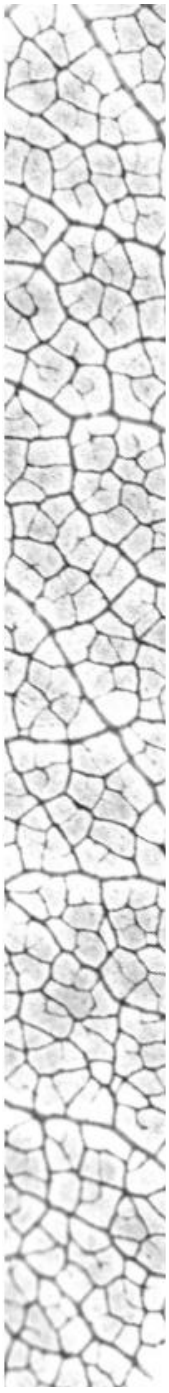

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Molecular detection of Quorum sensing genes in *Pseudomonas aeruginosa* isolated from CSOM patients and their relationship to biofilm ability

Amer Alwan Khuris Al-Kilabi [1], Thanaa Shamsulddin Al-Turaihi [1*], Habeeb Shuhaib Al Mohammed [1]

[1] Faculty of Medicine, University of Kufa, IRAQ
*Corresponding author: thanaa.alturaihi@uokufa.edu.iq

Abstract
CSOM is considered as a complex infectious and inflammatory diseases that affect middle ear. The complications are due to CSOM including, intracranial like meningitis and brain abscess which are the most common causes of death in patient with CSOM.
Pseudomonas aeruginosa are difficult to treat because the majority of isolates present high level of resistance to many types of antibiotics this is associated to its ability for biofilm production.
Biofilm-associated infections cause considerable problems, thus the major objective of the present study is to evaluate the frequency of phenotypic biofilm production and the role of the selected Quorum Sensing Genes in *pseudomonas aeruginosa* that is the most common and serious bacterial type isolated from CSOM patients.
Material and Methods: In a cross sectional study, a total of (31) isolated strains of *Pseudomonas aeruginosa* from (82) patients suffering from CSOM were collected between January to the end of April 2019 admitted at the main hospitals in Al-Najaf province.
Results: Among 31 isolates of *Ps. aeruginosa* collected from CSOM patients Detection of In-vitro biofilm formation in these isolates were carried out by tissue-culture plate TCP method (the most sensitive method for detection biofilm production) the method detected (45%)as strong, (42%)as moderate, (13%) weak adherent and (0%) non biofilm production. Screening for some selected genes responsible for biofilm formation was done by PCR (*LasI, LasR, rhlI, Rh IR*) were present in most of the *Ps. aeruginosa* isolates, 27(87%) of isolates give positive results for *LasI;* 25(80.64%) of isolates give positive results for *LasR* gene; 25(80.6%) of isolates show positive result for *rhlI* and 28(90.3%) %) of isolates show positive result for *RhlR* gene.
Conclusion: The results revealed that the presence of Quorum sensing genes (*lasI, lasR, rhlI, RhlR*) which are responsible in biofilm formation were present in most of the *Ps. aeruginosa* isolates.

Keywords: chronic supportive otitis media, polymerase chain reaction, pseudomonas aeruginosa, quorum sensing, tissue culture plate

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INTRODUCTION

CSOM, chronic inflammation of the middle ear with tympanic membrane perforation, with or without prolonged otorrhea (lowest possible 2-6 weeks of discharge) (Qureishi et al. 2014). CSOM takes place in the patients with chronic dry-perforated otitis media or going to follow the Acute Otitis media episode, during which treatment is either failing or not initiated (Reid et al. 2009)

Pseudomonas aeruginosa it is ubiquitous, human an opportunistic pathogen correlated usually to nosocomial infection especially in immunocompromised (Moradali et al., 2017) *Pseudomonas aeruginosa* is an important chronic suppurative otitis media (CSOM) pathogen that exhibits multiple resistances to antibiotics with increasing frequency, making patient treatment more difficult (Sahu et al, 2019) this pathogenic bacteria uses different virulent factors to assist in its pathogenicity and to evade from host defense mechanisms, among these many factors one can focus attention on is the formation of biofilm, it can interact within a biofilm by means of diffusible molecules through a process called quorum sensing (Harmsen et al, 2010; Li YH & Tian, 2012) Quorum sensing (QS) is a bacterial cell–cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs) and it's a type of cell

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signalling pathway that initiates the production of virulence factors**,** Quorum-sensing systems (QS) regulate the formation of Biofilm, in *Ps. aeruginosa,* play a key role in the differentiation process which increases concentration depending on density of the cells (Verhoeff et al,2006). Auto inducers (AIs) accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene expression. QS controls genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Pérez-Velázquez et al, 2016) The formation of biofilms follows event sequence: microbial surface fixation, proliferation of the cell, production of matrixes, and detachment (Sauer et al,2008). *Pseudomonas aeruginosa* are difficult to be treated like the majority of isolates, and considered the most widely distributed bacteria forming biofilm and exhibit high level of innate resistance to many antibiotics. Indeed, they are extremely difficult or impossible to eliminate (Miller & Bassler,2001. Ciofu & Nielsen,2019) *P. aeruginosa* employs three major interconnected QS systems that function independently and dependently involving *las*, *rhl*, *pqs* pathways as well as novel candidate *iqs* pathway regulated by several QS signal molecules (Malešević et al 2019) This study aimed to evaluate the biofilm formation capabilities of *P. aeruginosa* isolates from CSOM patients genotypically by the presence of Quorum sensing genes (*LasI, LasR, Rh1I, Rh1R*) and phenotypically by Tissue Culture Plate method TCP.

MATERIALS AND METHODS

This cross sectional study was conducted from January to end of April, 2019 at the main hospitals in AL-Najaf province. All study participants suffered from perforated tympanic membranes which had effective pus release, the diagnosis of CSOM was carried out according to clinical examination under supervision of specialist of ENT, extensive data on age, gender, residence, duration of discharge, antibiotic therapy were collected from each study participant prior to data collection.

The study was confirmed by institutional ethical committee.

For bacterial detection, the clinical samples of ear swabs (from patients in hospitals) were submitted besides routine bacterial research findings were collected and analysed standard methods were used to process the total samples(Cheesbrough,2000) Patients were tabulated by age, gender as well as place of residence.

Pseudomonas aeruginosa Isolation and Identification

Clinical samples were transported to the laboratory immediately, the cultivation of collection sample was done at the department of Microbiology, faculty of Medicine, University of Kufa. Ear swabs Samples were planted directly on the culture media including Blood agar, MacConkey agar plates and incubated at 37 °C for 24-48 hours colonies were diagnosed initially depending on the phenotypic and culture characteristics, then colored by Gram stain (Kadhim et al 2009), the isolates of *Pseudomonas aeruginosa* were also biochemically confirmed by using VITEK2-automated system.

Phenotypic characterization of biofilm formation Method for tissue culture plates (TCP)

TCP test described by (Christensen et al, 1985) defined as semi-quantitative microplate reader test (biofilm screening test) TCP it was regard as standard test for observation of biofilm formation (Abdelshafy et al, 2015) after isolation fresh *Pseudomonas aeruginosa* strains from agar plates were inoculated in 10% ml of (TSB tryptic soya broth with 1%glucose), incubated for 72 hours at 37C° and then mitigate to 1:100 with fresh TSB.

A 96 well of flat bottom tissue culture plates, were loaded, with 150µl aliquots diluted cultures each isolate was inoculated in triplicate broth, and one of the well loaded without bacterial sample, it was served as negative control to examine non-specific binding of media, all plates were aerobically coated and incubated within 24 hours at 37 ° C. The well contents were aspirated after incubation. Each well was washed 3 times by using physiological sterile saline then were exposed to air -dry. The same plate had been vigorously shaken to take off bacteria which do not adhered to it. The residual attached bacteria were fixed for 15 minutes using 0.2 ml of absolute methanol per well. Then, methanol was removed and was dried by air, by 200 µL of 0.1% crystal violet the wells were stained for 30 minutes at room temperature, in order to remove the unbounded dye the plates were washed with distilled water and allowed to dry .The adhered stain was solubilized by addition 200µl of 95% ethanol. In order to obtain the correct reading of Optical densities (OD) of stained adherent biofilm we were using micro ELISA auto reader at wavelength 630nm as shown in **Table 1**.

Table 1. Bacterial adherence classification and biofilm formation by tissue culture plate method (TCP) according to (Saffari et al 2017)

Mean of negative control value at 630nm	Formation of Biofilm
Less than Negative Control (N.C)	Non
N.C *2	Weak
N.C * 4	Moderate
Above N.C *4	Strong

Table 2. Sequence of quorum sensing gene primer

Primer Type	Primer sequence (5'-3')	Amplicon size (bp)	Reference
Las I	5'-CGTGCTCAAGTGTTCAAGG-3' 5'-TACAGTCGGAAAAGCCCAG-3'	295	**CostaLima et al,2018**
Las R	5'-AAGTGGAAAATTGGAGTGGAG-3' 5'-GTAGTTGCCGACGACGATGAAG-3'	130	**CostaLima et al,2018**
rhII	5'-TTCATCCTCCTTTAGTCTTCCC-3' 5'-TTCCAGCGATTCAGAGAGC-3'	155	**CostaLima et al,2018**
rhIR	5'-TGCATTTTATCGATCAGGGC-3' 5'-CACTTCCTTTTCCAGGACG-3'	133	**CostaLima et al,2018**

Table 3. Volumes of the Monplex PCR protocol reaction

Mixture Solution	
Master mix	(8) µL
DNA template	(5) µL
Forward primers	(1.5) µL
Reverse primers	(1.5) µL
De-ionidied water (d d water)	(4) µL
Master mix	(8) µL
Final total volume 20 µL	

Table 4. Amplification of the PCR reactions using the conditions of the genes.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
Las I, Las R, rhII and rhIR	94 Cº for 2 min.	30	95 Cº for 40 Sec	50Cº/60 for 1 min	72 Cº for 2min.	72Cº for 10min.

Detection of QS genes

By using a system for commercial extraction kit (Genomic DNA promega kit), DNA extraction from *Pseudomonas aeruginosa* isolates was performed.

Estimation of DNA Concentration

A reading of the optical density can also calculate the concentration as well as the pureness of DNA of the sample in spectrophotometry at 260 and 280 nm by adding 5 µl of each DNA sample to 2ml of distilled water The sample was well mixed. Ultraviolet absorbance probably used in order to measure the purity of the extracted DNA. The absorbance ratio was at 260 nm and the absorbance at 280 nm (A260 / A280) was 1.8 for a pure DNA sample, if the ratio less than 1.8 that mean the sample is contaminated with organic solvent such as phenol that is frequently used in procedure of DNA extraction or protein (CostaLima et al, 2018)

Molecular detection of the quorum sensing genes

The (*LasI, LasR, rhlI, Rh IR*) genes were amplified by PCR using a specific set of primers that listed in **Table 2**. It was used according to the protocol instructions that given by the manufacturer of the Promega Biosystem. Singular reaction (final volume of reaction 20 µl) is shown in **Table 3**.

The PCR's tubes were all placed mostly in the machine, the amplification program was shown in **Table 4**.

Table 5. Demonstrates the distribution of bio adherent isolates which used the method of tissue culture plate (TCP).

Ps. aeruginosa / **N.O. isolates**	**Mean of OD value at 630nm (N.C= 0.1)**	**Biofilm formation**
Nil	≤ 0.1	non
2, 4, 8, 13	>0.1and ≤ 0.2	weak
1, 3, 6, 9, 10, 12, 14, 15, 17, 22, 26, 28, 30	>0.2 and ≤ 0.4	Moderate
5, 7, 11, 16, 18, 19, 20, 21, 23, 24, 25, 27, 29, 31	Above 0.4	strong

Table 6. Biofilm production of *Ps. aeruginosa* by tissue culture plate method

Bacteria Type	NO	Non-adherent	Weak-adherent	Moderate-adherent	Strong-adherent	p-value
Ps. aeruginosa	31	0 0.0%	4 12.9%	4 12.9%	14 45.2%	0.043

Electrophoresis of an agarose gel

All of the requirements, techniques as well as planning and preparation for the detection and identification of DNA for agarose gel electrophoresis were carried out (Nakayama et al, 2016) Products with PCR amplicons were observed by visualizing the electrophoresis of the agarose gel through dyeing with Red Safe. The results of electrophoresis were detected through the use of gel documentation system. The positive outcomes were seen when the target product size is comparable with DNA band base (Bartlett & Stirling,2003) by using Biometra gel documentation system, the gel was ultimately photographed

Statistical Analysis

The data were analyzed using Microsoft Excel computerized programs (Paulson, 2008). The electrophoresis results were analyzed; the size of DNA bands (PCR amplicons) were measured by using gel analyzing program (UV Band Software, Version12.14) as compared with DNA ladder.

RESULTS

In a cross section study a total of (31) clinical specimens of *Pseudomonas aeruginosa* from patients suffering CSOM were collected between January to the end of April 2019 admitted at the main hospitals in Al-Najaf province, Iraq.

Biofilm production by TCP methods was seen in 31 (100% isolates) (**Table 5**).

Molecular detection of Quorum sensing gene in Ps. aeruginosa which is responsible for biofilm formation by PCR Detection of the LasI gene

In fact, 31 isolates of *Ps. aeruginosa* tested for presence of *LasI* gene that responsible for biofilm

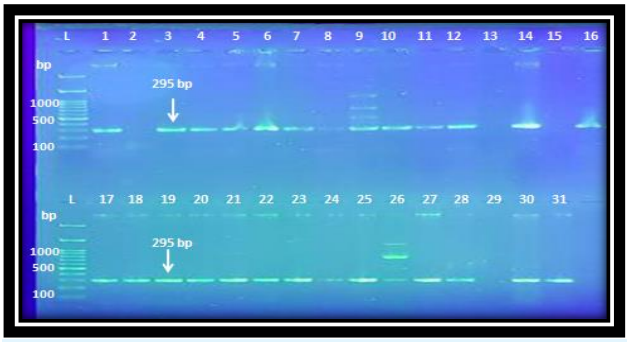

Fig. 1. Red safe dye stained agarose gel electrophoresis of PCR amplification product of *Ps. aeruginosa* isolates that amplified with *LasI* gene primer with product 295 bp (1.5% agarose gel, 75 V, 1.20 hours), L:Ladder,

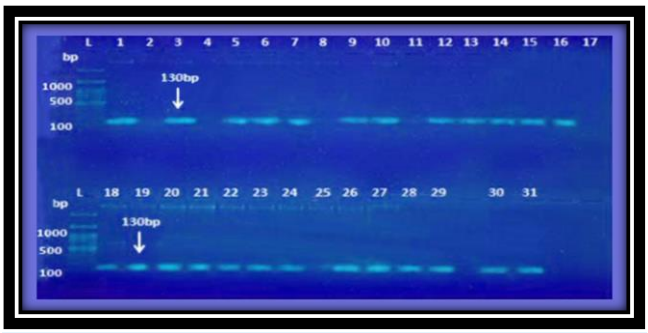

Fig. 2. Red safe dye-Stained stained agarose gel electrophoresis of PCR amplification product of bacterial isolates that amplified with LasR gene primer with product 130 bp. (1.5% agarose gel, 75 V, 1.20 hours), L:Ladder,

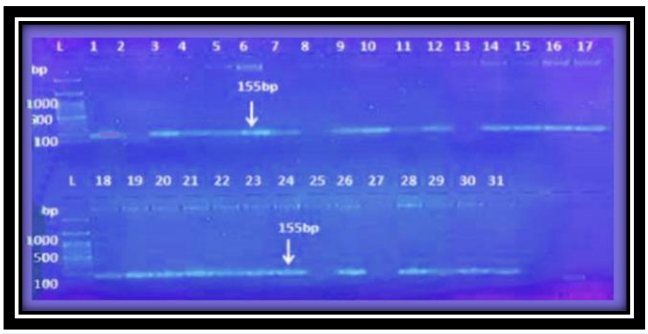

Fig. 3. Red safe dye agarose gel electrophoresis staining of the PCR amplification product of bacterial samples that was amplified with product 155bp with RhlI gene priming. (1.5% agarose gel, 75 V, 1.20 hours) L Ladder.

production capabilities by use PCR, 27 (87 %) of isolates show positive as show in **Fig. 1**.

Detection *LasR* gene

All isolates were analyzed to find out LasR genes, that encode for quorum sensing system QS manage for transcriptional regulatory proteins by using PCR technique with specific forward and reverse primers. according to the result shown in **Fig. 2** of the present study it appears that to LasR gene represented 25(80.64%) in bacterial isolated.

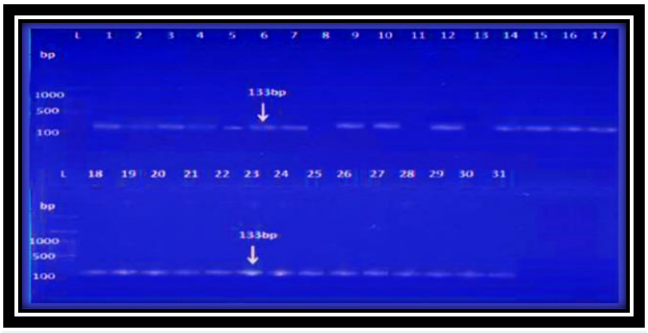

Fig. 4. Red safe dye – PCR amplification product stained agarose gels of bacterial isolates that was amplified with product 133bp with *RhlR* gene priming. (1.5% agarose gel, 75 V, 1.20 hours) L-Ladder.

Detection of the *RhlI* gene

31 isolates have been tested for the presence of the *RhlI* gene which is responsible for its ability to produce biofilm using PCR, 25(80.6%) of isolates showing positive results and confirm the presence of this gene by showing band on 155bp as shown in **Fig. 3**.

RhlR gene produce transcriptional regulatory proteins that activate target genes by using PCR technique with specific forward and reverse primers. according to the result shown in **Fig. 4** of the present study it appear that *RhlR* gene represented 28(90.3%) in bacterial isolated.

DISCUSSION

CSOM is one of the most important concerns regarding public health, especially in developing countries. In particular, accurate diagnosis and better management of these cases is crucial in alleviating the difficulties associated with this disease. Globally, CSOM has been one of the major preventable hearing losses; however, in most cases a sequelae of badly organized of AOM. Therefore effects on personal life quality (Adoga & Nimkur, 2010)

In the present study we have found that *Ps. aeruginosa* isolates reached (31)37.8% from CSOM patients, most of these isolates were resistant to antibiotics, this result concordance with other recent studies conducted by (Deshmukh & Manthale, 2017) who reported that the most common bacterial isolate from CSOM was *Ps. aeruginosa* 35% followed by *Staphylococcus aureus*, *Ps. aeruginosa* is perhaps the most repeatedly isolated organism in chronic supportive otitis media from various regions of the world (Bhat et al, 2020)

In the present study, all isolates of *Ps. aeruginosa* were biofilm producers detected by TCP method, similar results were obtained by (Elhabibi & Ramzy, 2017) biofilms are more often associated with many pathogenic forms of human diseases

The QS system take part in pathogenicity of *Pseudomonas aeruginosa*. These genes were mostly

observed in clinical isolates. The relationship between these genes and drug resistance were determined (Salehi, 2017) almost all research investigate the function of QS genes in the virulence of *P. aeruginosa* and the formation of biofilm, also they focused on the role of LasI / R system because of its found at the top of the QS signal transduction stream (Perez et al, 2011) in the present study all strains showed to have always different contribution of individual QS genes, a highest frequency of results belongs to *lasI* (87%), *lasR* (80.6%), *RhlR* (90.3%), and *RhlI* (80.6%),this results come in similar with previous studies found by (Salehi et al,2017. Lima et al,2018)and also by (Perez et al,2011)who demonstrate that the detection of QS genes was high for *lasI* and *lasR* genes (81.25%), *RhlI* gene (68.75%), and *RhlR* gene (62.5%) Also a study (Sabharwal et al,2014) carried out in India by Z.Salehi et al they found that the occurrence of *Ps. aeruginosa* QS genes were as follows *lasI* and *lasR* genes 75%, *RhlI* genes 41.6%, and *RhlR* genes 58.3%.

CONCLUSIONS:

We have found out that Quorum sensing genes (*lasI, lasR, RhlI, RhlR*) responsible for biofilm formation were present in most of the isolates of *Ps. aeruginosa* This is a good evidence that QS system take a critical part in pathogenicity of *Ps. aeruginosa* and it's an excellent way for bacteria to increase growth and resist antibiotics.

RECOMMENDATION

The tendency of bacteria to form biofilms enhances the spread of antibiotics resistance and the accumulation of virulence genes. However, we need to find out a new anti-biofilm strategies in order to deal with chronic inflammations associated with biofilm. Thus, this assumption might be addressed in future studies.

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