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Research Article

Isolation and evaluation of *Acinetobacter baumannii* from hospitals in Mosul city and detect their ability to produce acylated homoserine lactones

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Abstract

One of the characteristics that distinguish living things is communication or the simplest form of talking and sharing information. Gram-negative bacteria use the AHL autoinducer for their quorum sensing, and this quorum sensing has been a major factor in the development of pathogenic conditions in a specific host. This study aimed to isolate, identify *A. baumannii*, and detect N-acyl-homoserine lactone (AHL) produced by *A. baumannii*, which was isolated from patients admitted to Mosul hospitals in Iraq. A total of 125 samples were taken from patients who were being treated at Mosul hospitals. The isolates were characterized using the Vitek-2 and API 20 E systems as well as microscopy and biochemistry. Only 25 isolates were confirmed as *A. baumannii*. Patients with burns had the highest prevalence of *A. baumannii* (40%) while those with blood and throat swabs had the lowest prevalence (8%). This study assessed the sensitivity of all *A. baumannii* isolates to thirteen different antibiotic classes using the disc diffusion method. The three categories of *A. baumannii* infection's antibiotic sensitivity (MDR), extensively drug-resistant (XDR), and non-multi-drug resistance (non-MDR) were calculated to be, respectively, 34.61%, 53.84%, and 11.53. The -galactosidase assay was used to quantify AHL activity during a 24-hour growth phase in order to determine the association between AHL production by *A. baumannii* isolates and their phase's growth. Using the biosensor strain *Agrobacterium tumefaciens* KYC55, all isolates displayed the quantitative and qualitative activity of acyl homoserine lactones (AHL) signals.

Keywords: *Acinetobacter baumannii*, MDR, Quorum Sensing, *Agrobacterium tumefaciens*

Introduction

Acinetobacter baumannii is a gram-negative, non-motile Gammaproteobacterium in the Pseudomonadales class. *A. baumannii* is a prominent nosocomial pathogen that can cause a variety of ailments, including ventilator-acquired pneumonia, urinary tract infections, wound infections, and bloodstream infections. (Fournier et al., 2006) This bacterium has caused numerous illnesses. The rising prevalence of multidrug-resistant strains of *A. baumannii*, which offer few, if any, therapeutic choices for therapy, is of particular concern. (Rice, 2006).

Quorum sensing (QS) is a bacterial communication system that enables the control of specific methods, involving the genes expression of virulence factors, the production of secondary metabolites, biofilm formation, and ways to deal with stress methods like bacterial secretion systems (SS) and

competition systems. In bacterial communication, these play a crucial role. They perform both general tasks like heme-binding proteins, transporting proteolytic enzymes, and adhesins, as well as more specialized ones like the release, transport, absorption, and of DNA; secretion of effectors to create an infectious niche; environmental adaptability; and synthesis of proteins in infected cells. Each of these activities may support pathogenesis and virulence (Pena et al., 2019). Cell-to-cell communication in gram-negative bacteria is frequently carried out by the creation of N-acyl-homoserine lactone (AHL) signaling molecules. A number of bacterial biosensor strains have been developed to detect these signals. (Winson et al., 1998). *A. tumefaciens* KYC55 is used as the AHL bioassay strain in both qualitative and quantitative protocols in this study, and was reported to detect a broad range of AHL derivatives and to show great susceptibility (Cha et al., 1998). This strain's galactosidase expression

system has been employed as a sensitive gene expression indicator. The galactosidase gene in *A. tumefaciens* KYC55 is turned on by a *traI* promoter, allowing QS signals (AHLs) to control how much of the enzyme is expressed. When active forms of AHLs are present, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) is hydrolyzed and cleaved by galactosidase, resulting in the formation of a blue residue. The blue precipitate's accumulation is then observable by spectral absorbance at 635 nm (Kawaguchi et al., 2008).

Materials and method

Approval of the ethical

According to Ref number (21/130), the ethical committee of the Biology department collage of science/university of Mosul accepted this work, the samples were taken after the patients gave their consent.

Sample collection

Pathological samples were collected from Mosul city hospitals from the first of October to December 2021. The specimen took from Mosul hospital (Asalam teaching hospital, Mosul general hospital, Aljumphuri teaching hospital, Ibi sina teaching hospital, and Specialized burn center) and the specimen reached 125 samples taken from the wound (16), urine (13), csf (3), blood (2), sputum (11), swab (54), burn (26). All of these specimens were taken with care and high accuracy. Burns, wounds, and throat swabs were taken by using a wet sterile cotton swab in order to keep the sample wet and not dry so that we could transfer it to the laboratory and not be damaged or contaminated. The urine and sputum specimens were collected in a sterile cup and were closed tightly. Sterile culture tubes containing brain heart infusion broth were used to collect specimens Blood and CSF samples were collected by an experienced doctor, and then the samples were transferred to the laboratory for necessary inoculation.

Gathering of bacterial isolates

After being transferred to the laboratory all samples were cultured on MacConkey agar, which were then incubated at 37° for 24 hours under aerobic conditions. The colonies that did not appear to be lactose fermenting were subcultured on a MacConkey agar plate and incubated for another 24 hours at 37°C.

Identification of *Acinetobacter baumannii* isolates

All the obtained bacterial isolates (125 isolates) were tested for gram stain capacity, and shape by light microscope using oil emersion at a power of 100x magnification.

Biochemical tests

The standard biochemical tests were used to identify all gram-negative coccobacilli or bacilli from probably isolated bacteria. Carried out according to (Forbes et al., 2007).

Oxidase test

This test was performed to determine whether or not bacteria can produce an Oxidase enzyme. It was done by soaking a filter paper in an oxidase reagent (1 %-N-NN-tetramethyl paraphenel diamin dihydrochloride). With a sterile wooden stick, a single colony was transferred to filter paper and rubbed over the reagent. A positive reaction is when a purple color develops within 20-30 seconds; a negative reaction is when the colony's color remains the same (Brown et al., 2005).

Catalase test

The catalase enzyme activity was measured by using the slide method. To experiment a single colony of recently activated bacteria was transferred to a glass slide containing 3 % hydrogen peroxide. This test was performed to see if the microorganisms under investigation could produce catalase enzyme. The presence of bubbles was interpreted as a catalase-positive result (Benson, 2002).

Identification of *A. baumannii* with Analytical Profile Index (API) 20 E system

The strips were read using the interpretation table after 24 hours of incubation. One drop of each of the reagents was added to the VP test, and the results were recorded. Results were listed as positive or negative, with the equivalent values (1, 2, or 4) for a positive test and 0 for a negative test being given. The results of the three tests were put together to produce a final number (the code-code), which has seven numbers. By using the API standard catalog, the results were compared and reported.

Vitek-2 System

Vitek-2 (bioMerieux) was used to identify Gr- bacterial isolates. The 25 isolates were cultured before being incubated at 37°C overnight (Bagudo et al., 2020).

Sensitivity test

The Kirby-Bauer method was used to perform an antibiotics susceptibility test for 13 antibiotics, including Amikacin (AK:30µg), Tobramycin (TOB:10µg), Tigecycline (TGC:15µg), Tetracycline (TE:10µg), Clarithromycin (CL:15µg), Azithromycin (AZM: 15µg), Imipenem (IPM:10µg), Ciprofloxacin (CIP: 10µg), Cefazidime (CAZ: 30µg), Colistin (CL:10µg), Piperacillin/Tazobactam (PIT:100/10 µg), Meropenem (MEM:10 µg), and Trimethoprim/Sulfamethoxazole (SXT:25µg), followed as described by WHO, (2003). Bacterial suspension for the 25 *A. baumannii* isolates and ATCC (19606) was created by selecting one to two separated colonies of *A. baumannii* and adding them to normal saline (85 %), which resulted in a moderately turbid bacterial suspension as opposed to the standard turbidity that McFarland equals to 1.5×10^8 CFU/ml. Using sterile cotton, a part of the bacterial suspension was carefully transferred and distributed equally on the nutrient

agar medium. The antimicrobial discs were placed on the agar and firmly pressed with sterile tongs to ensure contact with the agar. The plates were then turned over and allowed to remain for 24 hours at 37°C. According to the Clinical Laboratories Standards Institute, the inhibition zones were measured in millimeters (mm) (HiAntibiotic Zone Scale) (CLSI, 2021).

Screening of bacterial isolates for the production of AHLs

1- Determination of AHLs

The quantity of AHLs was estimated according to the level of β -galactosidase activity as previously described (Joelsson and Zhu, 2006) with some modification.

A) Preparation of AHL bioassay *A. tumefaciens* KYC55 strain

A. tumefaciens was cultured in LB medium supplemented with antibiotics and incubated at 28°C.

B) Preparation of *A. baumannii* Am1 isolate

A. baumannii Am1 isolate was cultured to different time points in a brain heart infusion broth, including the growth phases to study the AHL-production kinetics. Cells were micro centrifuged for 2min at 16,000x/g.

2- Cross-streaking method with *A. tumefaciens* KYC55

The methods described by (Paulk Tierney and Rather, 2019) were modified to detect the range of AHLs produced by *A. baumannii* (Am1-Am24). If AHL molecules were present in the agar, they would diffuse to the reporter strain and be detected as a result of TraR activating the traI-LacZ fusion. Co-culturing the reporter strain *A. tumefaciens* KYC55 with the same strain served as the negative control.

Results and Discussion

Description of study samples

A 25 *A. baumannii* were isolated and identified from all the total number of collected specimens, whereas 25 specimens gave negative result for culturing and 75 specimens gave positive bacterial cultures for other bacterial species as shown in Fig. 1.

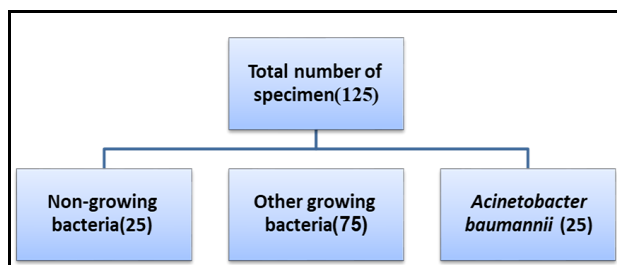


Fig. 1 The count of *A. baumannii* isolates from the total number

The distribution of the studied clinical specimens (125) was as follows: the maximum number of specimens were collected in Mosul General Hospital 44 (35.2%) and the lowest number was collected in Ibn Sina Teaching Hospital 10 (8%), As for the other hospitals, the number of samples collected from them was as follows, Aljumphurii teaching hospital 30(24%), Specialised burns center 26 (20.8%), Alsalam teaching hospital 15(12%). as shown in Fig. 2.

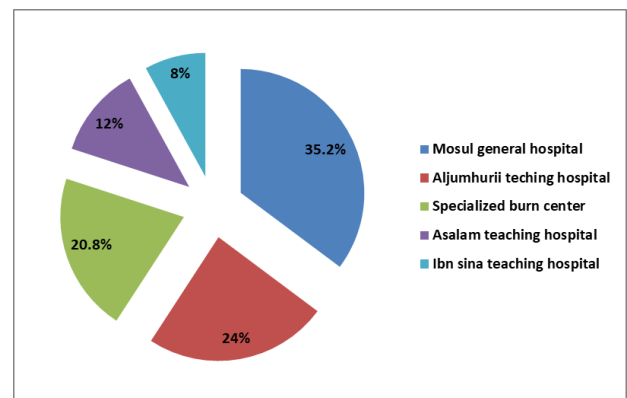


Fig. 2 Distribution of clinical specimens from different hospitals in Mosul

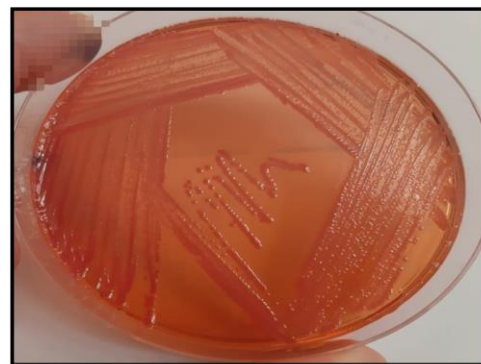


Fig. 3 Colonies of *Acinetobacter baumannii* on Maconkey agar



Fig. 4 Colonies of *Acinetobacter baumannii* on blood agar

Colonies appeared in the MacConkey medium as pale yellow colonies due to not fermenting the lactose, with a smooth round shape, about 2-3 mm in diameter, as shown in Fig.3, as well as when culturing samples on the medium of blood agar, colonies were white and did not lyse red blood cells because it does not produce hemolysin. As it appears in the following Fig. 4.

Microscopic examination (Gram Stain)

Laboratory Diagnosis Microscopic examination of the gram-stained slides revealed that all of the isolates were gram-negative coccobacilli that occasionally formed diplococci Fig. 5

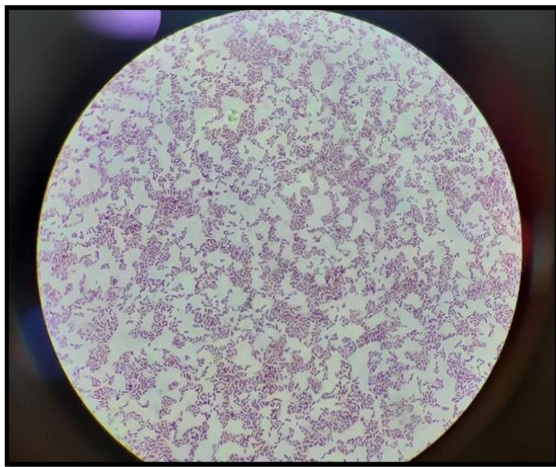


Fig. 5 *A. baumannii* cells under the light microscopic

Diagnosis by biochemical tests

1- Oxidase and catalase enzymes assay

A. baumannii isolates were negative for the oxidase test, and thus they were distinguished from other bacteria such as *Pseudomonas aeruginosa*, which are positive for the oxidase test. At the same time, the catalase was positive and the colony cells produced air bubbles.

2- API 20E system

The biochemical test diagnosis was completed using the API 20E system, which is one of the rapid tests for detecting bacterial species. After 24 hours of incubation, the results were read and the necessary reagents were added. The

positive and negative results (seven numbers were called the code) were recorded. The complete scientific name of the bacterial isolate was then retrieved by entering this code into the system isolates directory. All isolates were identified by the API 20E system. The obtained results for *A.baumannii* are shown in Fig. 6

Antibiotics susceptibility profile

Using the disc diffusion method, 13 antibiotics were tested for antimicrobial susceptibility against all 25 *A. baumannii* isolates and an ATCC isolate, and the statistical analysis showed a significant correlation between the groups at ($P < 0.01$), as illustrated in Fig. 8.

It was observed that the vast majority of *A. baumannii* strains were resistant to a wide range of antibiotics, including Amikacin (88.4%), Tobramycin (84.6%), Tetracycline, Tigecycline, Ceftazidime, Trimethoprim / Sulfamethoxazole (96.1 %), Clarithromycin (92.3%), Azithromycin (88.4%), Meropenem (92.3%), Imipenem (46.1%), Ciprofloxacin (88.4%), and Piperacillin/Tazobactam (73%). However, the isolates had extremely low resistance to Colistin antibiotic (0%). Thus, the bacterial isolated divided into three categories: XDR, MDR, and non-MDR, and were calculated to be 34.61%, 53.84%, and 11.53 respectively as showed in Table 1 and the statistical analysis showed a significant correlation between groups ($P \leq 0.05$).

Table 1. Three categories: XDR, MDR and non-MDR of bacterial isolated

Antibiotics Phenotypic resistant patterns	No (%)	Chi ²	Sig.
XDR	(14) 53.84%	7.00*	0.030
MDR	(9) 34.61%		
Non MDR	(3) 11.53%		
Total	(26) 100%		

The current result on Azithromycin resistance was agreed with (Abdullah and Merza, 2019) who discovered that *A. baumannii* is resistant to many antibiotics, including azithromycin with a percentage of (83%). Also, the results showed the similar to the (Ahmed and Mohammad, 2020) results which also mentioned that *A. baumannii* were resistant to Clarithromycin with percentage (89.4%).

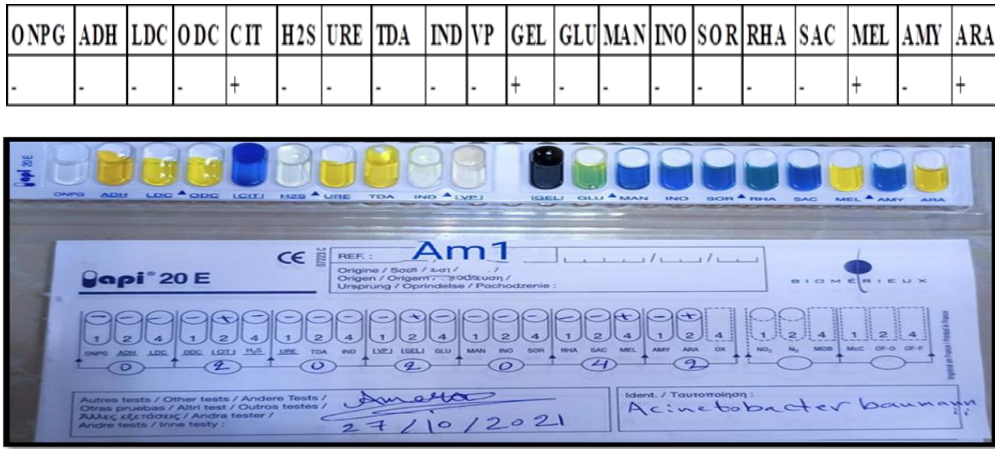


Fig. 6 Reading of API 20E system used for identify of *A. baumannii* (Positive result)

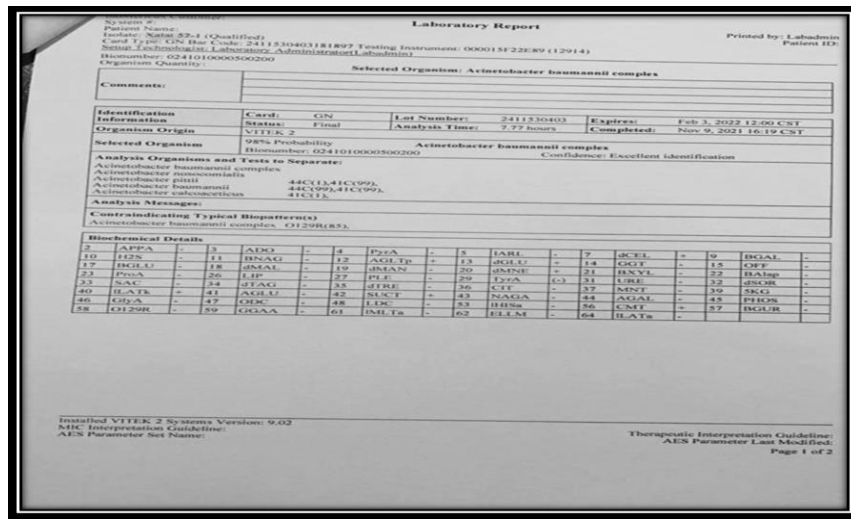


Fig. 7 Identification of *A. baumannii* using Vitek 2

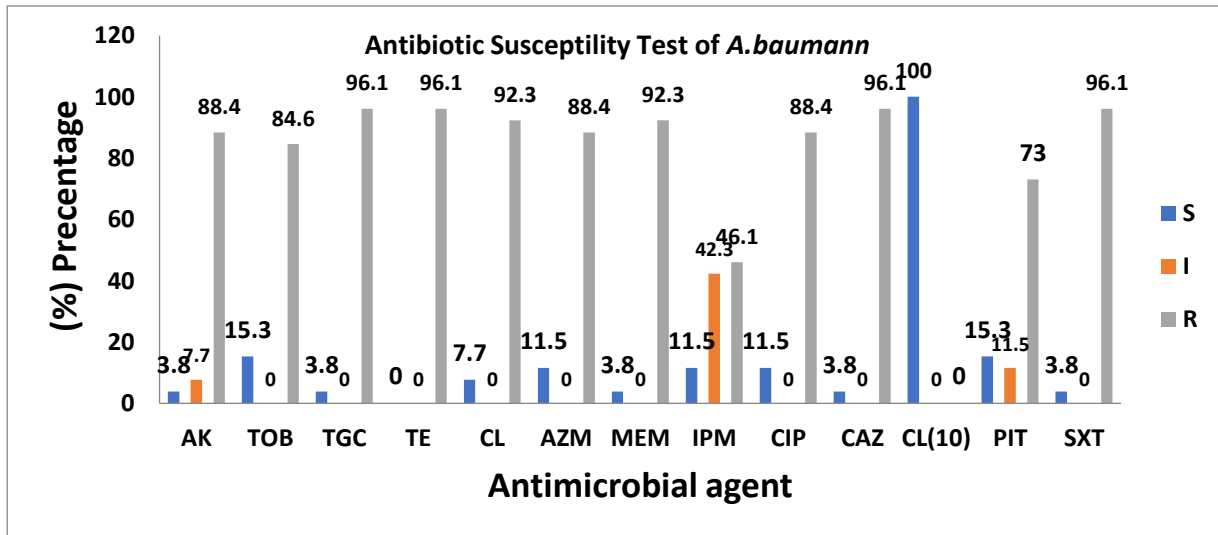


Fig. 8 Antibiotics susceptibility test for *A. baumannii* clinical isolates and ATCC

While current study disagreed with the work of (Sehree et al., 2021) who recorded the percentage of Tetracycline was (29.2%) and Tigecycline (2.4). Besides, the resistance of *A. baumannii* to Tobramycin and Amikacin this percentage was not consistent with the works of (AL-Baroodi and AL-Ghanimi, 2020) who demonstrate that the percentage of Tobramycin and Amikacin resistances was (66.6%, 46.6%) respectively. And the result of the current study was consistent with the work of (Arjmand et al., 2020). Who showed resistance of *A. baumannii* to 55 % of Trimethoprim and 45.5% Ceftazidime. According to the current study, the percentage with percentage (89.4%) of Colistin resistance was similar to the percentage obtained by (Sehree et al., 2021), who shows the percentage of Colistin resistance was (0%). The results of the current study revealed a high resistant rate to Meropenem and Trimethoprim, similar findings exhibited by a study conducted by (Hassan and Khider, 2019) on Meropenem (92.31%) and Trimethoprim (92.31%).

There are a variety of factors that have resulted in these bacteria being resistant to nearly all antibiotics. The presence of efflux pump families is one of the most important of these explanations. There are various families of efflux pumps in *A. baumannii*, but the most important family is the existence of Resistance Nodulation cell Division (RND) family, which is divided into three groups of the pump, each group responsible for resisting a set of antibiotics. Other factors that cause *A. baumannii* to become antibiotic-resistant include the existence of β -lactamases, an enzyme that breaks the beta-lactam ring in antibiotic molecules this converts the molecule inactive and makes *A. baumannii* resistant to antibiotics with the β -lactam ring (Monem et al., 2020).

A. baumannii is remarkably capable of developing antibiotic resistance to numerous classes of antibiotics through a variety of mechanisms, including decreased outer membrane permeability to limit antibiotic entry, multi-drug efflux pumps, antibiotic modification, and acquisition of mutations in antibiotic targets. For instance, the membrane transporter superfamily of resistance-nodulation cells is highly represented in *A. baumannii*. It is known that these transporters contribute to the resistance to fluoroquinolones, aminoglycosides, macrolides, trimethoprim, and beta-lactam antibiotics (Gellings et al., 2020).

The present study showed that strains demonstrated 100% sensitivity to Colistin, and this indicates that the targets the outer membrane of bacteria, that Colistin antimicrobial is a cationic polypeptide antibiotic by an early charge-based interaction between the negatively charged lipid A component of lipopolysaccharide (LPS) in bacteria and the positively charged Colistin. (Khoshnood et al., 2020) causing alterations in the phospholipid bilayer composition via causing an osmotic imbalance, this mechanism leads to cell death (Cai et al., 2012).

Detection of Acyl-Homoserine Lactone (AHLs) Quorum-Sensing Signals

The experiment's method principle was to describe a simple procedure for the rapid assay of AHL activity in selected isolates by quantitatively measuring the β -galactosidase activity of a reporter AHL bioassay strain grown with different concentrations of isolates growth broth filtrates.

Correlation between AHL activity by *A. baumannii* Am1 isolate and growth phases

The amount of AHLs produced by an AHL-positive isolate was determined previously by measuring β -galactosidase activity with X-gal as a substrate. The multi-drug resistance isolate *A. baumannii* Am1 was chosen to conduct the correlation between AHL activity and its growth phases and the results are shown in Fig. 4 and 5.

According to the experiment, the AHLs enzymatic activity of *A. baumannii* Am1 growth phases was performed to pinpoint when the bacteria might produce an autoinducer, and after the experiment was conducted these times, we found that AHLs is secreted in the middle and end of the logarithmic phase, where the color of the enzymatic reaction changes to blue, while the stationary phase with the control in which AHLs was not secreted, the color remained yellow and did not change. Through the results and statistical analysis, it was found that the relationship between growth and autoinducer secretion is inverse, as shown in Fig.9

The correlation between AHL activity average and growth phases refers to a highly significant correlation ($P < 0.01$).

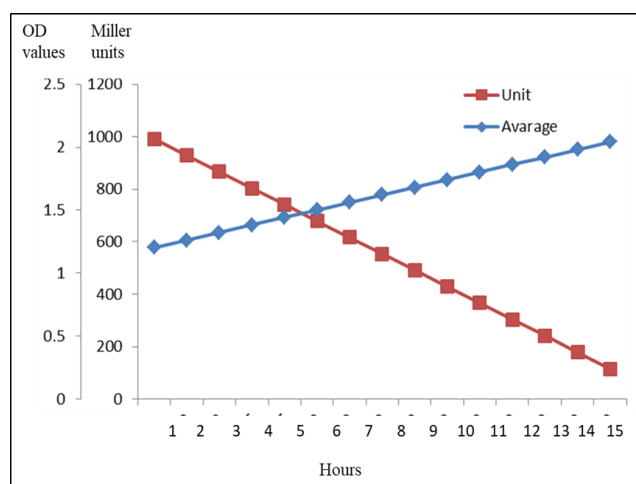


Fig. 9 Experimental points clarify the Correlation between average AHL activity and growth phases

AHLs were produced by *A. baumannii* Am1 isolate, with levels reaching a maximum of 798 and 850ml/U in the middle of the log phase and late log, beginning of the stationary phase, respectively, and gradually decreasing to 565ml/U in the late stationary phase, followed by a rapid decrease to 400 ml/U at the end of the stationary phase. At the same time, the growth of the bacterial culture increased

gradually during incubation and reached 1.75, followed by keeping steady.

Fig. 10 demonstrates that all *A.baumannii* isolates possessed galactosidase activity, but with variable activities compared with the ATCC(19606) isolate as a positive control and with negative control (fresh LB broth was used instead of bacterial culture supernatant). The higher activity in all isolates was in the middle of the logarithmic phase, and the isolates (Am6, burn), (Am15, burn), and (Am21, sputum) were the most active among the isolates.

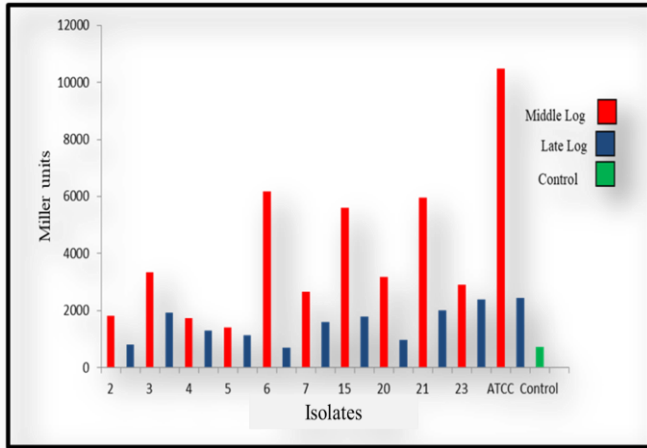


Fig. 10 β -galactosidase activity average into Middle and Late Log \ beginning stationary phase for the ten *A. baumannii* isolates

Previous research has suggested that after the late log phase, the quorum sensing system may play a role in the physiology of bacteria. In the first incubation, the pattern of AHLs activity corresponded to the growth of bacteria. This suggested that QS pathways could interact with starvation-sensing pathways, allowing cells to transition from the log to the stationary phase (Han et al., 2010; Lazazzera, 2000). AHLs activity diminishes as the pH of the culture increases, which is consistent with the hypothesis that AHLs are unstable under alkaline circumstances (Li et al., 2016).

Cross-streaking method with *A.tumefaciens* KYC55

A 24 isolates of *A. baumannii* were tested for producing AHLs, including 10 from burn infections (Am3, Am6, Am9, Am10, Am15, Am17, Am19, Am20, Am22, Am23), eight from sputum (Am1, Am2, Am5, Am7, Am12, Am16, Am18, Am21), three from wounds (Am4, Am13, Am14), two throat swabs (Am8, Am11), and one isolate from blood (Am24). All of the isolates changed color to a clear blue, indicating that they produced AHLs molecules when assayed with *A. tumefaciens* KYC55 as the reporter strain shown in Fig. 11

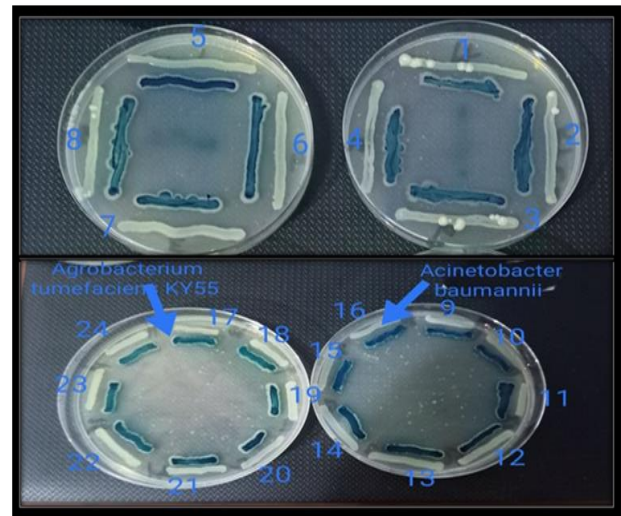


Fig. 11 Detection of long AHL signals production of *A. baumannii*. Isolates suspensions cross-fed with biosensor *A. tumefaciens* KYC55

All *A.baumannii* bacterium isolates appear to be capable of producing AHLs particles during their growth in LB medium, which then spreads in the medium and the detective strain reaches the cultivated adjacent strain. It leads to the stimulation of gene expression of the LacZ reagent gene and the production of the enzyme beta-galactosidase, which hydrolyzes the X-gal reagent into a blue product.

The strain KYC55 was chosen for this investigation because it detects all 3-oxo-HSL compounds as well as C6- 3-hydroxy-HSL, C8-3-hydroxy-HSL, and C10-3-hydroxy-HSL, making it especially helpful for detecting extremely low amounts of AHLs (Zhu et al., 2003; Bitrian et al., 2012) investigated the virulence markers on environmental and nosocomial isolates of Acinetobacter species and discovered that all of them secrete AHLs with medium-to-long chains, but no C4-C6 short-chain AHLs were discovered.

(Gonzalez et al., 2001) investigated *A. calcoaceticus* in two clinical isolates from hospitalized persons and discovered that each *A. cinetobacter* isolate has a number of signaling molecules with autoinducer activity.

Conclusions

In this study, a large number of local medical specimens of *A. baumannii* were found to be highly drug-resistant bacteria to most of the antibiotics used in their treatment in Mosul hospitals, which identified it as one of the primary sources of infection. Using *A. tumefaciens* KYC55, the strain used for the AHLs bioassay, all isolates demonstrated quantitatively and qualitatively active AHL, and a relationship between the AHL activity and the growth phases of *A. baumannii* revealed an inverse correlation.

Conflict of Interest

The author hereby declares no conflict of interest.

Consent for publication

The author declares that the work has consent for publication.

Funding support

The author declares that they have no funding support for this study.

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