

Detection of quorum sensing activity among biofilm forming *Acinetobacter baumannii* isolates

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Abstract

Bacteria communicate with each other by producing chemical signals (acyl homoserine lactones, AHLs) through the quorum sensing (QS) signaling circuits, which control the expression of virulence genes. Also QS plays a significant role in biofilm formation, therefore it is important to develop new strategies to inhibit QS and get rid of biofilm. *Acinetobacter baumannii* is a major nosocomial pathogen that has a high resistance to antimicrobials and desiccation. In addition, it has a strong ability to form biofilm in chronic infections.

In the present study, quantification of biofilm and screening of MDR *A. baumannii* for QS signal molecules (AHLs) were performed. Out of the 52 MDR *Acinetobacter baumannii* isolates, 42 isolates were found to be strong biofilm formers. Out of the 42 strong biofilm forming isolates, 20 isolates produced long chain AHL signals and none of them produced short chain AHL signals. The results revealed that 81% of the isolates formed strong biofilm and 48% of the strong biofilm forming isolated have QS activity, which indicates that QS has a potential role in the control of *A. baumannii* biofilm formation.

Keywords: *Acinetobacter baumannii* ; MDR; biofilm; quorum sensing (QS); acyl homoserine lactone (AHL)

1. INTRODUCTION

Bacteria have a tendency to adopt economic processes in order to conserve resources and avoid wasteful expression of genes using quorum sensing to control the gene expression (Fuqua *et al.* 1994; Cza'ra'n and Hoekstra, 2009). Expression of virulence genes is ineffective at lower cell densities as it fails to escape the host immune system (Bhargava *et al.*, 2010). Many opportunistic pathogens rely on quorum sensing (QS) circuits as central regulators of virulence expression. Therefore, QS is an obvious target for novel classes of antimicrobial drugs through the inhibition of quorum sensing signals that regulate biofilm production and other virulence genes (Sperandio, 2007). Mis-regulation or inhibition of quorum sensing can be achieved using plant extracts

(Adonizio *et al.*, 2008), synthetic compounds (Rasmussen *et al.*, 2000) or AHL degrading enzymes (Dong and Zhang, 2005). As QS is not directly involved in processes essential for growth of the bacteria, inhibition of QS does not impose harsh selective pressure for development of resistance as with antibiotics. Numerous chemical libraries of both natural and synthetic origin have been screened and several QS-inhibitory compounds have been identified (Rasmussen and Givskov, 2006). *Acinetobacter baumannii* is emerging as one of the major nosocomial infectious pathogens, facilitated by tolerance to desiccation and multidrug resistance (Dijkshoorn *et al.*, 2007; Gayoso *et al.*, 2014). The infected or colonized patients serve as reservoirs for disease transmission

and may be responsible for sustained circulation of outbreak strains in healthcare facilities (Maragakis and perl, 2008). *A. baumannii* clinical isolates have been observed to possess a strong ability to form biofilms (Rodríguez-Baño *et al.*, 2008). It is obvious that this trait is responsible for chronic infections due to the extra contribution of biofilms to bacterial persistence and colonization (Von Eiff *et al.*, 2005; Kennedy *et al.*, 2010). In *A. baumannii*, the quorum sensing is mediated by the production of acyl homoserine lactone (AHL) signaling system through two proteins, LuxI (autoinducer synthase) and the cognate LuxR protein, which regulates the expression of QS target genes (Egland and Greenberg, 2001; Bhargava *et al.*, 2010). AHL signal molecules in *A. baumannii* have been shown to influence biofilm formation which is an important virulence factor responsible for its outstanding antibiotic resistance and survival properties (Gaddy and Actis, 2009).

The present study aimed to detect biofilm formation among MDR *A. baumannii* clinical isolates and to screen the strong biofilm-forming isolates for the production of quorum sensing signals.

2. MATERIALS AND METHODS

2.1. Clinical isolates

A total of fifty two clinical isolates were collected from different surgical intensive care units (ICUs) of Zagazig University Hospitals, Sharkia, Egypt, except eleven strains were collected from the International Medical center, Cairo, Egypt in the period from November 2011 to March 2015. All strains have been identified and confirmed to be MDR *A. baumannii*. The isolates were of different sources and comprised of tracheal aspirate, wound, blood and urine samples.

2.2. Quantification of Biofilm formation using microtiter plate method

For determination of biofilm production by *Acinetobacter baumannii* strains, the modified method of Stepanovic *et al.* (2007) was used. Overnight cultures of the isolates were prepared, diluted with fresh Muller-Hinton broth (MHB), and adjusted to a cell density of 1×10^6 CFU/mL. Aliquots of 100 μ L of the adjusted bacterial suspension were inoculated in the wells of sterile 96-well polystyrene microtiter plates with rounded bottom; wells containing media only were also included as negative control. After incubation for 24 h at 37°C, the contents of the wells were gently aspirated and the wells were washed three times with sterile phosphate buffered saline (PBS, pH 7.2). The adherent cells were fixed with 100 μ L of 99% methanol for 20 min and then stained with 100 μ L crystal violet (1%) for 20 min. The excess dye was then removed under running distilled water, and then the plates were left to air dry. The bound dye was extracted by the addition of 80 μ L of 33% glacial acetic acid and the optical densities of the stained adherent films were read with a microplate reader (Biotek Spectrofluorimeter, Biotek, USA) at a wavelength of 600 nm. The test was repeated three times, and the mean optical densities were calculated. The cut-off OD (OD_c) was defined as three times standard deviations above the mean OD of the negative control. According to the criteria of Stepanovic *et al.*, the test isolates were categorized into four groups; non-biofilm forming (OD \leq OD_c), weak biofilm forming (OD > OD_c, but \leq 2x OD_c), moderate biofilm forming (OD > 2x OD_c, but \leq 4x OD_c), and strong biofilm forming (OD > 4x OD_c).

PAO1 strain was tested also as positive control for biofilm production.

2.3. Screening for quorum sensing AHL signal molecules production

2.3.1. Extraction of culture supernatant

The culture supernatants were extracted according to the method of Ravn *et al.* (2001). Extractions of bacterial cultures were done on sterile supernatants derived from bacterial cultures grown in MHB for 24 h at 37°C. Cells were harvested and the supernatants were sterile filtered before extraction. Ten-milliliter volumes of sterile supernatants were extracted with an equivalent volume of ethyl acetate (acidified with 0.5% formic acid). The mixture was shaken vigorously for 30 s and the phases were allowed to separate. The shaking was repeated three times before the ethyl acetate containing fraction was removed and another 10-mL fraction added. The whole extraction process was repeated three times. The combined ethyl acetate fractions were evaporated in oven to dryness at 40 °C and reconstituted in 1 mL acidified ethyl acetate, transferred to an eppendorf and stored at -20°C.

2.3.2. Detection of long chain AHL using CV026 biosensor in a well-diffusion assay (CV026 inhibition assay)

Chromobacterium violaceum CV026 assay was performed as described by Renee and Gray (2000) with the modification of Tan *et al.* (2012). Overnight grown *C. violaceum* CV026 cells (15 mL) were added into 200

mL of molten Luria-Bertani (LB) agar that has been supplemented with N-hexanoylhomoserine lactone (C6-HSL, 0.25µg/mL) purchased from Sigma-Aldrich chemie GmbH, steinheim, Germany. *C. violaceum* CV026 agar suspension was poured into Petri dishes and allowed to solidify. Wells were then made using sterile pipette tips. Culture extracts to be tested for QS activity were placed in sterile tubes, dried under stream of sterile air to evaporate ethylacetate, then redissolved in 50 µL of sterile LB broth. Culture extract (10 µL) was placed in each well and LB broth served as negative control. The plates were incubated at 28°C for 24 hours. Halo formation on a purple background suggested that the culture extract has QS activity due to the production of long chain AHL which inhibit violacein pigment production around cups.

2.3.3. Detection of short chain AHL using CV026 biosensor in a well-diffusion assay (CV026 induction assay)

For detection of short chain AHL, *C. violaceum* CV026 assay was performed as in long chain AHL detection assay without supplementing the molten LB agar with N-hexanoylhomoserine lactone. A dark purple pigmentation around any cup suggested that the culture extracts contain short chain AHL which induce violacein production from CV026.

3. RESULTS

3.1. Quantification of Biofilm Formation Using Microtiter Plate Method

Quantification of biofilm production by the 52 clinical isolates of *Acinetobacter baumannii* revealed that 50 (96%) isolates formed biofilms. Fig (1) shows the biofilm formation among some isolates in a microtiter plate. Quantification of biofilm formation showed that in all the 50 isolates,

42 (81%) isolates formed strong biofilms compared to 8 (15%) moderate biofilm forming isolates (Fig. 2). Two isolates with OD less than the ODC of the control were classified as non-biofilm formers (4%). PAO1 standard strain demonstrated a strong biofilm forming capacity. The microtiter plate assay was done in triplicates and the data were pooled from these experiments.

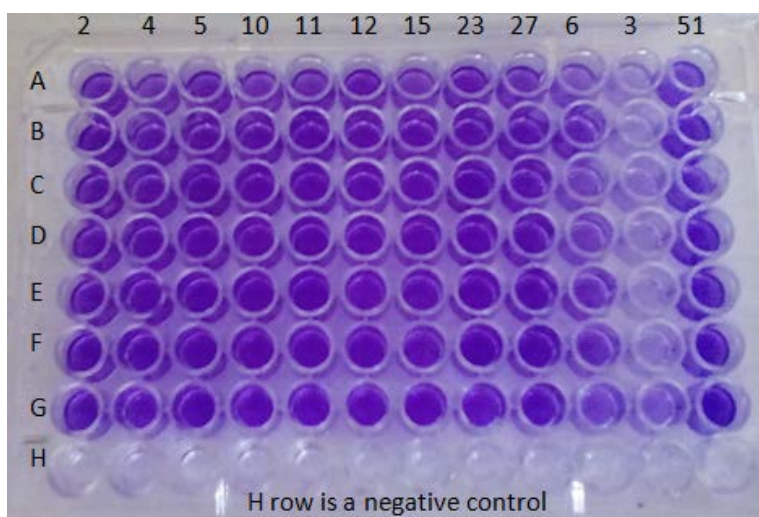


Figure 1: A representative microtiter plate shows examples of a strong, moderate and non biofilm forming isolates. Isolate number 3 is non biofilm former, isolate 6 is moderate, and the rest are strong biofilm formers.

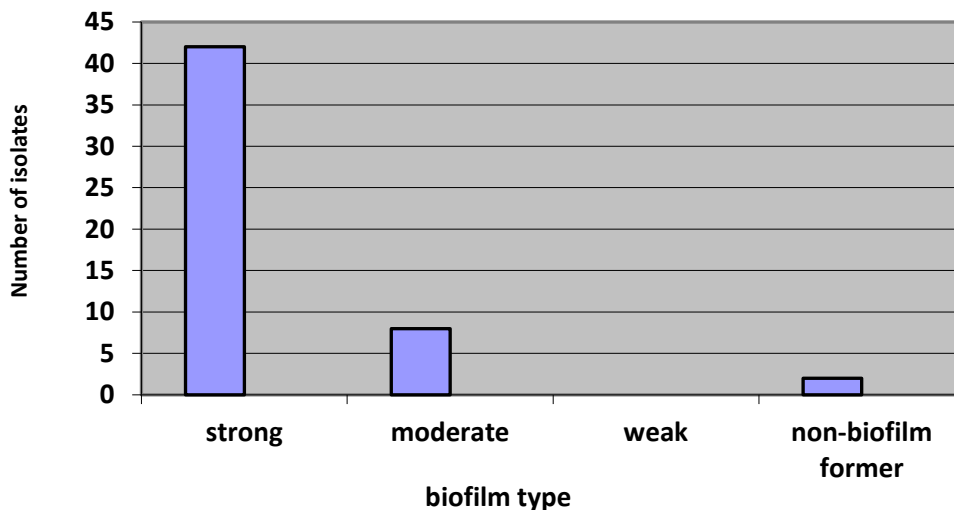


Figure 2: Biofilm formation in MDR *A. baumannii* isolates.

3.2. Detection of long chain AHLs using CV026 agar plate assay

The assay of *Acinetobacter baumannii* culture extracts using the CV026 inhibition assay demonstrates the ability of twenty isolates (48%), out of 42 strong biofilm formers, to produce long chain AHLs (fig 3a).

3.3. Detection of short chain AHLs using CV026 agar plate assay

The assay of *Acinetobacter baumannii* culture extracts using the CV026 induction assay demonstrates that all 42 strong biofilm forming isolates (100%) are unable to produce short chain AHLs (fig 3b).

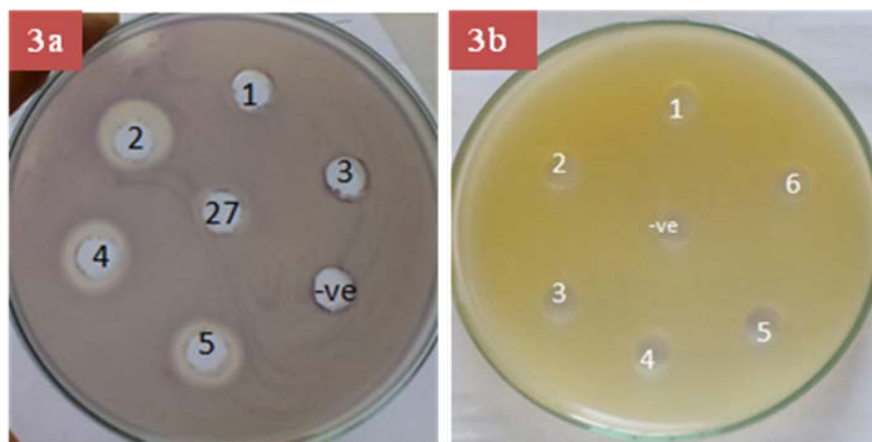


Figure 3: Fig 3a; shows the long chain AHL producing isolates (2, 4, 5, 27) as clear halo around the wells, and isolates (1, 3) not produce it. Fig 3b; shows the production of short chain AHL, all isolates are negative, no purple pigment around the wells. -ve; the broth as negative control.

4. DISCUSSION

The most important problem of biofilms is its high tolerance to antimicrobial chemotherapy. Bacteria living in the biofilm mode are often up to 1000-fold more tolerant to antibiotics, biocides and heavy metals than planktonic cells (Anwar *et al.*, 1990; Allison *et al.*, 1993; Teitzel & Parsek, 2003). The effective control of biofilm infections requires a combined work to develop new therapeutics that target the biofilm phenotype and community signalling-based agents that may prevent the formation, or enhance the detachment, of biofilms (Costerton *et al.*, 1999).

In our study, it is observed that about 96% of the 52 clinical MDR isolates of *Acinetobacter baumannii* significantly formed biofilms. These results are similar to that of Qi *et al.* (2016) study, as they reported that 91% of their isolates were positive for biofilm formation. Our results revealed 81% of the biofilm forming isolates were strong biofilm formers whereas Anbazhagan *et al.* (2012) found a high percentage (60%) of strong biofilm formation among *A. baumannii* isolates. On the other hand, Qi *et al.* (2016) and Modarresi *et al.* (2015) found that 23% and 35% of their isolates were strong biofilm formers, respectively.

In our study, the strong, moderate and weak biofilm forming isolates represented 81%, 15% and 4% of the isolates, respectively. The obtained results are inconsistent with Hendiani *et al.* (2014) who reported higher percentage for weak and non-biofilm formers. The results revealed a high biofilm forming capacity in our MDR isolates, which is compatible with the results reported by Babapour *et al.* (2016) but are incompatible with results obtained by Qi *et al.* (2016) and Rodriguez-Bano *et al.* (2008).

In this study, the 42 strong biofilm forming isolates were screened for the QS signal molecule production where 48% of these isolates effectively produced long chain AHL molecules by using the CV026 biosensor monitor system, while none of the strong biofilm formers produced short chain AHL signals. Our results are inconsistent with that obtained by Anbazhagan *et al.* (2012) who reported a lower percentage for long chain AHL production, but are consistent with their obtained results for short chain AHL production.

From the present findings, it seems that QS could play a role in the control of biofilm formation in *A. baumannii* beside many other factors that affect biofilm formation like chaperone-usher pili assembly system (Tomaras *et al.*, 2003), biofilm-associated protein (Bap) (Loehfelm *et al.*, 2008), the pgaABCD operon responsible for the production of poly- β -1,6-Nacetylglucosamine (PNAG) exopolysaccharidic matrix (Choi *et al.*, 2009) and the outer membrane protein OmpA of 38 kDa (Choi *et al.*, 2005). This conclusion is compatible with that of He *et al.* (2015) and

Niu *et al.* (2008) who confirmed the role of *abaI* gene in biofilm formation.

Conclusion

From our results, we concluded that QS may have a partial role in the control of biofilm formation in *A. baumannii*, as half of the strong biofilm forming isolates do not have QS activity. So, biofilm formation may be due to the collaboration of other factors beside QS. Consequently, the fact that QS plays a role in the control of biofilm formation in *A. baumannii* could be considered in attempts to control antimicrobial resistance by employing quorum sensing inhibitors as a potential therapeutic alternative for the traditional antimicrobial therapy.

Aknowledgments

The authors are grateful to Massara Mohamed (assistrant lecturer at Department of microbiology, faculty of pharmacy, Ain Shams University) for providing a copy of CV026 biosensor. Appreciation goes to Islam Mohsen and Fatma Yehia (assistant lecturers at microbiology and immunology department, faculty of pharmacy, Zagazig University) for providing some of their *Acinetobacter baumannii* isolates, ten isolates from each one.

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تعيين نشاط استشعار النصاب بين عزلات ميكروب الاسنيتوبكتر بوماني المكون للغلاف الحيوي

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تتواصل البكتيريا فيما بينها بانتاج اشارات كيميائية (الاسيل هوموسيرين لاكتون) من خلال دوائر ارسال استشعار النصاب و التي تتحكم في التعبير عن جينات الشراسة. و يلعب استشعار النصاب دورا هام في تكوين الغلاف الحيوي و لذلك فانه من المهم ان تطور استراتيجيات جديدة لكي يثبط استشعار النصاب و يتم التخلص من الغلاف الحيوي. و ان ميكروب اسنيتوبكتر بوماني ممرض انتهازي يمتلك مقاومة عالية للمضادات الحيوية و ايضا مقاوم للجفاف. بالاضافة الى قدرته العالية لتكوين الغلاف الحيوي في العدوى المزمنة.

و في هذه الدراسة تم تعيين تكوين الغلاف الحيوي في عزلات الاسنيتوبكتر ذات المقاومة المتعددة للمضادات الحيوية كمي و تم فحص هذه العزلات لانتاج اشارات استشعار النصاب. و وجد ان من بين 52 عزلة, 42 عزلة كانت ذات غلاف حيوي قوى و من بينهم 20 عينة منتجة لاشارات استشعار النصاب ذات السلسلة الطويلة. وهذا يدل على ان استشعار النصاب يمكن ان يكون له دور في تكوين الغلاف الحيوي في ميكروب اسنيتوبكتر بوماني.