World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Available online at: http://www.wjpsonline.org/
Original Article

The effect of Ahl-1 recombinant lactonase on the resistance of *Pseudomonas aeruginosa* clinical isolates

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Received: 19-04-2018 / Revised Accepted: 17-05-2018 / Published: 01-06-2018

ABSTRACT

Quorum quenching has lately become a focal point in research as a method of controlling infection and reducing virulence in pathogenic bacteria like *P. aeruginosa.* To investigate whether the ability of quorum quenching lactonase to reduce virulence was accompanied by any change in the resistance profile of *P. aeruginosa* clinical isolates, lactonase gene from a *Bacillus* isolate cloned and expressed in *E.coli* Bl21 (DE3) was used in this study. The biological activity of the produced Ahl-1 lactonase was validated using a standard signal and *Chromobacterium violaceum* CV026 as a biosensor. Results showed that the resistance profile of the tested *P. aeruginosa* isolates was not changed by Ahl-1 lactonase. Antibiogram analysis revealed that Ahl-1 quorum quenching activity had no influence on antibiotics prone to resistance mechanisms involving the expression of the chromosomally encoded AmpC, cephalosporinases nor OprD. Determination of the MIC of azithromycin alone and in combination with Ahl-1 lactonase displayed no change in the susceptibility of the tested isolates to azithromycin when Ahl-1 lactonase was added.

Key words: Quorum quenching, lactonase, resistance, *Pseudomonas aeruginosa*, azithromycin

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How to Cite this Article: Masarra M. Sakr, Khaled M. Aboshanab, Walid F. Elkhatib, Mahmoud A. Yassien, Nadia A. Hassouna. The effect of Ahl-1 recombinant lactonase on the resistance of *Pseudomonas Aeruginosa* clinical isolates. World J Pharm Sci 2018; 6(6): 101-106.

INTRODUCTION

One of the recent approaches in infection control is quorum quenching, a term which describes the interference with the quorum sensing and consequently, interrupting the expression of different bacterial functions including numerous virulence traits [1]. Such approach is of great benefit in highly resistant pathogenic bacteria like *P. aeruginosa*. Lactonase enzyme represents one of the tools of quorum quenching of this harmful bacteria. It degrades acyl homoserine lactone (AHL) signal molecules which normally bind to the LasR and RhlR transcriptional regulators and direct the expression of genes in the bacterial population [2]. In addition to playing role in the virulence of bacteria, the effect of quorum sensing in controlling the expression of resistance genes in bacterial cells have been a concern. Some previous studies have focused on the effect of quorum quenching on *P. aeruginosa* resistance [3, 4, 5].

The importance of studying the effect of quorum quenching on resistance in *P. aeruginosa* lies in the characteristic intrinsic resistance of this pathogen. *P. aeruginosa* has an outer-membrane with very little permeability, constitutively expresses various efflux pumps and produces antibiotic-inactivating enzymes (e.g., cephalosporinases) [6]. Another important trait of *P. aeruginosa* is that it can develop or acquire new mechanisms of resistance to antibiotics. This may be related to the large size and the versatility of its genome [7]. So far, the correlation between quorum quenching and resistance in *P. aeruginosa* remains unclear.

Although, in other bacteria like *Acinetobacter baumannii* and *Escherichia coli* it was reported that quorum-sensing signalling molecule induces the expression of drug-resistance genes [8] and [9], until today no study has proved that resistance in *P. aeruginosa* is among the functions controlled by QS. A previous study reported the increased formation of tolerant persister *P. aeruginosa* cells in response to quorum sensing signaling molecule [10].

Accordingly, the aim of the present study was to investigate whether quorum quenching lactonase had an effect on the resistance of *P. aeruginosa* clinical isolates. To achieve this, an Ahl-1 recombinant lactonase expressed in our lab was first tested for its acyl homoserine lactone (AHL) degrading activity. Then, the effect of the Ahl-1 lactonase on the Antibiogram of the tested isolates was studied to detect any change in the resistance profile and the expression of genes responsible for resistance. MIC determination in the presence and absence of Ahl-1 lactonase was also done to detect any variation attributed to quorum quenching.

MATERIALS AND METHODS

Chemicals and media: Lauria Bertani agar and broth, Mueller Hinton agar and broth were obtained from LabM, England. IPTG and Hexanoyl homoserine lactone (HHL) were purchased from Sigma-Aldrich Co., USA.

Ahl-1 lactonase: The recombinant enzyme Ahl-1 lactonase was expressed from *E.coli* BL21 (DE3) harbouring the recombinant plasmid pET22b/*ahl*-1 produced in our lab. Induction of expression was achieved by IPTG and Ahl-1 was purified using Ni-NTA spin column (Qiagen, Germany). Quantification of the produced enzyme was then done by Pierce ^{TM} protein assay kit (ThermoFisher, Massachusetts, USA).

Bacterial strains

Clinical isolates: Two *P. aeruginosa* clinical isolates were obtained from Microbiology lab at Ain Shams hospital. They were routinely grown in LB broth. The effect of Ahl-1 lactonase on the virulence of the two isolates was studied and Ahl-1 was found to reduce protease, rhamnolipids and pyocyanin production in the two isolates in addition to reducing swarming and biofilm formation (data not shown).

Chromobacterium violaceum CV026: The strain was purchased from NCTC (NC 13278.T). *Chromobacterium violaceum CV026* was used as a biosensor to verify the biological activity of the recombinant produced lactonase, Ahl-1. It produces the purple pigment violacein in response to the presence of AHL in the growth medium.

Testing the biological activity of the recombinant Ahl-1 using CV026 as a biosensor: Checking the AHL degrading activity of Ahl-1 protein was done according to Ravn and his coworkers [11] with some modifications. A 10 ml volume of LB agar contained in a petri dish was overlaid by 10 ml semisolid LB agar containing synthetic hexanoyl homoserine lactone (HHL) at concentration 100 nM seeded with the biosensor strain (CV026). Wells were punched in the agar after its solidification. Aliquots (150 µl) of the prepared enzyme (3 mg/ml) were pipetted into the wells. Control was done using 150 µl NPI-500 buffer. After incubation at 28°C for 24 h, the growth of CV026 showed purple color in the whole plate except the zones around the wells where degradative enzymatic activity against HHL caused no purple color development.

Testing the effect of Ahl-1 on the resistance profile of *P. aeruginosa* clinical isolates: To determine the susceptibility of the *P. aeruginosa* clinical isolates, the Kirby-Bauer disk diffusion

method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI M100-S27, 2017). In brief, the inoculum preparation was done by suspending freshly isolated colonies grown on Mueller Hinton agar (18 to 24 h incubation period) in isotonic saline (0.9% NaCl). Turbidity was then adjusted to match 0.5 McFarland standard suspension. Surface inoculation of Mueller Hinton agar plates was then done using a sterile swab, then the antimicrobial discs (Table 1) were applied on the inoculated surface. After incubation at 37°C for 18 h, the formed inhibition zones were measured and interpretation of the results was done according to the CLSI standard table.

Determination of MIC in the presence and absence of Ahl-1: This was done by agar dilution and broth dilution methods according to the CLSI guidelines (CLSI M100-S27, 2017) as follows;

Determination of MIC by agar dilution method

To prepare the dilutions of the antimicrobial agent to be tested in the presence and absence of Ahl-1 lactonase, double strength Muller Hinton broth was used. Then, Ahl-1 purified lactonase in NPI-500 buffer was added to a final concentration of 1.5 mg/ml in the antimicrobial dilutions in the test group and plain NPI-500 buffer was added to the antimicrobial dilutions in the control group.

For determination of MIC, inoculum preparation was done as previously described by direct colony suspension equivalent to 0.5 McFarland standard followed by surface inoculation on Mueller Hinton agar plates. Wells were then punched into the agar and filled with the prepared dilutions of the antimicrobial agent. Then, plates were incubated for 18 h at 37°C. After incubation, the diameters of the inhibition zones formed were measured and MIC was determined.

Determination of MIC by broth dilution method

A stock solution of 2048 µg/ml of azithromycin was prepared. In the wells of a microtitre plate, sterile 100 µl of double strength Mueller Hinton, were dispensed in the first well of each micro dilution series. In the remaining wells, 50 µl aliquots of double strength Mueller Hinton plus the same volume of either the prepared enzyme (final concentration 1.5 mg/ml) or of NPI-500 buffer (as a control) were dispensed in the remaining wells. Then, 100 µl of the tested antimicrobial stock was added in the first well, then two fold serial dilution was carried out till reaching the tenth well where 100 was discarded after mixing. Well number 11 contained culture media with no antimicrobial agent and was used as control for organism viability (positive control) and number 12 was used for medium sterility (negative control). For inoculation of the wells, direct colony suspension equivalent to 0.5 McFarland standard was prepared and further diluted in saline twenty folds (final inoculum count was 5×10^5 cfu/ml). Then 100 µl of the prepared inoculum was added to each well (except the negative control well). The plate was then incubated at 37ºC for 18 h. The MIC was considered as the lowest antimicrobial agent concentration showing no visible growth.

Statistical analysis: Experiments were carried out in triplicates. Data were analysed using Graph pad Instat-3 software (Graph Pad Software Inc., USA) and the results were represented as respective average values ± Standard deviation.

RESULTS AND DISCUSSION

Ahl-1 lactonase produced in this study proved to have quorum quenching activity as it successfully prevented the accumulation of HHL signals making CV026 fail to produce the purple pigment violacein as shown in figure 1. Although Ahl-1 showed ability to suppress virulence of the tested *P. aeruginosa* isolates, Antibiogram analysis proved that resistance profile was not changed secondary to quorum quenching. Results displayed in Table 2 showed that both tested *P. aeruginosa* isolates were resistant to penicillin/clavulanate combinations, cephalosporins, Co-triamoxazole and lincomycins. Isolate P2 was also resistant to flouroquinolones, displayed intermediate sensitivity to erythromycin and doxycycline and was sensitive only to azithromycin. *P. aeruginosa* P1 displayed sensitivity to ciprofloxacin and azithromycin, intermediate sensitivity to erythromycin, doxycycline and levofloxacin. Displaying resistance to different classes of antibiotics is common among *P. aeruginosa*. Even during the course of treating an infection, *P. aeruginosa* can develop resistance to antimicrobial agents [12]. The three most commonly studied chromosomally encoded resistance mechanisms in *P. aeruginosa* are the AmpC, the cephalosporinase, the OprD outer membrane protein, and the multidrug efflux pumps [12]. Until today, no study has proved any of these mechanisms to be controlled by AHL quorum sensing. Accordingly, it is no weird that results in the present study showed that Ahl-1 lactonase, despite proving to be capable of degrading AHL signals, did not affect expression of any of these resistance mechanisms. Results in this study come in accordance with a previous study which reported that Expression of the *las* and *rhl* quorum-sensing systems in clinical isolates of *P. aeruginosa* doesn't correlate with efflux pump expression or antimicrobial resistance [13].

However, another study on resistance of *P. aeruginosa* suggested that enzymatic quorum quenching increased antibiotic susceptibility [4].

Findings of that study were based on that minimum Bactericidal Eradication Concentration (MBEC) of some antimicrobial agents against *P. aeruginosa* biofilm decreased when lactonase was added. A third study that tested susceptibility of bacterial biofilms to antibiotics reported an increase in the susceptibility of the biofilm secondary to quorum quenching inhibition. Results of that study were based on using already sensitive isolates of *P. aeruginosa* and *Burkholderia cenocepacia* [14]. Results of these two studies however do not confirm any change in the gene expression of resistance genes and findings can be more attributed to our previous finding of the role of Ahl-1 lactonase as an anti-biofilm agent.

Displaying susceptibility to azithromycin by both isolates, determination of MIC of azithromycin alone and in combination with Ahl-1 lactonase was done to investigate the effect of lactonase on the susceptibility of the isolates when used in combination with the antibiotic. For more relevant data, determination of MIC was done by two methods. Results displayed in figure 2 showed that using agar dilution method, azithromycin alone displayed an MIC of 19.8 and 15.5 µg/ml against P1 and P2, respectively. When used in combination with lactonase, recorded MIC against P1 and P2 was 17.9 and 13.4 µg/ml, respectively. The calculated fractional inhibitory concentration (FIC), shown in Table 3, when lactonase was added was 0.9 and 0.86 indicating that addition of lactonase did not have an effect on susceptibility of the tested *P. aeruginosa* isolates to azithromycin. The same result was observed when MIC was determined using broth dilution method. Azithromycin displayed an unchanged MIC of 32 µg/ml against the two isolates when used alone and in combination with lactonase. Macrolides such as azithromycin work by binding to 50S ribosomal subunit thus blocking the protein translation. It is clear therefore from findings of this study that quorum quenching does not have an influence on protein translation machinery and verifies that Ahl-1 lactonase exerts its effect away from protein translation pathway. In the same manner, resistance to azithromycin was reported to be primarily mediated by mexCD-oprJ efflux pump [15]. Since results here showed that MIC of azithromycin remained unchanged when azithromycin was used in combination with lactonase, this implies that expression of mexCD-oprJ efflux pump was not affected by quorum quenching.

In the two previous related studies which reported quorum quenching to increase the susceptibility of *P. aeruginosa* biofilms [4] and [14], azithromycin was not among the tested antimicrobial agents. To the best of our knowledge, this is the first study to investigate the effect of using lactonase in combination with azithromycin on the susceptibility of *P. aeruginosa* clinical isolates.

Having no effect on resistance of *P. aeruginosa*, Ahl-1 lactonase therefore has a better opportunity as an antipathogenic drug with no fear of emergence of resistance. By avoiding provoking resistance mechanisms in *P. aeruginosa*, we believe that lactonase can successfully combat *P. aeruginosa* infections by exerting its anti-virulence effect leaving the pathogen to be easily eliminated by the host immune system. Results in this study still cannot confirm that there is no correlation between quorum sensing as a whole and resistance in *P. aeruginosa.* As mentioned before, quorum sensing in *P. aeruginosa* is a very complex network where some functions are regulated by multiple factors so further studies are still required to determine if by any means quorum sensing can affect resistance of such opportunistic pathogen.

CONCLUSION

Quorum quenching lactonase supresses the virulence of *P. aeruginosa* apart from its antibiotic resistance pattern. Ahl-1 lactonase neither upregulated nor downregulated the chromosomally encoded AmpC, cephalosporinases nor OprD. It also had no affect the expression of mexCD-oprJ efflux pump. Thus, Ahl-1 lactonase quorum quenching can be used for infection control with little or no fear of emergence of resistance.

Table 1. Antimicrobial sensitivity discs used in the study, their concentrations and sources

Antimicrobial agent	Amount per disc (µg)	Source
Co-amoxiclav (AMC)	30	Bioanalyse ®, Turkey
Cefotaxime (CTX)	30	
Ciprofloxacin (CIP)	5	
Doxycycline (DO)	30	
Levofloxacin (LEV)	5	
Co-triamoxazole (SXT)	25	
Azithromycin (AZM)	15	Oxoid®, UK
Erythromycin (E)	15	
Clindamycin (DA)	2	

Table 2. Results of Antibiogram analysis for the *P. aeruginosa* clinical isolates against tested antimicrobial agents.

Antimicrobial agent	Susceptibility to antimicrobial agent	
	P1	P2
Co-amoxiclav (AMC)	R	R
Cefotaxime (CTX)	R	R
Ciprofloxacin (CIP)	S	R
Doxycycline (DO)	I	I
Levofloxacin (LEV)	I	R
Co-triamoxazole (SXT)	R	R
Azithromycin (AZM)	S	S
Erythromycin (E)	I	I
Clindamycin (DA)	R	R

Table 3. Fractional inhibitory concentration (FIC) when Ahl-1 lactonase was used with azithromycin

Isolate code	FIC determined by agar dilution method	FIC determined by broth dilution method
P. aeruginosa P1	0.9 ±0.14	1±0.0
P. aeruginosa P2	0.86 ±0.2	1±0.0

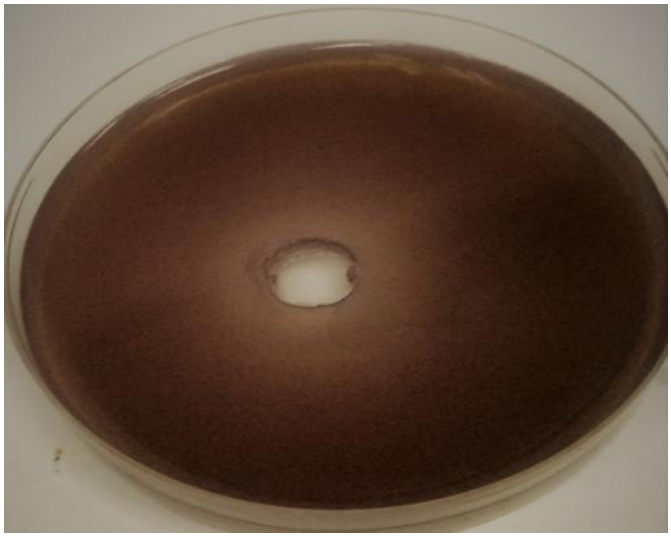

Figure 1. Testing the AHL degrading activity of the recombinant overexpressed lactonase using CV026 as a biosensor, the zone with no purple colour indicates degradation of AHL.

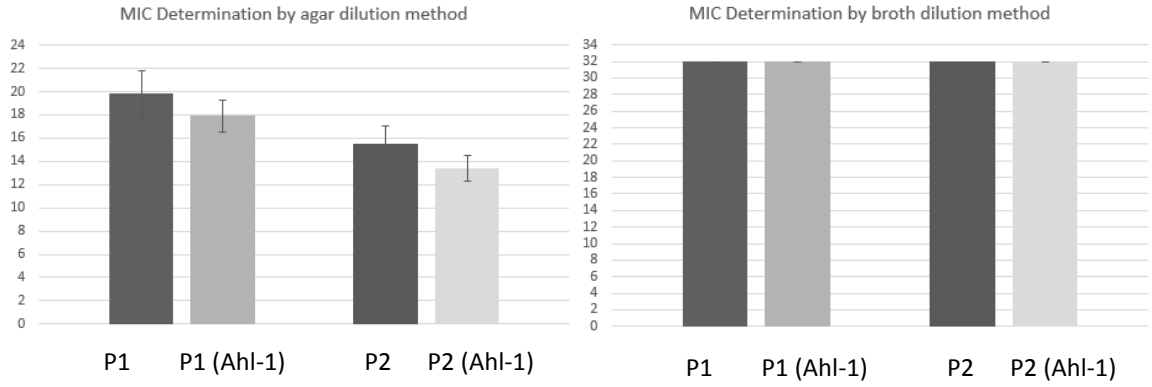

Figure 2. Determination of azithromycin MIC alone and combination with quorum quenching Ahl-1 lactonase.

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