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The Suppressive Effect of Quorum Sensing Related to *Pseudomonas aeruginosa* (PQS) Against Pro-inflammatory Biomarkers of Gene Expression in Immune Cell Culture

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Abstract

Pseudomonas aeruginosa is a gram-negative, aerobic, non-spore forming rod which is capable of causing a variety of infections in both immunocompetent and immunocompromised hosts. Quorum sensing (QS) is the process through which gram-negative bacteria control the expression of the proinflammatory genes based on cell density, that allows communication and signaling between cell and bacteria. Our study was focused on the modulatory role of QS against inflammation in different immune cell cultures in vitro including splenocytes, lymphocytes and Bone marrow. The results of the current study suggested an immune modulatory role of QS against all inflammatory markers of interest including NF_KB, IL-2 ,IL-6, IL-1β and TNFα where all of these markers were downregulated in QS treated groups compared with the control group (untreated group). The results of our study indicated that the response started from the lowest amount of QS which was 5%, while the downregulation was shown with the highest concentration of QS which was 25%. All in all, our results suggested an anti-inflammatory role of QS which may be lead to a future investigation about their future role as a targeted therapy against inflammation.

Keywords: Quorum sensing, Inflammation, Gene expression, Immune suppressive effect.

Introduction

Pseudomonas aeruginosa is an aerobic Gram-negative rod shape bacterium which is able to cause pathogenicity in human an animals [1]. As this type of bacteria has such highly virulence factors, patients with immunocompetent could be exposed to several disorders such as pneumonia, septic shock, urinary tract infections, gastrointestinal infections, skin and soft tissues infections in cystic fibrosis, neutropenic, premature infants, burn victims and immunosuppressed [2].

QS used by *Pseudomonas aeruginosa*, like many other bacteria, these chemical signals have a functional communication between cells [3]. QS allows groups of bacteria to sense population density, thus in response for changing in cell densities, to coordinate behavior while complete circuits are forming the Quorum sensing that involve acyl-homoserine lactone signals and a third system that uses quinolone signals. Together, these three QS circuits regulate the expression of hundreds of genes, so for this reason QS has been considered as a model of study for molecular biology [4].

Quorum sensing (QS) has the ability to make communication within a single bacterial species or among bacterial species which means that they may mediate a cross talk between microorganism and different signals in the microenvironment [5]. On the other side, studies find that *Pseudomonas aeruginosa* uses quorum sensing to coordinate several functions including modulation and attenuation of the immune response [6].QS produced by *Pseudomonas aeruginosa* has been know of their controversial effect as an immune modulator [7]. A wide range of QS related molecules called autoinducers or

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pheromones have been showed to induce signaling, these include oligopeptides which is used by Gram-positive bacteria, N-acyl homoserine lactones (AHL) used by Gram-negative bacteria, and a family of autoinducers known as autoinducer-2 (AI-2) used in both Gram-negative and positive bacteria. On the other hand, bacteria also contain a specific receptors which interacts with autoinducers leading to the activation of a specific signaling that at the end lead to such an activation of few genes [8].

Inflammation reaction is a stress response by which a number of activities and signaling including cytokines, chemokines, biogenic amines and eicosanoids that induce diverse biological processes, affecting the local or systemic homeostasis (9). Inflammation can be caused due to different reasons, such as bacterial, viral, physical injury, cancer and other conditions and that inflammation may be far causing different consequences such as cancer and autoimmune disease as the frequent or chronic inflammation may potentially assist in that [10]. Understanding inflammation is considered very important for understanding the target pathway in order to achieve the meaningful therapies [11].

Mouse Mus tissue has been found that the genetic mice identical with human genome with more than 85% [12].*Mus musculus* have well known to be used to test the hypothesis and treatments related to understanding the mechanisms of diseases in human as the recent data of human genome project suggested a significant genetic homology between human and mice as that has been verified by the Encyclopedia of DNA Elements (ENCODE) as well as National Human Genome Research Institute (NHGRI) revealed that a huge similarity in protein coding, non-protein coding as well as regulatory elements that control gene expression which was achieved by using a number of genomic approaches such as RNA seq , DNA seq as well as Chip seq [13].

Spleen is one of the immune organs that plays an important role in the cellular and molecular levels as it regulates innate and adaptive immune response, control antigen tolerance as protected the host as well as its contribution in disease response [14].

Mesenteric lymph node (MLN) is considered the key for the immune tolerance and is the most common organ assaulted by antigens as it has been considered that MLNs one of the major immune spots that responsible for the mucosal immunity in the body which in turn have been found responsible for immune homeostasis [15].

On the other side, evidences are growing up by the time showing that bone marrow is the site where the active function and trafficking of all immune cells including T cells, B cells, dendritic cells, natural killer T (NKT) cells, neutrophils, myeloid-derived suppressor cells and mesenchymal stem cells [16].

Based on the information above it has been shown that the primary and secondary immune organs are considered the most typical parts for studying the immune response and the mechanism of different immune response and pathways.

The study aimed to investigate the immune modulatory role of QS specifically their suppressive effect through the regulation of the immune - inflammatory gene expression.

Material and Methoda

Samples Collection

A total of two hundred and forty-five samples of urine were collected from patients suffering from urinary tract infections (UTIs) with different ages and from both gender. The source and location was the teaching hospitals in Mosul city during the period between March 2023 and September 2023.

P. aeruginosa Inoculation, Isolation and Identification

Two types of agars including blood agar (Oxoid,UK) as well as MacConkey agar (Oxoid, UK) were used to inoculate the samples which were then incubated aerobically at 37°C overnight, then non-lactose ferments and oxidase positive colonies were selected and sub cultured on *Pseudomonas* cetrimide agar (Himedia, India) and incubated using the same conditions. Analysis Profile Index (API) was used for identification of the isolates on the basis of their colony morphology, cultural characteristics, Gram stain, as well as multiple biochemical tests [17]. Molecular diagnosis was done using the universal primers and PCR conditions mentioned elsewhere [41] while the QS crude extracted was obtained from another study [42].

Mice

A mouse of age 8-10 weeks was brought (Mus musculus, obtained from the Animals House in the College of Veterinary Medicine at the University of Mosul).

Preparation of Cell Culture Media (Complete Medium)

RPMI media with a volume of 9 ml were added to 1 ml of Fetal Bovine Serum (FBS) which the last represents 10 % of the whole mix, then 0.5 ml of the whole volume of penicillin-streptomycin solution was added to the final volume. The mixture was mixed well before using[18].

Preparation of Immune Cells Isolation

The immune cells were collected from different organs, including spleen lymph nodes and bone marrow, which were all obtained through dissection by a specialized veterinarian.

Isolation of Splenocytes

After the mouse was euthanized, the spleen was obtained under aseptic conditions. In brief, the spleen were removed then single cell suspension was prepared by smashing the tissue using a stomacher blender (Biobase, China) in complete media, then cells were filtered through a 70 µm filter after that RBC lysis (Promega/ USA) solution was added to get rid of the erythrocytes. The cells were then washed twice with Phosphate Buffer Saline (PBS) at 1300 rpm for 5 minutes. Then the cells were seeded in a 96-well microtiter plate with 500,000 cells/well [19, 20].

Isolation of Mesenteric Lymph Node (MLN)

Several mesenteric lymph nodes were collected from the abdominal area of the mouse then isolated as described by[20]. In brief, single cells were prepared in a complete medium by smashing the tissue using a stomacher blender. The cells were then filtered using a 70 µm filter and centrifuged at 1300 rpm for 10 minutes. The pellets were then resuspended in a complete RPMI media.

Isolation of Bone Marrow Immune cells

Bone marrow was isolated from the femur bone of the mice where the cells collected by pushing syringe with 2mm gauge inside the whole of the femur bone then the cells were washed with PBS and centrifugated with 1300rpm/min for 7 minutes then the pellets were resuspended in RPMI complete media. [19].

RNA Extraction and cDNA Synthesis

Transzol up plus RNA Kit was employed following the manufacturer's instructions. RNA was eluted in 20 µL of RNase-free water and adjusted according to the size of the precipitate. The quality and concentration of the isolated RNA was then assessed using a Nanodrop device (Thermo Fisher Scientific, United States). Subsequently, 500 ng of the extracted RNA was used to synthesize the complementary strand (cDNA) using Transcript First-Strand cDNA Synthesis super Mix Kit (Beijing, China) and following the steps recommended by the company.

Quantitative PCR Amplification

Regarding this step, Trans Start Green qPCR super mix, qPCR master mix containing Trans Start Taq DNA Polymerase SYBR Green I, dNTPs, PCR enhancer and stabilizer, were utilized. The qTower3 qPCR machine from analytika jena (Germany) was used for the experiment and all qPCR work was done in the central laboratory located at the College of Science/ University of Mosul. The genes of interest studied are listed in table (1). Gene expression was quantified, and specifically relative gene expression was quantified based on housekeeping gene (GAPDH) and the genes studied from treated and control group. Three different treatments using the crude extract were used (5%, 12.5%, and 25%) and compared to the untreated control. The treatments were conducted in all immune cells from the three organs mentioned above. The conditions toward the cycling environment were as follows: 45 cycles which consist of three steps at 98°C for 30 seconds,98°C for 10 seconds, and 60°C for 30 seconds.

cDNA concentrations were determined and normalized to the [21] housekeeping gene in each sample using the ΔΔCT method [20, 22].

Results

The current study complemented a previous study [40], as it actually began by obtaining the QS Crude from the aforementioned study. The results of our study indicated a sharp downregulation in the gene expression of the inflammatory cytokines NF_KB, IL-2, IL-6, IL1-β and TNFα in different immune cells treated with different concentrations of QS crude extract isolated from *P. aeruginosa* compared with the control group (Control-untreated group) as listed below in figs. 1, 2 and 3 in details.

Discussion

A total of 245 samples of UTI was collected from both gender, were 8 of these samples only identified for *P.aeruginosa* with a percentages 4%.The identification of isolates was done on the basis of their colony morphology, cultural characteristics, Gram stain, and biochemical tests using API meanwhile, several studies have mentioned that API is considered a quality way for isolation of different types of bacteria including *Pseudomonas aeruginosa* [26].

Sequencing on 16S rRNA has already been confirmed for detection of the isolates of different types of microorganism which is considered one of the most typical methods for molecular identification of bacteria particularly *P. aeruginosa. lasI*, *LasR* genes are both considered as a finger print for molecular detection of *P. aeruginosa* [27], this method was used in our study to confirm the identity of *P. aeruginosa* using polymerase chain reaction technique (PCR).

Cell culture is defined as an isolation and processing the cells from their natural environment (In vivo) to a controlled artificial environment [28, 29]

NFK B marker is well known as an immune modulator for the immune response in the body and plays an essential role in the immune inflammatory response [30, 31]. The results of our study in all of (Figs 1, 2) of spleen, lymph node have indicated a huge downregulation in all of mRNA gene markers (NFKB, IL-2, IL-6, IL1β and TNFα). On the other side, our results showed that the downregulation was

undetectable in the bone marrow immune cells (Fig. 3), which needs further investigation in the future.

Interlingually, our study showed that downregulation of mRNA gene expression started at the lowest amount which was 5%, in all the three different organs then continued with a volume of QS of 12.5% and 25%, these results have been supported with another study that showed that quorum sensing related to *Pseudomonas aeruginosa* modulated and suppressed the inflammatory response in mammalian cell culture [32].

While the modulation of immune response is mediated by the transcription factor NFKB by translocation to the nucleus where it binds to such specific target genes and then regulates some transcription factors our results identified with [31], where this study have been suggested the role of QS as it has been decreased the gene expression of NFKB suggesting the inhibitory role of QS against inflammation.

Another biomarker, IL-2, which is on one side, has been responsible for the proliferation of immune cells [33], while on the other side has been found that IL2 in a combination with CD28 costimulatory signaling that both are responsible for the proliferation and activation of T cells, However, both these molecules were inhibited in human T cells in vitro after treatment with QS molecules [34]. These results suggest the inhibitory role of PQS which is identified with the results of our study as we have found that the fold expression of mRNA IL-2 has been severely downregulated in PQS treated group compared with control group (untreated group) in spleen and lymph node in figs.(1,2) suggesting the inhibitory role of QS which is identified also with the previous study that we have mentioned previously as well as with another study which suggested the immune modulatory role of QS against IL-2 (34). IL-2 was also severely downregulated in the bone marrow immune cells compared with untreated group (Fig.3), our results identified and supported with another study which found that PQS plays such an important role in decreasing the IL-2 gene expression in bone marrow derived cells, while decreasing the T cell proliferation [35].

IL6 is one of the interleukins which plays an important role in cell signaling of chronic inflammation and autoimmunity [36], at the same time, another inflammatory signals such as IL1 β, which is considered a potent inflammatory cytokine and a key mediator of inflammatory response [37]. Along with that TNFα also is considered a proinflammatory cytokine as well [38]. All these studies suggested the inflammatory role of these cytokines, however it has been well investigated that the immune suppressive role of QS related to *Pseudomonas aeruginosa* , the results of our study has been shown a significant decrease in the gene expression of the immune cell culture of splenocytes and lymphocytes and bone marrow in all of the three different concentration 5%, 12.5% and 25% compared with control group which these studies are totally supported with previous studies that have indicated the immune modulatory and anti-inflammatory role of PQS in human endothelial cells which showed a sharp downregulation in the expression of the genes of IL-6, TNFα and IL1β [39].

Our results indicated that there is such a significant decrease in the fold change of the gene expression of each inflammatory gene signaling including NFK B ,IL2, IL6, 1L 1β and TNFα, as listed below in different treated concentrations including 5%, 12.5 % and 25 %, of quorum sensing, even though the down regulation of the mRNA gene expression was at the concentration of 12.5% was less than 5% and 25 % respectively in all the genes of the three organs which are spleen, lymph node and bone marrow which suggested further investigations and more mechanistic studies which may needed for this purpose in the future, meanwhile, the downregulation overall for the inflammatory genes was with the highest concentration of 25 % of QS in compare with the control group, our results identified with another study that have mentioned the potential immune modulatory of QS related to *Pseudomonas aeruginosa*. The concentration of 25 % was the most suppressor dose that led to downregulation of NF-KB gene expression, this result is identified with results from other study suggesting that Nuclear Factor kappa B (NF-κB) transcription factor regulates over 500 genes involved in inflammation, cell proliferation and apoptosis [40].

In general, the results of our study indicated that there were significant changes in the fold change of the gene expression which happened in 100 % of the genes of interest of our study specifically in the groups of the immune cells including Splenocytes, Lymphocytes and Bone Marrow immune cells treated with QS compared with the control group, thus suggested primarily that QS has a significant effect on gene expression in all cultured immune cells of different organs of our study which may explain the future role of the QS as a target therapy for inflammatory diseases in the future and tremendous immune modulatory and suppressor role of QS against the inflammation and that pretreatment with QS as a preventive immune supportive natural compounds may prevent possible inflammation and infection in the future [41, 42].

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Author`s contribution: The first researcher participated in designing the research and carried out the practical aspect. The second researcher completed the task of statistical analysis, making tables, and writing.

TABLE 1. Primers used in the current study

Gene	Primer	Sequence 5'-3'	References
GAPDH	Forward	AGGTCGGTGTGAACGGATTTTG	[20]
	Reverse	TGTAGACCAAGTAGTTGAGGTCA	
NFƘB	Forward	CGCAAAAGGACCTACGAGAC	[23]
	Reverse	TGGGGGAAAACTCATCAAAG	
IL-2	Forward	AACCTGAAACTCCCCAGGAT	[21]
	Reverse	CATCATCGAATTGGCACTCA	
IL-6	Forward	GAACAACGATGATGCACTTGC	[24]
	Reverse	TCCAGGTAGCTATGGTACTCC	
IL-1 β	Forward	GCCCATCCTCTGTGACTCAT	[25]
	Reverse	AGGCCACAGGTATTTGTCG	
TNFα	Forward	ATTCGAGTGACAAGCCTGTAGCCCAC	[25]
	Reverse	CTGGGAGTAGACAAGGTACAACCCA	

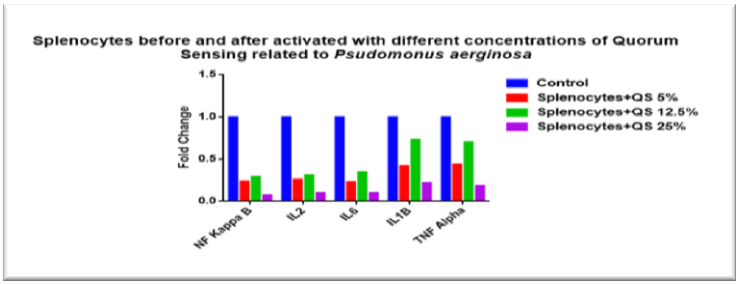

Fig. 1. Splenocytes before and after treatment with different concentrations of QS including 5%, 12.5% and 25%, respectively. Gene expression was studied in five different markers including NFƘB, IL-2,IL-6 , IL1β and TNFα, in addition to GAPDH, the housekeeping gene which used as a source of normalization for the calculations.

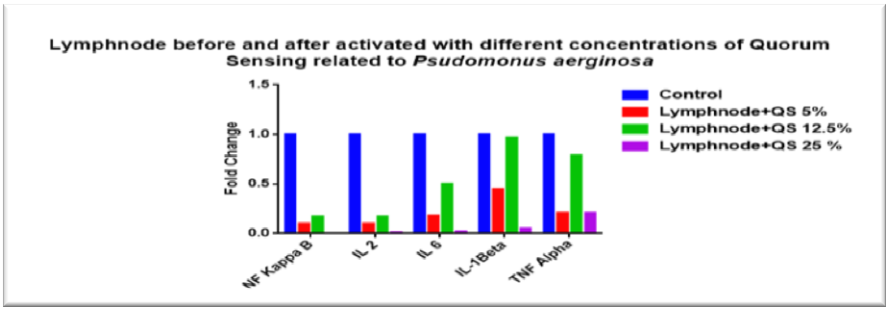

Fig. 2. Lymph node immune cells before and after treated with different concentrations of QS including 5%, 12,5% and 25% respectively .Gene expression which include five different markers including NFƘB, IL-2 , IL-6 ,IL-1β and TNFα, in addition to GAPDH, the housekeeping gene which used as a source of normalization for the calculations.

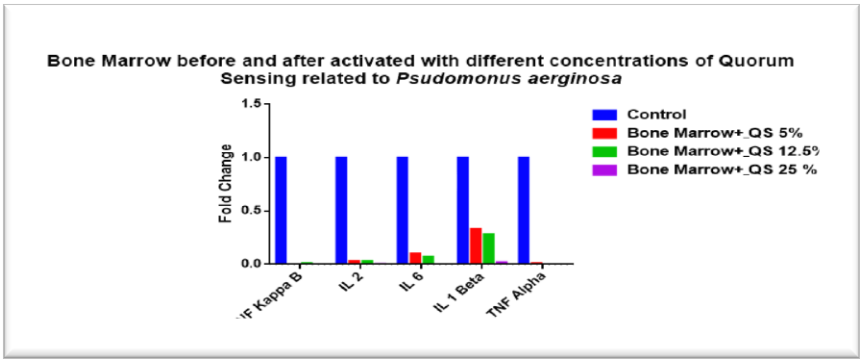

Fig. 3. Bone marrow immune cells before and after treated with different concentrations of QS including 5%, 12.5% and 25% respectively .Gene expression which include five different markers including NFKB, IL-2 , IL-6 ,IL-1β and TNFα, in addition to GAPDH, the housekeeping gene which used as a source of normalization for the calculations.

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التأثير القمعي لاستشعار النصاب المتعلق ببكتيريا الزائفة الزنجارية على المعلمات الحيوية البادئة للالتهابات عند التعبير الجيني في مزارع الخلايا المناعية

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الملخص

الزائفة الزنجارية هي عصية سلبية الجرام، هوائية، غير مكونة للسبورات وهي قادرة على التسبب في مجموعة متنوعة من الالتهابات في كل من المعرضين للإصابة سواء كانوا ذوي كفاءة المناعية او لديهم امراض متعلقة بنقص المناعة . استشعار النصاب هو العملية التي تتحكم من خلالها البكتيريا سالبة الجرام في التعبير عن الجينات المسببة للالتهابات بناءً على كثافة الخلية ، مما يسمح بالاتصال والإشارة بين الخلية والبكتيريا. ركزت دراستنا على الدور المعياري لـ استشعار النصاب ضد الالتهاب في مزارع الخلايا المناعية المختلفة في المختبر بما في ذلك الخلايا الطحالية والخلايا الليمفاوية ونخاع العظام.

اقترحت نتائج الدراسة الحالية دورًا مناعيًا تعديليًا لـ استشعار النصاب ضد جميع معلمات الالتهابات محل الاهتمام بما في ذلك NFKB و IL-2 و IL-6 و IL-1β و TNFα حيث تم تنظيم كل هذه المعلمات في المجموعات المعالجة بـ استشعار النصاب مقارنة مع مجموعة السيطرة (المجموعة غير المعالجة).

أشارت نتائج دراستنا إلى أن الاستجابة بدأت من أقل كمية من استشعار النصاب وهي 5%، في حين ظهر انخفاض كبير في التعبير الجيني مع أعلى تركيز من استشعار النصاب وهو 25%، اي كلما زاد تركيز استشعار النصاب في المزارع الخلوية انخفض التعبير الجيني اكثر . وبشكل عام، تشير نتائجنا إلى ان هناك دور مضاد للالتهابات لـ استشعار النصاب والذي قد يؤدي إلى رؤية مستقبلية حول دورها كعلاج مستهدف ضد الالتهاب.

الكلمات المفتاحية: استشعار النصاب، الالتهاب، التعبير الجيني، التأثير المثبط للمناعة.