Original Research Article

A rapid method to screen for Multi Drug Resistance (MDR) traitin clinical isolates of *Acinetobacter baumannii*-Acyl Homoserine Lactone (AHL) screening in CRAB and CSAB isolates.

ABSTRACT

Aims: Our proposal aimed to evaluate AHLs(*Acinetobacter baumannii*-Acyl Homoserine Lactone (AHL)as a functional marker for Multidrug resistance (MDR) potential in clinical isolates of *Acinetobacter baumannii*. We investigated the AHL production potential of clinical isolates using a biosensor assay directly on a commonly used agar media.

Study design:

Place and Duration of Study: Department of Molecular Diagnostics and Biomarkers, Gleneagles Global Hospitals, Lakdikapul, Hyderabad-500004.

Methodology: Antimicrobial drug sensitivity testing (AST) was performed on72 clinical isolates of *A. baumannii*against two front-line antibiotics, Imipenem (10µg) and Meropenem (10µg), by Kirby-Bauer disk diffusion method.Production of long chain Acyl Homoserine lactone (AHLs) in the clinical isolates of *A. baumannii* was tested by cross streaking with the biosensor *Chromobacterium violaceum* mutant strain CV026 and *Agrobacterium tumefaciens* (NTL4pZLR4) by agar plate diffusion assay. Screening and identification of the quorum sensing mediator gene *abaI* was done by PCR to confirm its presence in all the 72 clinical isolates.

Results: Out of the 72 clinical isolates, 58 were Carbapenem resistant(CRAB)and 14 were Carbapenem sensitive(CSAB)for AST by agar disc diffusion method. None of our isolates produced short chain AHLs whereas, all the isolates could produce varying amounts of long chain AHLs. Genotypic confirmation of AHL gene was obtained by *abaI* gene PCR.

Conclusion:
Carbapenems are the front-line antibiotics used to treat gram negative bacterial infections in emergencies and in the critical care units of hospitals. Clinical isolates *A. baumannii* has innate resistance to several antibiotics due to various mechanisms, biofilms forming the first line of defense against antibiotics for the bacterium. Our study used AST to carbapenem as the leading marker for MDR, assuming the innate resistance of *A. baumannii* to other beta lactam antibiotics. Our study brought out certain important observationsnamely: a) All clinical isolates of *A. baumannii*produced Quorum Sensing signal molecules, the AHLs b) the clinical isolates of *A. baumannii* did not produce any short chain AHLs b) All the clinical isolates of *A. baumannii* produced long chain AHLs c) AHL production is not specific to carbapenem drug resistance because even CSAB isolates produced AHL d) AHL production is inherent to all clinical isolates of *A. baumannii*and it apparently indicates an underlying biofilm potential and MDR trait in these *A. baumannii* isolates. e) AHLs could be a universal marker for revealing MDR trait and biofilm potential in clinical microbiology AST profiling protocols.

Comment [Dm1]: authors must give the meaning of abbreviations as soon as they appear in the texte from the abstract. Exemple*Acinetobacter baumannii*-Acyl Homoserine Lactone (AHL)

Comment [Dm2]:

Comment [Dm3]: Multi drug resistant (MDR)

Comment [Dm4]: What does CARB means ?

Comment [Dm5]: What does CSAB means ?

Keywords:Antimicrobial resistance, Quorum sensing, Acyl Homoserine lactone, Anti-microbial Susceptibility Testing, Acinetobacter baumannii

1. INTRODUCTION

Antimicrobial resistance (AMR) is the single most important threat of this century, and it requires immediate attention and urgent action. It renders most treatable infections untreatable but also results in almost 700,000 deaths annually. Most of the antimicrobial therapies involve empirical/inappropriate antibiotics frequently, mandated by the clinical condition of the patients or emergencies. The protracted time required for Anti-microbial Susceptibility Testing (AST) adds to this ineptitude. The mortality due to AMR infections is estimated to reach 10 million by 2050 according to UN report (2016) on AMR. This mortality is compounded by a heavy morbidity caused due to such AMR infectious diseases [1]. Recent research has shown how *Acinetobacter baumannii* biofilm-forming capacity exerts its effect on resistance phenotypes, development of resistomes, and dissemination of resistance genes within biofilms by conjugation or transformation, thereby making biofilm a hotspot for genetic exchange. Various genes control the formation of *A. baumannii* biofilms and a beneficial relationship exists between biofilm formation and "antimicrobial resistance" (AMR) in the microorganism [2].

Pathogen identification has been made a lot easier and affordable now with the introduction of many rapid diagnostic techniques.Some of them also help to detect/identify drug resistance genes or proteins. Usually, isolated pure cultures are needed for AST. Though molecular techniques have made detection of drug resistance genes much easier, assaying the gene product/marker helps to confirm the exact mechanism and facilitates designing alternate therapeutic strategies. Emergence of opportunistic pathogens from the environment causing nosocomial infections in immunocompromised patients in the hospitals is posing new challenges to the clinicians because these pathogens have acquired newer mechanisms not just to inactivate the antibiotics and but also to evade the immune system of the host. Quorum sensing, one such mechanism usually seen in aquatic environment, has been identified and implicated as a cause for failure of antimicrobial therapies.

Quorum sensing leads to formation of bacterial communities and biofilms. These biofilms not only protect the members within them from antibiotics but also help in transfer of the resistance genes within the species and across other species in the community. While there are established laboratory methods to evaluate the biofilm potential of a pathogen, it takes several days to know the result. It will help the clinician if the AST profile of the pathogen is made available quickly to design an effective antimicrobial strategy.

Quorum sensing (QS) potential and the ability to form biofilms are interlinked. We are proposing that an assay which detects QS signal molecule would reveal the multi drug resistance potential of the pathogen and such an assay would give rapid and functionally reliable information to the clinician. *A. baumannii* has an active QS system and strong biofilm potential. It produces Acyl Homoserine lactone(s) (AHL) of different side chains as signal molecules for QS and biofilm synthesis. Dou et al, [3] found that N-3-hydroxy-dodecanoyl-homoserine lactone (N-3-OH-C12-HSL) produced by *A. baumannii* could induce the expression of drug-resistance genes such as *OXA-51, AmpC*, *AdeA* and *AdeB*. Our proposal is to evaluate the potential of clinical isolates of *A. baumannii* to produce AHLs as a functional marker for MDR potential/trait. Several methods have been reported for the detection and quantitation of AHLs. We have investigated the AHL production potential of clinical isolates directly on a commonly used agar media in presence of a biosensor bacterium.

2. MATERIAL AND METHODS

2.1 Clinical isolates source

Clinical isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex were kindly provided by the Department of Clinical Microbiology of Gleneagles Global hospital, Hyderabad (n=72). These isolates were initially isolated from different clinical specimens, which included respiratory secretions, blood, wound swabs, sputum, body fluids including urine and aspirated fluids. Each isolate was confirmed as *Acinetobacter baumannii* by PCR genotyping [4]. *Acinetobacter baumannii* ATCC 19606 was used as the reference strain.

2.2 Bacterial culture media

The isolates were grown at 37°C on Mueller Hinton Agar (MHA) plates (Himedia Laboratories Pvt Ltd, India, Cat. No. SM173 HM infusion B from Beef infusion 300 g/L, Acicase Casein acid hydrolysate 17.5 g/L, Starch 1.5 g/L, Agar 17.0 g/L, final pH 7.3±0.1 at 25°C). 38.0g of the dehydrated culture media was suspended in 100 mL distilled water in a flask. The medium was heated to boiling to dissolve completely. It was sterilized by autoclaving at 15lb(121°C) for 15 minutes. The medium was cooled to 40-45°C, mixed well and poured into sterile petri plates.

UNDER PEER REVIEW

Agrobacterium reporter strain NTL4pZLR4 was cultured in LB agar plates containing the antibiotic tetracycline 4.5 µgmL^{-1}. Luria Bertani culture media, 25g was suspended in 1000 mL distilled water. It was sterilized by autoclaving at 15lb (121°C) for 15 minutes. Agar-agar, 15g was added to prepare LB agar plates.

2.3 Bio-monitor strains used for screening of AHL production

Two reporter strains were used in the present study. *Chromobacterium violaceum* mutant strain CV026 (cvil::mini-Tn5-mutant of *Chromobacterium violaceum* ATCC 31532) was the monitor strain used to screen for short chain (C4 to C8) AHL-producing *Acinetobacter* species. *Chromobacterium violaceum*, ATCC 12472 is a wild type strain which produces a characteristic purple pigment called violacein and it serves as a positive control. These strains were procured from National Centre for Microbial Resource (NCMR, Pune). The bio-monitor strain CV026 detects AHL signals by the activation of a reporter gene such as lacZ or lux or by the production of a purple pigment. The CviR (*Chromobacterium violaceum* Repressor) [5] regulates the production of a purple pigment when induced by external AHLs.

Agrobacterium tumefaciens NTL4 (pZLR4) (Kindly given by Dr. Clay Fuqua, Indiana University, Indiana, USA) monitor strain [6] was utilized to detect AHLs with long acyl chains (>C6). This strain carries the plasmid pZLR4, which contains a traG::lacZ fusion and traR. In the presence of AHLs with long acyl chains the TraR protein is activated, transcription of the traG::lacZ fusion is turned on, and translating LacZ to produce β-galactosidase which can be used as a reporter of traG transcription. *A. tumefaciens* NTL4 (pTiC58ΔaccR), an AHL over producing strain [7] was used as positive control for the screening assays.

2.4 Antimicrobial drug sensitivity testing by disc diffusion method

Antimicrobial drug sensitivity testing (AST) was performed on all the clinical isolates against two antibiotics, Imipenem (10µg) and Meropenem (10µg) (Himedia, Mumbai, India) as per BSAC guidelines, 2015 [8] by Kirby-Bauer disk diffusion method. A single isolated colony of *A.baumannii* was inoculated into 3 mL of Mueller Hinton broth (MHB) and incubated at 37°C over-night with shaking at 150 rpm and the turbidity was adjusted to 1.0 McFarland. A sterile cotton swab dipped in the broth culture was used to prepare a uniform lawn of *A. baumannii* on Mueller Hinton agar (MHA) plate. Imipenem (10 µg) and Meropenem (10µg) discs were placed in the centre of the freshly prepared lawn of cells and incubated at 37°C for 24 hours. The diameter of the zone of inhibition of growth produced by the antibiotic discs was measured. The results were interpreted according to the BSAC guidelines (2015) and the *A. baumannii* isolates were classified as Carbapenem Resistant (CRAB) and Carbapenem Sensitive (CSAB) based on the growth inhibition zone diameters.

2.5 Screening for Quorum sensing signal molecules, the Acyl Homoserine Lactones (AHLs)

***2.5.1 Chromobacterium violaceum* CV026 induction assay to detect short chain AHL (C4-C8 molecules)**

A. baumannii clinical isolates were screened for short chain AHLs by cross streaking with *Chromobacterium violaceum*. Screening of the *A. baumannii* for production of AHLs was carried out by agar plate diffusion assay with minor modifications [9]. Briefly, QS reporter strain CV026 was cultured in LB agar plates containing the antibiotic kanamycin at 20 µgmL^{-1}. Single colonies of *A. baumannii* clinical isolates were grown on MHA plates. With the help of a sterile cotton swab, *A. baumannii* single colony was cross streaked perpendicularly to the CV026 reference culture in an LB agar plate such that they had a 0.5 cm gap between them. The *Chromobacterium violaceum* strain ATCC 12472 was used as positive control for AHL production. Streaked plates were incubated for 24 h at 28 °C. Development of purple pigmentation on the colonies of *C. violaceum* CV026 suggests that the tested isolate produces and secretes short chain AHLs.

2.5.2 Biosensor Assay to detect long chain AHL (>C6 molecules)

Common methods for identifying AHLs involve a combination of thin-layer chromatography and biosensors [10, 11]. Production of long chain Acyl Homoserine lactone (AHLs) in the clinical isolates of *A. baumannii* was detected by cross streaking with the biosensor *Agrobacterium tumefaciens* (NTL4pZLR4) by agar plate diffusion assay [12]. *Agrobacterium* reporter strain NTL4pZLR4 was cultured in LB agar plates containing the antibiotic tetracycline 4.5 µgmL^{-1}. As a visualizing agent, 40 µgmL^{-1} of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside, Thermo Scientific, Cat. No. R0404) was incorporated into the LB medium. Single colonies of *A. baumannii* clinical isolates were grown on MHA plates. With the help of a sterile cotton swab, a single colony was cross streaked perpendicular to NTL4pZLR4 streak in an LB agar plate such that they had a 0.5 cm gap between them. *Agrobacterium tumefaciens* pTiC58 strain, a hyper producer of AHL, was used as the positive control. Inoculated plates were incubated for 24 h at 28 °C. After 24 h of incubation, development of blue-green pigmentation on the colonies of NTL4pZLR4 suggests that the tested isolate produces long chain exogenous AHLs.

2.6 Genotyping by PCR for detection and confirmation of the presence of key regulatory QS gene – *abaI*

UNDER PEER REVIEW

The *A. baumannii* QS system consists of AbaI/AbaR, a two-component system. The *abaI* gene encodes the auto-inducer AHL synthase enzyme. Screening and identification of the quorum sensing mediator gene *abaI* were done by PCR to confirm its presence in all the 72 clinical isolates.
The primers and PCR conditions used for screening of the quorum sensing gene are described in Table1. PCR assay was performed with genomic DNA in 20µl total reaction volume. The PCR reaction mixture contained 20pmol of each primer, 200µmol of dNTPs, 1U of Taq DNA polymerase (KAPA Taq DNA Polymerase, KAPA Biosystems Inc), 2µl 10X buffer, 1.65 mM $MgCl_2$ and 100 ng of genomic DNA lysate.

Table1: List of primers and PCR conditions used in *abaI*screening.

Primer Sequence (5'-3')	Amplicon size	PCR conditions	Reference
abaI **F: AATGCCTATTCCCTGCTCAC** ***abaI*** **R: ATTGCTTCTTGCAGAATTGC**	132 bp	95°C - 5min 95°C - 30s ⎫ 55°C - 40s ⎬ 35 cycles 72°C – 60s ⎭ 72°C – 10min	[13]

3. RESULTS

3.1 Antimicrobial Sensitivity Testing (AST) profile

The 72 clinical isolates were characterized for carbapenem sensitivity and grouped as Carbapenem Resistant (CRAB) (n=58) and Carbapenem Sensitive (CSAB) (n=14). The isolates were considered CRAB if the inhibition zone diameter was ≤13mm (Imipenem) and ≤12mm (Meropenem). CSAB isolates showed an inhibition zone of ≥ 25mm (Imipenem) and ≥ 20mm (Meropenem) as depicted in Fig.1.

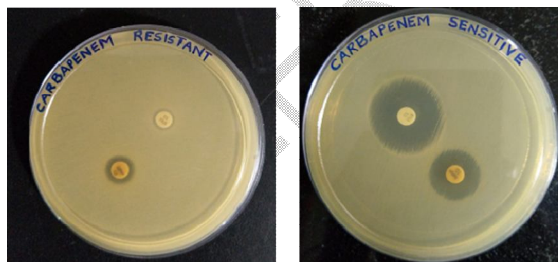

Fig.1. Kirby-Bauer disc diffusion test on *A. baumannii* isolate to determine the AST to Meropenem and Imipenem

3.2 Biosensor assay to screen for AHL productionby*A. baumannii* isolates.

<u>3.2.1Short Chain AHL screening</u>

The preliminary screening for the production of AHLs by all the 72 clinical isolates of *A. baumannii* was done using *Chromobacterium violaceum* CV026. In CV026, violacein is inducible by all the commercially available AHL compounds with N-acyl side chains from C_4 to C_8 in length, with varying degrees of sensitivity. None of our isolates couldinduce purple pigmentation in the biosensor strain which implies that our isolates did not produce short chain (C_4-C_8) AHLs (Fig. 2).

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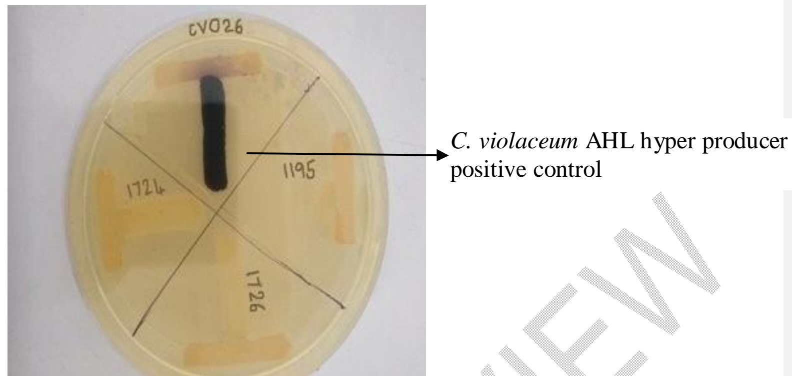

Fig. 2.*Chromobacterium violaceum* CV026 induction assay.

A. baumannii isolates (1724, 1726, 1195) were streaked perpendicular to the indicator strain CV026 to check for the ability to induce violacein pigment production by *A. baumannii* isolates.

3.2.2 Long Chain AHL screening

The *A. baumannii* clinical isolates were streaked perpendicular to the *A. tumefaciens* biosensor strain. Induction of blue-green pigmentation in the *A. tumefaciens* biosensor indicated production of the long chain AHL molecule by the test isolate (Fig. 3). Positive pigment production was observed for all 72 clinical isolates of *A. baumannii.* These strains were categorized as strong and weak AHL producers based on the visual colour intensity score. The isolates which scored between 2+ to 4+ were considered as strong AHL producers as they produced intense blue-green pigmentation in the monitor strain. The isolates which triggered less pigment production (scored ± and 1+) were considered as weak AHL producers. Analysis revealed that 80% of the isolates were strong (2+ to 4+) AHL producers and 20% of the isolates were weak (± and 1+) AHL producers (Table2).

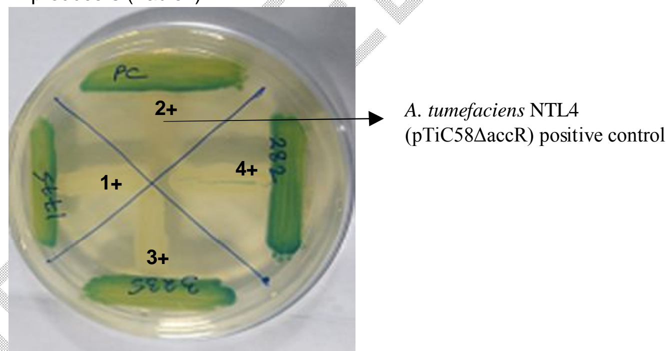

Fig. 3. Cross-streak of *Agrobacterium tumefaciens* indicator strain NTL4 (pZLR4) and *A. baumannii.*

A. baumannii (vertical line) was streaked perpendicular to the *A. tumefaciens* biosensor (horizontal line). Blue-green pigmentation of the biosensor strain indicates production, secretion, and diffusion of AHL through the medium. *A. tumefaciens* NTL4 (pTiC58ΔaccR), an AHL over producing strain was used as positive control in the screening assays.

***3.3 abaI* gene genotyping by PCR**

The *abaI* gene encodes the autoinducer synthase enzyme which synthesizes the AHL signal molecules. All the 72 isolates showed the presence of *abaI* gene. Amplicons of apparent size 132 bp (Fig. 4) were detected in agarose gel after electrophoresis as expected.

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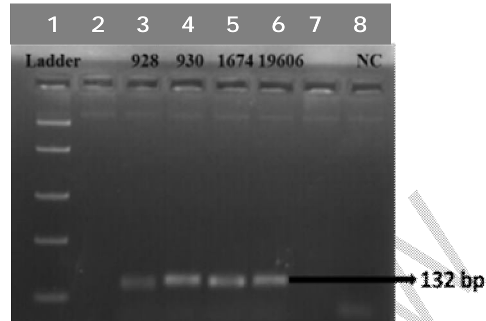

Fig. 4. Demonstration of the presence of autoinducer synthase gene *abaI* (132 bp) in clinical isolates of *Acinetobacter baumannii*.

Lane-1: Middle range Ladder (100-5000bp); Lane-3, 4 and 5: clinical isolates; Lane-6: Positive Control; Lane-8: Negative Control

Table2: Categorisation of clinical isolates of *A. baumannii*based on long chain AHL production.

S.No.	AHL Score	No. of isolates	AHL Category	AST profile		*abaI* PCR
				CRAB	CSAB	
1.	±	6	Weak	**5**	**1**	**Positive**
2.	1+	19	Weak	**14**	**5**	**Positive**
3.	2+	22	Strong	**19**	**3**	**Positive**
4.	3+	17	Strong	**13**	**4**	**Positive**
5.	4+	8	Strong	**7**	**1**	**Positive**
Total		**72**				

4. DISCUSSION

Acinetobacter baumannii has intrinsic resistance to several antibiotics and one of the important mechanisms contributing to this multi drug resistance is the innate ability to form biofilms. This may be because *A. baumannii* originated from the aquatic natural environment [14], only to emerge as one of the most dreaded opportunistic pathogens causing several nosocomial infections [15]. In the natural environment and in the hospitals *A. baumannii* must survive hostile factors, chemicals, and drugs and hence its potential to form biofilms. The biofilm formation rate in *A. baumannii* at the solid-liquid interface is 80–91%, which is 3 times higher than other *Acinetobacter* species (5–24%) [16,17]. Surface disinfection and empirical antibiotic treatment are very common in hospitals especially while treating emergency patients both in the ER and in the ICUs [18]. Clinicians are compelled to go for empirical antibiotic treatmentsdue to the protracted turn-around time to get pathogen identification and AST profiling from the clinical microbiology laboratories [19]. Quorum sensing facilitates biofilm formation through mediators called signal molecules and AHLs are the main mediators of QS in *A. baumannii*[20]. Therefore, detecting these signal molecules is perhaps a rational method to detect biofilm potential and hence MDR profile. Reports indicate that AHLs may strengthen drug resistance by moderating the expression of drug-resistance genes and result in pan-resistance [21].

UNDER PEER REVIEW

Currently there are no protocols used in clinical microbiology to screen for QS signal molecules for MDR potential/trait of a bacterial isolate. Screening for AHL by the cross-streak biosensor method on MHA plates (16-24h) and *A. baumannii* species confirmation by Ab-ITS PCR in 4-6h as published from our laboratory earlier[4] together would enable the clinician to quickly design an appropriate antibiotic strategy. We have shown in this study that AHL could be detected easily in an agar plate-based biosensor method, and it correlated well with the MDR profile of the clinical isolates. Since AHL is produced by several Gram-negative bacteria, this protocol could be used as a general screen for MDR profiling of GNB in the routine microbiology laboratory.

The absence of short chain AHLs in our isolates lead us to select another biosensor strain *Agrobacterium tumefaciens* NTL4 (pZLR4), which can detect long chain AHLs (C8-C16). Cross-streaking of *A. tumefaciens* biosensor against these isolates confirmed the production of long chain AHLs by *A. baumannii*. Positive pigment production was observed for all 72 clinical isolates of *A. baumannii*. *A. baumannii*has already been reported[22] to produce long chain AHL which is confirmed in our study also.

5. CONCLUSION

Clinical isolates of *A. baumannii* in our study did not produce any short-chain AHL molecules. This assay not only will help to screen for MDR potential of the clinical isolates of gram-negative bacteria,the identification of long chain AHLs in our *A. baumannii* clinical isolates will allow screening for biomimetic or chemical analogs that can block QS mechanisms. This will hopefully lead to a discovery of anti-QS drugs for the treatment of infections by multidrug resistant *A. baumannii*.Carbapenems are the front-line antibiotics used to treat gram negative bacterial infections in emergencies and in the critical care units of hospitals. Clinical isolates *A. baumannii* has innate resistance to several antibiotics due to various mechanisms, biofilms forming the first line of defense against antibiotics for the bacterium. Our study used AST to carbapenem as the leading marker for MDR, assuming the innate resistance of *A. baumannii* to other beta lactam antibiotics. Our study brought out certain important observations namely: a) All clinical isolates of *A. baumannii* produced Quorum Sensing signal molecules, the AHLs b) the clinical isolates of *A. baumannii* did not produce any short chain AHLs b) All the clinical isolates of *A. baumannii* produced long chain AHLs c) AHL production is not specific to carbapenem drug resistance because even
CSAB isolates produced AHL d) AHL production is inherent to all clinical isolates of *A. baumannii* and it apparently indicates an underlying biofilm potential and MDR trait in these *A. baumannii* isolates. e) AHLs could be a universal marker for revealing MDR trait and biofilm potential in clinical microbiology AST profiling protocols.

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UNDER PEER REVIEW

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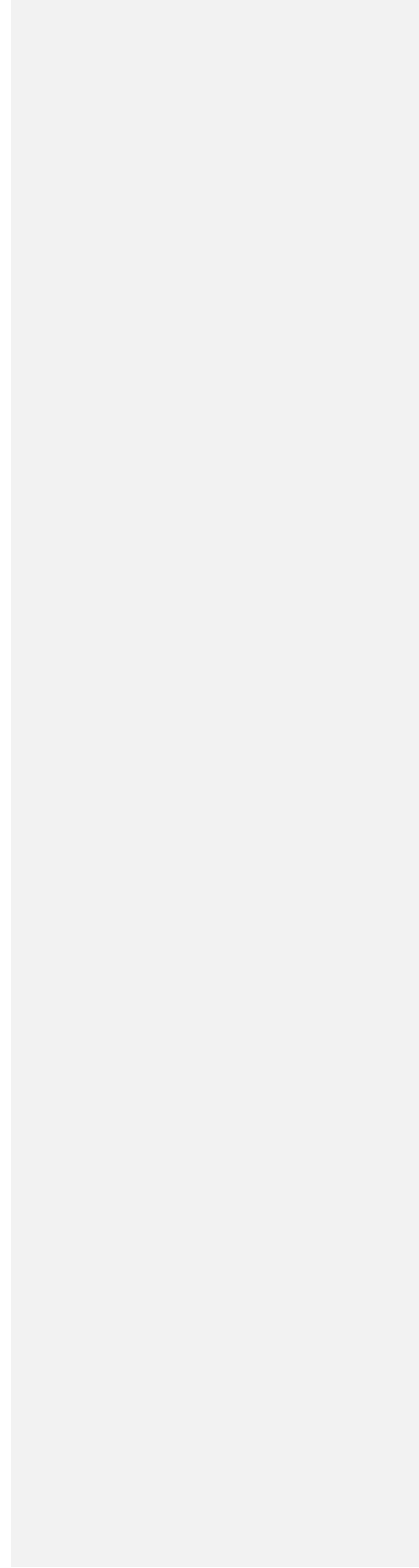

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