*(23).

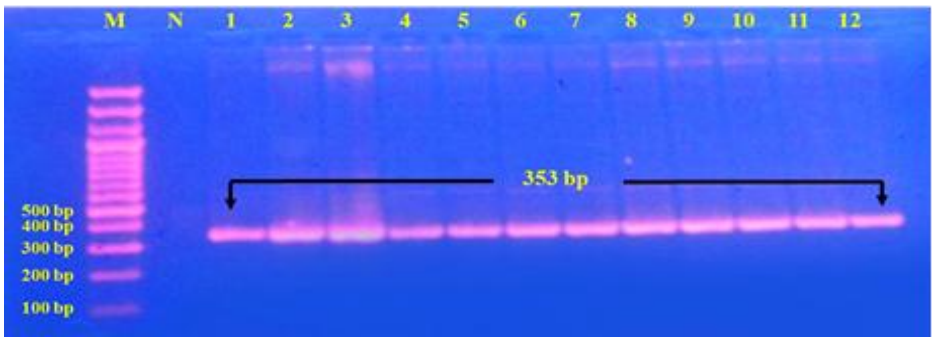

Figure (5): Agarose gel electrophoresis of PCR products (1% agarose,75 volt for 90 min) for blaOXA-51-like gene product using DNA template of *A.baumannii* isolates. Lane (M): (100 bp DNA Ladder). lane N: Negative control. All lanes (1-12) of *A.baumannii* isolates show positive results (amplified size 353bp).

Table (4): Prevalence of PCR amplified detection genes in *A. baumannii* clinical isolates.

No.	Gene name	Number and the percentage of positive isolates n= 83		Number and the percentage of negative isolates n= 83		Chi-Square (χ2) (P-value)
1.	*16SrRNA*	83	(100%)	0	(0%)	15.00 ** (0.0001)
2.	*blaOXA-51*	83	(100 %)	0	(0%)	15.00 ** (0.0001)
**** (P<0.01).**						

Detection of the Bacterial Ability for Adherence and Biofilm Formation:

In this study we determine their ability of 83 *A.baumannii* isolates for adherence and to produce slime layer (Biofilm formation) were experienced by using three conventional phenotypic methods, including Congo-red agar (CRA), Tube method (TM) and Microtiter plate (MTP) by ELISA reader Figures (6 ,7, 8).

The results shown that the difference was statistically significant at P<0.01between the 83 *A.baumannii* isolates to produce biofilm by using these methods, the highest value of biofilm formation 74(89.15%) from isolates have the ability to adherence and produce slim layer while 9(10.84%) from isolates which have no the ability to adherence and produce slim layer Table (5). The positive results for each method divided in to three types according to amount of slime layer that present in Table (6) as the fallowing: (Strong, Moderate and week) producer biofilm, and shown difference significant between these methods.

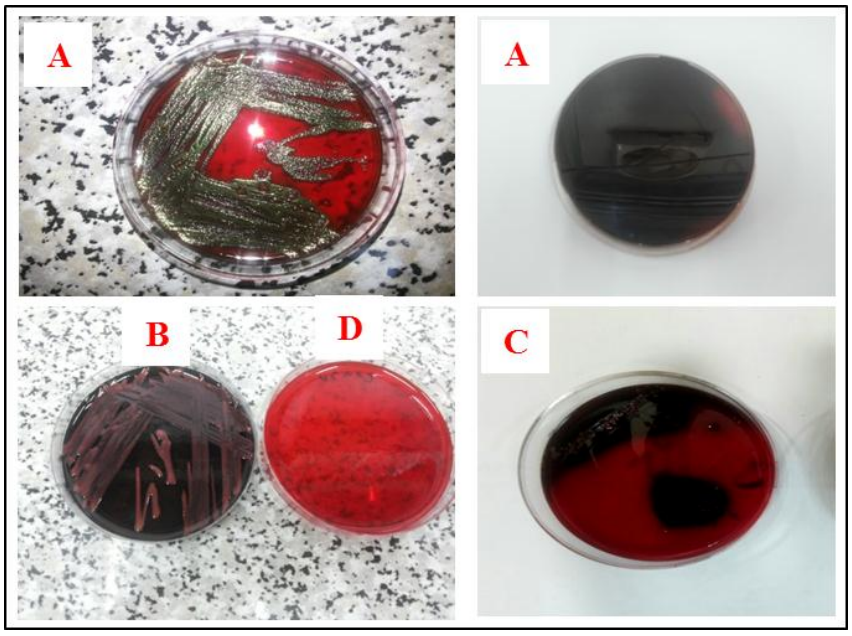

Figure (6): Detection of slime layer producing *A.baumannii* isolates colonies on Congo red agar method. A- Strong producer biofilm, B- Weak biofilm producer, C- Moderate biofilm producer and D- Non biofilm producer.

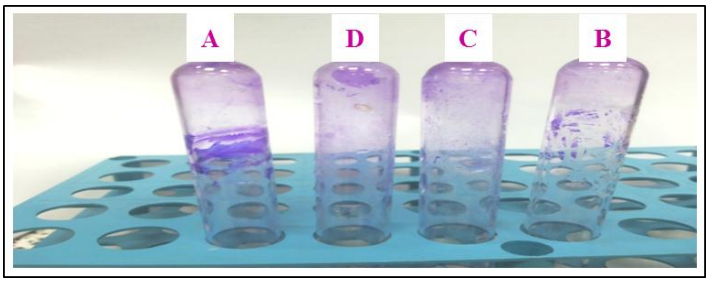

Figure (7): Detection of biofilm producers in *A.baumannii* isolates by Tube method. A- Strong biofilm, B- Moderate biofilm producer, C- Weak biofilm producer and D- Non biofilm producer.

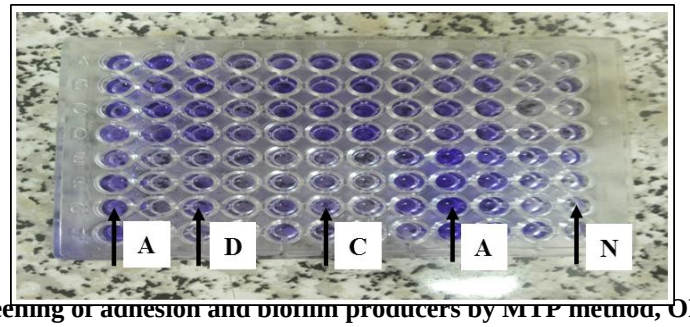

Figure (8): Screening of adhesion and biofilm producers by MTP method, OD (measured at wavelength of 630 nm). A: strong producer biofilm, B: moderate producer biofilm, C: week producer biofilm, D: non biofilm producer and N: negative control.

Table (5): Total *A.baumannii* isolates which have the ability and non ability to adherence and produce slim layer.

solates	Number of isolates	Percentage (%)
Isolates which have the ability to adherence and produce slim layer in three methods (CRD, TM and MTP)	74	89.15%
Isolates which Non-have the ability to adherence and produce slim layer in three methods (CRD, TM and MTP)	9	10.84%
Total number	83	100%
Chi-Square (χ2) (P-value)	**---**	**14.06 ** (0.0001)**
**** (P<0.01).**		

Table (6): Number and Percentage of 83 *A.baumannii* isolates which have the ability to produce slime layer by using three phenotyping methods

Biofilm produce	Congo red agar (CRA) n= 83		Tube method (TM) n= 83		Microtiter plate (MTP) n= 83	
Strong (+++)	48	(57.83%)	45	(54.21%)	58	(69.87%)
Moderate (++)	16	(19.27%)	15	(18.07%)	10	(12.04%)
Weak (+)	10	(12.04%)	14	(16.86%)	6	(7.22%)
Non (-)	9	(10.84%)	9	(10.84%)	9	(10.84%)
Total	83	100%	83	100%	83	100%
Chi-Square (χ2) (P-value)	**---**	**10.72 ** (0.0001)**	**---**	**10.35 ** (0.0001)**	**---**	**12.25 ** (0.0001)**
**** (P<0.01)**						

These results nearly compatible with the study by (16) which found more among *A.baumannii* isolates showed strong slime layer on the Congo red agar. Furthermore also agreement with the study by (24) shown the strains are found positive as well as slime-forming onto CRA.

The CRA is selected in an attempt to improved its capability to identify adhesion and biofilm formation by *A.baumannii* isolates using a specially prepared solid medium by production changes in the procedure and djusting different physical parameters. Thus the CRA method is simple, sensitive and fast to carry out and the results are usually based on the colony color produced, which ranges from red for non-biofilm producing strains to black for biofilm producing strains, and reproducible and has the advantage that colonies remain viable on the medium (25).

The results of Tube method (TM) show that there was complete agreement with the results of (16) who reported from the detection of adherent by this method was positivity results among *A.baumannii* isolates. It's noted for this procedure the presence of high variability among repetitions of this method.

Tube method is also called traditional Christensen method this method is asimple and widely use to determine the ability of bacteria to adherence to the smooth surface such as glass tubes (11). The adhenece growth on smooth surface of class tubes results from the presence of polysaccharide on the smooth surface of class tubes because *A.baumannii* uses glucose which is found in culture media to produce aggressively sticky glucan polymers that facilitate the attachment of cells and adherent to smooth surface lead to promoting biofilm formation (4).

But we in agreement with the preceding studies, Tube method (TM) metod cannot be suggested as general screening test to identify biofilm producing isolates. Similarly, it was difficult to differentiate between strains weak biofilm producers and non-producers (24).

In this and data obtained from biofilm formation were study biofilm was formed on polystyrene microplates parallel with (26) whom found that all *A.baumannii* have the ability to formation of biofilm in this method. And another by (27) they showed that 12 isolates from 15 total isolates have ability to produce biofilm formation.

Furthermore the results was parallel with the results of other study by (16) and (15) whom evaluation of biofilm formation of *A.baumannii* isolates by microtiter plate method and gave higher rate of biofilm formation in this method. Microtiter plate is an important instrument for the study of the early stages in biofilm adhesive and allows for the formation of a biofilm on the wall and bottom of a microtiter plate (28). It has been is most widely used and was considered as standard test for detection of biofilm formation because it allows a simple, more accurate, and rapid to quantify contact cell attachment and biofilm formation of different bacterial strains (29).

The variation in biofilm thickness may be ascribed to the differences in isolates ability to produce biofilm, Formation of biofilm plays an important role in the pathogenesis of pathogens

and development of these biofilms is support on the signal mediated QS system, the QS systems play an important role in the pathogenesis and regarding the role in biofilm formation of *A.baumannii* infections (30).

Even though the principle of adherence detection by MTP depends on a variety of conditions, they remain better and more accurate than CRA and TM for biofilm detection (31).

In conclusion, this study showed the CRA and TM tests could be successfully used to detect *A.baumannii* isolate adhesion and biofilm producing, observed in this study when the CRA and TM were compared with the MTP test, indicated the MTP test should be the first choice because this test was more sensitive than the CRA and TM tests, MTP allowed an easy and quantitative classification of the *A.baumannii* isolates. This method MTP gave the best discrimination between strong, moderate producers and non-producers of adherence.

References:

1. Tomaras, A.P.; Dorsey, C.W.; Edelmann, R.E. and Actis, L.A. (2008). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone usher pili assembly system. Microbiology; 149(12): 3473–84.
2. Shamsizadeh, Z.; Nikaeen, M. ; Esfahani, B.N.; Mirhoseini, S.H. ; Hatamzadeh, M. and Hassanzadeh, A. (2017). Detection of antibiotic resistant *A.baumannii* in various hospital environments: potential sources for transmission of *Acinetobacter* infections. Environmental Health and Preventive Medicine201722:44.
3. Bardbari, A.M.; Arabestani, M.R.; Karami, M.; Keramat, F.; Alikhani M.Y. and Bagheri, K.P. (2017). Correlation between ability of biofilm formation with their responsible genes and MDR patterns in clinical and environmental *Acinetobacter baumannii* isolates. Microbial Pathogenesis. 108: 122-128.
4. Senadheera, M.D.; Lee, A.W.; Hung, D.C.; Spatafora, G.A.; Goodman, S.D. and Cvitkovitch, D.G. (2007). The *Streptococcus mutans vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. J. Bacteriol. 189:1451–1458.
5. Pereira, C.A.; Costa, A.C.B.P.; Liporoni, P.C.S.; Rego, M.A. and Jorge, A.O.C. (2016). Antibacterial activity of Baccharis dracunculifolia in planktonic cultures and biofilms of *Streptococcus mutans*. Journal of Infection and Public Health, 9, pp. 324–330.
6. Badave, G.K. and Kulkarni, D. (2015). Biofilm producing multidrug resistant *Acinetobacter baumannii*: an emerging challenge .Journal of Clinical and Diagnosis Research , 9(1) : 8-10.
7. Ryder, M.A. (2005). Catheter related infections: It's all about biofilm. Advanced practice nursing e J. 5(3): 1- 15.
8. Weissensteiner, T. and Thomas, W. (2003). PCR Technology: Current Innovations, Second Edition. Boca Raton, Florida: CRC Press. USA. p. 93-150.
9. Sambrook, J. and Rusell, D.W. (2001). Molecular Cloning: a Laboratory Manual. 3th ed. Cold Spring Harbor Laboratory Press., New York. USA.
10. Freeman, K.; Woods, E.; Welsby, S.; Percival, S.L. and Cochrane, C.A. (2010). Biofilm evidence and the microbial diversity of horse wounds. Canadian Journal of Microbiology 55, 197-202.
11. Christensen, G.D.; Simpson, W.A.; Younger, J.J.; Baddour, L.M.; Barrett, F.F.; Melton, D.M., *et al*. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of *staphylococci* to medical devices. Journal of Clinical Microbiology, 22: 996–1006.
12. Sharma, S.; Mahajan, B.; Patidar, R.K.; Sahare, K.N.; Khare, M. and Singh, V. (2013). Molecular detection of

antimicrobial resistance genes in biofilm forming *Escherichia coli*. American J. Pharmacy and Health Res. 1(6): 2321-3647.

13. Stepanovic, S.; Vukovic, D.; Dakic, I.; Savic, B. and Svabic-Vlahovic, M. (2000). A modified microtiter-plate test for quantification of *staphylococcal* biofilm formation. J. Microbiol. Methods .40:175-179.
14. Urdan, T.C. (2005). Statistics In Plain English, 2nd ed. Lawrence Erlbaum Associates, London : 130-143.
15. Al Sehlawi Z.S.; Almohana, A.M. and Al Thahab, A.A. (2014). Isolation and Identification of *Acinetobacter baumannii* Clinical Isolates using Novel Methods. Journal of Babylon University/Pure and Applied Sciences, 22: (3).
16. Fallah, A.; Rezaee, M.A.; Hasani, A.; Barhaghi, M.H.S. and Kafil, H.S. (2017). Frequency of *bap* and *cpaA* virulence genes in drug resistant clinical isolates of *Acinetobacter baumannii* and their role in biofilm formation. *Iran J Basic Med Sci*, 20: (8).
17. Babapour, E.; Haddadi, A.; Mirnejad, R. and Amirmozafari, N. (2016). Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pacific Journal of Tropical Biomedicine, 6(6):528-533.
18. Lahiri, K.K.; Mani, N.S. and Purai, S.S. (2004). *Acinetobacter spp*. as Nosocomial Pathogen: Clinical Significance and Antimicrobial Sensitivity. MJAFI, 60(1): 7-10.
19. Misbah, S.; Hassan. H.; Yusof, M.Y.; Hanifah, Y.A. and Abu Bakar, S. (2005). Genomic species identification of *Acinetobacter* of clinical isolates by *16SrDNA* sequencing. Singapore Med J. 46: 461-464.
20. Patwardhan, R.B.; Dhakephalkar, P.K.; Niphadkar, K.B. and Chopade, B.A. (2008). A study on nosocomial pathogens in ICU with special reference to multiresistant *Acinetobacter baumannii* harbouring multiple Plasmids. Indian J. Med. Res. 128: 178-187.
21. Reeve, E.C. R. (2013). Encyclopedia of Genetics. Routledge. New York.
22. Srinivasan, R.; Karaoz, U.; Volegova, M.; MacKichan, J.; Midori Kato-Maeda, K. and Miller, S. *et al*. (2015). Use of *16SrRNA* Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. PLoS One, 10(2):112-121.
23. Ghaima, K.K.; Saadedin S.M.K and Jassim, K.A. (2016). Isolation, molecular identification and antimicrobial susceptibility of *Acinetobacter baumannii* isolated from Baghdad hospitals. Int. J. SciRes Public., 6(5): 351.
24. Khalilzadegan, S.; Sade, M.; Godarzi, H.; Eslami, G.; Hallajzade, M.; Fallah, F. and Yadegarnia, D. (2016). Beta-Lactamase Encoded Genes blaTEM and blaCTX Among Acinetobacter baumannii Species Isolated From Medical Devices of Intensive Care Units in Tehran Hospitals. Jundishapur J Microbiol; 9(5):e14990.
25. Neel, V.H.; Parvathi, T. and Krishna, P.B. (2016). Study of Biofilm Production and Anti-microbial susceptibility pattern of bacterial and fungal from urinary catheters. Int. J. Curr. Microbial. App. Sci., 5(2): 415-424.
26. Pandey, K.P.; Verma, P.; Kumar, H.; Bavdekar, A.; Patole, M.S. and Shouche, Y.S. (2012). Comparative analysis of fecal microflora of healthy full-term Indian infants born with different methods of delivery (vaginal vs cesarean): *Acinetobacter sp*.prevalence in vaginally born infants. Journal of Biosciences. 37(1): 989–998.
27. Bahreini, Z.; Hosseini, F. and Salehi, M. (2014). Molecular investigation of the factors Involved in the Biofilm Formation in clinical Isolates of *Acinetobacter*: *CsuA/BABCDE* Gene cluster. Bulletin of Environment, Pharmacology and Life Sciences. Bull. Env. Pharmacol. Life Sci., 3(8): 37- 42.
28. Abdallah, M.; Benoliel, C.; Drider, D.; Dhulster, P. and Chihib, N.E. (2017). Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Archives of Microbiology, 196: 453–472.
29. O'Toole, G.A. (2011). Microtiter dish biofilm formation assay. J. Vis. Exp. 47: 2437.

30. Khudhur, I.M. (2013). Investigating the ability of some bacterial species to produce slime Layer. Raf. J. Sci. 24(1):36-49.
31. Holling, M.; Komuro, Y. and Tochihara, H. (2014). Cucumber green mottle mosaic virus. Descriptions of Plant Viruses. No. 154. Onwards URL http://www.dpvweb.net/dpv/show-adpv.php?dpvno=154.