149

Egypt. J. Chem. **Vol. 64,** No. 3 pp. 1615 - 1625 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Biofilm formation by clinical *Acinetobacter baumannii* strains and its effect on antibiotic resistance

Abdulaziz Abdullah Othman Almosa[1], Ali A. Al-Salamah[2], Dina A. Maany[3], Mostafa Abd El-Hameed El-Abd[3]*; Abdelnasser S.S. Ibrahim[2,3]

[1]Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia,
[2]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia,
[3]Department of Chemistry of Natural and Microbial Products, Pharmaceutical Industries Research division, National Research Centre, El-Buhouth St., Dokki, Cairo, Egypt,

Abstract

Acinetobacter baumannii is one of the most important nosocomial, multidrug resistant pathogens. The most important virulence factor is its ability to form biofilms. Microtiter-plate method was used to detect the ability of twenty *A. baumannii* clinical strains to form biofilm in polystyrene wells. Biofilm formation was assessed by staining with a crystal violet solution 0.1% then elution by an ethanol-acetone mixture (80:20) and O.D measurement. In general, among the twenty isolates, 2 isolates were strong biofilm formers, 13 were medium and 5 were weak. It is worthy to mention that the strong biofilm forming strains were those isolated from tracheal aspirates and wound swabs. When examining environmental factors' effects on biofilm formation, results revealed that biofilm formation was enhanced in the presence of lactose as a carbon additive. Production of biofilm was maximum at pH 6 and 7.4 as indicated from O.D values. Biofilm formation was better at all temperatures with a slight increase at 40ºC and was maximum after 72h. The effect of addition of serum on biofilm formation varies among the isolated samples giving rise to high O.D values in sample 2 and 4. Non-biofilm producing mutant generated by 70% CV treatment of strain no. 1 was tested and found to be as antibiotic resistant as the wild type strains indicating the contribution of other factors in addition to biofilm to resist antibiotics.

Keywords: *Acinetobacter baumannii,* nosocomial infection, biofilm, multidrug resistant pathogens, mutagenesis

Introduction

Acinetobacter baumannii is one of the most important nosocomial pathogens in healthcare-associated facilities. It is an opportunistic, multidrug resistant pathogen (MDR); specially to carbapenem and most commercially available antibiotics, causing infections and is responsible for a lot of in Intensive Care Unit (ICU) outbreaks worldwide [1,2,3]. In addition, *A. baumannii* is one of the most detected nosocomial pathogens, causing skin, bloodstream, urinary tract, and other soft tissues infections [4,5]. Hospital acquired infection by *A. baumannii* is the most frequent but recently community acquired infections are increasing. In addition, mortality rates associated with general hospital population and intensive care unit (ICU) patients are scientifically increasing [6,7].

The abundance of *A. baumannii* in hospitals can be attributed partly to its ability to survive and persist in the harsh conditions found within hospital environments such as; desiccation, sudden changes in temperature and other environmental conditions [8,9]. In addition, the MDR of *A. baumannii* is mostly due to the presence of several antibiotic resistance mechanisms including secretion of enzymes that can break the antibiotic molecules, modifying the structure of the active sites, inhibiting or minimizing the cell membrane permeability of antibiotic molecules, and the efflux pumps that extruding the antibiotic molecules out of the cell using efficient efflux pumps [10,11,12]. However, biofilm formation by *A. baumannii* is considered as one of the most important mechanisms of antibiotic resistance. *A. baumannii* has the gene cluster called the K locus, which is

responsible for the production of a biofilm matrix of exopolysaccharides and Lipopolysaccharides (LPS) protecting the pathogenic cells against outer environmental conditions and representing one of the most important virulence factors of *A. baumannii* [13,14].

The biofilm matrix signal transduction mechanisms in bacteria play an important role in adapting to environmental conditions, therefore modifying their susceptibility to antibiotics [17].

The main objective of this work is to investigate the biofilm formation in local clinical strains of *A. baumannii* isolates under different environmental conditions. In addition to testing the effect of biofilm formation on the antibiotic resistance of these strains.

Materials and Methods
Clinical samples collection
Clinical specimens were collected from 20 patients, including 12 males and 8 females, mainly from the ICU along with other hospital departments like neurology, urology and surgery in King Khalid University Hospital and Armed Forces Hospital (Riyadh, Saudi Arabia). The samples listed in **Table 1** were collected under sterile conditions using cotton swabs and were delivered to the laboratory in an ice box within 1 to 2 h. The clinical samples were taken from tracheal aspirates, sputum, urine and wound swabs.

Table 1: Clinical samples collected from different hospital sources

Sample no.	Sample Source	Gender	Age
1	Tracheal aspirate	M	62
2	Urine	F	65
3	Tracheal aspirate	M	70
4	Wound swab	F	68
5	Wound swab	M	28
6	Urine	F	76
7	Sputum	M	21
8	Wound swab	F	80
9	Wound swab	F	45
10	Sputum	M	69
11	Urine	M	44
12	Sputum	F	52
13	Wound swab	F	67
14	Tracheal aspirate	M	63
15	Wound swab	M	37
16	Tracheal aspirate	M	54
17	Tracheal aspirate	F	50
18	Urine	M	81
19	Wound swab	M	54
20	Wound swab	M	55

Isolation of *A. baumannii* strains
Each sample was inserted in 10 ml sterile phosphate buffered saline solution (PBS, pH 7.2 - 7.4). The sample-saline mixture was vortexed, then centrifuged for 10 minutes at 6000 rpm. The filtrate of each sample was serially diluted in the same buffer. Aliquots of 1 ml (dilution 10^{-4} of each sample) were inoculated on blood agar medium and incubated for 24 to 48 h at 37 °C. After the incubation period, the obtained single colonies were individually picked up and sub-cultured several times in fresh blood agar medium plates until obtaining homogeneous pure cultures.

Identification of *A. baumannii* strains
The preliminary identified *A. baumannii* isolates on blood agar medium were confirmed by API 20NE and/or MicroScan Walk- away® automated systems (Dade Behring, CA). These tests were performed according to the manufacturer's protocol for Enterobacteriaceae and non-enteric bacteria. Wells of biochemical test were inoculated with an overnight 0.5 McFarland bacterial suspension and incubated at 37 °C for 24 hrs. The results were read after addition of reagents, as a seven-digit number that was identified by API 20 analytical index [18].

Biofilm formation by *A. baumannii* strains
Microtiter-plate method (96-well microtiter ELISA plate Sarstedt, Germany) was used to detect the ability of isolated clinical strains to form biofilm in polystyrene wells (which is used in medical devices such as catheters and respiratory tubes). Biofilm

formation was assessed by crystal violet staining as described previously [19]. Twenty clinical strains of *A. baumannii* were inoculated in Luria Bertani medium (LB, OXOID)**.** To detect their ability to form biofilm, each strain was inoculated in 3-5 ml LB liquid medium and incubated at 37°C for 48h. Cultures were diluted (1:100) in the same medium and 100 µl of each diluted culture was inoculated as triplicates in microtiter plate wells and incubated at 37 °C for 48 h.

Assay of biofilm formation
After the proper incubation period, the microtiter wells were rinsed thoroughly by distilled water. The cells attached to the well walls of the microtiter plate were visualized and quantified by crystal violet staining method [19]. Briefly 125 µl of 0.1% crystal violet (CV) was added to each well and incubated for 10 min at room temperature then washed several times with distilled water to remove all the excessive stain. The plates were left to air-dry. Thereafter, 200 µl of ethanol and acetone mixture (80:20 v/v) was added to each stained well. Dye was allowed to solubilize by covering plates and incubating for 15 min at room temperature.The contents of each well were mixed then centrifuged at 6000 rpm for 10 min. Aliquots of 125 µl of the crystal violet/ethanol filtrate were transferred from each well to a separate well in an optically clear flat-bottom 96-well plate. 125 µl of the solubilizing solution was added to one well as a blank. Optical density (O.D) of each sample was measured at a wavelength of 500 to 600 nm using a microtiter plate reader (Biotech).

Factors affecting biofilm formation
Effect of different carbon source additives
Microtiter-plate method was used to study the effect of different carbon source additives on the ability of *A. bumannii* to form biofilm. In this study we assessed the effect of addition of glucose (0.25%), sucrose (5%), and lactose (0.5%) to LB medium in microtiter wells inoculated by different strains of *A. baumannii* (n=20).

Effect of incubation temperature, pH, and incubation period
To evaluate the effect of the incubation temperature on biofilm formation, the *A. baumannii* strains inoculated in LB were incubated at different temperatures (30, 35, 40 °C), and the biofilm formation was evaluated as described above. The effect of pH on biofilm formation was evaluated by incubating the clinical strains in LB medium with different pH values adjusted to 5.5, 6, and 8.5, and the biofilm formation was evaluated as described above. To evaluate the effect of incubation period on biofilm accumulation, microtiter plates were incubated for different incubation periods including 24, 48, and 72 hours. and the biofilm formation was evaluated as described above.

Effect of addition of blood serum.
Serum is known to encourage the growth of bacteria as it contains several growth factors such as glucose, salts, proteins, vitamins and hormones. To assess the role of serum in biofilm formation, LB medium injected with 10% serum was used as previously described [20]. Briefly, whole blood (250 ml) was obtained from the Blood Bank (King Khalid University Hospital, Riyadh, Saudi Arabia). For plasma recovery, the blood was centrifuged at 10000 rpm for 5 min under aseptic conditions. To ensure that the supernatant (plasma) was free of cells the sample was centrifuged again in the same conditions and supernatant was recovered. The serum was added to LB medium with final concentration of 10%.

Generation of non-biofilm producing *A. baumannii* mutant
Non-biofilm producing *A. baumannii* mutant was obtained by random mutagenesis using crystal violet as a mutagen [21]. Briefly, one of the most biofilm producing clinical strain of *A. baumannii* was grown on LB agar medium containing different concentrations of crystal violet (CV) as a mutagen including 42, 56 and 70 µg/ml of CV to induce mutation. Then, the plates were incubated at 37°C for 24 h. After the incubation period, 16 single colonies were picked up and cultured individually in fresh LB medium. Thereafter, the ability of each mutant (n=16) to produce biofilm was evaluated as described above.

Antibiotic sensitivity test of wild and mutant strain
The susceptibility of the wild and mutant *A. baumannii* strains to a wide range of antibiotics were assayed using a fully automated microbiological system; VITEK 2 Compact (BIOMERIEUX). The system performs microbial identification via growth-based technology by colorimetric reagent cards that are incubated and interpreted automatically. In vitro susceptibility testing was then performed by determining the minimum inhibitory concentration (MIC) of the following 7 antibiotics using broth microdilution methods: Ampicillin, Amoxicillin, Clavulanic acid, Cefotaxime, Ceftazidime, Cefepime ,Imipenem. The results were interpreted according to the criteria recommended in the Clinical and Laboratory Standards Institute guidelines (CLSI), [22].

Results and Discussion
Isolation and identification of *A. baumannii*
Isolation of *A. baumannii* from the clinical samples (tracheal aspirates, sputum, urine and wound swabs) resulted in isolation of 20 strains. The colonies that grew in blood agar and showed the typical morphology of *A. baumannii* were selected, including non haemolytic, non-pigmented small colonies. The cells were gram negative, non-motile, non-spore forming coccobacilli. Moreover, the isolated strains (n=16)

were able to grow at 44°C, which is a unique feature of *A. baumannii.* The morphology of the colony and cells of the isolated bacterial strains were consistent with those reported for the typical strain of *A. baumannii*[4]. Thereafter, the selected bacterial strains were further identified as *A. baumannii*(n=20) using API 20NE and MicroScan Walk- away® automated systems (Dade Behring, CA). All isolates were catalase positive, oxidase-negative, gelatinase negative, esculin test negative, nitrate reductase negative, indole production positive, urease positive. However, the isolates showed slight variation in carbon sources consumption. The biochemical features of the isolated clinical strains (n=20) of the isolated bacterial strains were in agreement with those reported for typical strains of *A. baumannii*[4].

Origin and distribution of isolated clinical *A. baumannii* strains

Twenty clinical strains of *A.baumanii* were isolated from 20 patients including 12 males and 8 females.

The highest recovery of *A. baumannii* strains was from wound (n=8), followed by tracheal aspirates (n=5), and urine (n=4) and sputum (n=3), respectively. These results are in agreement with that previously reported high recovery *of A. baumannii* from wounds infections [4].

Evaluation of biofilm production by the isolated *A. baumannii*

Evaluation of biofilm production by the isolated *A. baumannii* (n-20) was carried out using the crystal violet method as described earlier. Microtiter wells presented in **Figure 1** indicated that all isolated clinical *A. baumannii* strains (n-20) were capable of biofilm formation in polystyrene wells, as an example of abiotic surfaces which is used in medical devices such as catheters and respiratory tubes. Due to the lack of oxygen at the bottom of the microtiter wells, bacterial strains formed the biofilm near the surface (air-liquid interface) in the form of a ring.
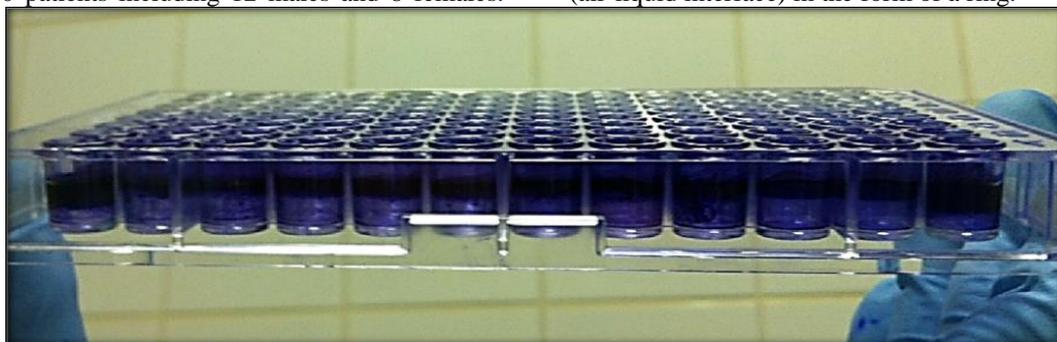
Figure 1: Microtiter plate after staining with crystal violet dye showing the formation of biofilm ring by *Acinetobacter baumannii* clinical strains (at the air-liquid interface)
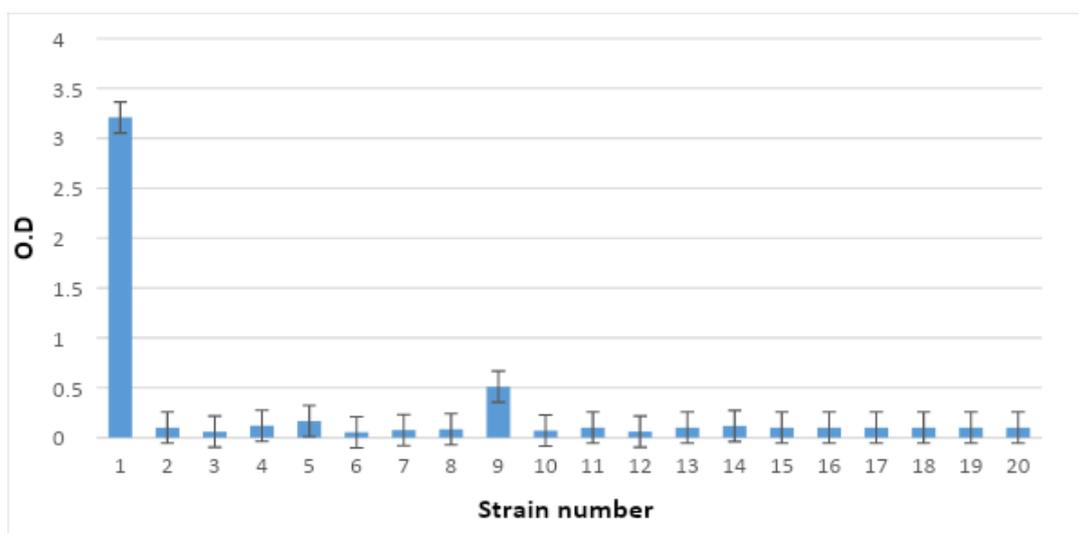
Figure 2. Biofilm formation in microtiter wells by the 20 clinical strains of *Acinetobacter baumannii.* - $O.D_{590}<0.050$, non-biofilm forming; $0.050<OD_{590}<0.09$, weak; $0.1<OD_{590}<0.5$, medium; and $0.5<OD_{590}$, strong biofilm forming. The data presented are the mean of triplicate for each sample and the standard deviations are shown as error bar.

High amount of biofilm was developed by *A. baumannii* on different surfaces as stainless steel, polystyrene, and polycarbonate thermoplastic materials that are often used to construct medical devices [15]. Ezel *et al.* reported that *A. baumannii* strains isolated from ICU in a hospital showed resistance to almost all types of antibiotics due to the production of a biofilm [16].

The results shown in **Figure 2** revealed the biofilm production is variable among *A. baumanni* strains. Two of the isolated *A. baumannii* strains were strong biofilm forming (strains no. 1& 9), 13 medium (strains no. 2, 3, 4, 5, 11, 13, 14, 15, 16, 17,18, 19, 20) and 5 weak biofilm forming strains (strain no. 6,7,8,10,12). Oure findings are in agreement with previous research which reported that the biofilm production is variable among *A. baumanni* strains [20]. On the contrary in another research, *A. baumanni* was found to be able to form biofilm on most abiotic surfaces with no significant variation [25].It is worthy to mention that the strong biofilm forming strains were those isolated from tracheal aspirates followed by wound swabs and urine samples, respectively (**Table 2**). These results were consistent with the results of Babapour *et al.* [26] who reported that *A. baumannii* isolated from tracheal aspirates were the strongest biofilm producing strains followed by wound swab. On the other hand, there was no difference on the biofilm production by *A. baumannii* strains recovered

from different sources including blood, urine, sputum, catheters, cerebrospinal fluid, bronchoalveolar lavage, exudates, and burned skin [27].

Factors affecting Biofilm formation
It was reported that *A. baumannii* is responsible for a number of hospital acquired infections as well as community acquired infections [23]. Moreover, multidrug-resistant *Acinetobacter spp.* was reported to cause long term persistent infections in hospital settings after a long time of starvation because of biofilm formation [24]. Therefore, understanding the environmental factors affecting its biofilm formation is fundamental in finding suitable procedures for its eradication.

Effect of different carbon source additives
The effect of different carbon sources (glucose, sucrose and lactose) on biofilm formation by *A. baumannii* was investigated. As shown in **Figure 3**, biofilm formation was enhanced in the presence of glucose, sucrose and lactose. However, highest biofilm formation in most isolates was in the presence of lactose followed by glucose and sucrose, respectively. Lin *et al.* reported that biofilm formation by *A. baumannii* was enhanced by addition of 20% sucrose [27]. However, Andrew *et al.* reported that different carbon source has no significant effects on biofilm formation by *A. baumannii* [28].
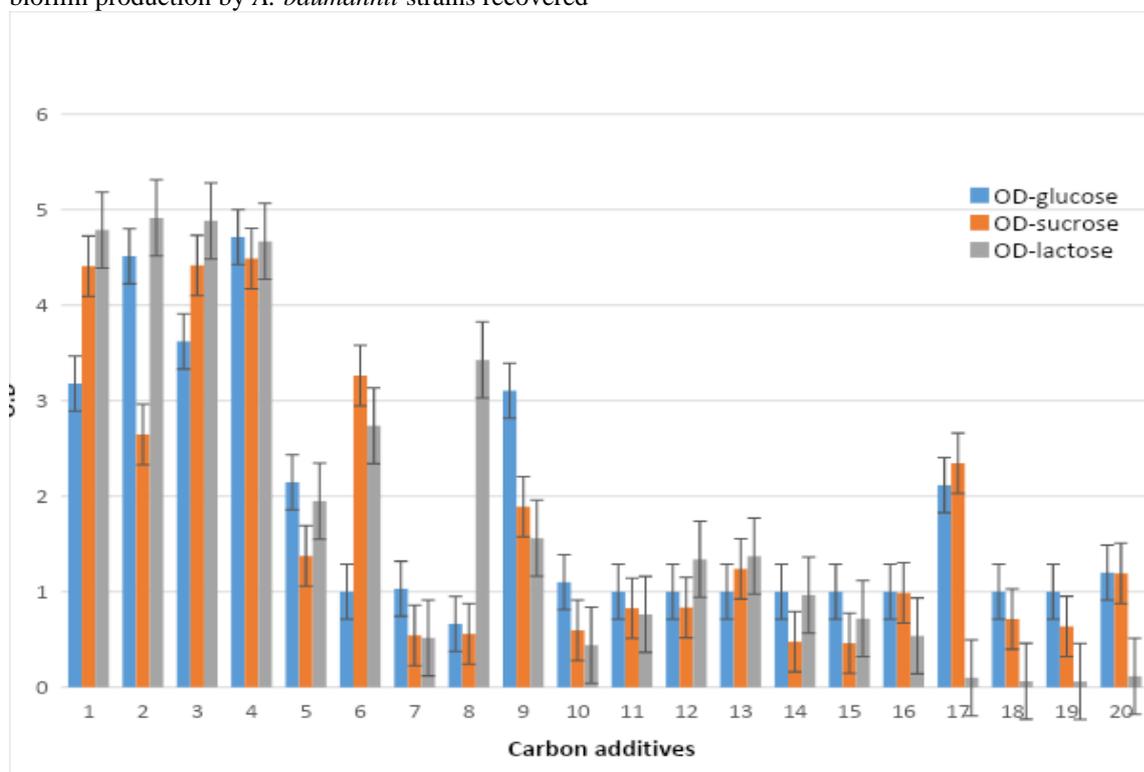
Figure 3. Effect of different carbon additives on biofilm formation by *Acinetobacter baumannii* clinical isolates (n=20). The recorded data is means of triplicates test and the standard deviations are shown as error bar.

_____

Table 2: The degree of biofilm formation by *Acinetobacter baumannii* clinical strains according to isolation spot

Clinical sample source	Number of Biofilm Forming strains			Total no.
	Weak	Medium	Strong	
Tracheal aspirate	1	3	1	5
Wound swab	-	7	1	8
Sputum	3	-	-	3
Urine	-	4	-	4
Total	**5**	**13**	**2**	**20**

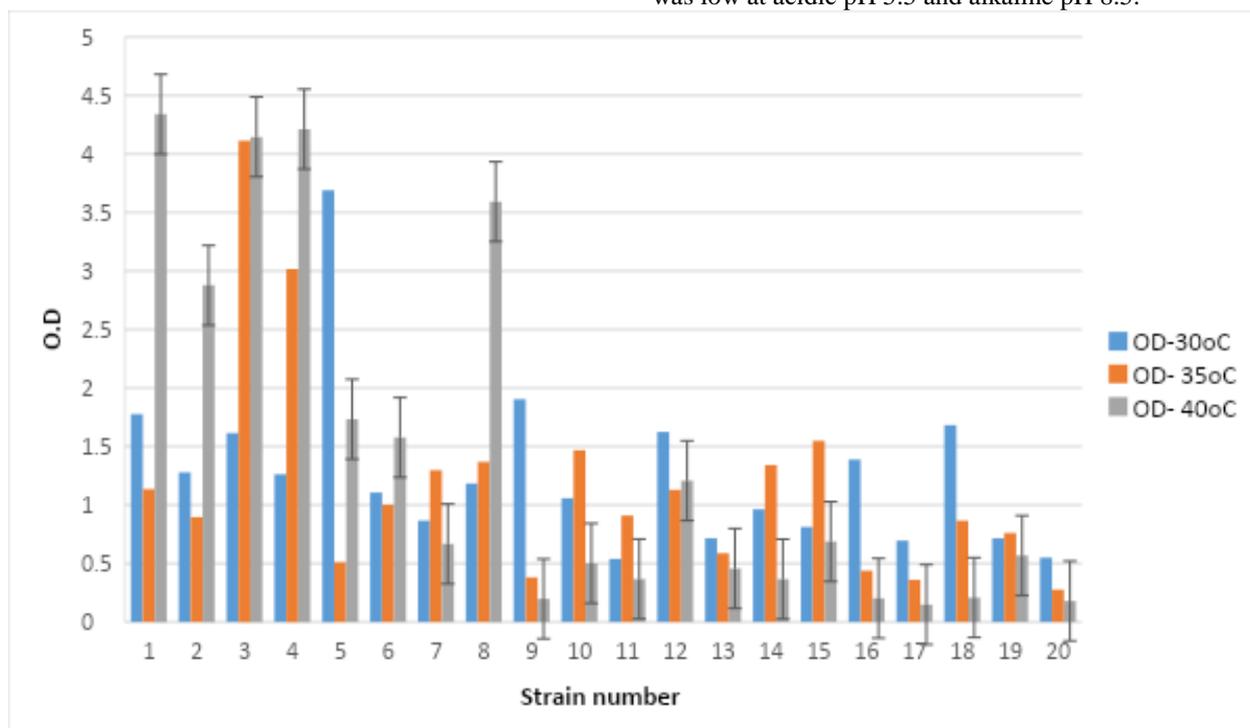*Effect of incubation temperature on biofilm formation*

Results of the biofilm formation by *A. baumannii* strains at different incubation temperatures are illustrated in **Figure 4**. The results indicated that the different strains were able to form biofilm at all temperatures with a slight increase of biofilm formation at 40ºC.

It has been previously reported that high temperature enhances cell hydrophobicity which results in biofilm formation, increases nutrient uptake and induces the production of polysaccharide forming the biofilm matrix [29,30,31]. However, De Silva *et al.* recently reported that biofilm formation on plastic surfaces was high at 28 °C due to the up-regulation of certain biofilm-associated proteins (Baps) such as Csu and iron-uptake proteins in *A. baumannii* strain [32]

Effect of different pHs on biofilm formation

The effect of pH on biofilm formation was measured by inoculating *A. bumannii* isolates (n=20) in microtiter plates containing LB medium with different pH values (5.5, 6, 7.4 and 8.5). The results shown in **Figure 5** indicated that the production of biofilm was maximum at pH 6 and 7.4. In the current study, we assessed biofilm production at different pH values (5.5, 6, 7.4 and 8.5). As shown in **Figure 5**, among the twenty tested isolates biofilm production was maximum in isolate numbers 1 and 4 at pH 7.4 and isolates 11 and 14 at pH 6. While biofilm production was low at acidic pH 5.5 and alkaline pH 8.5.

Figure 4. Effect of incubation temperature on biofilm formation by *A. bumannii* clinical strains (n=20). The recorded data is means of triplicates test and the standard deviations are shown as error bar

_____
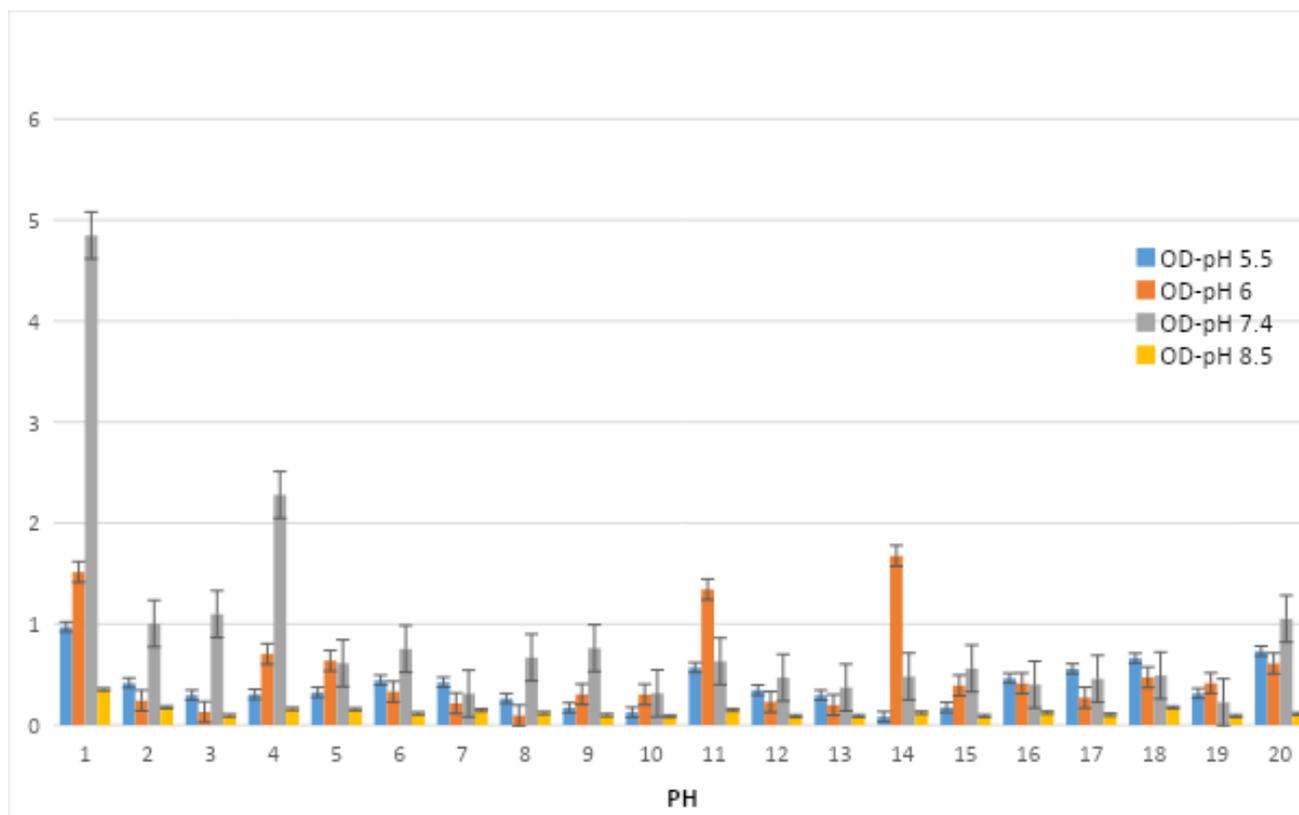
Figure 5. Effect of different pHs on biofilm formation by *A. baumannii* clinical strains (n=20). The recorded data is means of triplicates test and the standard deviations are shown as error bar.
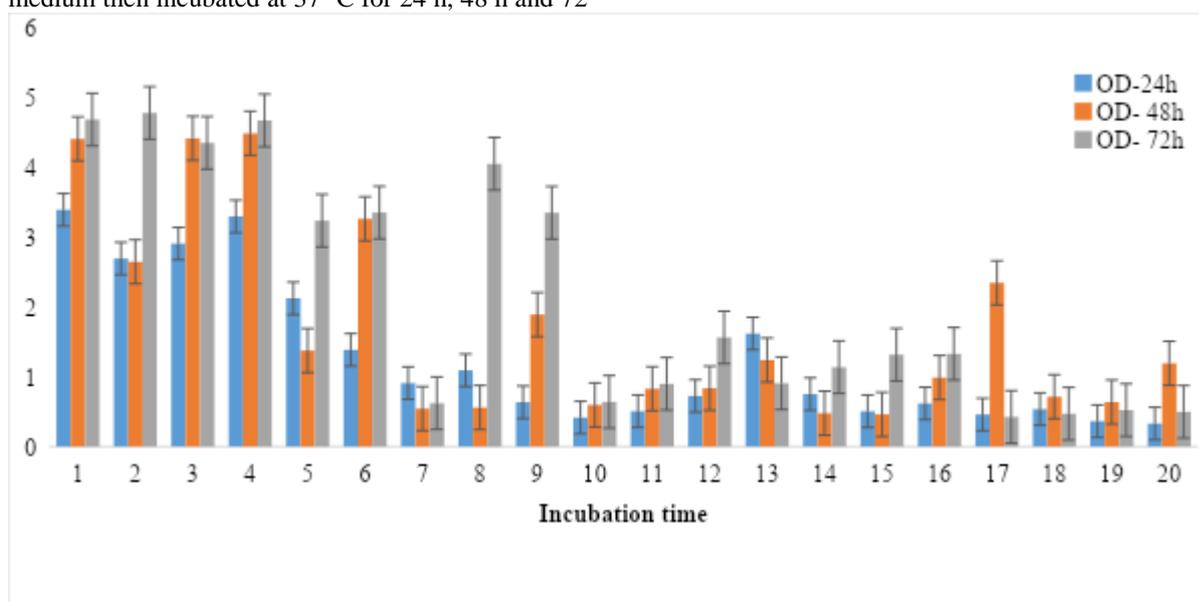
Effect of incubation period on biofilm formation
To evaluate the effect of incubation period on the accumulation of biofilm, *A. bumannii* clinical strains (n=20) were inoculated in microtiter wells on LB medium then incubated at 37 ºC for 24 h, 48 h and 72

h. As shown in **Figure 6**, biofilm formation is enhanced by increasing the incubation period in most strains. This result is matched with previous reports that the biofilm production is increased by increasing the incubation period [30,32].

Figure 6. Effect of incubation periods on biofilm formation by *A. bumannii* clinical strains. The recorded data is means of triplicates test and the standard deviations are shown as error bar.

_____

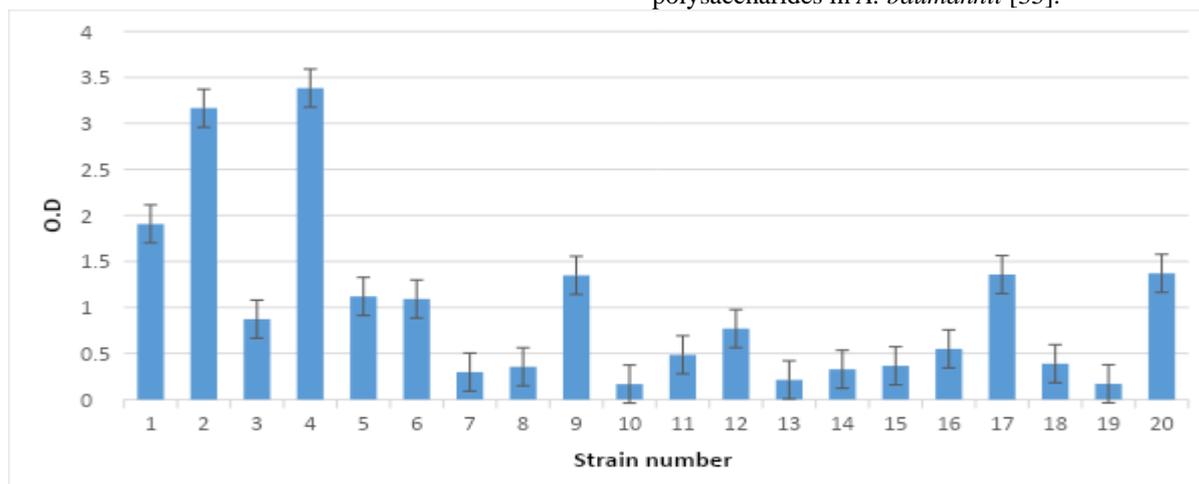**Effect of addition of blood serum on biofilm formation**

Serum contains many growth factors that encourage the growth of bacteria. To investigate the effect of serum on accumulation of biofilm, *A. bumannii* strains (n=20) were inoculated into microtiter plates containing LB medium with 10% serum. The results shown **Figure 7** revealed that the presence of serum in the media enhances biofilm production for all strains with varying degrees. Serum contains many growth factors that encourage the growth of bacteria. However, serum contains the proteins required for complement activation which is fatal for bacteria. However, it was reported that *A. baumannii* have the ability to resist the complement system and this feature is usually associated with mortality of patients [20]. Researchers reported that *A. baumannii* develops a mature biofilm when grown in complement-free human serum (HS). Unfortunately, complement activation is inhibited due to the presence of some regulatory proteins which can attach to bacterial lipopolysaccharides, lipo-oligosaccharides, and a variety of membrane proteins. These regulatory proteins are factor I and cofactors H and FHL[-1] [20]

4. Assessment of the role of *Acinetobacter baumannii* biofilm in its susceptibility to different antibiotics

**4.1. Generation of non-biofilm producing *A. baumannii***

From the isolated clinical strains of *A. baumannii* strain no. 1 was selected to generate non biofilm producing strains. The non-biofilm producing mutant strains were then picked up, inoculated in LB medium and tested for the ability to form biofilms by the biofilm assay using crystal violet method. As shown in **Figure 8**, the microtiter wells showed a number of mutant colonies that have almost lost their capability to produce biofilm including mutant no. 6,7,8, 9 and 10. (**Figure 9**). Inhibition of biofilm formation can be attributed to the down regulation of genes involved in the synthesis and transport of poly-β-1,6-N-acetylglucosamine (PNAG); the genes responsible for synthesis and transport of the biofilm-associated exo-polysaccharides in *A. baumannii* [35].

Figure 7. Effect of addition of blood serum on biofilm formation by *A. baumannii* clinical strains (n=20). The recorded data is means of triplicates test and the standard deviations are shown as error bar

Figure 8 Growth of *A. baumannii* on mutant strains in microtiter wells
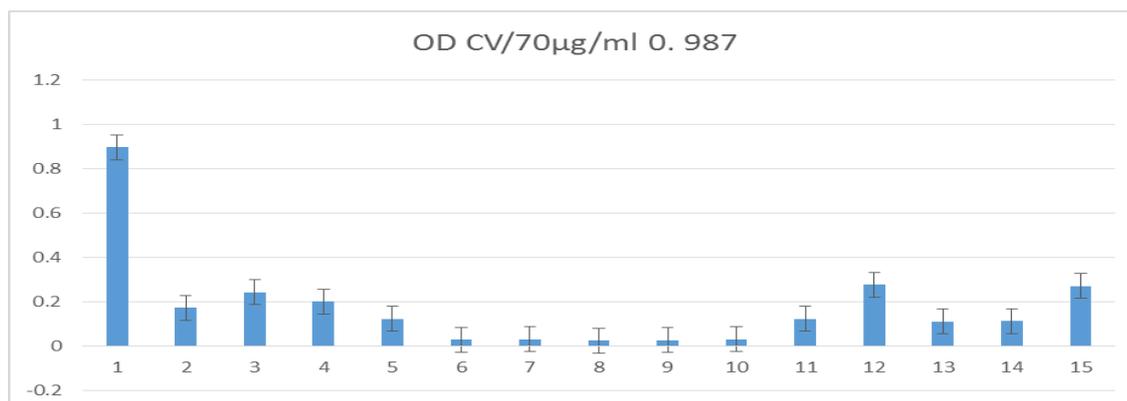
Figure 9. Biofilm formation by 15 mutant strains of *Acinetobacter baumannii*

Antibiotic sensitivity test.

The antibiotic susceptibility of wild and mutant *A. baumannii* strains was carried out. The results shown in **Table 3** indicated that both wild type strains (biofilm forming) and mutant strains (non-biofilm forming) *A. baumannii* were high resistant to all of the antibiotics tested based on the MIC of each antibiotic. The antibiotic resistance of the mutant strain which lacks the ability to form biofilm refers that other mechanisms in addition to biofilm formation are involved in antibiotic resistance. Moreover, Chen *et al.* showed no significant differences in resistance rates among the different groups of antibiotics [36]. Moffatt *et al.* found that lipopolysaccharide-deficient mutants resist higher concentrations of colistin.

Therefore, mutants generated in this study may bear the two features; the ability to resist antibiotics and inability to form biofilms [37].

A. baumannii resistance is linked to the capacity of the bacteria to produce biofilms [38] However, no statistical significance was observed between the ability to form biofilms and antibiotic resistance [39]. In another research, only 74.7% of the MDR isolates from a Portuguese hospital showed biofilm forming ability [40]. A strong biofilm-forming MDR *A. baumannii* strain collected from a Chinese hospital showed that resistance to levofloxacin, cefepime, and gentamicin significantly decreased when biofilm-forming ability was strong [41].

Table 3: Antibiotic sensitivity tests for *Acinetobacter baumannii*

Antibiotics	MIC	Interpretation	Antibiotics	MIC	Interpretation
Ampicillin	<=32	Resistant	Gentamicine	<=16	Resistant
Amoxicillin/ Clavulanic acid	<=32	Resistant	Ciprofloxacin	<=4	Resistant
Cefotaxime	<=64	Resistant	Norfloxacin	<=16	Resistant
Ceftazidime	16	Resistant	Fosfomycin	<=256	Resistant
Cefipime	32	Resistant	Nitrofurantion	<=512	Resistant
Imipenem	<=16	Resistant			

Conclusion

The antibiotic resistance mechanisms in *A. baumannii* are very complex, mainly involving the expression of active efflux pumps, inactive enzymes production and mutation of outer membrane proteins. There was no clear relationship between antibiotic resistance and biofilm formation in *A. baumannii*.

References

1. Dijkshoorn L, Nemec A and Seifert H (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*.Nat Rev Microbiol.; (12):939-51.

2. Yang CH, Su PW, Mio SH and Chuang LY (2019). Biofilm Formation in *Acinetobacter baumannii*: Genotype-Phenotype Correlation. Molecules; 24, 1849; doi:10.3390/molecules24101849

3. Fournier PE and Richet H (2006). The epidemiology and control of *Acinetobacter baumannii*in health care facilities. Clin. Infect. Dis. 42, 692–699. doi: 10.1086/500202

4. Kulah C, Mooij MJ, Comerta F, Aktas E, Celebi G, Ozlu N, Rijnsburger MC and Savelkou PM (2010). Characterisation of carbapenem-resistant Acinetobacter baumannii outbreak strains producing OXA-58 in Turkey. Inter. J. Antimicrob. Agents. 36: 114–118.

_____

5. Lin MF and Lan CY (2014).Antimicrobial resistance in *Acinetobacter baumannii*: From bench to bedside. World J Clin Cases. 2(12): 787–814.

6. Falagas ME, Koletsi PK and Bliziotis IA (2006).The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa.* J Med Microbiol. 55(12):1619-29.

7. Sunenshine RH, Wright MO, Maragakis LL, Harris AD, Song X, Hebden J, Cosgrove SE, Anderson A, Carnell J, Jernigan DB, Kleinbaum DG, Perl TM, Standiford HC and Srinivasan A (2007). Multidrug-resistant Acinetobacter infection mortality rate and length of hospitalization. Emerg Infect Dis. 13(1):97-103.

8. Antunes LC, Visca P and Towner KJ (2014). *Acinetobacter baumannii:* evolution of a global pathogen. Pathog Dis. 71(3):292-301. doi: 10.1111/2049-632X.12125.

9. Gordon NC and Wareham, D. W. (2010). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. Int. J. Antimicrob. Agents 35, 219–226. doi: 10.1016/j.ijantimicag.2009.10.024

10. Gordon NC and Wareham, D. W. (2010). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. Int. J. Antimicrob. Agents 35, 219–226. doi: 10.1016/j.ijantimicag.2009.10.024

11. Lee CR, Lee JH and Park M (2017). Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol. 7:55.

12. Abdulla AA, Althahab AA, Abed TA, Mahdi RK and Fadhil S (2015). Screening of virulence factors in *Acintobacter baumannii* isolated from clinical samples. International Journal of Current Research and Academic Review; 3(6):124-138

13. Geisinger E and Isberg RR (2015). Antibiotic Modulation of Capsular Exopolysaccharide and Virulence in *Acinetobacter baumannii*. PLOS pathogens DOI: 10.1371/journal.ppat.100469 p: 1-27.

14. Wood CR, Mack LE and Actis LA (2018). An update on the *Acinetobacter baumannii* regulatory circuitry. Trends Microbiol. 26:560–562.

15. Kroger C, Kary SC, Schauer K and Cameron AD (2016). Genetic regulation of virulence and antibiotic resistance in *Acinetobacter baumannii* Genes (Basel) 8:12.

16. Eze1 EC, Chenia HY and El Zowalaty ME (2018). *Acinetobacter baumannii* biofilms:

17. Kenney LJ (2018). The role of acid stress in Salmonella pathogenesis. Curr. Opin. Microbiol. 47, 45–51. doi: 10.1016/j.mib.2018.11.006.

18. Al-Arfaj AA, Ibrahim ASS, Somily AM and Al-Salamah AA (2011).Genetic basis of carbapenem resistance in Acinetobacter clinical isolates in Saudi Arabia. African Journal of Biotechnology; 10(64):14186-14196.

19. Tomaras AP, Dorsey CW, Edelmann RE and Acts LA (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone usher pili assembly system. Microbiology 149, 3473–3484.

20. Runci F, Bonchi C, Frangipani E, Visaggio D and Visc P (2017). *Acinetobacter baumannii* Biofilm Formation in Human Serum and Disruption by Gallium Antimicrobial Agents and Chemotherapy, 61(1) e01563-16.

21. Salamah AA and Charnetzky WT (1986). Effect of the virulence associated 47 megadalton plasmid of Yersinia pestis on permeability to gentian violet and sensitivity to novobiocin. Acta Microbiol Hung, 33(3):193-201.

22. Wayne, PA (2014). Clinical and Laboratory Standards Institute. Performance standards for antimicro-bial susceptibility testing. Twenty-fourth international supplement. Document M100-S24.: CLSI.

23. Seng R, Kitti T,Thummeepak R, Kongthai P, Leungtongkam U,Wannalerdsakun S and Sitthisak S (2017). Biofilm formation of methicillin-resistant coagulase negative staphylococci (MR CoNS) isolated from community and hospital environments. PLoS One. 12, e0184172.

24. Chapartegui-González I, Lázaro-Díez M, Bravo Z, Navas J, Icardo J and Ramos-Viva J(2018). *Acinetobacter baumannii* maintains its virulence after long-time starvation. PLOS ONE. https://doi.org/10.1371/journal.pone.0201961 August 22, 1-14.

25. Hendiani S, Abdi-Ali A, Mohammadi P and Kharrazi S (2015). Synthesis of silver nanoparticles and its synergistic effects in combination with imipenem and two biocides against biofilm producing *Acinetobacter baumannii*. Nanomed J. 2(4):291–298.

26. Babapour E, Haddadi A, Mirnejad R, Angaji S and Amirmozafari N (2016). Biofilm formationin clinical isolates of nosocomial *Acinetobacter*

_____

baumannii and its relationship with multidrug resistance. Asian Pac J Trop Biomed. 6(6):528–533.

27. Lin MF, Lin YY, Yeh HW and Lan CY (2014). Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii ade AB* genes and its correlation with tigecycline susceptibility. BMC Microbiol. 2014; 14: 119.

28. Andrew P, Tomaras CW, Richard E and Luis A (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. Microbiol. 149: 3473–3484.

29. Assaidi A, Ellouali M and Latrache H (2018). Effect of temperature and plumbing materials on biofilm formation by Legionella pneumophila serogroup 1 and 2-15. J Adhes Sci Technol. 1–14.

30. De Silva PM, Chong P, Fernando DM, Westmacott G and Kumar A (2018). Effect of incubation temperature on antibiotic resistance and virulence factors of *Acinetobacter baumannii* ATCC 17978. Antimicrob Agents Chemother. 62(1):e01514–01517.

31. Han N, Mizan MFR, Jahid IK and Ha S-D (2016). Biofilm formation by *Vibrio parahaemolyticus* on food and food contact surfaces increases with rise in temperature. Food Control. 70:161 166.

32. De Silva PM and Kumar A (2019). Signal Transduction Proteins in *Acinetobacter baumannii*: Role in Antibiotic resistance, Virulence and Potential as Drug Targets. Frontiers in Microbiology, Mini Review. doi: 10.3389/fmicb.2019.00049

33. Pour NK, Dusane DH, Dhakephalkar PK, Zamin FR, Zinjarde SS and Chopade BA (2011). Biofilm formation by Acinetobacter baumannii strains isolated from urinary tract infection and urinary catheters. FEMS Immunol Med Microbiol. 62(3):328-338.

34. Pour NK, Dusane DH, Dhakephalkar PK, Zamin FR, Zinjarde SS and Chopade BA (2011). Biofilm formation by *Acinetobacter baumannii* strains isolated from urinary tract infection and urinary catheters. FEMS Immunol Med Microbiol, 62:328–338.

35. Henry R, Nuwan V, Paul H, Torsten S, Scott C and Jennifer H (2011). Colistin-Resistant, Lipopolysaccharide-Deficient *Acinetobacter baumannii* Responds to Lipopolysaccharide Loss through Increased Expression of Genes Involved in the Synthesis and Transport of Lipoproteins, Phospholipids, and Poly-_-1,6-N-Acetylglucosamine. Antimicrob Agents and Chemother. 59–69.

36. Chen L, Li H, Wen H, Zhao B, Yujia Niu Y, Mo Q and Wu Y (2020). Biofilm formation in *Acinetobacter baumannii* was inhibited by PAβN while it had no association with antibiotic resistance. Microbiology Open. 1 of 12, 00:e1063.

37. Moffatt JH, Harper M, Mansell A, Crane B, Fitzsimons TC, Nation RL, Li J, Adler B and Boyce JD (2013). Lipopolysaccharide-deficient Acinetobacter baumannii shows altered signaling through host Toll-like receptors and increased susceptibility to the host antimicrobial peptide LL-37. Infect Immun. 81(3):684-689.

38. Azizi O, Shakibaie MR, Modarresi F and Shahcheraghi F (2015). Molecular detection of class-D OXA carbapenemase genes in biofilm and non-biofilm forming clinical isolates of *Acinetobacter baumannii*. Jundishapur J Microbiol. 8(1).

39. Longo F, Vuotto C and Donelli G (2014). Biofilm formation in *Acinetobacter baumannii*. New Microbiol. 37(2):119–127.

40. Zhang D, Xia J and Xu Y (2016). Biological features of biofilm-forming ability of *Acinetobacter baumannii* strains derived from 121 elderly patients with hospital-acquired pneumonia. ClinExp Med. 16(1):73–80.

41. Han X, Li Q, Shen L, Hu D and Qu Y (2014). Correlation between the biofilm- forming ability, biofilm-related genes and antimicrobial resistance of *Acinetobacter baumannii*. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue, 26(9):639-643.