Membrane Technology

ISSN (online): 1873-4049

Effect of Bovine Serum on Quorum Sensing Genes Expression in MDR Resistant P. Aeruginosa

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Abstract

Background: *Pseudomonas aeruginosa, similar to several bacteria, use chemical signals for intercellular communication via use of quorum sensing (QS) mechanisms. QS enable the bacteria groups to detect population density and, in reaction to variations in cell density, to synchronize their behaviors. The current study intended to investigatethe effectof bovine serum on quorum sensing genes expression in Pseudomonas aeruginosaduring early stages of growth.*

Methodology: *Pseudomonas aeruginosa isolates were obtained from burns, wounds, urine, and ears. Bacteria isolates were identified in accordance to their biochemical reactions, then antibiotic susceptibility and The isolates' ability to build biofilms was examined, and each bacterial isolate's genomic DNA was extracted in order to find quorum sensing genes (rhlR, rhlI, lasR, and lasI).*

Result: *From different clinicalspecimens, twenty-five Pseudomonas aeruginosa isolates were identified,antibiotic resistance was present in these isolates., and it was found that only 10(40%) of these isolates were biofilm producers. Biofilm virulence genes lasR, lasI, rhlR, and rhlI were detected in all Pseudomonas aeruginosa isolates examined.*

Conclusion: *lasR, lasI, rhlR, and rhlI genes were common inPseudomonas aeruginosa isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.*

Keywords: *Quorum sensing, Biofilm virulence genes, Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a prevalent infection that increases morbidity and death in patients with cystic fibrosis (CF), Its extraordinary potential for adaptation makes it more easier for it to spread chronic infections(1). Through the release of several virulence factors, and may adjust to the unfavourable conditions in its hosts, making diseases and infections easier (2).Another, even more complex system of signal molecules and their receptors has been identified in *P. aeruginosa* ; two fully, but semi-independently functioning Quorum-Sensing (QS) circuits, namely *las* and *rhl*, are also found in this bacterium. Whereas, *LasI* controls the synthesis of Autoinducer PAI-1, N-(3-oxododecanoyl)-l-homoserine lactone, *RhlI* controls the synthesis of Autoinducer PAI-2, N-butyryl-l-homoserine lactone (3). Other factors such as activator proteins in the protein secretory genes, *rhlR*, alkaline proteases, P and A exotoxin, and *xcpP* and *xcpR*, have been established to be controlled by the las system (4). In this regard, it has been established that *rhl* QS controls the identified genes for genes for pyocyanin, rhamnolipid, alkaline protease, *LasB* protease, and other vital genes such as *xcp*R, *xcpP* and *rpoS*genes(5).Cell density controls the expression of virulence factors through the QS autoinducers of essential *Pqs, Iqs, Las,* and *Rhl* mass communication system (6). Surprisingly, As was noted, the expression of the secretion system varies greatly depending on the QS system. Similarly, QS is found to be involved in the regulation of many virulence factors in P. aeruginosa such as expression of efflux pumps (7), extracellular proteases(8), biofilm formation(9), iron sequestering agents(10), swarming(11)and response to host immune system (12). Therefore, the aim of the current study was to evaluate how serum albumin, which is essential for *P. aeruginosa* virulence in the early stages of infection, affects the expression of genes regulated by iron via a Fur-independent mechanism unrelated to albumin-associated iron.

Materials and Methods

Clinical Specimens

Between September and November of 2023, this research was done at Al-Nahrain University's College of Biotechnology.

At Imamein Kadmia Medical City, Baghdad Teaching Hospital, Al-Karkh General Hospital, and Al-Yarmouk Teaching Hospital (hospitalised at the general surgery department and burn unit), 155 clinical isolates of P. aeruginosa were obtained from various patients with burn, UTI, otitis, and surgically infected wounds.Using sterile swabs, samples were taken, and they were then examined bacteriologically. Following the cultivation of each sample on Cetrimide agar medium, the isolated organisms were identified using:

1- Common microbiological methods(13) include Gramme staining (for Gram-negative bacilli), biochemical responses, and colony morphology (pale yellow colonies on MacConkey and green exopigment on Nutrient agar and Cetrimide agar).

2- VITEK 2 identification system: ID-GNB cards were used to identify on the VITEK 2 system in compliance with the manufacturer's instructions. 18 tests for sugar fermentation, 18 tests for sugar assimilation, two decarboxylase tests, and three miscellaneous tests (for tryptophane deaminase, urease, and malonate utilisation) are among the 41 tests on the 64-well plastic ID-GNB cards. Fluorescence is monitored every 15 minutes, and the identification results are determined three hours later.

Antimicrobial susceptibility testing by disc diffusion method

Using commercially available discs and the disc diffusion method, the antibiotic susceptibility of each isolate was assessed. Three to five well-isolated colonies from the pure culture of bacterial isolates were suspended in three millilitres of sterile saline (0.9%) using a sterile loop. Clinical and laboratory standard institute standards were followed to select antibiotics and the assessment of antimicrobial susceptibility data(14).

Biofilm quantification

To investigate the in vitro formation of biofilms in 96-well microtiter plates (MTP) with nutritional broth, the following methods(15) were employed: The isolated bacterial strains (initial turbidity of 0.05 at 600 nm) were diluted 1:100 in nutritional broth and then their overnight broth cultures were injected onto a microtiter plate (200 µ per well). Three separate tests were conducted to evaluate each isolate, and the average was calculated. A blank of eight wells was utilised. Sterile LB broth was found in the blank wells.After that, For twenty-four hours, the plates were incubated at 37 °C in an aerobic incubator. The contents of the wells were decanted after incubation. Two hundred microlitres of sterile saline (0.9%) were used to wash each well three times. Each MTP well's adhering biofilm layer was dyed at room temperature, for fifteen minutes using 150 µ of 0.1% crystal violets. Following staining, excess dye was washed off by submerging the MTP in a large container of water and aspirating the stain with a pipette. In order to facilitate the interpretation of the findings, the strains were categorised using the criteria of Stepanović et al. (15)separated into four categories: biofilm producers that are mild, moderate, strong, and none.

Growth curve and conditions

Under the previously mentioned conditions, aliquots of bacterial cultures were cultivated in LB or LB supplemented with 10% (v/v) adult bovine serum (LB/S), and samples were obtained at the designated intervals. We determined that the early exponential phase of growth (early phase) was represented by a growth index of OD600 1.0–1.2 at about 6 hours after inoculation, and the late stationary phase of growth (late phase) by a growth index of OD600 3.0–4.0 at around 16 hours after inoculation.

Gene expression

Using the oligonucleotide primers shown in table (1), isolates of *P. aeruginosa* were examined for *lasI*, *rhlI*, *lasR*, and *rhlR* gene expression using real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Table (1): Oligonucleotide primers used in this study

Gene	Primer Sequence (5'→ 3')	T_m (℃)	Amplicon size (bp)	Reference
lasI	F:GTGTTCAAGGAGCGCAAAG R:GAAACGGCTGAGTTCCCAGA	62	240	Lima *et al*. (16)
lasR	F:AGATCCTGTTCGGCCTGTTG R:CTGCTTTCGCGTCTGGTAGA	62.5	194	

rhlI	F:GCTACCGGCATCAGGTCTTC R:GTTTGCGGATGGTCGAACTG	63.5	100	
rhlR	F:ACCAGCAGAACATCTCCAGC R:CATTGCAGGATCTCGCGTTC	64.5	157	

Detection of quorum genes expression using RT-PCR

RT-PCR reaction was achieved to determine quorum genes expression based on the subsequent procedures

RNA extraction

In short, *P. aeruginosa*isolates were cultured for 24 hours in LB wells. To attain a 0.05 turbidity level at 600 nm, in fresh medium, the cells were diluted and suspended. before being centrifuged at 8000 rpm. After removing the supernatant, RNA was extracted from the pellet. Following the GoScript Reverse Transcription System kit's manufacturer's instructions, total RNA was extracted from the deposit. RNAs extracted from a minimum of two separate cell cultures were subjected to triple qPCR testing. RNA's purity and concentration were evaluated using a Nano-drop spectrophotometer (Fisher Scientific, USA, Hampton).

One-step Sybr green kits were used for RT-PCR in an ABI 7500 device (Applied Biosystems, USA, Massachusetts). A real-time RT-PCR reaction was produced with a final volume of 20 µl in accordance with the manufacturer's instructions: Two microlitres Template: 0.4 µl 0.4 µl of forward primer Ten microlitres of PerfectStart Green qPCR SuperMix (2X); seven microlitres of nuclease-free water; 0.2 microlitres of reverse transcriptase; and reverse primer. As a reference gene, we used the Acyl-homo serine lactone acylase-pvdQ housekeeping gene to relative quantify the PCR data. Table 2 summarised the circumstances of the reaction.

Table (2): PCR cycles conditions for quorum genes expression

Step	Temperature (°C)	Time (minute)	Cycle
Initial denaturation	94	2	1
Denaturation	94	0.5	40
Annealing	58	0.5	
Extension	72	0.5	
Melting curve	65 – 95	0.5	1

Each gene's calibrators and treatment groups Signals reached a threshold cycle within the logarithmic phase, and the cycle number (Ct) at that point was calculated. The variations in fold changes and cycle threshold (ΔCt) between these values were normalized to House Keeping gene (Acyl-homo serine lactone acylase-pvdQ) expression as shown below(17):

Relative quantification:

- Folding = $2^{-\Delta\Delta CT}$
- ΔΔCT = ΔCT Treated − ΔCT Control
- ΔCT = CT gene − CT House Keeping gene

Statistical analysis

The SPSS software for Windows (version 20 statistical software; Texas Instruments, IL, USA) was used for all statistical analyses.

Results and discussion

ISSN (online): 1873-4049

Bacterial isolates

One hundred and fifty five clinical samples were collected from Burns, Wound, Urine, and Ears.Identification of *P. aeruginosa*was initially performed according to their morphological and cultural characteristics, and biochemical reactions. Then,identification of isolates was confirmed by using automated VITEK2 system (BioMérieux, France).Out of 102 bacterial isolates, only 25 isolates (25.5%) of *P.aeruginosa* were identified.In addition to that *P. aeruginosa* shown to be prevalent in clinical samples as 13(52%) fromBurns, 6(24%) fromWounds, 4(16%) fromUrine, and 2(8%) fromotitis infections. The other bacterial agents shown to be etiological were mainly, *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli*, and *Proteus mirabilis*.

Antibiotic Susceptibility

The VITEK2 compact system was used for testing for antimicrobial susceptibility. The isolates' resistance to various antibiotics varied, according to the results. Results showed that although all (100%) of *P. aeruginosa*isolate from burn, wound, UTI, and otitis infections were multiple drug resistance, and they were also categorized as resistant, moderate, and sensitive (figure 1).

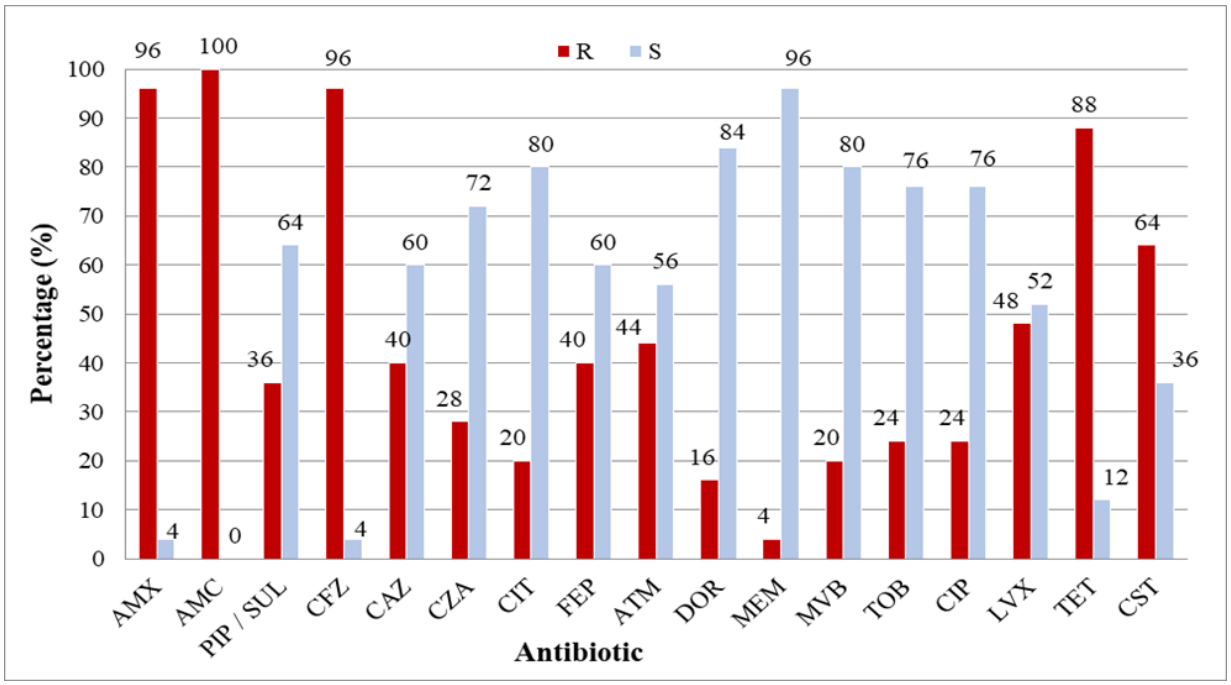

Figure (1): Antibiotic susceptibility of *P.aeruginosa* isolates against different antibiotics. Amx: Amoxcillin; Amc: Amoxcillin-clavululanate; Pip/sulb:Piperacillin sulbactum; CFZ: Cefazolin; CAZ:Ceftazidim; CZA:Ceftazidime-avibactam; C/T:Ceftolozane-tazobactam; FEP:Cefepime; ATM: Aztreonam; DOR: Doripenem; MEM: Meropenem; MVB:Meropenem-vaborbactam; TOB: Tobramycin; CIP: Ciprofloxacin; LVX: Levofloxacin; TET: Tetracycline; CST: Colistin. R: resistant; S: sensitive.

Results also showed that all *P. aeruginosa*100% wereresistant to Amoxcillin, 96% were resistant toAmoxcillin and Cefazolin, 88% to Tetracycline, 64% to Colistin, 48% to Levofloxacin ,44% to Aztreonam , 40% to Ceftazidim andCefepime, 36 % to Piperacillin-sulbactum,28% to Ceftazidime-avibactam,, 24% toTobramycin and Ciprofloxacin, 20 % to Ceftolozane-tazobactam and Meropenem-vaborbactam 16% to was resistance Doripenem.

Biofilm formation

The capacity of isolates of *P. aeruginosa* to produce biofilm was assessed using the microtiter plate technique. Results illustrated in figure (2) showed that all bacterial isolates (100%) were biofilm producers with variable degrees, as there are

21(84%) weak biofilm producers, 3(12%) were moderate biofilm producers, and only 1 isolate (4%) was strong biofilm producer.

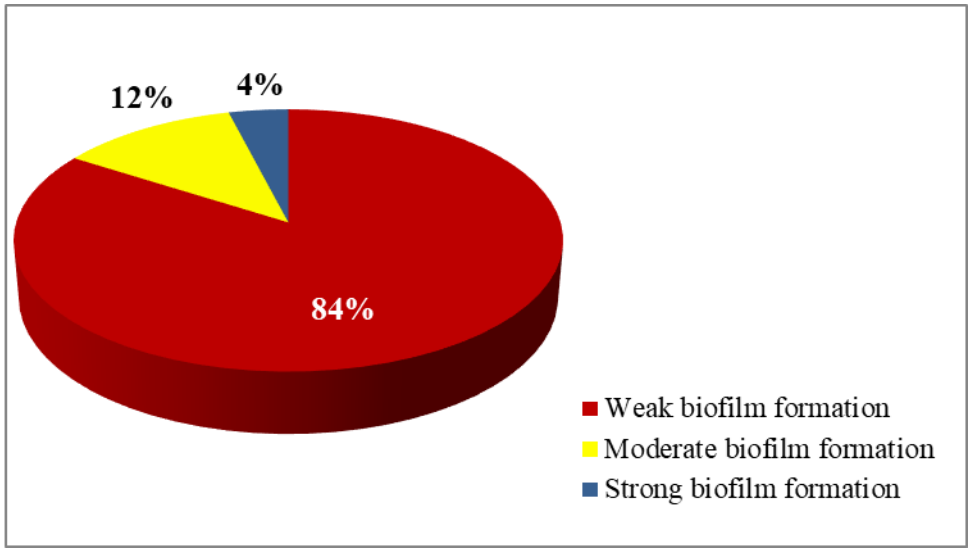

Figure (2): Ability of *P. aeruginosa* isolates in biofilm formation

Effect of bovine serum on expression of quorum sensing genes

Relative gene expression of *lasI, rhlI, lasR, and rhlR* in different exponential growth phaseswas investigated. For this purpose, the MDR and the strong biofilm producer isolate PA-10 was selected and grow in Luria Bertani medium at 37°C under ambient aeration using shaker incubator. The growth was tracked by measuring OD at 600 nm for 19 hours of incubation for 2 hours intervals.Results illustrate in figure (3) showed the bacterium was stay one hour at lag phase,then enterearly, mid, and late log phasesafter two, four, and eight hours respectively.The exponential phase seems to have ended by the end of ten hours, with strong growth occurring between two and six hours, followed by a slower growth rate until, around twelve hours later, maximum density was attained.

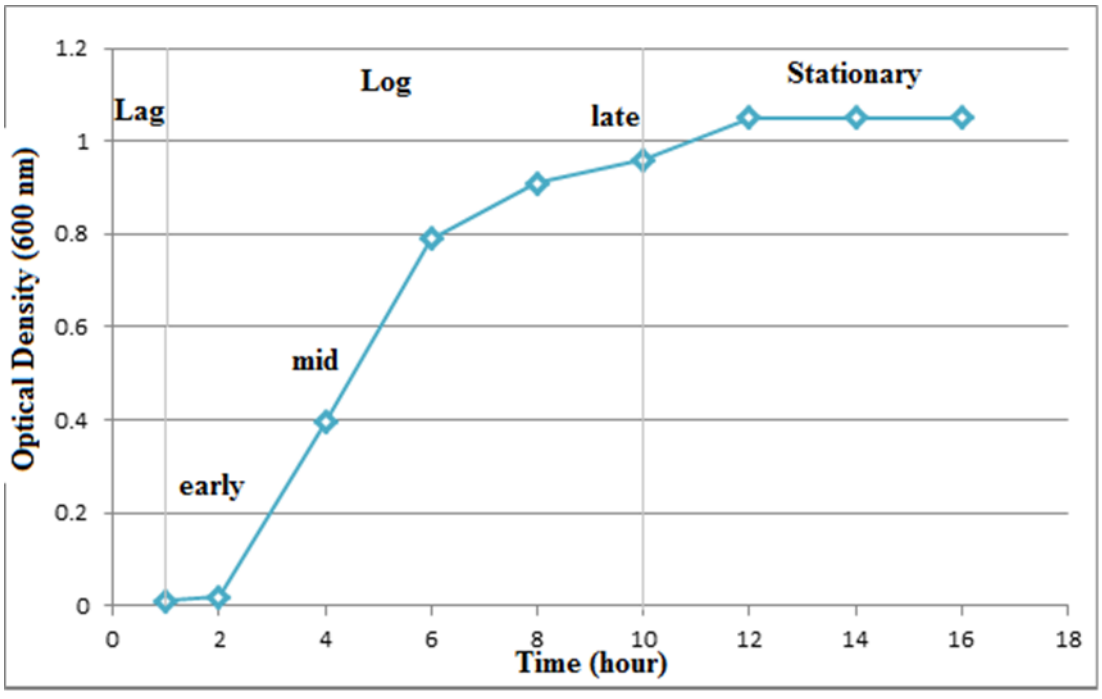

Figure (3): Growth curve of *P.aeruginosa* PA-10 after 18 hours incubation at 37°C in shaker incubator at 100 rpm

To investigatethe effect of bovine serum on the expression of QS genes (*lasI*, *rhlI*, *lasR*, and *rhlR*),*P. aeruginosa* PA-10 was incubated at 37 °C with bovine serum (10% v/v) in a shaker incubator at 100 rpm along the exponential phase time periods.Fold change of expression was determined at each time interval.Results illustrated in figure (4) and table (3)

ISSN (online): 1873-4049

showed that *lasI* gene expression was significantly increase (P<0.01) after two and four hours of incubation with bovine serum at each stages of exponential phase as the fold change expression was increased to 3.07 at early log phase, then to 3.86 at mid log phase, and then slightly decreased to 3.41 and 3.14 at late phase of exponential growth but still highly increased. On the other hand, results also showed that*lasR* gene expression was also significantly increased (P<0.01) at early and mid-log phases after two and four hours of incubation with bovine serum, then the level of *lasR* gene expression was slightly decreased during mid and late log phase of exponential growth but also still highly increased.

Effect of bovine serum on *rhlI* gene expression during different stages of the exponential growth of *P.aeruginosa* PA-10 was also studied. Results illustrated in figure (4) and table (3) showed that *rhlI* gene expression was significantly increased (P<0.01) two fold at mid log phase of exponential growth, while it was not changed in both the early and late stages of growth..

Finally, results showed that *rhlR* expression was unchanged during early and mid-log phases of exponential growth (non-significant different with control treatment), then, expression was significant increased (P<0.01) at late log phase.

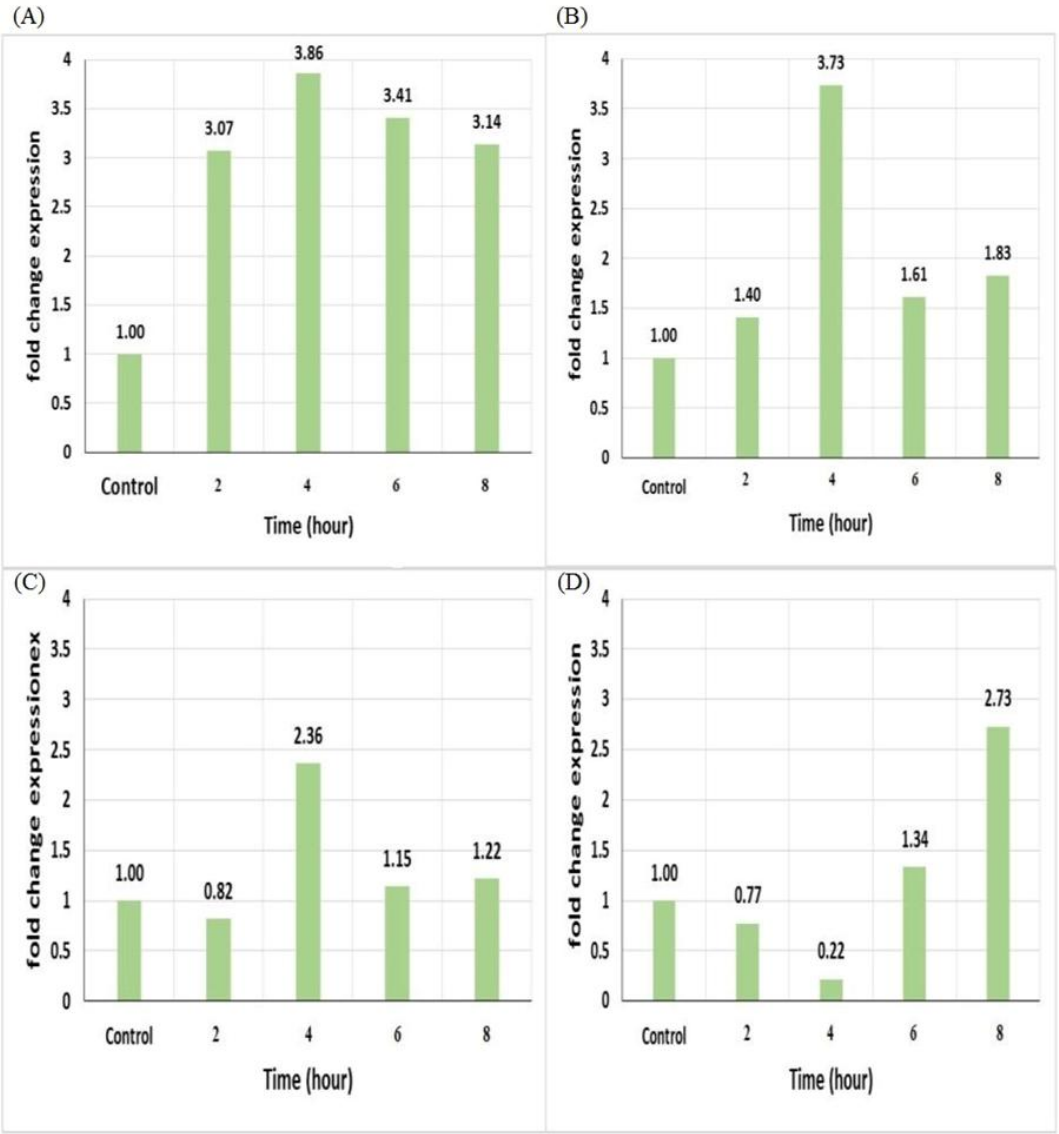

Figure (4): Quorum sensing genes expression after incubation of *P. aeruginosa* PA-10 with 10% bovine serum along the exponential growth phase period. (A): *lasI*; (B): *lasR*; (C)*: rhlI*; (D): *rhlR*

Table (3): Over expression of *P. aeruginosa* PA-10 quorum sensing genes after incubation bovine serum along the exponential growth phase period.

Time (hr)	Exponential phase	PvdQ CT	CT	ΔCT	ΔΔCT	Folding ±SD
lasI						
Control	---	22.96	20.72	-2.24	0	1.00^{a} ±0.00
2	Early	23.66	19.8	-3.86	-1.62	3.07^{b}±0.41
4	Mid	26.87	22.68	-4.19	-1.95	3.86^{b}±0.67
6	Late	23.89	19.88	-4.01	-1.77	3.41^{b}±0.38
8		22.07	18.18	-3.89	-1.65	3.13^{b}±0.37
lasR						
Control	---	22.96	20.16	-2.8	0	1.00^{a} ±0.00
2	Early	23.66	20.37	-3.29	-0.49	1.40^{a} ±0.29
4	Mid	26.87	22.17	-4.7	-1.9	3.73^{b} ±0.57
6	Late	23.89	19.4	-3.49	-0.69	1.61^{a} ±0.28
8		22.07	18.4	-3.67	-0.87	1.82^{a} ±0.41
rhlI						
Control	---	22.96	19.63	-3.33	0	1.00^{a} ±0.00
2	Early	23.66	20.61	-3.05	0.28	0.82^{a} ±0.25
4	Mid	26.87	22.3	-4.57	-1.24	2.36^{b} ±0.51
6	Late	23.89	19.36	-3.53	-0.2	1.14^{a} ±0.33
8		22.07	18.45	-3.62	-0.29	1.22^{a} ±0.24
rhlR						
Control	---	22.96	19.48	-3.48	0	1.00^{a} ±0.00
2	Early	23.66	19.85	-3.11	0.37	0.77^{ac} ±0.19
4	Mid	26.87	21.66	-1.3	2.18	0.22^{c} ±0.07
6	Late	23.89	19.06	-3.9	-0.42	1.33^{a} ±0.38
8		22.07	18.03	-4.93	-1.45	2.73^{b} ±0.61

Discussion

The present work intended to determine how serum albumin, which is essential for *P. aeruginosa* virulence in the early stages of infection, affects iron-controlled gene expression via a Fur-independent mechanism unrelated to albumin-associated iron.

The results show that 100% of *P. aeruginosa* isolates were MDR. These results in agreement with Chika *et al*. (18)that

reported that multi-drug resistance was prevalent inclinical isolates of*P. aeruginosa*. The high rate of multidrug resistance caused by improper use of antibiotics. Moreover, MDR *P.aeruginosa* can develop such resistance through different

processes like the production of β-lactamases, modification enzyme of aminoglycosides, efflux pumps that are active with multiple drugs and decreased permeability of the outer membrane, besides, *P.aeruginosa* is intrinsically resistant to some classes of drugs, frequently forms biofilms and develops resistance to antibiotics swiftly within 48hours of exposure(19).

Furthermore, the current findings showed that all MDR and XDR isolate se produced biofilms. Biofilms possess remarkable capacity with regard to the physical and physiological resistance to antimicrobial agents. From their approach to enhance the antimicrobial tolerance, the biofilms are capable of enduring longer exposure to the conventional antimicrobial therapies while maintaining their cell integrity and survival (20).

Relative gene expression of *lasI, rhlI, lasR,* and *rhlR* during various phases of exponential growth phase presented in figure (3) of the present study was corroborated with the Alayande *et al.*(21) while studying biofilms formation and quorum sensing signal molecules.

The effect of bovine serum on QS gene expression of *LasI, Rhl, RHl* and *LaS* revealed that *lasI* gene expression only increased after incubation with bovine serum at each stage of the exponential phase for two and four hours ($P<0.01$). These findings pointed out that serum effectively increased the *lasI* gene expression difficult the early exponential phase, this has been known as the serum iron-binding protein (Apo transferrin) increase *lasI* gene expression as mentioned by Kruczek *et al.*(22).

Data also revealed that *lasR* was induced up to a statistically significant level ($P<0.01$) during early and mid-log phase in response to two and four hour incubation with bovine serum.These results are comparable to those of Kruczek *et al.*(22) who reported that serum induces *lasR* transcription in the early stages. Factors or compounds found in bovine serum influence the amount of autoinducers 30C12-HSL and C4-HSL that are synthesised by the quorum sensing system.

Results showed that *rhlI* gene expression was significantly increased ($P<0.01$) two-fold at mid log phase of exponential growth. Kruczek *et al*. (22) mentioned that bovine serum increased the expression of *rhlI* in the late phase but had no discernible influence on it in the early phase. Given that *rhlI* encodes the synthase that produces the C4-HSL inducer, serum variably controls the expression of its target *rhlI* gene, boosting it during the mid-stage of development but representing its expression at the early stage of exponential growth.

Bovine serum caused induction in *rhlR* transcription in late growth of exponential phase, at this stage *rhlR* was activated by their respective autoinducer due to the synthesis of enough C12-HSL and C4-HSL to completely activate *rhlR*, which in turn results in the highest expression of the genes encoding quorum sensing-controlled virulence factors. The expression levels of the *lasR*/*lasI* and *rhlR*/*rhlI* systems increased when they entered the Lag phase of growth, which corresponded to the highest cell density as show in (figure 4). Compared to their respective regulatory protein genes (*lasR* and *rhlR*), the autoinducer synthase genes (*lasI* and *rhlI*) in the las and *rhl* systems expressed at noticeably greater relative concentrations and earlier periods. High quantities of *LasR* and *rhlR* bind to their respective N-acyl homoserine autoinducer molecules, and the resulting complex then acts as a transcriptional regulator of several P. aeruginosa genes. Most *P. aeruginosa* virulence factors are produced in a manner that is regulated by the QS system.

In conclusion *lasR, lasI, rhlR,* and *rhlI* genes were common in *Pseudomonasaeruginosa* isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.

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