Studies on Cross Kingdom Signaling by *Pseudomonas sp.* Quorum Sensing Molecules on Mammalian System

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Certificate

This is to certify that the research work embodied in this thesis entitled **"Studies on Cross Kingdom Signaling by *Pseudomonas sp.* Quorum Sensing Molecules on Mammalian System"** has been carried out in the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110067, India. The work is original and has not been submitted so far, in part or full for any degree or diploma of any university.

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Dedicated to my Parents, Family and Teachers

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Miles to go before I sleep!

Pallavi

TABLE OF CONTENTS

ABBREVIATIONS

β-ME	β-Mercaptoethanol
µg	Microgram
µL	Microlitre
µM	Micromolar
ATCC	American Type Culture Collection
HSL	Acyl Homoserine Lactone
AQs	Alkyl Quinolones
AU	Acid Urea PAGE
CID	Collision Induced Dissociation
DMEM/F12	Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EPS	Extracellular polysaccharides
ESI-MS	Electrospray Ionisation Mass Spectrometry
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
GFP	Green flourescence protein
hBD	Human beta defensin
HRE	HIF response element
HRP	Horse Radish Peroxidase
kD	Kilo Dalton

Kb	Kilo Base pair
LB	Luria-Bertani Broth
M	Molar
Mg	Miligram
Min	Minute
mL	Millilitre
Mm	Milimolar
mRNA	Messenger RNA
MALDI	Matrix assisted laser desorption ionization mass spectrometry
NCCS	National Centre for Cell Science
µM	Micromolar
nM	Nanomolar
OMV	Outer membrane vesicles
PPAR	Peroxisome proliferator activated receptor
PQS	Pseudomonas Quinolone Signal
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
QS	Quorum sensing/signaling

qPCR	Quantitative RT-PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TE	Tris EDTA
Tris	Tris (hydroxylmethyl)–aminometh

Section 1

REVIEW OF LITERATURE

REVIEW OF LITERATURE

1.1 The *Pseudomonas sp.* – a versatile opportunistic pathogen.

The genus *Pseudomonas* includes Gram-negative, flagellated, aerobic Gammaproteobacteria in the family Pseudomonadaceae. The genus is characterized by the largest number of species with more than 200 known; with significant genomic, proteomic and metabolic plasticity which allows competitive fitness in extremely diverse ecological niche[1, 2]. This explains the successful colonization of *Pseudomonas sp.* in soil, water, animals or plants worldwide. Many members of this genus exhibit remarkable fitness in extreme environments like the artic or in soil and water with high petrochemicals, related organic materials, salt or metal content[3]. This unique ability has witnessed the emergence and growth of *Pseudomonas* biotechnology in bioremediation of pollutants by using species like *P. fluorescens*, *P. alcaligenes*, *P. stutzeri*, *P. putida*, *P. cepacia* and others[4]. Whereas most species in this genus are non-pathogenic, many others are opportunistic pathogenic to human, animals and plants. Particularly, the latter encompass a large group of phytopathogens including the highly characterized *P. syringae* that affects wide range of economically important plans. Similarly, both *P. viridiflava* and *P. cichorii* can infect broad range of plants; whereas others target specific hosts like *P. marginalis* (lettuce), *P. cannabina* (hemp), P. marginalis (lettuce, and crucifers) or *P. corrugata* (tomatoes). Together these indicate horizontal evolution of *Pseudomonas sp.* from inanimate soil to living plants forms and natural selection of virulence determinants to target specific plant species[5]. The evolution of pathogenic determinants is evident in other host targets as well, for example in other *P. entomophila.* This true entomopathogen is unique since it is naturally selected to target many insect hosts including the model organism *Drosophila melanogaster*[6]. The genome of *P. entomophila* encode many insecticidal toxins, diffusible hemolytic factors, adhesins and extracellular proteases but lacks type III secretion systems that are signature factors in *Pseudomonas sp.* human pathogen[7]. This category includes species like *Pseudomonas oryzihabitans*(epticemia, peritonitis, endophthalmitis, and bacteremia)[8, 9] and by far one of the most important human opportunistic pathogen *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa was first reported by the French microbiologist Carle Gessard in 1882[10]. Since then it continues to be one of the most studied bacterial pathogen in human. Recent studies support *P. aeruginosa* as constituent of the human lung, skin and

intestinal microbiota in immuno-competent individuals. Community acquired or nosocomial infections of *P. aeruginosa* this pathogen occurs only in immunocompromised or clinically predisposed individuals. These continue to support *opportunistic pathogenecity* to define *P. aeruginosa* association with human host[11]. However, the molecular determinants and mechanisms of host-microbe relationships in opportunistic pathogens are arguably more complex and less understood than true pathogens. One of the many limitations is the poor understanding of triggers and mechanisms of commensal to pathogen switch in *P. aeruginosa* despite its abundant arsenal of virulence determinants.

The genome size of *P. aeruginosa* is unusually large (5.5–6.8 Mbp) and encodes between 5,500 and 6,000 open reading frames, which is close to simple eukaryotes like *Saccharomyces cerevisiae* (12 Mbp)[12]. The large number of ORFs allows inducible translation of many genes in response to environment and consequently unprecedented adapation that is key to the success of *P. aeruginosa* as an opportunist pathogen. However arguably the more significant determinant of its evolutionary fitness is its ability to form quasi- multicellular biofilms in axenic or multi-genera complex ecosystems[13].

P. aeruginosa, a facultative anaerobe and versatile pathogen can proliferate in conditions of partial or total oxygen depletion. Mechanisms to achieve anaerobic growth by the pathogen are via nitrate or nitrite as a terminal electron acceptor.[8] Certain strains of *P. aeruginosa* adaptation to microaerobic or anaerobic environments is essential for its survival. For example, during lung infection in cystic fibrosis and primary ciliary dyskinesia due to thick layers of lung mucus and alginate surrounding mucoid bacterial cells can limit the diffusion of oxygen. *P. aeruginosa* growth within the human body can be asymptomatic until the bacteria form a biofilm, which overwhelms the immune system[14, 15].

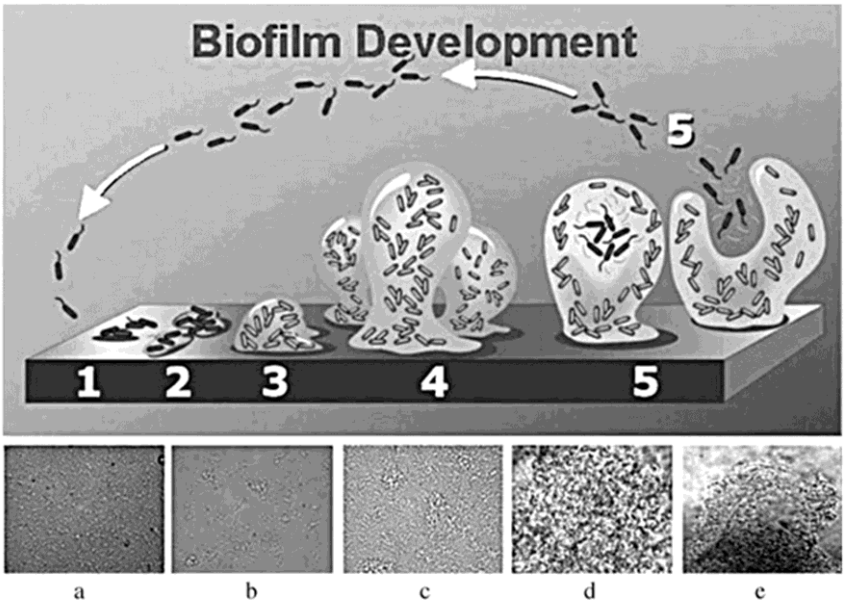
1.2 The biofilm paradigm and *Pseudomonas sp.* infections.

Microbial biofilms are surface attached sessile microbial communities that grow in air-water interface in micro-colonies within hydrated polymeric matrices (extracellular polymeric matrix or EPS) synthesized by themselves[16-18]. It has a characteristic slimy layer and can form on abiotic (catheter, hospital set-up etc) to biotic (prokaryotic/eukaryotic cells) surfaces. The existence of microbial biofilms were known since the early days of microbiology; Anton Van Leeuwenhoek described biofilms

scraped from dental plaque as termed them *"animalculi"* or a microbial community[19]. However the overwhelming amount of microbiology research beginning from last century concentrated on the planktonic, single-cell, free living forms of bacteria than their biofilm counterparts. The significance of this have begun to be appreciated only in the last two or three decades. It is now known that biofilms are much more than sessile microbial aggregates within an EPS. Biofilm bacteria not only exhibit often dramatically different morphology; they also enjoy many other remarkable features distinct from their planktonic counterparts. Presence of highly hydrated extra-cellular polymeric substances (EPSs) is characteristic feature of microbial biofilms. The (EPSs) comprised of polysaccharides, proteins, lipids, and nucleic acids. The biofilm developmental program usually starts as soon cells population reaches quorum on chemically inert exopolymer matrix as a response to external stress (pH, osmolarity, starvation, shear)[20] . Biofilm formation is beneficial to the cell population as a whole, since it allows cells to endure within highly stressful environments that would avert the survival of free-swimming cells. Stoodely et al., has demonstrated all the stages of biofilm formation starting from early adherent phase to dispersal of the bacterial cells in late stationary phase **(Fig 1.1)**. In fact, it has been proposed that since gene expression varies tremendously between planktonic cells and sessile cells in biofilms, they may even be seen as two different types of differentiated cells within a multicellular organism. Indeed this remarkable machinery of *P. aeruginosa* biofilms allows it to infect more physiological sites than any other bacterial pathogen . The pathogen can survive on dry surfaces and colonise on floors, sinks, toilet surfaces and equipments like dialysis machine and other in-dwelling appliances in human host with altered host defence[3]. Unlike many other pathogens *Pseudomonas aeruginosa* (PA) enjoys remarkable diversity in invasion and infection; leading to lung/respiratory, urinogentital, skin/burn injuries, eye, gastrointestinal and systemic septicemia most commonly in immunocompromised patients[11] **(Table1)**. Some of the major infections caused by *P. aeruginosa* biofilms are described in the subsequent section.

Respiratory Tract Infections: *P. aeruginosa* is a major cause of human lung infections and second most common pathogen associated with ventilator associated pneumonia (VAP)[21, 22]. Respiratory infections are classified under two categories; **Acute** and **Chronic** lung infections which can be easily transmitted through hospital or community acquired infections. However chronic lung infection is always associated through

compromised/defects in host immune system. Acute nosocomial lung infections are generally through direct damage to patient lung epithelium due to intubation or other factor like smoking etc. Whereas chronic infections arises in conditions when patient's medical situation is not capable for mounting an effective immune response as seen in Cystic Fibrosis cases[23].



Adopted from *P. Stoodley et al*.; 2002

Fig1.1 Developmental stages of biofilm formation and maturation.

Urinary Tract Infections: Urinary tract infection is second most common infection and presents serious health problem worldwide. 30- 40% of Catheter associate urinary tract infections (CAUTI) are caused by *Pseudomona*[24]*s*. Along with all the secreted virulence factors, PA has enormous capability to form biofilm on urinary catheters. Indwelling catheter can then act as direct entry of pathogens to bladder[25]. Persistent bacterial biofilm on the catheter cause continuous damage to the host by releasing the secretory factors as well as breaching the mucosal epithelium.

Ocular Keratitis: Microbial keratitis is an infection of cornea which causes morbidity and sight threatening complications like corneal scrapping, perforation and ultimately leads to blindness. Approximately 50% of microbial keratitis is caused by bacteria and *Pseudomonas aeruginosa* comprises 10% of microbial keratitis cases. PA keratitis can cause widespread collagenolysis within few days of onset of the disease and ultimately

leads to corneal perforation[26]. Much concern is for developing resistance of PA to new generation fluroquinolones and tropically used aminoglycosides. Thereby emergence of MDR, XDR and PDR (Pan Drug Resistance) is becoming a threat due to limited safe option for tropical antibiotics. There are reports where XDR strain of PA has lead to systemic infection and MDR strains has been reported in severe ocular infection[27]. Along with these, contact has also been a risk factor in PA keratitis. The hydrophobic nature of the silicone hydrogel forms a good matrix for PA biofilm formation on the posterior surface of contact lens. Biofilm formations worsen the disease and leads to chronic infection[28]. Mechanism of this is still not completely understood.

Ecthyma gangrenosum: (EG) is a rare but well-recognized cutaneous infection typically associated with *Pseudomonas aeruginosa* bacteremia. Critical ill and immunocompromised patients are suspected to EG and is always associated to a sign of pseudomonal sepsis. In contrast *Pseudomonas* folliculitis which is a community-acquired skin infection exclusively results from the bacterial colonization of hair follicles after exposure to contained, contaminated water[29].

Disease	**Affected organ & prognosis**	**Infectious Species**	**Assoc. with MDR/XDR strains**	**Patient demographics& Epidemiology**	**Reviews/ Reference**
Ocular Keratitis	Eye/cornea. Blindness	*P. aeruginosa*	Y	Global	Willcox et al.[30] Garg et al.[31]
1. Gastrointestinal infections 2.Necrotizing enterocolitis . Irritable Bowel Syndrome 4.	GI tract	*P. aeruginosa*	XDR	1. Neonates. Global. 2. Adults. Global.	. Morrow et al. [32] . Kerckhoffs et al. [33]
Pneumonia 1. Community acquired 2. Nosocomial	Respiratory tract	*P. aeruginosa*	Y	3. Neonates. Global	1. Cystic fibrosis 2. Ventilated patients
UTI	Urinary Tract	*P. aeruginosa*	Y	Adults	All
Ear (Otitis externa)	Ear	*P. aeruginosa*	N	Global	Chandle A Wright
1.Ecthyma 2.gangrenosum 3.pyoderma4. folliculitis	Skin	Fulva *P. aeruginosa*	Yes	1. Critical-Care Patients, Burn patients 2. Community accquired	Percival et al. [34]
Meningitis	CSF	*P. stutzeri* *P. aeruginosa*	Y	1. Critical-Care Patients	G Karagoz Huang CR

Table 1 Diseases mediated by *Pseudomonas sp.* biofilms

Virulence factor	Function
Colonisation Flagella	Adherence, motility
Pili	Adhesin, Type III secretion
Exopolysaccharides (EPS)	Alginates (Biofilms, Antibiotic and AMP resistance)
Lipopolysaccharide (LPS)	Endotoxin, adherence and biofilms
Invasion Lipase A and C	
Phospholipase C	Haemolytic, Disrupts lung surfactant
Protease	Degrades immune system components for ex complement factors.
Pyocyanin	Inhibits lymphocyte proliferation; apoptosis of neutrophils; impairs mucocilliary motility by stimulating production of H2O2 by polymorphonuclear (PMN) cells.
Siderophores Pyoverdine and pyochelin	Iron scavengers Pyoverdine regulates secretion of exotoxin A and proteases.
Pathogenesis Exotoxin A	Unknown role - possibly causes apoptosis of eukaryotic cells
Biofilm	Confers protection against biocides and immune system effectors as impenetrable to antibodies (Ab), antibiotics, and biocides.
Rhamnolipids	Dissolve phospholipids; cause apoptosis of leukocytes; impair mucocilliary motility and are solublising agents.
Type III secretion Exoenzyme (Exo) S, T and Y, and exotoxin U	Exoenzyme S inhibits phagocytic motility; induces death of macrophages and PMNs and disrupts tight junctions of epithelial cells. Exotoxin U is cytotoxic to eukaryotic cells.

Table 2 List of virulent factors from *P. aeruginosa.* Biofilms

Mechanisms of Antibiotic Resistance in *P. aeruginosa.*

P. aeruginosa enjoys three types of mechanisms for resistance against antibiotics which include intrinsic, acquired and adaptive. Intrinsic mechanisms in *P. aeruginosa* provide low permeable membrane with constitutive expression of efflux pumps and constitutive expression of lactamases enzyme. Whereas adaptive mechanisms include high level of beta lactamases and upregulation of multi-efflux pumps like MexA-MexB-OprM which provides resistance to cephalosporins[35]. Along with this expression of gyrase and topoisomerase IV is also regulated which leads to reduce binding of fluroquinolones and changes in membrane leads to resistance against colisitin. Acquired resistance includes most threatening with expression of extended ß lactamases (ESBL) and metallo-ß lactamases and aminoglycosides modifying enzymes. *P. aeruginosa* acquires antibiotic resistance through genetic transfer from intra and inter- species bacterial cells population. Biofilms environment is idea for these antibiotic resistance gene transfers among bacterial population. Along with theses biofilm formation itself involve several mutation within the cells which has also led to development of MDR strains. Emergence of fluroquinolones resistance strain from aquatic flora has been reported; whereas genetic transfer between *P. aeruginosa* and *Enterobacteriaceae* spp. has led to emergence of ß lactamases resistant strain[36-38].

1.3 Quorum Sensing in *Pseudomonas aeruginosa*

The planktonic to biofilm transformation is a highly regulated process which employ "quorum sensing" – a prokaryotic signaling mechanism mediated by small, chemically distinct classes of messengers which modulate large gene sets within target bacteria; affecting as much as 6% of the *Pseudomonas aeruginosa* genome. 'Quorum sensing' ensue a series of events by which planktonic bacteria detect a critical bacterial density required for dedicating itself to sessile/biofilm growth mode. Functionally, quorum sensing (QS) result in remarkable genetic, proteomic, metabolic and morphological changes which can modulate pathogenecity islands, coordinated "multicellular-like" behaviour in bacteria[39, 40]. QS has been widely studied in genus *Pseudomonas*. The planktonic-biofilm transformation alters the expression profiles of numerous genes that include those involved in bacterial virulence, pathogenesis, antibiotic resistance and others[18]. Quorum signalling molecules, also called as QS Autoinducers, are small MW (200-1500 m/z) bacterial metabolites, which include several chemically discrete families

of Lactones (Acetyl Homoserine Lactones, HSL), Quinolones (*Pseudomonas* quinolone signal (PQS), Furanones, Peptides (Autoinducing Peptides, AIP), fatty acids, metabolic intermediates and others (**Fig 1.2**). Many QS classes exhibit taxonomically discrete expression profiles[41]. At least two major families of QS metabolites exist in *Pseudomonas aeruginosa*: the acyl homoserine lactones (HSLs) and the Quinolones (PQS).

P. aeruginosa utilizes three signalling pathways viz. Las, Rhl and alkylquinoline pathway.

The Las and Rhl pathways involves HSLs (N-acylhomoserine lactones); N-(3-oxo-dodecanoyl)-L-homoserine lactone (3OC12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) respectively[42]. Whereas, alkylquinoline pathway utilizes 2-heptyl-3-hydroxy-4-quinolone also known as PQS(*Pseudomonas* quinolone signal). 2-heptyl-4-quinolone (HHQ) forms the precursor molecule for PQS[43]. As the concentration of this quorum signalling molecules reaches a threshold, QS molecules consequently bind to their cognate transcriptional factor such as 3OC12-HSL binds to LasR, C4-HSL to RhlR and PQS/HHQ to PqsR, which results in the subsequent induction of virulence gene expression and also translates into the up-regulation of signal biosynthetic genes triggering autoinduction [44]. One of the virulent factor production facilitated by QS molecule is rhamnolipid which inhibits the function of host polymorphonuclear leukocytes (PMNLs), thereby conferring antibiotic resistance in *P. aeruginosa*[45]. Other virulent factors linked to Quorum sensing system in *P. aeruginosa* include HSL (inducing vasodilatation in host), PQS, elastase etc[46].

Recently, therapeutic strategies based on use of QS inhibitors/quenchers such as azithromycin have also been elucidated as a treatment against *P. aeruginosa* infections, proving the principle role of QS in pathogenicity[47, 48]

1.3.1 Global regulation of QS network

Global regulation of QS network is regulated bia two orphan transcription factor VqsR and QscR as illustrated in **Fig No. 1.3**. QscR regulates the QS network by repressing/activating both Las/RhI under specific stimulation to prevent synthesis of QS controlled gene. Whereas, VqsR always positively drives Las/RhI QS network[49].

1.3.2 Environmental regulation of QS network

P. aeruginosa adaptability and regulation of QS network largely depend on environmental factors. Under certain environmental condition like hypoxia as a host defence mechanisms for removal of pathogen, the QS signaling processes are changed and significantly up-regulated. While low phosphate conditions, also directs independent activation of Las/Pqs QS pathway via use of PhoB regulator[49, 50].

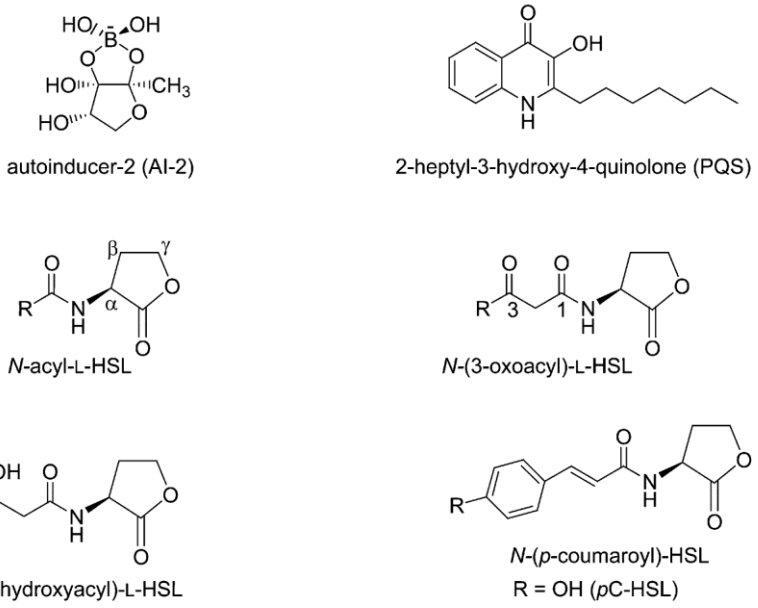


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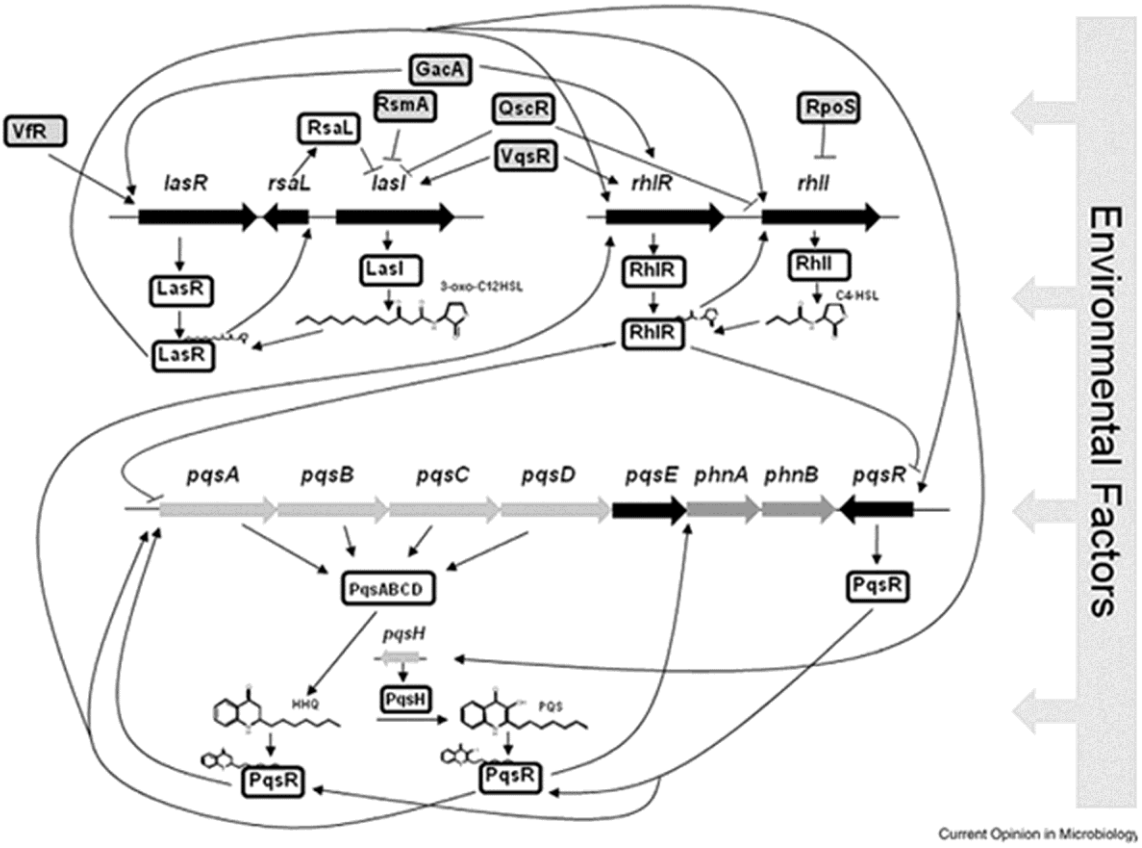
Fig. 1.2. Structure of different class of QS signaling molecules.

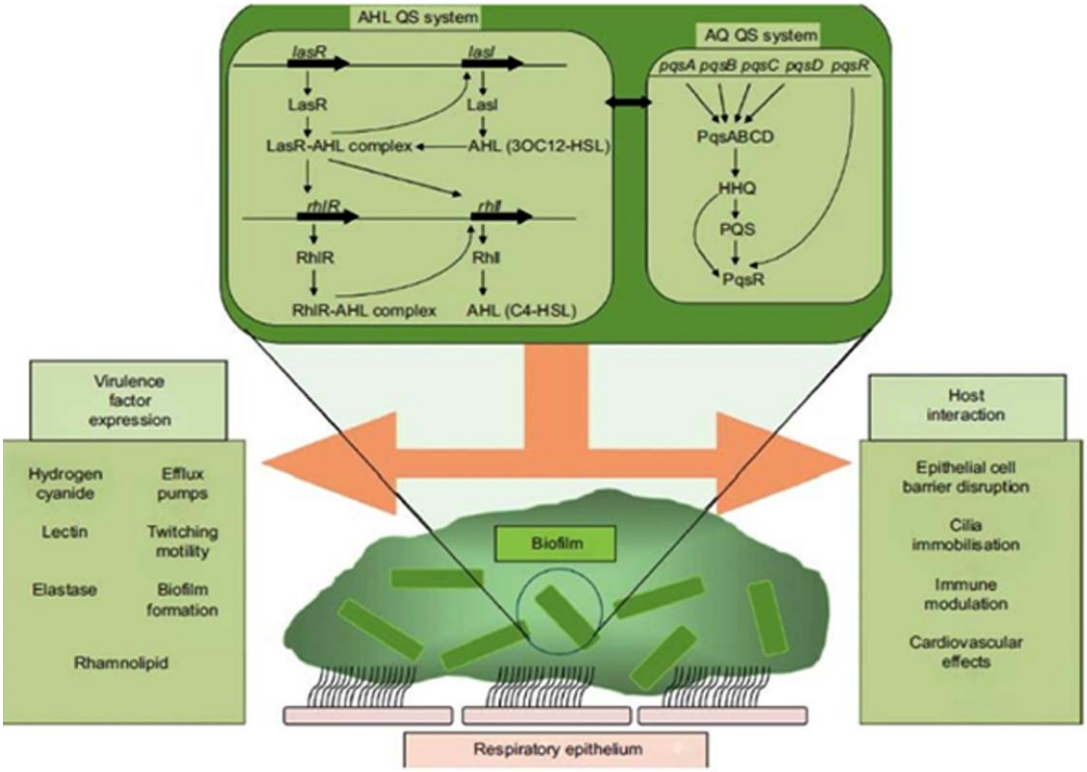


Adopted from William et al,; 2010

Fig. 1.3 HSL and AQ dependent QS network in *P .aeruginosa* which controls expression of virulent determinants , secondary metabolites and biofilm development.

Global regulatory system regulates the QS network and is depended on environment and/or site of infection. QscR and VqsR are transcriptional regulators of the QS network. LuxR homologue LasR, being regulated via Vfr (Virulence factor regulator) and GacA (Global antibiotics and cyanide) which is a two component regulatory system, induces LasI expression to produce 3-oxo-C12 HSL. RsaL acting as negative regulator

counteracts with LasR feedback loop and promotes 3-oxo-C12 production homeostasis. LasR/LasI complex via synthesis of 3-oxo-C12 HSL promotes synthesis of QS dependent target genes along with giving positive signal to second HSL dependent QS system RhlR/Rhll and Alkyl Quinolone (AQ) dependent system. AQ synthesis occurs through pqsR and pqsH that is homologue for LasR type regulator. The latter give rise to HHQ and PQS (co-inducer of PqsR). Las and Rhl QS network relationship is environmental dependent and regulated at both transcriptional and post transcriptional level mainly by luxR homologue regulator (QscR, VscR), RmsA, Sigma factor Rpos and riboregulator. On the other side AQ network operates via pqsABCDE operon and depend on induction of anthranilate pathways (via anthranilate synthase, PhnAB) and activation of Pqs A, B, C, D on anthranilate to synthesize HHQ. Later on PQS is produced from HHQ via pqsH. HHQ and PQS are inducer of pqsR and promotes the expression of pqsABCDE operon. C4 HSL negatively regulates pqsA and pqsR. Function of pqsE is not known but independently, it gives rise to expression of virulence determinants pyocyanin.



Adopted from Hurley et al., Camara et al. 2012.

Fig. 1.4. Quorum sensing signaling pathways of *P. aeruginosa.*

1.4 Inter-species and cross-kingdom signalling of *Pseudomonas sp*. quorum sensing molecules and the relationship with diseases

The QS network of *Pseudomonas* controls approximately 11% of the PA genome, mainly comprised of gene which controls the expression of virulence factor. For example the Las system controls not only biofilm related virulence gene but also expression of virulence factors such as alkaline protease, Elastase, lipase, Swimming, swarming and twitching Motility, exotoxin A, alginate etc. Similarly the RhI system controls expression of gene related to alkaline protease, elastase, lipase, pyocyanin, hydrogen cyanide, motility, siderophores, Type III secretion system and rhamnolipids. PQS system exclusively controls the expression of pyocyanin, siderophores, elastase, rhamnolipids and HAQs genes.

Significantly QS molecules and similar bacterial small MW metabolites has recently been shown to signal across kingdom and directly modulate mammalian host behaviour[51]. There are more than 10-12 members in HSL family and more than 56 Quinolones involved in Quorum sensing in *Pseudomonas*. Till now two of these: 3-oxo-C12-HSL and PQS are reported to be significant in cross-kingdom signaling with human host resulting in many different type of pathogenesis [51, 52]. Transcriptomic analysis of human lung epithelial cells exposed 3-oxo-C12HSL was reported to modulate ~40,000 total sequences (including known genes, predicted genes and transcribed sequences adding up to 11% of the transcriptome) in the human genome[53]. Recently it has also been added that 3-oxo-C12 interact with a type of nuclear receptor family i.e PPAR. According to a previous report, 3-oxo-C12-HSL act as agonist of PPARß/d and antagonist of PPARY[54]. PPARY is a ligand activated nuclear receptor and is widely known for its anti inflammatory roles and in maintenance of innate immunity of the host[55]. 3-oxo-C12-HSL interaction with mammalian cells has been shown to induce apoptosis in various cells including macrophage, neutrophils, fibroblast, monocytes etc in high concentration whereas others HSLs with similar concentration did not show any effect[56, 57]. Along with apoptotic activity 3-oxo-C12-HSL also showed loss of cell integrity when exposed to mammalian cells by rearrangement of actin cytoskeleton and regulating phosphorylation of various tight junction proteins.

Immune modulatory activity of 3-oxo-C12-HSL falls under two divisions, one of which represents pro-inflammatory and the other represents suppression of the immune

response. The differences solely depend upon the specific cell type and concentration of HSL used. Several studies reported that 3-oxo-C12-HSL indcues pro-inflammatory response which includes expression of chemokines, cytokines, and several interleukins (IL-6, IL-1 and IL-8) along with TNF alpha, IFN gamma and also other immunomodulatory factors like Cox-2 etc. The response was dose dependent with higher concentration (upto 200μM) and also was reported on epithelial cells, endothelial cells, T-cells, macrophage, neutrophils and mouse dermis. This suggests that HSL induced inflammation can cause significant damage to host and also favours *Pseudomonas* infection as seen in case of acute infection[58-62].

On the contrary it has also been reported that 3-oxo-C12-HSL can also downregulate/suppress immune system in order to develop environmental factors which actually favours chronic infection. 3-oxo-C12-HSL when administered in low concentration (<10μM) inhibits mitogen/antigen stimulated T cell proliferation. Overall the data indicates that 3-oxo-C12-HSL can induce antibody production from B cells and also repress cytokine production in LPS stimulated macrophage cells. Altogether it has been employed the capability of 3oxoc12 to modify host immune system to anti-inflammatory Th-2 response[63-66].

The second class of QS molecule, PQS is involved in the production of HAQs possessing antimicrobial activity[67]. Recent study has showed that PQS is involved in homeostasis of iron and act as iron trap for facilitating siderophores mediated iron delivery[68]. Apart from this PQS has been detected from CF patients' sputum and bronchoalveolar lavage fluid, suffering from *Pseudomonas* infection. In a recent study Hooi et al. Reported the PQS can also modulate the immune system like 3-oxo-C12HSL [69]. However the immune suppression activity of PQS was relatively more than 3-oxo-C12-HSL. PQS inhibited cell proliferation of human peripheral blood mononuclear cells (hPBMC) and without affecting release of interleukin (IL-2) when activated by lectin. PQS and HSL also have synergistic effect on T-cell proliferation[43, 70, 71]. Thereby, also proves theses two molecules have different target inside mammalian cells. In addition to this Kim et al. has showed PQS down regulates NF-κB signalling pathway by inhibiting its binding to target DNA and expression of NF-κB targeted genes and hence suppress host immune response[72, 73]. Overall studies opens a door to for application of these molecules as a therapeutics purpose as immune modulators. Although detailed temporal study of these molecules and their interaction with the host is yet to be discovered. The fact that specific

QS metabolite play role in host pathogenesis suggest these molecules may also contribute to pathogenicity of emerging drug resistant *Pseudomonas* [51, 74]. However there are no systematic studies on profiling these metabolities in accordance to pathogenecity or drug resistance. Therefore species specific analysis of QS is very crucial in order to determine the *Pseudomonas* infection and novel mechanisms of pathobiology.

QS Inducer	**Bacteria**	**Biological Roles**	**Molecular formula**	**Molecular Weight; m/z ()**
C4 HSL	*P. aeruginosa*	Cell to cell signalling and biofilm formation, cystic fibrosis	$C_8H_{13}NO_3$	171.0895
C6 HSL	*P. aeruginosa*	Regulation of virulence in disease like cystic fibrosis, biofilm reduction in commercial agriculture, food spoilage prevention, In UTI	$C_{10}H_{17}NO_3$	199.1208
C8 HSL	*P. aeruginosa*	Cystic fibrosis, UTI	$C_{12}H_{21}NO_3$	227.1521
3-oxo C6	*P. aeruginosa*	UTI	$C_{10}H_{15}NO_4$	213.1001
3-oxo C8	*P. aeruginosa*	Cystic fibrosis	$C_{12}H_{19}NO_4$	241.1314
3-oxo C12	*P. aeruginosa*	Cell to cell signalling, Interkingdom Signalling, Gram positive interference, Fungal development inhibition, Immune modulation, IgE production, Apoptosis, Epithelial cell barrier disruption & Cardiovascular effect	$C_{16}H_{27}NO_4$	297.194

Table 3 List of reported QS molecule (HSLs) from *Pseudomonas aeruginosa* and their biological roles.

QS Inducer	Strains	Biological Role	Molecular formula	Molecular Weight; m/z ()
PQS	*P. aeruginosa*	Oxidative Response, Autolysis, Membrane Vesicle Formation, Metal Chelation, Immune modulation, Biofilm formation, Bacterial Cross talk	C16H21NO2	259.1572
HHQ	*P. aeruginosa*	Bacterial Signalling , Immune modulation	C16H21NO	243.1623
HQNO	*P. aeruginosa*	Gram positive interference	C16H21NO2	259.15

Table 4 List of Reported QS molecule (Quinolones) from *Pseudomonas aeruginosa* and their biological roles.

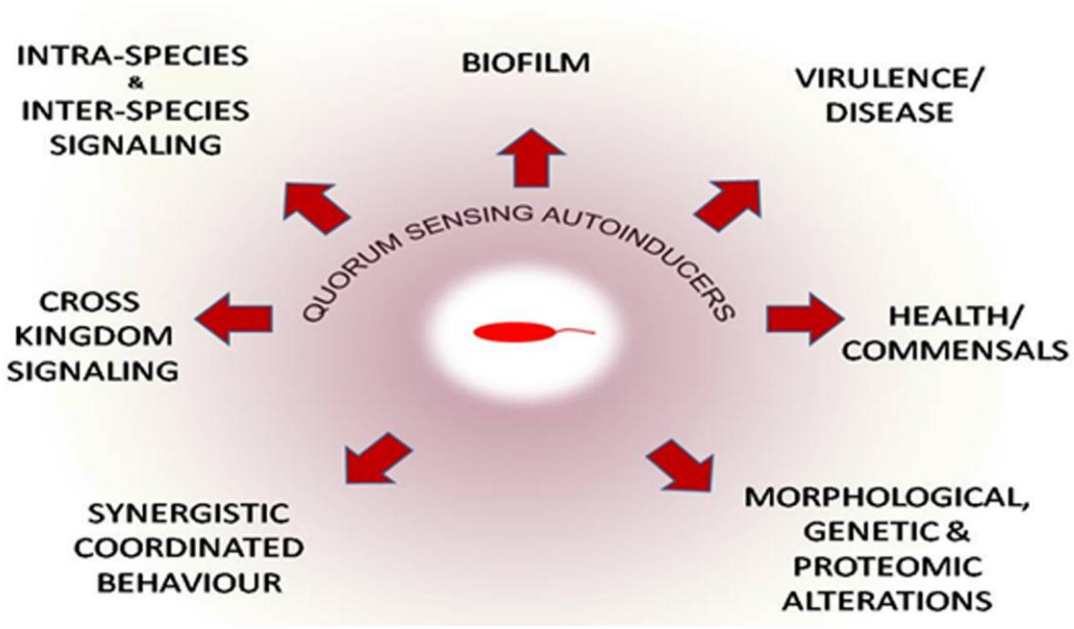


Fig 1.5. Schematic diagram: The many role of Quorum Signaling (QS)

1.5 Alkyl Quinolones (AQs) and membrane vesicle trafficking

Unlike HSL, PQS are hydrophobic in nature and there trafficking is required for these molecules between the cells. Recently, it has been reported that several of these identified QS molecules especially quinolones are packaged into outer membrane vesicles (OMVs) released by *P. aeruginosa*. Gram negative bacteria have been reported to secrete small particles composed of outer membrane components, called as outer membrane vesicles (OMVs), into the extracellular milieu[75]. OMVs derived from *P. aeruginosa* have been extensively studied. This bacterium secretes many virulence factors that are packed in OMVs which includes phospholipase C, proteases, alkaline phosphatases and hemolysins[76]. OMVs have been found to serve multiple functions in physiology and pathogenicity of the bacterium. These are involved in the transfer of proteins as well as genetic material in polymicrobial communities. In a study, OMVs derived from *P.*

aeruginosa showed beneficial effects to their own group by transferring an antibiotic resistance protein, β-lactamase, to increase survival[75, 77]. Later on it was demonstrated that QS molecules especially AQs (Alkyl Quinolones) are being trafficked via OMVs. The significance of this packaging may be attributed to the fact that these molecules are hydrophobic in nature. So the use of MVs as a shuttle enables transfer of these compounds in a stable form to the extracellular environment efficiently[78]. Another significant study on OMVs as "vesicle trafficking systems" was conducted by Mashburn and Whiteley. They proposed that *P. aeruginosa* MVs encapsulates QS molecules that might play a very significant role in cross-kingdom signalling. Using TLC and LC-MS/MS they reported that *P. aeruginosa* MVs contains about 86% PQS, trace amount of (1%) 3OC12-HSL and C4-HSL in addition to quinolines which is responsible for anti-microbial property of MV against Gram-positive bacteria[77, 79]. They also showed that PQS alone is required and sufficient for MV formation in *P. aeruginosa* and that exogenous PQS mediates its own packaging and the packaging of other quinolones into these vesicles. It has also been proposed that the hydroxyl at position-3 of PQS is important for its interaction with lipidA of LPS. Along with this the alkyl chain also interact with lipid A and results to a less fluidic membrane. Thereby PQS interaction with LPS cause asymmetric growth of outer membrane and ultimately leads to budding of the membrane from the cell as OMVs. Albeit little is known about OMVs fusion and PQS signaling and how specific PQSs are transported to target cells to initiate signaling pathway further.

1.6 Host – Microbe interactions between *P. aeruginosaP. aeruginosa* biofilms and the Epithelium

Microbial biofilms are surface attached sessile microbial communities that grow in air-water interface in micro-colonies within hydrated polymeric matrices (extracellular polymeric matrix or EPS) synthesized by themselves[16-18]. It has a characteristic slimy layer and can form on abiotic (catheter, hospital set-up etc) to biotic (prokaryotic/eukaryotic cells) surfaces. The existence of microbial biofilms were known since the early days of microbiology; Anton Van Leeuwenhoek described biofilms scraped from dental plaque as termed them *"animalculi"* or a microbial community[19]. However the overwhelming amount of microbiology research beginning from last century

concentrated on the planktonic, single-cell, free living forms of bacteria than their biofilm counterparts. The significance of this have begun to be appreciated only in the last two or three decades. It is now known that biofilms are much more than sessile microbial aggregates within an EPS. Biofilm bacteria not only exhibit often dramatically different morphology; they also enjoy many other remarkable features distinct from their planktonic cells.

The innate immune system is the first line of host defence mechanisms against pathogens which can further initiate adaptive immunity to protect against re-infection from the same pathogens. Three types of defence mechanisms have been proposed in innate immunity[80]. First is anatomical which includes skin, epithelial layers and the chemicals they secrete. Second is cellular and third is humoral which includes innate immune cells. These cells are activated by Pattern recognition receptor (PRRs) upon interaction with Pathogen assistant molecular pattern (PAMPs). PRRs are expressed on immune cells like macrophages, B-cells, dendritic cells (DC) etc. These PRRs are very specific and recognize highly conserved structures known as PAMPs. Till date most studied PRRs is TLRs which downstream leads to activation of innate immune effector molecules like defensins etc. and signals to adaptive immunity as well[81].

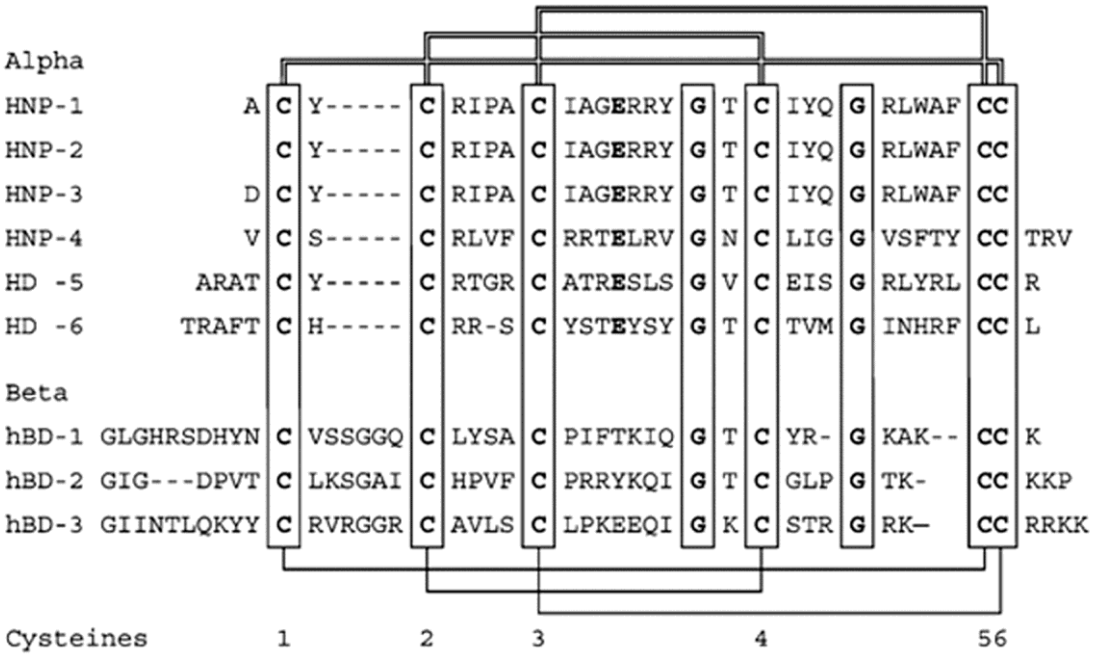
1.7 DEFENSINS

Defensins are small, cysteine rich cationic peptide with molecular mass 3-5k. Defensins are large members of antimicrobial peptide. Defensins are categorized by β-sheet structure linked by three disulfide bonds, formed specifically by six cysteine residues[82]. Defensins have been mainly classified into two classes, α- and β-defensins **(Fig1.6).** There is one more new defensin class recently identified in rhesus macaque leukocytes and named as θ-defensins. Classifications of defensins are solely based on linking pattern of cysteine residues. α-defensins composed of 29–35 amino acids in length with disulphide bond between the cysteine arrangement has been identified as C1–C6, C2–C4 and C3–C5. On the other hand β-defensins are 38–42 amino acids chain length with the disulfide linkage at cysteine residues at C1–C5, C2–C4 and C3–C6[82, 83]. The α-defensins (HNP1-4) are expressed and concentrated in granules of neutrophils (PMNs) as well as non granulocytes which include monocytes and lymphocytes. Whereas, HD-5 and HD-6 are expressed in Paneth cells of the small intestine. The β-defensins on the other hand are

expressed and secreted by mucosal surface epithelia, including cell populations found in the eye, skin, oral mucosa, urogenital and respiratory systems[84].

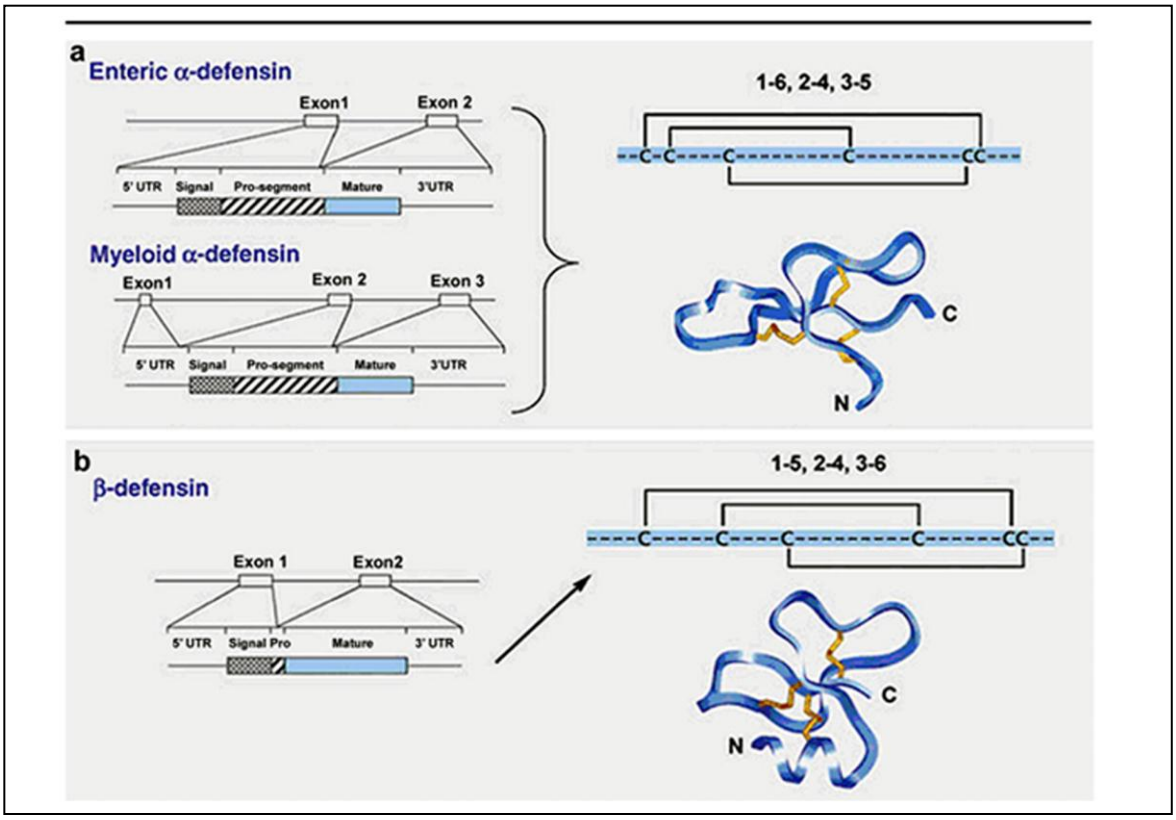
Defensins are generally produced in inactive pre-proform. Thereby to achieve the active form or mature peptide pre-prodefensins undergoes post translational modification with removal of pre and pro sequences **(Fig1.6)**. The pro-sequence is a signal peptide which is being cleaved in Golgi apparatus. Once the removal of pro-sequence is achieved HNP1-4 concentrate and resides in PMN granules. In contrast with other HNPs, HD-5 resides as proform in ileal mucosa and it is proteolytically processed by pancreatic trypsin to mature HD-5 after secretion[85] **(Fig1.7)**.

More than 28 genes have been found for human ß defensin gene on the basis of computational search. However till date only few are known to us. Human beta defensins (hBD) 1-4 are most studied. They are expressed in epithelial and mucosal tissue and are constitutive/or inducible in expression in response to certain stimulus[84]. The anatomical distributions of beta defensin are very significant and are responsible for clearing out infection/pathogen from the site. For example hBD-1-4 are expressed in respiratory tract, urogenital as well as intestinal epithelium with constitutive expression of hBD-1 and inducible expression of hBD-2-4 in response to infection/inflammation. hBD-2 is highly expressed in lung; hBD3 is expressed in skin and tonsillar tissue where as hBD4 is expresses in testes and epididymis and some immune cells. Expression of hBD-1 is constitutive, however hBD-2-4 are expressed in response to bacteria or bacterial products. In keratinocytes hBD-2 expression is induced LPS and other bacterial epitopes along with various pro-inflammatory cytokines TNF or IL-1ß. hBD3 and hBD4 is induced with TNF, interferons (IFN-Y)LPS or phorbolmyristate acetates and TLR ligands. hBD-1 gene does not contain any regulatory transcription factor binding sites like NF-κB and results in constitutive expression whereas hBD-2-3 contains site for NF-κB as well as other transcription factor like AP-1 and STAT-3. hBD4 contains NF-κB. The synthesis of these genes are highly regulated under activation and binding of these transcription factor[86-90].



Adopted from Ganz et. al 2003

Fig. 1.6. Amino acids sequences of human defensins



Adopted from Linda et al,; 2011

Fig 1.7: Structure and alignment of alpha and beta defensin gene and peptide

1.7.1 ANTIMICROBIAL ACTIVITIES OF BETA DEFENSINS

There are considerable number of reports which suggest antimicrobial activity of defensins against bacteria, fungi, viruses and parasites which act as a part of early host defence mechanism system. Ericksen et al. showed antibacterial properties of alpha defensins (HNPs) against Gram positive bacteria *S. aureus* and Gram negative *E. coli*. Beta defensins (hBD) 1-4 have strong microbicidal activity against many reported strains. hBD-2 preferably is highly active against gram negative bacteria for example *P. aeruginosa* than gram positive strains[84]. For antiviral activities there are reports that alpha defensin are active human immunodeficiency virus (HIV) and herpes simplex virus (HSV) infections [91]. Also it has been reported that hBD (2-3) are highly expressed in HIV positive individuals. Mechanisms are still unclear. Including this intestinal defensins HD-5 and HNP posses significant parasiticidal activity against *Toxoplasma gondii* and *Trypanosoma cruzi* (Madison et al.2007), respectively. Also it has been reported that HBD1 and HBD2 peptides showed microbicidal activity against *Cryptosporidium parvum* [92 93].

Mechanisms for defensin mediated antimicrobial activity are not completely known. However different model have been proposed. First is carpet model which described via opsonisation of the antimicrobial peptide mediated process on the surface of the pathogen and ultimately brings necrosis by disturbing the charges on the membrane. Second is pore model where the antimicrobial peptide forms pore in membrane by oligomerization of the peptide complex ultimately leads efflux of nutrients and important ions[94, 95].

1.7.2 ß-DEFENSINS IMMUNE MODULATORY ACTIVITY

Role of defensins in pro-inflammatory response and as well as recently in immunosupression is being investigated. It has been reported that ß defensins can attract/recruit immature dendritic cells (DCs) as well as memory T cells to the localised site of inflammation/infection and thus connects the link between innate and adaptive immunity. Mechanisms proposed for this is involved by chemokine receptor CCR6 expressed on TH $_{17}$cells which promotes chemotractant function of ß defensins. Along with this TH_{17} cytokines IL-22 and IL-17 induces expression of beta defensin in various cells including keratinocytes. In addition to this defensin recruitment of monocytes and macrophages to the site, where these cells lack CCR6, employs via CCR2 receptors present on the cells for hBD-2 as well as hBD-3[96-98].

hBDs signals not only through chemokine receptor but also via TLRs. hBD-2 act as a ligand for TLR-4 present on immature dendritic cells which results in maturation of DCs as well as helps in proliferation and maturation and survival $CD4^+$ T cells.

1.7.3 EPITHELIAL IMMUNITY AGAINST PA INFECTION.

Epithelial Innate Immunity plays a significant role in host defense against *Pseduomonas* infections [99]. Antimicrobial peptides constitute an important component of mammalian epithelial immunity. Whereas alpha defensins are found in polymorphonuclear leukocytes and paneth cells of small intestine, beta defensins are produced in all major epithelial cells. The epithelial cells of the respiratory tract, skin and the intestine contain an array of innate immune sensors like Toll Like Receptors (TLRs). Upon recognition of Pathogen Associated Molecular Patterns (PAMPs) of bacterial like *Pseudomonas*, the TRLs engage the NFkB mediated induction of human beta defensin-2 (hBD-2) [100]. An abundance of reports strongly support the important role of defensins like hBD-2 against *Pseudomonas* in various infections eiher independently or in tandem with antibiotics [101]. Ongoing work indicates that specific homorserine lactone QS molecules of *Pseudomonas aeruginosa* are recognized by host innate immunity [102, 103].

Among beta defensin family hBD-2 is remarkable in its way, that it is one of the strongest inducible AMP in response to various stimuli and thus helps maintaining host defence by eliciting strong antimicrobial response against pathogens.

However it is not known if multiple drug resistance in *Pseduomonas*, specifically against peptide antibiotics, result in concurrent resistance against host defensins. Many lines of arguments support this possibility. First, biofilm derived Extracellular Polymeric Substances (EPS) are known to quench or deter antimicrobial agents penetrating the EPS barrier and killing embedded bacteria [104]. This indicates strong biofilm forming strains and/or chronic infections and/or multiple insults from mature biofilms indirectly associated with the host (e.g. medical devises like catheters) may result in compromised defensin axis. Second, host genetic factors like SNPs in defensin genes may reduce innate host defence [105]. Although there are sufficient literatures to support the critical importance of researching this area, there is very little direct work exploring relationships of defensins against drug resistant *Pseduomonas* biofilms. Further the relationship of QS metabolites from *Pseudomonas* biofilm modulating the axis of defensin is still unknown.

Keeping all these facts in mind the inherent motivation of the program has been set to investigate and expand current knowledge in the following areas:

Objectives:

1: Identify and characterize Microbial Quorum Signalling molecules using Mass Spectrometry based untargeted metabolomics platforms.

2: Screen QS molecules with cross kingdom signalling functions with specific emphasis on the following mammalian determinants (a) Peroxisome proliferator-activated receptor (PPAR) gamma nuclear receptor (b) Hypoxia-inducible factor 1 (HIF-1) (c) Human beta-defensins.

3: Investigate the outcome of bacterial cross kingdom (quorum) signalling in human diseases.

Section 2

MATERIALS AND METHODS

Materials and Methods

Chemicals and Reagents

All the reagents and chemicals were acquired from Sigma-Aldrich (Millipore-Sigma Ltd., India) unless otherwise mentioned. The purified quorum signalling standards including the N-acyl-homoserine lactones: N-butyryl-DL-HSL, N-hexanoyl-DL-HSL, N-heptanoyl-DL-HSL, N-octanoyl-DL-HSL, N-decanoyl-DL-HSL, N-dodecanoyl-DL-HSL, N-tetradecanoyl-DL-HSL, N-3-oxo-hexanoyl-DL-HSL, N-3-oxo-octanoyl-DL-HSL, N-(3-Oxododecanoyl)-L-HSL and the quinolone standards, 2-Heptyl-4-quinolone (HHQ) and 2-Heptyl-3-hydroxy-4(1H)-quinolone (PQS) were all procured from Sigma Aldrich. The 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO) was procured from Santa Cruz Biotechnology, USA. Stock solutions (0.5 M) of all standards were made in ethyl acetate acidified with 0.5% acetic acid and stored in -80°C before use.

All solvents for mass spectrometry including HPLC or MS grade methanol, acetonitrile and ethyl acetate were accquired from Thermo Fisher Scientific, USA. Ultra pure water (18 MΩ) was generated from a Milli-Q system (Merck-Millipore, USA). The MALDI matrices used for analyses were α-cyano-4-hydroxycinnamic acid (CHCA ; 5 mg/mL) and 2,5-dihydroxybenzoic (DHB ; 150 mg/mL). Saturated stock solutions of both matrices were prepared fresh in 70% acetonitrile in water containing 0.1% Trifluoroacetic acid (TFA). The SBA-15 mesoporous silica used for the mass spectrometry was developed using a proprietary process in house.

The cell culture plasticware were obtained from Corning Inc. (USA). TRIzol Reagent, Halt-protease inhibitor, Turbofect transfection reagent (R0532) was bought from Invitrogen™ Thermo Fisher Scientific. Phusion® High-Fidelity DNA Polymerase and Restriction enzymes KpnI and HindIII were procured from New England Biolabs (NEB). DNA Ladders, PageRuler Prestained Protein Ladder were obtained from ThermoFisher Scientific. TMB/H2O2 Reagent for ELISA was obtained from Merck Millipore. HRP-conjugated anti-IgG was purchased from Sigma Aldrich. Sep-Pak C18 Classic Cartridge was procured from Waters. Biochemical kits used are listed in Table 1, Antibodies used are listed in Table 2 and all the primers are listed in Table 3.

Dried droplet method

Bacterial strains and growth conditions

Pseudomonas aeruginosa ATCC PAO1 was a kind gift from Dr. Santasabuj s National Institute of Cholera and Enteric Diseases (NICED), Kolkata and *Pseudomonas*

aureofaciens was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. The *Pseudomonas aeruginosa* LasI/RhII-/- double mutant was a kind gift from Dr. Subhadeep Chatterjee, Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India. The clinical strains of *P. aeruginosa* used in these analyses were isolated from extubated and rejected endotracheal tubes (ETT) from patients of ventilator associated pneumonia (VAP) during routine course of treatment. The latter and related clinical samples were collected and processed in collaboration with Prof. Rakesh Lodha, Department of Paediatrics, All India Institute of Medical Sciences, New Delhi, in accordance to institutional ethical clearance (IEC 62/03.03.2017; RP-20/2017). All the strains were cultured in Tryptic Soy Broth (Hi-Media, India) at 37° C and stored in glycerol stocks at - 80° C. All bacterial cultures were carried out in a Biological Safety Level - II (BSL-II) laboratory in compliance with Institutional Biosafety Committee permissions (IBSC/JNU/2014/1/7).

Table 1: Biochemical kits

Kit details	**Company**	**Catalogue No.**
Genomic DNA extraction kit	RBC	YGB100
Plasmid DNA extraction kit	Promega	A1223
Gel elution kit	Promega	A9281
Dual luciferase assay kit	Promega	E1910
PowerUp SYBR Green	Applied Biosystem, ThermoFisher Scientific	A25742
High capacity cDNA reverse transcriptase kit	Applied Biosystem, ThermoFisher Scientific	4367659
Live/dead™ BacLight Bacterial Viability Assay™	Invitrogen, ThermoFisher Scientific	L7007
CellTracker™ Green CMFDA	Invitrogen, ThermoFisher Scientific	C7025

Table2: Antibodies list

Antibodies details	Company	Catalogue No.
hBD-2	Abcam	ab66072
p65	Santa Cruz	sc372
Phospho p65	Cell Signaling Technology	3033
Phospho Ikßα	Cell Signaling Technology	2859
PPARϒ	Santa Cruz	sc-7273
Hifα	Novus Biologicals	NB100-105
Actin	Cell Signaling Technology	49705
GAPDH	Cell Signaling Technology	21185

Table 3: Primer List

Primers	Company	Sequence
hBD-2 promoter	IDT	5'GGATGGTACCCAGTACAGCAGCAGTGATAG3' 5'TTCGAAGCTTGGGGAGGACATCAAGCCTT3'
hBD-2	IDT	5'TTCCAGGTGTTTTTGGTGGT3' 5'GAGACCACAGGTGCCAATTT3'
hBD-1	IDT	5'CGCCATGAGAACTTCCTACC3' 5'ACAGGTGCCTTGAATTTTGG3'
PPARϒ	IDT	5'TTCAGAAATGCCTTGCAGTG3' 5'CACCTCTTTGCTCTGCTCCT3'
mBD3	IDT	5'TCAGATTGGCAGTTGTGGAG 3' 5'GCTAGGGAGCACTTGTTTGC 3'
Actin	IDT	5'TGCATTGTTACAGGAAGTCC3' 5'ATGCTATCACCTCCCCTGTG 3'
GAPDH	IDT	5'GATGCTGGCGCTGAGTACGTCGTG3' 5'CCAGTAGAGGCAGGGATGATGTTCTG3'

Mass spectrometry

SBA-15 Silica based LDI-MS and MALDI

Mass Spectrometric studies on the samples were carried out on either AB Sciex MALDI-TOF/TOF 5800 MS (Sciex, USA) or a SYNAPT G2 LC MS (Waters, USA). The former is equipped with a 355 nm Nd:YAG laser. The accelerating voltage was set to 20 kV in the positive ion mode and the laser intensity was adjusted to obtain better spectral resolution and signal-to-noise ratio. All mass spectral analysis was carried out in reflectron mode with 4000 shots/spectrum. ta analysis was performed using ta Explorer.

The SYNAPT LCMS system is equipped with 8 k Quad operated in V mode for increased sensitivity over a mass range of *m/z* 1-5000 and *m/z* 1-10000 depending on the sample requirements in positive polarity. A 355 nm Nd: YAG laser is fixed to the instrument and the laser firing rate was kept at 200 Hz. The data is evaluated using MassLynx software after the data acquisition is done. Calibration performed using DIOS small molecule mix provided by Waters, Part no: 186002819 for the region *m/z* 100-1500 and the errors were within ~3ppm. The trap collision energies were varied and optimized to obtain the optimum fragmentation patterns for all the analyte molecules.

Sample preparation for Mass Spectrometry. The N-Acyl homoserine lactones (HSL) stock solutions (2 mg/mL) were prepared in ethyl acetate. A mixture of HSLs is prepared by mixing equal volumes of the individual HSL stock solutions and is prepared fresh prior to each experiment. SBA-15 particles were dispersed in methanol (5 mg/mL) followed by sonication for a brief period. 2, 5-Dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid were prepared in acetonitrile/ trifluoroacetic acid (0.1 %, 50:50 v/v, 10 mg/mL). Stainless steel target plate was sonicated for short time in acetonitrile/methanol/dichloromethane mixture (1:1:1) followed by rinsing with MilliQ water several times and air dried. For LDI MS, the samples were prepared by mixing equal amounts of analyte with SBA-15-LiCl mix matrix and 1 µl of this solution is deposited on the wells of the target plate. The samples are allowed to dry in air. For MALDI-MS, 1 µl of the sample solution is directly deposited onto the target plate. After air drying, 1 µl of standard matrix solution was placed onto the analyte. For LC-MS sample was dried and reconstituted in 70%ACN/70%MeOH 0.1% TFA and 20µL of the sample was injected to LC.

Liquid chromatography–mass spectrometry (LC-MS)

The analyses for QS molecules were performed using methods described by Cataldi et al and Lepine et al with specific modifications[106]. The LCMS was carried out on a Waters Synapt G2 UPLC-qTOF-MS (Waters Corporation, USA) system with an online Waters ACQUITY® UPLC system equipped with binary solvent delivery system and autosampler. The operating software was Masslynx® (Synapt, version 4.1 Waters, USA).

The UPLC used the Waters reversed phase UPLC BEH C18 column 2.1 mm × 100 mm, 1.7µm) with a Van Guard UPLC Cartridge The mobile phases were (A) 0.1% TFA in water and (B) 0.1% TFA in ACN. The UPLC elution conditions were optimized as

follows: linear gradient from 5 to 15% B (0–1 min), 15 to 35% B (1–10 min), 35 to 42% B (10–14 min) and 42 to 80% B (14–24 min). The flow rate was set at 0.5 ml/min. The column and autosampler were maintained at 40 and 8° C, respectively. Each wash cycle consisted of 200 µl of strong solvent (80% ACN) and 600 µl of weak solvent (30% ACN). The injection volume of 1 µl was used for the reference standards and samples. The chromatographic data were processed using TargetLynx Application Manager of MassLynx™ 4.1

The mass spectrometer used electrospray ionisation (ESI) with capillary voltage of 4 kV for positive mode; cone voltage 30 V; source temperature 130 °C; desolvation temperature 400°C; cone gas flow 50 L/h and desolvation gas flow 900 L/h. The scan range was m/z 100 –1500 . with resolution at 20,000 and accuracy within 1ppm Continuous calibration was carried out with the LockSpray™ system and leucine-enkephalin (200 pg/µl infused at 20 µl/min) $[M+H]^+$ ion as a reference lock mass (m/z 556.2771).

Extracted ion chromatograms (EICs) were generated for analytes by a 0.1 m chromatogram mass window on the expected m/z and mass resolution of 20,000. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the MassLynx™ 4.1 software. The calibration curve for oxo-C12 HSL and PQS were constructed by calculating the chromatographic peak area ratio of these standards at each concentration level. The accurate mass for precursor ions of oxo-C12 HSL or PQS and their corresponding product ions were analyzed using the MassLynx™ 4.1 software (Waters Co., Milford, USA). Centroided data were acquired from all peaks between 100 . to 1500 . for each injection. Linear regression analyses of these were based on three independent replicates of each data point. The limit of detection (LOD) for each metabolite was determined at the chromatography peak area compared to the blank sample with S:N > 3. The lower limit of quantification (LLOQ) was defined as the lowest determined concentration of the calibration curve.

Characterization of Pseudomonas quinolone isomers C7-PQS and HQNO by Synapt G2 HDMS System-Traveling Wave Ion Mobility Spectrometry

Purified standards of C7-PQS and HQNO (25µM) in 70% methanol 0.1%TFA or ethyl acetate extracts of *Pseudomonas aeruginosa* biofilm supernatants reconstituted in the 70%MeOH and 0.1%TFA were injected into UPLC and introduced into the ESI source

maintained at 3.0 KV. Nitrogen and Helium gas was used as the drift gas at 90 mL/min. The TWIMS cell in this study was operated at nominally 3 mbar N2 with a 40 V, 900 m/s T-Wave. The Masslynx 4.1 (Waters Corporation, Milford, MA, USA) was used to collect and process all the data.

Culture of *Pseudomonas sp.* biofilms *in vitro*

All strains of *Pseudomonas aeruginosa* and other *Pseudomonas sp.* were grown from glycerol stocks in 100 mL Brain Heart infusion medium (BHI) (HiMedia, India) in a closed microbiology shaker at 37° C overnight. After overnight growth, culture was centrifuged @ 5000 rpm for 10 min at 4 ºC and inculcated in modified M9 growth medium (Himedia, India). The media contained: 47.7 mM $Na_2HPO_4 \cdot 7H_2O$, 21.7 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, and 0.5% (wt/vol) Casamino Acids. Stock solutions of glucose (1M) $FeCl_2$ (100 mM) and $MgSO_4$ (100 mM) were filter sterilized and added to final concentrations of 11.1 100 µM and 1 mM, respectively to the autoclaved media. Whenever required, antibiotics were added in accordance to sensitivity assays. Biofilms were cultured using several systems, including static models using 96 well polystyrene tissue culture plates for quantitative assays using crystal violet[107]. For larger static cultures biofilms were grown in T-175 tissue culture flask (Corning USA). For quorum sensing metabolites assays, the biofilms were growth either in modified CDC bioreactor bottle(s) on polyurethane foam [108]or on fluidized bed bioreactor using polystyrene beads (Symbio Scientific, India) (vissagio) with some modifications. Sterile corona treated polystyrene beads were incubated with planktonic bacteria in M9 media (10^6 CFU/ mL) for 2 h for adherence. The beads were gently washed and added to 50 mL fresh media in a 100 mL flat glass bioreactor bottle. The system was equipped with two discrete ports for influent and effluent media and monitoring pH and temperature. The system was set on a shaker capable of 3 dimensional motion (*ncing shaker*™, Tarson, India) and incubated at 37° C. Effluent media and beads were collected at predetermined periods (12 h, 24 h, 48 h and further 72 h respectively). All experiments were carried out in BSL-II containment.

Extraction of homoserine lactone and cell culture conditions:

Acyl homoserine was purified from *P. aeruginosa* strains according to previous protocol with modifications[109]. Briefly, biofilm culture supernatant along with biofilm cells (using cells scraper) from 150mL culture was aspirated at determined time. To this supernatant

equal volume of -80 ^{0}C chilled acidified ethyl acetate was added and mixed vigorously for 1-2 h in 250 mL separating flask (Schott Duran) in cold. The extract was left on a stand for complete phase separation for 10-15 min at 4 ºC. The clear organic phase was collected and centrifuged at 15,000 rpm for 20 min at 4 ºC to remove cell debris or any macroparticles. The extract was then dried and concentrated in speed vac (preferred temperature 4 ºC) and reconstituted in 100uL of fresh ethyl acetate. For LDI-MS 1µL of the sample was directly spotted on MALDI plates. For LC-MS the extracts was dried completely and reconstituted in 70% Methanol 0.1% TFA. 20ul of the sample was injected for LCMS. Concentration of HSL was determined by relative quantitation using Mass Lynx (Waters). Typical concentration of HSL in the supernatants sample after extraction was found to be 600µM. HSL was frozen in aliquots at 80ºC after extraction and purification for further use.

Electron microscopy analyses.

Scanning electron microscopy was carried out according to the protocol described by Gil-Perotin *et al.* with some modifications[110]. Small sections (0.25 cm^2 ; tracheal ends and curve) of fresh ETT from patients or bioreactors were excised using titanium blades in a biosafety cabinet at 70% RH. Sections were fixed with 2·5% glutaraldehyde in 0·1 mol L^{-1} cacodylate buffer (pH 7·2) for 1 h. The latter were immediately post-fixed with 1% osmium tetroxide and 0·25% ruthenium red (0.22 µM filtered) in cacodylate buffer for 1 h at 4° C. The sections were dehydrated in graded alcohol series finishing with 100% acetone, mounted on aluminium stubs by carbon tape and sputter-coated with gold. Analyses were performed on a *Zeiss EVO40* Scanning Electron Microscope (*Zeiss, USA)* at 20 kV.Transmission electron microscopy of *P. aeruginosa* was perfomed according to the protocol described by Hartmann *et al.* with some modifications[111]. Bacterial samples were adsorbed on carbon coated copper grids, fixed with 2% glutaraldehyde and post-fixed with 1% osmium tetroxide. These were stained with 3% uranyl acetate and observed by *JEM 2100F* transmission electron microscope (JEOL, Japan) at 200 kV.

Cell Culture

The human lung epithelial cells A549 (ATCC CCL-185), human intestinal epithelial cells (HT-29), human corneal epithelial cells (HCEC) were cultured in Dulbecco Modified Eagle'sMedium (DMEM) high glucose (Sigma) and DMEM : Ham's F12 (1:1) medium (Gibco) with 1% penicillin, streptomycin, amphotericin solution (Hi-media) and supplemented with 10% fetal bovine serum (Gibco Invitrogen, USA) at 37°C; 95v/v% air with 5% CO_2. All experiments were carried out at 70-80% cell confluency. Before any treatment cells were kept in serum-free condition for 6h.

SDS PAGE Immunoblot analysis

Stimulated and unstimulated cells were washed in ice cold PBS. Cell lysates were prepared in RIPA lysis buffer and 1X protease inhibitor cocktail (Thermofisher Scientific) incubated in 2X laemmli buffer at 37°C for 15 min. Protein estimation was done with help of Bio-Rad assay (Sigma) kit. Equal amounts (50µg) of proteins from cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes (MerckMillipore). After blocking with 5% slimmed milk, the membranes were then probed with anti p65 (1:2000), Phospho p65 (1:1000), Phospo Ikß (1:1000), PPARϒ (1:500), Actin (1:2000), GAPDH (1:2000) overnight at 4°C. Then horse radish peroxidase- conjugated secondary antibodies (1:10000) were added for 1hr at RT. The protein bands were visualized using Super signal West Pico ECL (Thermofisher Scientific) detection method after exposure to X-Ray film.

Extraction of hBD2 and AU PAGE western blot analysis:

hBD-2 from the culture supernatant/condition media was extracted according to the previous protocol Ghosh et al. with slight modifications. Cationic protein in the stimulated medium was first adsorbed to a carboxy-methyl (CM matrix (Bio-Rad, Hercules, CA; 50% slurry in 25 mM ammonium acetate, pH 6.4) at a ratio of 15:1. After overnight incubation under constant shaking, CM matrix was washed 2 times in 25mM ammonium acetate buffer and eluted from the matrix with 10% acetic acid. Cationic eluted material was then lypholized and rsuspended in AU PAGE loading dye and subjected to 15% AU PAGE run. Culture supernatants from approximately 2×10^7 cells were used for the extraction. For equal loading equal amount of the extract was loaded on to the gel. Protein was transferred on 0.22µ PVDF membrane, fixed in formalin vapour blocked for 1hr in 5% skimmed milk and probed with monoclonal anti hBD-2 (1:2000) dilutions for 18h overnight at 4°C. The membrane was washed with 1%BSA in PBST

buffer and detected using HRP conjugated goat anti-mouse IgG (1:5000). Blots were developed using Super signal West Femto Chemiluminescent substrate (Thermo). Recombinant hBD-2 peptide was used for calibration control.

Immunocytochemistry:

A549 cells were grown on coverslips and treated with HSL according to pre determined concentration for hBD-2 induction. Cells were then fixed, permeabilized and blocked according to protocol as mentioned earlier (Reference) with slight modification. Cells were then probed with 1:100 dilution of anti hBD-2 and anti hBD-1 and anti HIF-1α (1:500) for overnight. Detection was performed with 1:500 dilution of Alexa fluor 488 Anti mouse IgG. Cells were then stained with DAPI and images were taken with the help of Confocal Microscope (Nikon).

Quantitative PCR: After stimulation of cells with HSL, total cellular RNA was extracted using Trizol. RNA was quantified and verified using nanodrop spectrophotometer. Total 1µg of RNA was reversed transcribed to cDNA in 20uL reaction using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to the manufacturer's instructions. Real-time PCR was performed using a Applied Biosystems® Power SYBR® Green Master Mix with 20ng of RNA correspond to cDNA template and 250 nM of hBD-2, hBD-1, PPARY and actin primer. Primer sets are listed in Table no.3. The thermal cycling conditions were 95˚C for 10 min, 95˚C for 15 s, 60˚C for 15 s, and 72˚C for 15 s and final extension 72 for 1min, repeated 40 times on AB Applied Biosystems® 7500 Real-Time PCR. To examine the identity of the PCR product melting curve was regularly check for single peak. mRNA expression was normalized to levels of beta actin and relative expression was determine by ratio of target gene expression and housekeeping gene expression using the comparative threshold cycle (ΔΔCt).

Preparation of nuclear extracts and EMSA: (NII INPUT)

HSL treated and untreated cells for indicated period of time were harvested and nuclear lysate was prepared according to the manufacturer's protocol (GE Biosciences). Protein concentration was determined by Bradford assay. The EMSA was performed according to protocol as described by Kim, J. et al. with modifications and manufacturer's instructions (Promega). Briefly, 10ug of nuclear extract was incubated with $Y^{32}P$-labelled oligonucleotide probe 5'-AGTTGAGGGGACTTTCCCAGGC3- 3' for NF-κB binding site for 30min. Followed by incubation; DNA was resolved on 5% polyacrylamide gels. For NF-κB p65 binding identification supershift assay was done as described earlier. EMSA was performed using Anti p65 (Rel A) antibody.

hBD2 ELISA

To detect the presence of hBD-2 in culture supernatant of A549 cells, a sensitive hBD-2 sandwich ELISA was performed. 96 well immunoplates (Nunc) were coated with 100µL of Rabbit anti-hBD-2 antibody (Abcam; 1:2000) at 4°C for overnight. After incubation plates were blocked with 2% BSA in PBS for 1 hour at room temperature. Subsequently plates were washed thrice with PBST (0.1% Tween20). Serial dilution of 100µL culture supernatant was added and incubated for 18h at room temperature. After three washes with PBST (0.1% Tween20), 100µL of mouse anti-hBD-2 antibody (1:2000) was added and incubated for 2 h at room temperature. After three washes, 100 µL of Goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Sigma, USA), diluted to 1:10,000 in 1XPBST was added for 1 hr at room temperature. Plates were again washed thrice and was developed using TMB/H2O2 for ELISA (Merck) for 20 min at room temperature in the dark. Reaction was stopped using 2M sulphuric acid. The absorbance was read at 450nm using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific). Standard curve was prepared using known concentration of recombinant hBD-2 peptide.

Cloning of hBD2 promoter region in pGL3 basic promoter less vector

The human hBD-2 promoter region of 2338 bp from the start codon was PCR-amplified from genomic DNA using following primers: forward, 5'GGATGGTACCCAGTACAGCAGCAGTGATAG3'and reverse 5'TTCGAAGCTTGGGGAGGACATCAAGCCTT3'. PCR-amplified product was cloned upstream to luciferase in pGL3 basic control vector between HindIII and KpnI restriction sites and the clone was confirmed by sequencing.

Transient transfection and dual luciferase assay

A549 cells seeded in 6-well plate were co-transfected with pGL3 containing hBD-2 promoter and pRL-TK plasmids (as internal control) using Turbofect^{TM} (Thermo scientific) transfection reagent. After 4h of transfection, serum recovery was done for additional 12h and then treated with HSL at different concentration for 6h in serum free medium. Luciferase assay was performed with dual luciferase reporter system (Promega) using luminometer. The transfection efficiency was normalized by Renilla luciferase.

Co-culture model for *P. aeruginosaP. aeruginosa* and lung epithelial cells and Antimicrobial Assay. The co-culture method was followed according to previous protocol as described by Cheryl J. Hertz et. al with slight modifications. Human lung

epithelial cell line A549 at 2.5 x 10^5 cells/ml were seeded in 24 mm diameter cell culture inserts (0.4µm pore polyester) in 6 well Transwell™ plates (Corning, USA) which does not allow bacterial migration between chambers. At constant trans-epithelial resistance (TER) ~800 Ohms, the cultures were activated by 3-oxo-C12 HSL (50-150µg/mL) for 24 h. Induction was confirmed by IL-8 ELISA of the basolateral medium. Bacteria were grown for overnight to mid log phase in Tryptic Soy broth and washed with sterile PBS. Bacteria were diluted to 5-10×10^3 C.F.U and 5-10 ×10^5 C.F.U (calculations based on O.D 600). 5µL of the inoculum which co-relates to 25-50 C.F.U was added to the apical compartment. The Transwell system was further incubated for 6h. Following incubation cells were washed thrice with 1XPBS and incubated with ceftazidime and gentamicin for an hr. Following incubation serial dilutions of the both internalized bacteria and bacteria in wash was plated. TSB plates were incubated for further 24h @37ºC and C.F.U was counted.

Gene Silencing: Gene silencing was done using Lipofectamine transfection reagent according to manufacturer's protocol. A549 cells knock down assay was done in 6 well Transwell plate using 50nM of hBD-2 siRNA diluted in 1 mL DMEM antibiotic free media. 24 h following transfection cells were induced with HSL with predetermine concentration for 24h. After induction cells were infected with PA for co-culture assay as described earlier. hBD-2 gene knock down was confirmed with RT PCR.. siRNA were synthesized by Santa Cruz. For control non targeting siRNA was used.

Microscopic evaluation of viabilities of *P. aeruginosa* biofilms

P. aeruginosa biofilm viability against Beta Defensin2 were analyzed using the *LIVE/DEAD BacLight Bacterial Viability Assay Kit*™(Invitrogen, USA) in accordance to manufacturer's instructions. Briefly, *PA* biofilms were grown on coverslips for 24h in 1:10 diluted TSB media and then charged with recombinant hBD-2 peptide 25ng/uL or induced basolateral medium from co-culture set for 8h. Following incubation biofilms were harvested, stained and subjected to microscopy. The kit reagents SYTO 9 and propidium iodide were diluted 1:40 (v/v) in deionized water and mixed 1:1 (v/v) for the working stock. Bacterial samples or biofilms samples were washed in 1XPBS and incubated with stock for 15 min in dark before viewing samples. Images were acquired on Olympus FV1000 confocal microscope (Olympus Corporation, Japan) with Kr/Ar laser using excitation at 488 nm or 594 nm and emission filters at 522–535 nm or 605–632 nm.

Animal Experiments

Studies on modulation of murine immunity by cross kingdom signalling mediated by *P. aeruginosa* quorum sensing molecules were conducted in collaboration with Prof. Durbaka Vijaya Raghava Prasad, Department of Microbiology, Yogi Vemana University College, Kadapa, Andhra Pradesh, India. Animal experiments on the C57BL/6 mouse model were performed in the Animal Research Facility in Yogi Vemana University College, in accordance to protocol (YVU/IAEC/DVR/12/1-15) approved by the Institutional Animal Ethics Committee (IAEC Reg. #1841/GO/Re/S/15/CPCSEA). The experimental design was based on protocol described by Lazenby et al. with some modifications. The experiments used minimum animal numbers required for statistical validity and animals were euthanized if signs of distress were detected. For the cross-kingdom signalling studies groups of four C57BL/6 mice (4 weeks old) were mildly anesthetized and 40 µL of oxo-C-12 HSL (150 µM), TNFα (10ng/mL) , non pathogenic *Pseudomonas aureofaciens* and clinical *P. aeruginosa* strain (1×10^3)C.F.U/mL was inoculated intra nasally. The process was repeated thrice over 12 h. The mice were housed under water and food ad libitum and euthanized after 24h. The trachea was isolated and subjected to further analyses for defensin (mouse beta defensin) expression.

Statistical analysis: All experiments were performed at least three times with similar results. ta for the quantitative real time PCR are presented as the mean ± standard deviation (SD). ta for the ELISA are presented as the mean ± standard error of the mean (SEM). Images were processed using Photoshop and Western blots were quantitated using ImagJ (http://rsb.info.nih.gov).

Section 3

CHAPTER I

INTRODUCTION

Quorum sensing in biofilms a term applied to a series of events by which planktonic bacteria detect a critical bacterial density required for dedicating itself to sessile/biofilm growth mode. The most important event in this series is bacterial secretion and response to, small molecular weight chemical messengers that are called as **autoinducers**. These inducers are often called primordial hormones, since they act in the same principle of chemical signalling through specific receptors that initiate complex series of downstream events. These signalling molecules termed autoinducers controls virulence gene expression in numerous micro-organisms. Bacteria communicate/talk with each other with the help of the autoinducers by regulating the expression of several genes which is necessary for them to survive in the corresponding environment so that can form a complex community[20]. Recently it has been also reported that QS Autoinducer can also cross talk with human host and thus are capable of mediating a large scale cross kingdom signalling[26, 27, 44]. QS molecules are various types depending upon which types of bacteria are producing it. For example Gram negative bacteria example *Pseudomonas sp.* produce Acyl Homoserine Lactone and Quninoles on the other hand Gram positive bacteria produce Autoinducing peptide (AIP) and there are other species like Vibrio which produces AI-2 furanosyl borate etc.

Quorum sensing molecules secreted from gram negative bacterial population i.e. acetylated homoserine lactones (HSLs) and has been widely studied in *Pseudomonas aeruginosa*. The pathogens employ sophisticated quorum responsive communication network that can sense and respond to multiple environmental cues and modulate it's gene expression. *Pseudomonas aeruginosa* uses at least two classes of QS systems for communications: the N-acylhomoserine lactone family including N-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C12-HSL) and the alkylquinoline family represented by 4-hydroxy-2-heptylquinoline (HHQ) and the corresponding dihydroxylated derivatives, such as 2-heptyl-3,4-dihydroxyquinoline (PQS, pseudomonas quinolone signal). There are at least thirteen different classes of HSLs; in part the diversity of HSL functions is contributed by the chemical variations in HSLs themselves[26, 27, 44, 112]. The chemical structure of HSLs is comprised of homoserine lactone moiety, which is derived from amino acid metabolism, linked to a variable acyl side chain, putatively derived from fatty acid synthesis. Whereas all HSLs have a conserved lactone moiety, it varies in the length of the acyl chain (C4 -C18), degrees of unsaturation at the C-7 or C-8 position and

oxidation at the 3 position. All HSLs are discrete gene products and many act as transcription factors. Receptors for HSLs include a number of transcriptional regulators called "R proteins," which function as DNA binding transcription factors or sensor kinases. The HSLs produced either freely diffuse or are pumped out of the cell depending on the length of the acyl side chain. With increasing growth and cell density, there is a simultaneous increase in the concentration of HSLs. At a certain critical threshold of HSL concentration, these HSLs bind to their respective regulatory proteins and initiate transcription of many proteins.[44, 70]

The analysis of QS molecules presents significant challenges. These molecules are small, often transient (chemically unstable) and secreted. Traditional methods include thin-layer-chromatography (TLC) based overlay assays using reporter bacteria [113]. Besides being labour intensive, these assays usually fail to detect global QS profiles since most reporters respond to only a narrow range of HSLs. Further, these platforms fail completely in identifying novel QS molecules. These problems can be largely overcome by chemical analytical platforms like Nuclear Magnetic Resonance (NMR) and Mass Spectrometry based platforms (GCMS or LCMS) which perform unbiased metabolomics analysis [74]. Now a day, QS autoinducers are largely determined exclusively on LC-MS platforms. The extremely robust and high-throughput nature of MALDI platforms has seen its global popularity in applications that require quick, effective analaysis. With the advances in MS/MS technology, MALDI can now routinely compete with best ESI platforms in accuracy and resolution. However it is difficult to apply MALDI in metabolomics research due to its noise generated by the matrix interference peak at <1000 m/z range (Saito, 2010). In this background, increasing interest has gained towards *matrix free* Laser Desorption and Ionization Mass Spectrometry (LDI-MS)[114, 115].

However till date matrix free LDI-MS platform has not been used for identification and detection of QS autoinducers. Thereby the inherent motivation of the objective is to analyse and detect autoinducer molecules by *matrix free* Laser Desorption Ionization Mass Spectromtery (LDI-MS) platform using a novel meso-porous silica based surface. Using the system on the AB-Sciex 5800 or the Waters Synapt (MALDI mode) can be analyzed for the *entire* range of bacterial autoinducers including homoserine lactones, quinolone, autoinducing peptide and AI-2 etc. MS/MS analysis on CID successfully allowed the confirmation of the respective identified autoinducer at picomolar concentration. This LDI-MS system therefore holds particular promise in single step,

high resolution analysis of bacterial Quorum sensing auotinducer molecule. Since it is clear now specific QS metabolite play role in host pathogenesis which suggest these molecules may also contribute to pathogenicity of emerging drug resistant *Pseudomonas.* However there are no systematic studies on profiling these metabolites in accordance to pathogenecity or drug resistance. Therefore species specific analysis of QS is very crucial in order to determine the *Pseudomonas* infection and novel mechanisms of pathobiology. Through this study, two potent QS molecule 3-oxo-C12HSL and C7PQS capable of mediating cross kingdom signaling functions in mammalian system has been screened and quantified in clinical strains of *P. aeruginosa*

The Extracellular secretory vesicles are produced by all living organisms from prokaryotes and eukaryotes[116-118]. This remarkably conserved mode of vesicular traffic allows all cells to modulate external environment by releasing clusters of molecular determinants for competitive advantage. Consequently, these secretory vesicles exhibit huge heterogeneity in size, cargo and functions. In bacteria, particularly the Gram – ve, these vesicles originate by external spherical bulges in the outer membrane (which justifies their name) and ultimately severalize into environment. The Gram-ve opportunistic pathogen *Pseudomonas aeruginosa* presents a classic example of OMV mediated communications. Significantly, many QS molecules contain variable alkyl chains and are hydrophobic in nature. Consequently these are largely trafficked via the OMVs. Therefore identification of these lipidic molecules in OMVs from infected samples or biofilm cultures hold promise in as novel diagnostic markers and mechanisms of pathobiology. Due to their lipid origin, all AQs are hydrophobic molecules; PQS is an order of magnitude more hydrophobic than the N-acylhomoserine lactone quorum signaling family and exclusively packaged in the OMV membrane [112, 119]. PQS is present in sputum and bronchoalveolar lavage (BAL) of chronic *P. aeruginosa* infections in cystic fibrosis [120, 121], ventilator-associated pneumonia (unpublished data), urinary tract infections [122] and other. The knowledge of HHQ and PQS implies that other OMV AQs may enjoy discrete, hitherto unknown functions and potential as biomarkers of specific diseases [123].

Analysis of OMV lipids and AQs have depended on biosensors [124], thin-layer chromatography (TLC) based reporter assays and liquid chromatography-mass spectrometry (LC-MS) [106, 125-127]. These assays require offline sample processing and are not adaptable for high throughput clinical analysis. Many vesicular metabolites and

membrane components are highly unstable when extracted. Metabolomics databases such as the METLIN do not cover all bacterial AQs since chemically pure standards are not available. However, native AQs are easily ionized and produce tandem mass signatures[106, 128]. In the second part of this objective, LDI-MS of HSLs and AQs is described by direct adsorption and ionization of OMVs on inert silica based ceramic ultrafiltration membranes. This technique enjoys several advantages. It is fast and offers targeted analysis of AQs on captured OMVs, concurrently filtering out soluble contaminants and ion-suppressing agents from cultures and bio-fluids. It is highly sensitive, capable of analysing picomolar quantities of AQs. Since it uses soft-ionization techniques, it produces negligible in source decay or fragmentation of native AQs. Since LDI-MS spectra predominantly contain singly charged AQs in either protonated, sodiated or potassiated forms, it is easy to interpret this data.

Results:

***P. aeruginosa* forms discrete biofilms and induces dramatic morphology modification on in-vitro in-dwelling device surfaces.**

P. aeruginosa form biofilms on several surface devices like fluidized bed bioreactors, polystyrene beads or polystyrene plate or Endotracheal tubes (ETTs) appeared in patches of multilayered structures of EPS embedded cells (**Fig. 3.1A).** High resolution electron microscopy of *Pseudomonas aeruginosa* biofilm determined highly developed three dimensional structures that aree clearly distinguished from their planktonic counterparts. The former when matured usually overlapped with mucus and EPS. In the assays *in vitro* the adherence of bacterial cells started within minutes (data not shown). hWithin 12 h bacterial cells start forming clusters within l endogenously secreted extracellular polymeric substances (EPS). . After 16 h of incubation, early biofilm formation started with cells embedding into the EPS matrix, shortening size and forming long interconnected mesh like structures. At this stage bacterial characteristics changed major motile/floating population to sessile forms (**Fig. 3.1B-C**). Higher magnification of the biofilm showed patches which often appeared as waves or ripple like patterns that was cultured *in vitro* (up to 48 h). Typical water channels and interspaced tower like structures containing EPS embedded cell clusters was observed (**Fig. 3.1D**). These spatial variations were largely absent in mature biofilms (>72 h), which appeared more homogeneous and isotropic. **Fig 3.E** showed crystal violet biofilm assay of PAO1 biofilms; lane 1 (E1 - E11) and gentamycin treated PAO1 biofilm (0 -150 µg/mL); lane 2 (E1-E11).

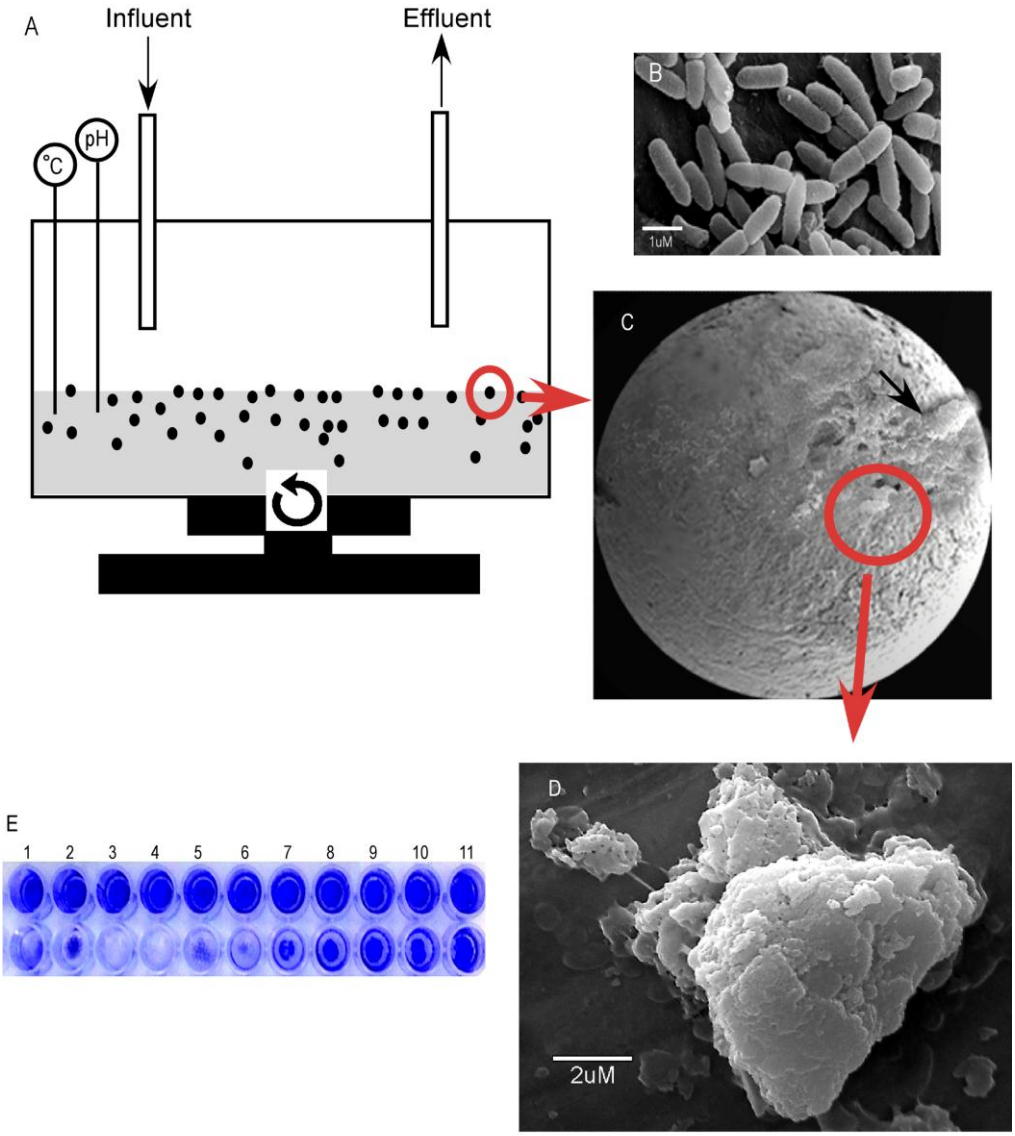


Fig. 3.1 Culture of *Pseudomonas sp.* biofilms *in vitro*.
Biofilms of *Pseudomonas sp.* with specific emphasis on *P. aeruginosa* strains were cultured in (A) fluidized bed bioreactors using (B) polystyrene beads (3 mm dia.) as support, in minimal media. Influent and effluent media were controlled via discrete ports and the system was incubated on 3D rotating shaker at 37° C. *P. aeruginosa* PAO1 and other clinical strains exhibited significant biofilm and transformation into (D) EPS-enclosed structures, which were different from (B) planktonic cells. Quantitative biofilm assays were carried out in (E) 96 well polystyrene plates using crystal violet staining Lane1: Control PAO1 biofilm Lane: 2 Gentamycin treated (0-150µg/mL) PAO1 biofilm in decreasing concentrations (E1-E11).

Discrete fragmentation pattern of representative *Pseudomonas* QS molecule 3-oxo-C12-HSL and C7-PQS using ESI-MS and MALDI-MS

3-oxo-C12 HSL and C7 PQS *Pseudomonas* pure QS molecule when subjected to MSMS using ESI-MS and MALDI-MS delivered different fragmented product ions. ESI-MS of 3-oxo-C12 HSL Precursor mass 298.20 (M+H) breaks down to product (signature) ion of lactone ring of m/z 102.1 and fragmented product of m/z 197.1 along with sodiated (M+Na) and potassiated peak (M+K) (**Fig. 3.2A**). Whereas MALDI-MS produced lactone ring of m/z 102.1 and along with this it also produced M-H_2O, m/z 280.19; M-CO, 270.1 and other fragmented product ions 80.0 and 198.1 (**Fig. 3.2B**). On the other hand ESI-MSMS of C7-PQS precursor mass (M+H) yields product (signature) ion of m/z 175.0 for Quinolone ring and fragmented ion of m/z 188.06 as reported by Lepine et al. The MALDI-MS produced quinolone ring, m/z 175.0 , also including M-H_2O, m/z 242.1; M-CO, m/z 232.19; M-CO_2, m/z 216.17; m/z 188.1 (**Fig3.2C-D**).

Global LC-MS profile of *P. aeruginosa* biofilm using Liquid Chromatography Mass Spectrometry (LC-MS)

To study the profile of QS molecules from *P. aeruginosa* biofilm sample was first subjected to routine and commonly used LC-MS technique. LC run of PAO1 biofilm detected the presence of all major HSL of *Pseudomonas* which includes C4HSL, C6HSL C8HSL, 3-oxo-C12 HSL, HHQ and C7PQS with respective elution time for 1.0, 3.3, 6.2, 4.8 min respectively. Sample was then run in MS mode and scanned for precursor ion mass (M+H). All the QS molecules precursor mass was detected with 20 ppm error, C4 HSL, m/z172. 0; C6 HSL, m/z 200.1; C8 HSL, m/z 228.1; 3-oxo-C12-HSL, m/z 298.2; HHQ, m/z 244.1; C7PQS, m/z 260.1. (**Fig 3.3**)

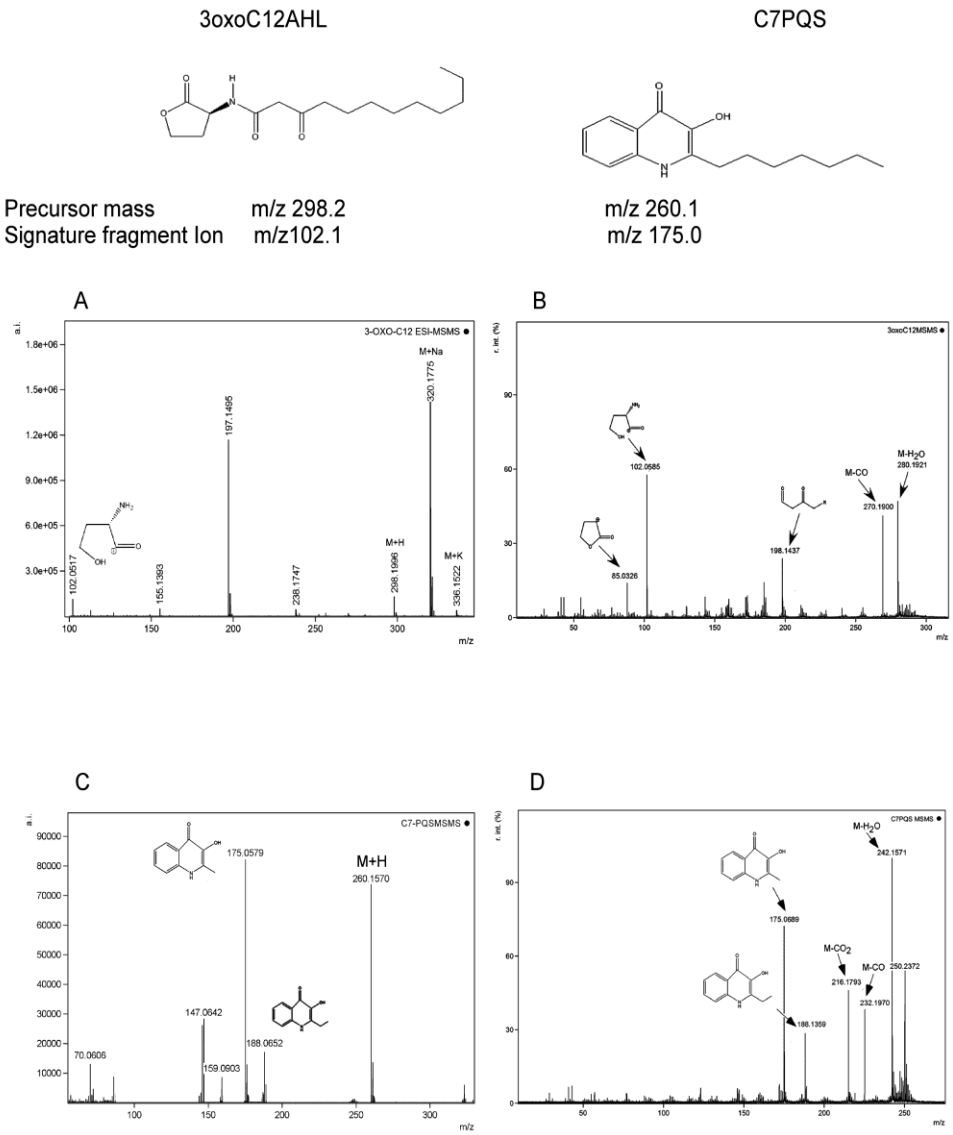


Fig 3.2 ESI and MALDI-MS/MS of representative QS molecules (3-oxo-C12-HSL and C7PQS). A. ESI-MSMS spectra of 3-oxo-C12 precursor mass (M+H) 298.2. B. MALDI-MSMS spectra of 3-oxo-C12 precursor mass. C. ESI-MSMS spectra C7 PQS precursor mass (M+H) 260.1. D. MALDI-MSMS spectra of C7 PQS precursor mass (M+H) 260.1.

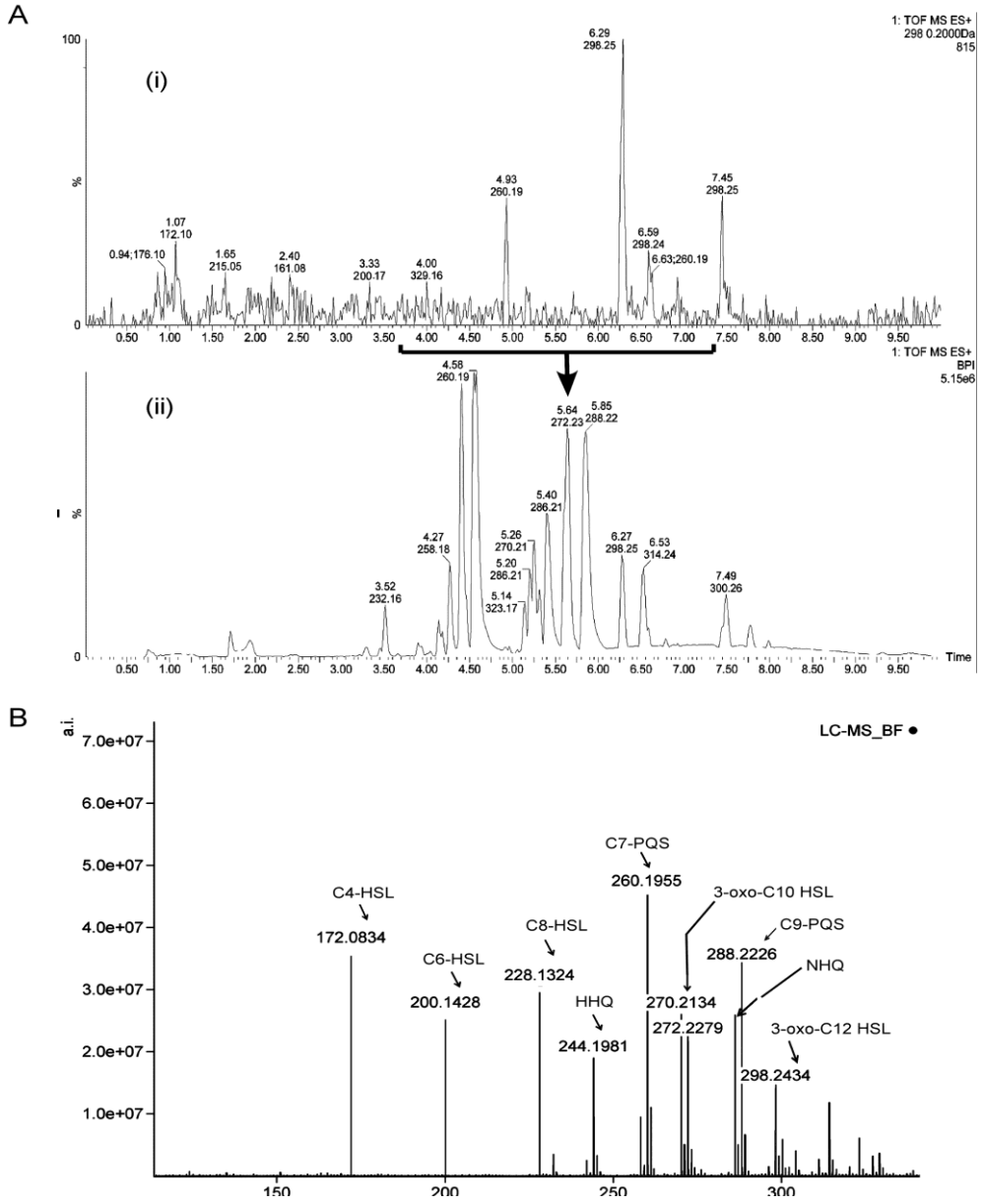


Fig 3.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of quorum signalling molecules in P. aeruginosa biofilms. (A)*P. aeruginosa* (PAO1) biofilm sample was extracted and reconstituted in MeOH 0.1%TFA and subjected to Liquid Chromatography run for 10min. C4HSL, C6HSL, 3-oxo-C12-HSL, HHQ and PQS was detected with retention time of 1.0, 3.3, 6.2, 4.8 min. (B) LC profile of the sample was acquired for precursor mass (M+H) scanning in MS mode and C4HSL, C6HSL, 3-oxo-C12-HSL, HHQ and PQS were detected within 20ppm error.

Comparison of Matrix free laser desorption ionisation mass spectrometry (LDI-MS) and MALDI-MS of *Pseudomonas sp.* QS molecules

In order to standardize the QS metabolites on matrix free MALDI platform, purified, chemically synthesized HSL molecules were used (**Table 4**) Samples were spotted on MALDI plates 3 spots per sample. Simultaneously in order to compare with conventional MALDI, HSL mix was also spotted with DHB. Spectra were acquired using 4000-5000 Hz laser per spot with delayed time extraction of 10 ns. LDI surface showed clean spectra and detection of all HSL mix molecules precursor mass (M+H) as shown in the table with higher intensities. Whereas, samples when spotted with DHB showed peaks with high noise (matrix peak interference) and poorly resolved HSL peaks (**Fig3.4A-B**).

LDI-MS platform as a tool to identify global Quorum sensing pattern in *Pseudomonas aeruginosa*

After successful detection of pure synthesized QS molecules using LDI-MS technology further identification of QS molecules from biofilm extract of *P. aeruginosa* PAO1 was carried out. QS Molecules from 24 h old biofilm was extracted and subjected to lithiation for improvement of signal. Lithiation of HSL molecule was done by addition of LiCl (7.5mg/mL) in 1:1 ratio. Using high resolution matrix free LDI MS post lithiation all the major QS Molecules of PA including HSL and PQS; C4-HSL, C6HSL, C8HSL, 3-oxo-C12-HSL, HHQ and C7 PQS was identified (**Fig 3.5**). Total number of QS molecules with their molecular mass (M^+) and identified mass (M+Li+) with ppm error is enlisted in (**Table 5)**. Lithiated C4 HSL of precursor mass 178.0 and C6HSL of precursor mass 206.1 obtained in MS mode was further subjected to MS/MS for further validation (**Fig 3.6A**). For Quinolone lithiated HHQ and C7PQS of precursor mass 250.1 and 266.1 was subjected to MS/MS (**Fig3.7B**). For homoserine lactone, lactone ring of m/z 102.1 after lithiation m/z 108.1 was considered as signature motif whereas for *Pseudomonas* Alkyl Quinolones (AQs), Quinolone ring of m/z 159.0 and m/z 175.0 and post lithiation of the ring m/z165.0 and m/z 181.0 was confirmed as signature product ion. Rest fragmented products obtained for both the group of molecules are ($M-H_2O$, M-CO, $M-CO_2$).

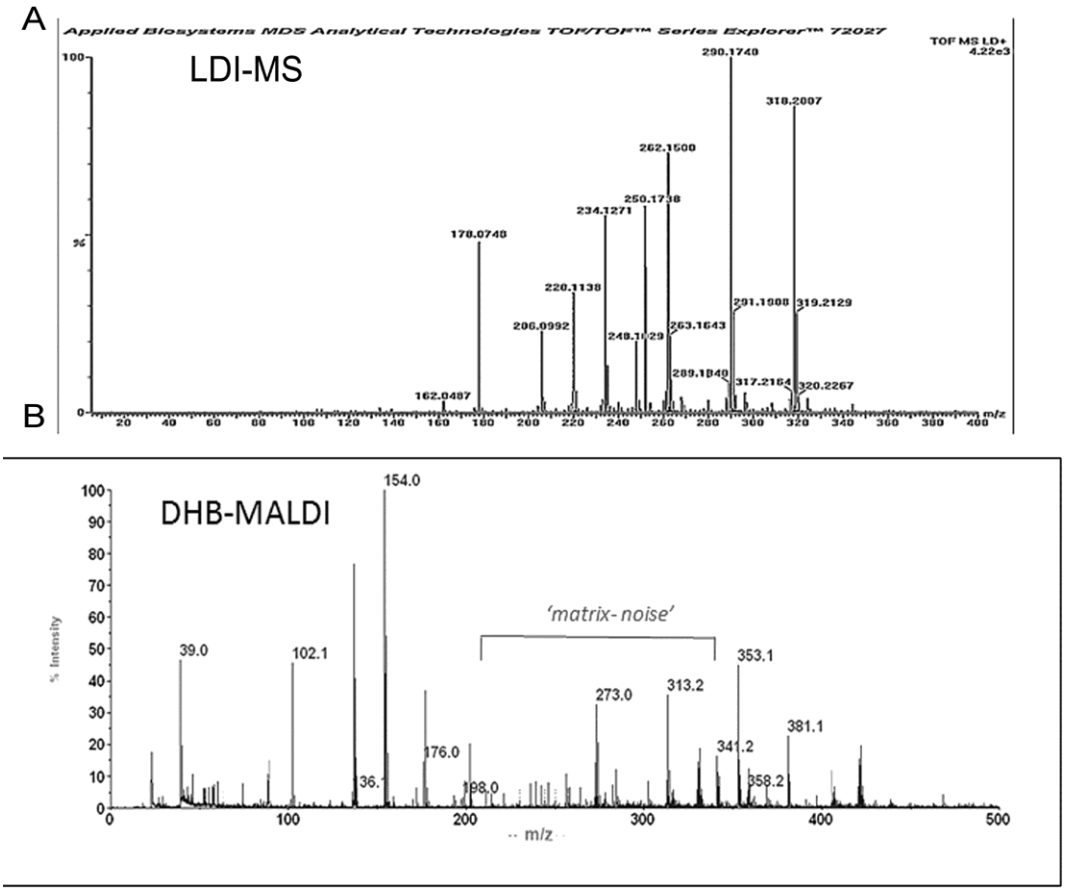
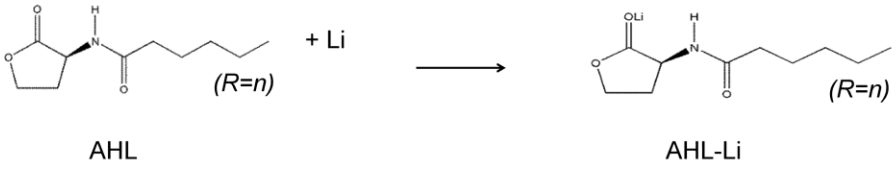


Fig 3.4 LDI-MS vs. MALDI-MS: a platform comparison study for *Pseudomonas sp.* QS molecules analysis. Pure *Pseudomonas* QS metabolite as listed in Table:4 was reconstituted in acidified 70%MeOH 5oµM. (A) For LDI-MS 1µL of the sample was mixed with SBA-15:LiCl and then spotted on to MALDI plate and targeted metabolite analysis was done for QS molecules. (B) For MALDI-MS 1µL of the sample was mixed with standard MALDI matrix i.e DHB and 1 uL was soptted for MALDI-MS run.

QS Molecule	Exact mass	(M+H)	(M+Li)	MALDI	LDI
C4 HSL	171.0895	172.0973	178.1055	+	+
C6 HSL	199.1208	200.1286	206.1368	+	+
C8 HSL	227.1521	228.1599	234.1681	-	+
C10 HSL	255.1834	256.1912	262.1994	-	+
C12 HSL	283.2147	284.2225	290.2307	+	+
C14 HSL	311.246	312.2538	318.262	+	+
3-OXO C6 HSL	213.1001	214.1079	220.1161	-	+
3-OXO C8 HSL	241.1314	242.1392	248.1474	+	+

Table 4: List of Pure HSL molecules identified using MALDI and LDI-MS



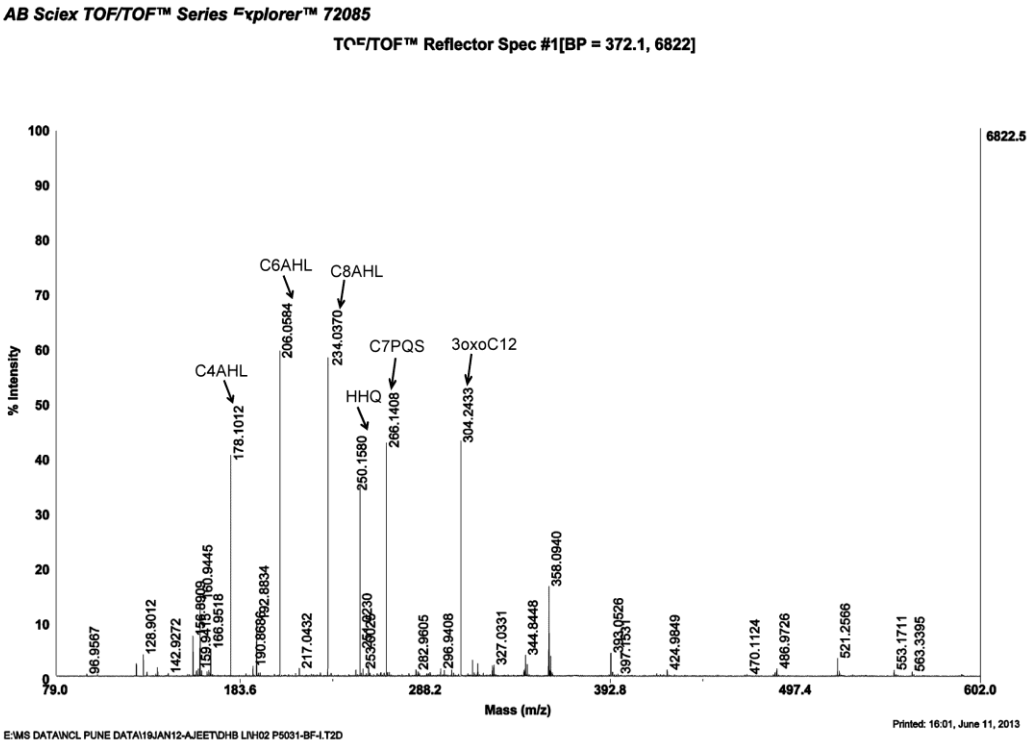


Fig 3.5: LDI-MS of QS molecules extracted from *Pseudomonas aeruginosa* PAO1 biofilms

24h old biofilm of *P. aeruginosa* was extracted with equal volume of ethyl acetate, dried and reconstitued in MeOH. Equal volume of the sample was then mixed with LiCl : SBA-15 (1:1). 1µL of the sample was spotted on 120 spotwell MALDI plate (Sciex, USA) and subjected to LDI-MS on Sciex 5800 MALDI-TOF/TOF instrument. Theprecursors were subjected to tandem MS for further confirmation.

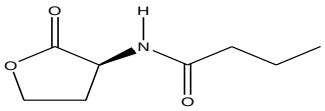
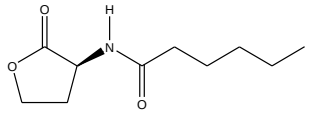
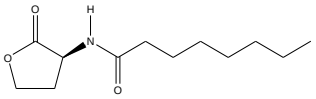
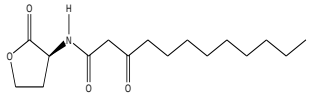
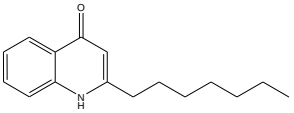
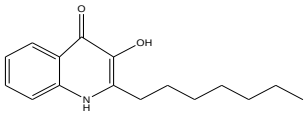
QS Autoinducer	Molecular Mass (M^+)	Theoretical m/z (M+Li)	Experimental m/z (M+Li)	Molecular Formula	Structure	MS MS Validation
C4 HSL	171.0895	178.1055	178.0411	$C_8H_{13}NO_3$		YES
C6 HSL	199.1208	206.1368	206.1024	$C_{10}H_{17}NO_3$		YES
C8 HSL	227.1521	234.1681	234.0824	$C_{12}H_{21}NO_3$		YES
3 OXO C12HSL	297.194	304.21	304.2404	$C_{16}H_{27}NO_4$		YES
HHQ	243.1623	250.1783	250.1101	$C_{16}H_{21}NO$		YES
C7 PQS	259.1572	266.1732	266.1024	$C_{16}H_{21}NO_2$		YES

Table 5: List of QS molecules detected and identified from PAO1 Biofilms using LDI-MS technology.

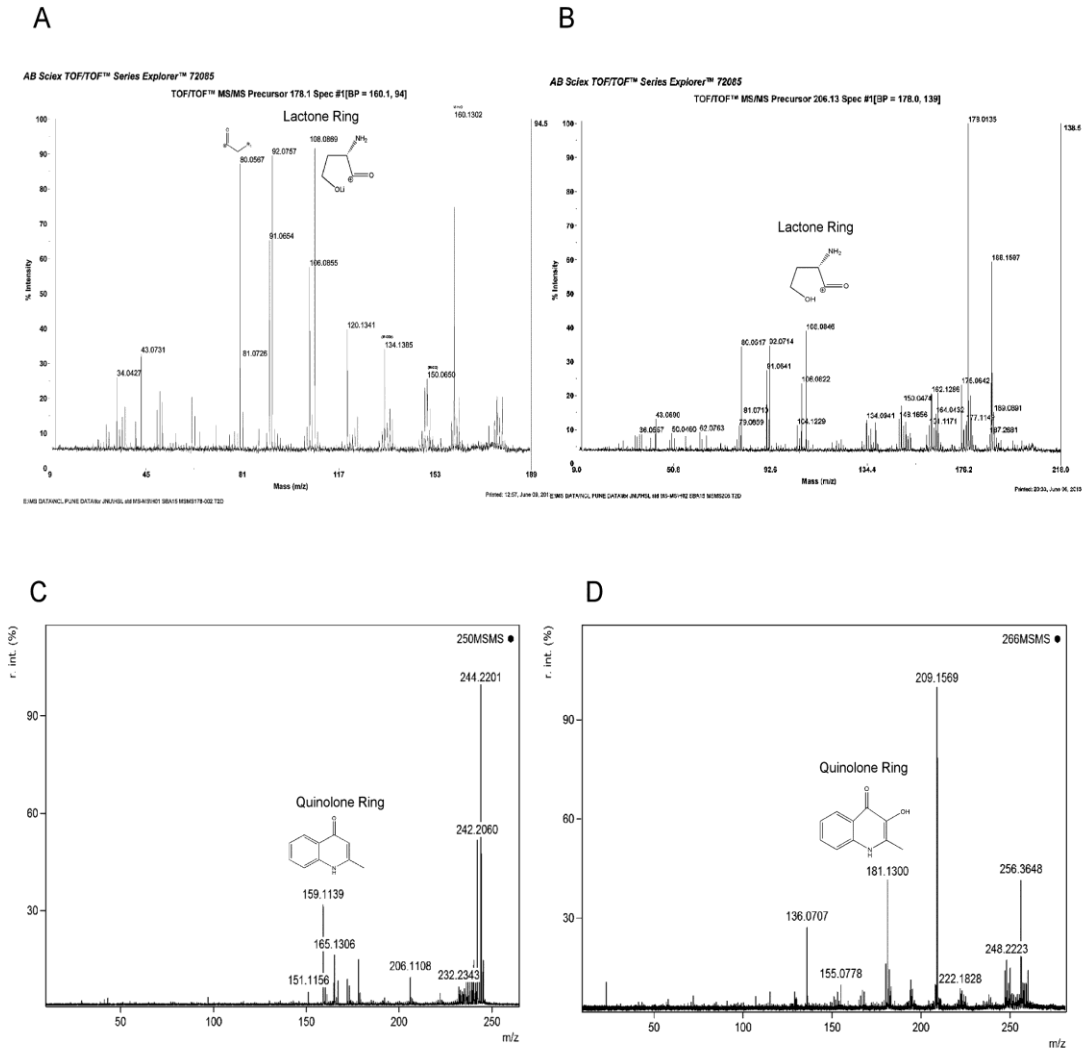


Fig3.6.1(A): **MS/MS spectra of N-Butyryl homoserine lactone**. Lithiated adducts of the HSLHSL via LDI-MS using SBA-15 particles as a matrix. **(B): MS/MS spectra of N-hexanoyl homoserine lactone** Lithiated adducts of the HSL via LDI-MS using SBA-15 particles as a matrix **(C)**: **MS/MS spectra of HHQ**. Lithiated adducts of the Quinolones via LDI-MS using SBA-15 particles as a matrix. **(D): MSMS spectra of C7-PQS** lithiated adducts of the HSL via LDI-MS using SBA-15 particles as a matrix

LDI-MS: Single step fast high resolution - technique to differentiate specific QS pattern of a pathogen- A key to understand the molecular mechanisms of pathogenesis

Matrix free LDI-MS allowed fast and high throughput selective screening and detection of low molecular weight metabolites. Through this technique, global QS metabolites of *Pseudomonas aeruginosa* were identified and in-house library was prepared. Confirmation of all metabolites was successfully achieved through CID dissociation MS/MS of the selected precursor mass. Further this technique was employed to understand the QS pattern of clinical PA strains. Clinical *P. aeruginosa* strains were acquired from different source (mentioned earlier), biofilms raised and QS metabolites were extracted using standardized protocol. In total 4 non-clinical PA strains and 5 clinical PA strains were taken for identification different QS specific metabolites pattern. Extracts from the clinical PA strains were then subjected to LDI-MS for precursor ion scanning **(Fig3.7)**. MS scanning reveals the expression of 3-oxo-C12 HSL was higher and was present in all the clinical strains. Further in Quinolones list HHQ and PQS expression was also higher and consistent in all of the strains. In addition to these 4 extra quinolones was detected in clinical strains. **Table 6A** enlists all the specific QS molecules associated with different clinical strains **Table 6B** shows the difference in the QS pattern between non clinical and clinical strains. All the QS molecules identified were confirmed through MS/MS.

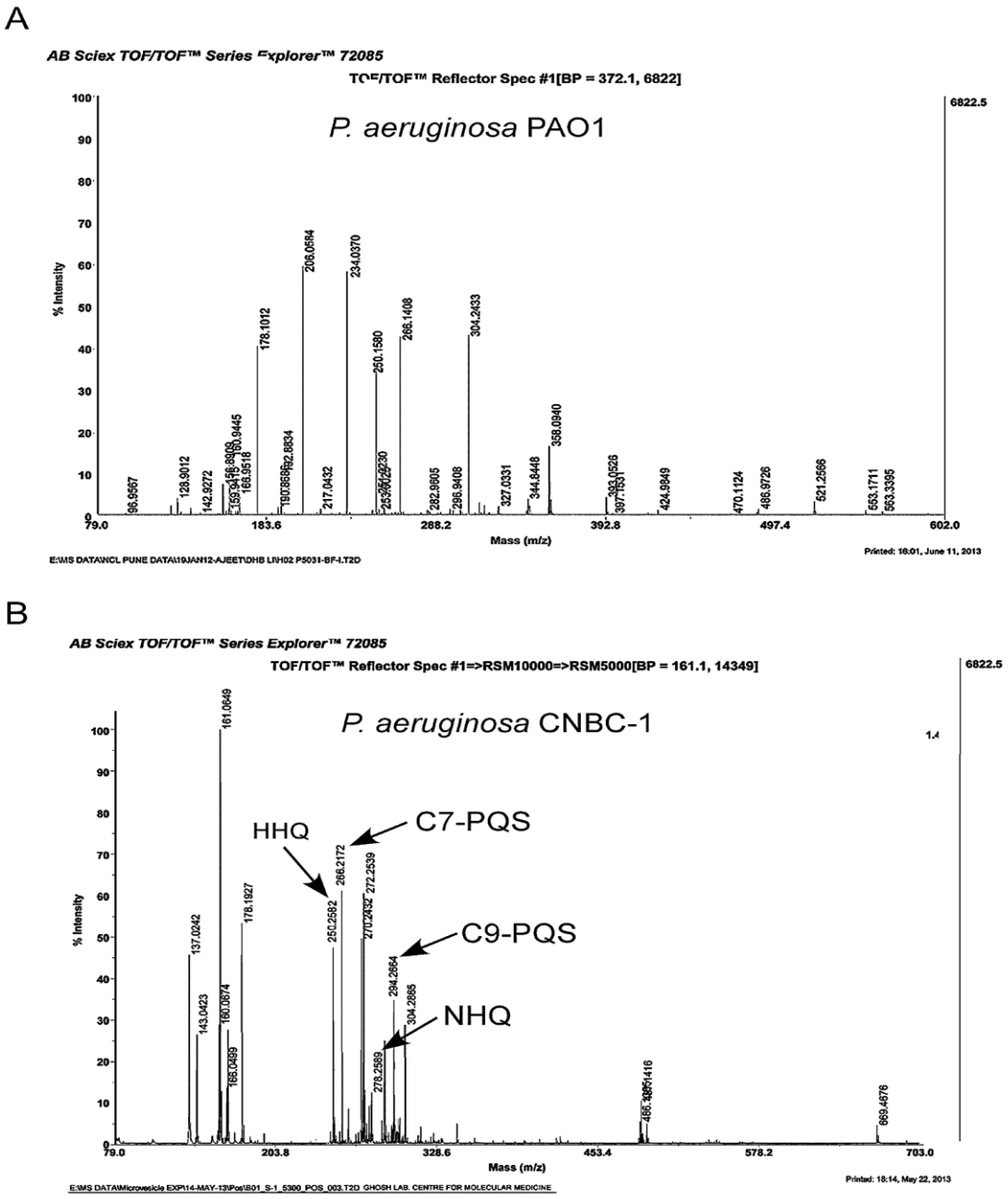


Fig 3.7 Comparative targeted metabolomics of *P. aeruginosa* biofilm extracts of clinical and type strains by LDI-MS. Identically cultured biofilm supernatants from clinical P. aeruginosa and P. aeruginosa PA01 were spotted on SBA-15 and analyzed by MS using external calibration and 20 ppm error. Putative QS were verified by tandem MS of the respective precursor ions. The presence of unique QS or higher expression of specific QS in clinical strain is indicated by black arrow. Result is representative of 10 clinical strains.

QS Class	M^+	Precursor Mass ($M+Li^+$)	PAv8	PAv-16	Pa-13	CNBC-I	CNBC-II
C4HSL	171.0895	178.1055	+	-	+	+	+
3-oxoC12	297.194	304.21	+	+	+	+	+
C6HSL	199.1208	206.1368	-	-	-	-	-
C8HSL	227.1521	234.1681	-	-	-	-	-
HHQ	243.1623	250.1783	+	+	+	+	+
C7PQS	259.1572	266.1732	+	+	+	+	+
C9 PQS	287.1885	294.2045	+	-	-	+	-
NHQ	271.1936	278.1045	-	+	+	-	+

QS Class	M^+	Precursor Mass ($M+Li^+$)	PAO1	PNCIM 5029	PA ATCC 27583	PAU	LasI/RhlI -/-
C4HSL	171.0895	178.1055	+	+	+	+	-
C6HSL	199.1208	206.1368	+	+	+	+	-
C8HSL	227.1521	234.1681	+	+	+	+	-
3-oxoC12	297.194	2304.21	+	+	+	-	-
HHQ	243.1623	250.1783	+	-	-	-	-
C7PQS	259.1572	250.1783	+	+	+	-	-
C9 PQS	287.1885	294.2045	-	-	-	-	-
NHQ	271.1936	278.1045	-	-	-	-	-

Table 6: List of all class of QS autoinducers obtained from (A) 5 different Clinical PA strains (B) 4 ATCC type strains and mutant strains. PA-I-PA-V: Clinical strains. PAU: *Pseudomonas aureofaciens.*

***P. aeruginosaP. aeruginosa* outer membrane vesicles-an emerging pathobiology**

Outer membrane vesicles of *P. aeruginosa* serves as Membrane encapsulated nanoscale delivery system which contains multiple pathogenic factor including QS molecules. Mechanisms of pathogenesis are completely unknown. Theses vesicles were successfully isolated from biofilm culture of *P. aeruginosa*. The morphology of the vesicles was examined over electron microscopy and closely resembled published reports. TEM analysis of Outer membrane vesicles showed that OMVs are produced by *P. aeruginosa* in large number. The images showed variable size OMVs in which majority of vesicles were found to possess a ring-like structure (**Fig3.8**). This experiment was performed to establish the basis of the study and to confirm the presence of enormous number of microvesicles in biofilm sample as it forms a validation study for this project.

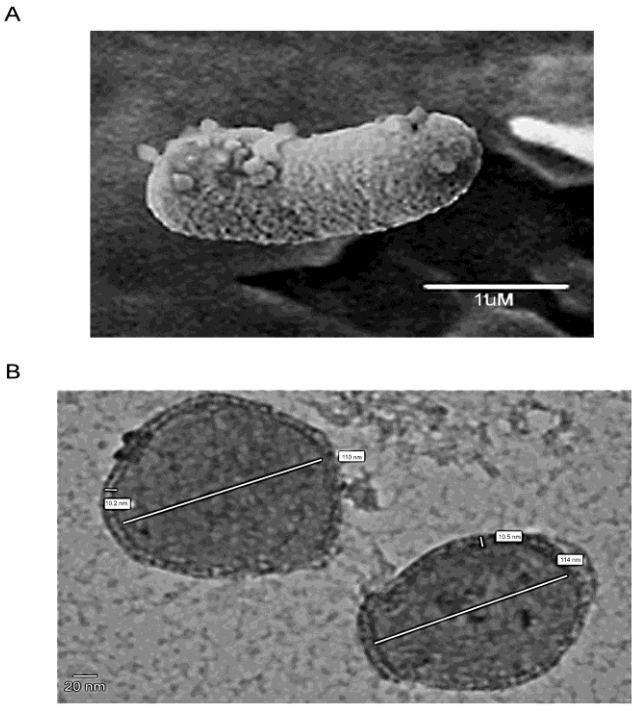
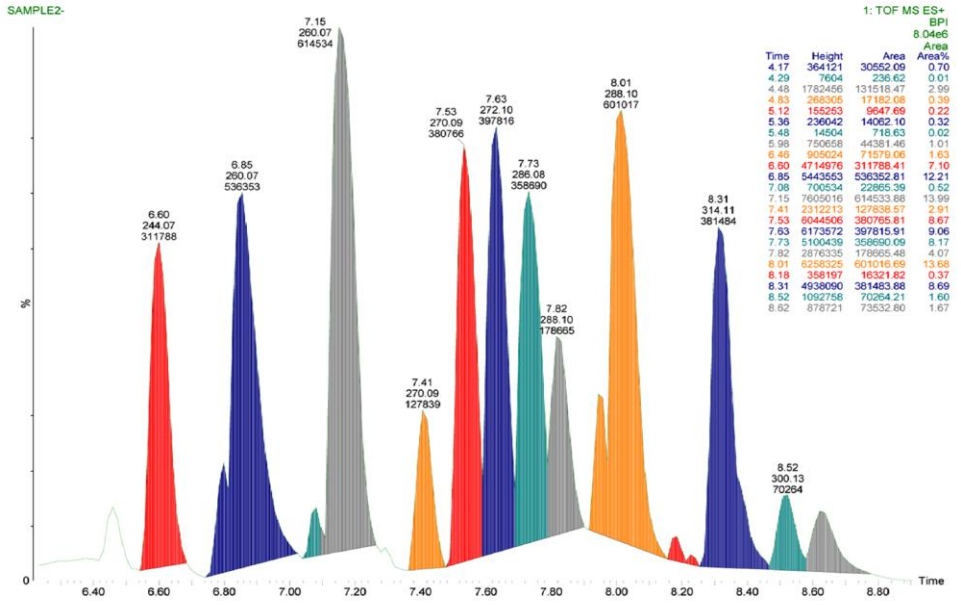


Fig 3.8: Electron microscopy analyses of Outer Membrane Vesicles (OMVs) from *P. aeruginosa*. Biofilm cells of P. aeruginosa (48 h) were analyzed by scanning electron microscopy. The cells exhibited (A) clusters of vesicular protrusions off the membrane. Detailed study of these purified vesicles by transmission electron microscopy at 8000x magnification, revealed discrete bi-layered membrane bound organelles ~100 nm diameter.

Global LC metabolite profile of *P. aeruginosa* OMV using Liquid Chromatography Mass Spectrometry

Pseudomonas outer membrane vesicles can also act as carrier of QS molecules and subsequently cause pathobiology. Earlier Whitely et. al has shown the presence of PQS molecule in *PseudomonasPseudomonas* OMVs with <1% HSL. Here, first the global LC-MS study of OMVs has been targeted and successfully identified 7 different classes of Quinolones other than PQS. **Fig 3.9** shows the total ion chromatogram of organic extract of PA OMV. We have 7 classes of AQs by looking at ($M+H^+$) and their retention time. Table XX presents retention time, intensities, peak area and precursor mass of the AQs identified. Further confirmation of the precursor ion was done through CID of the ($M+H^+$). Within each class the tandem MS spectra are identical as fragmentation occurs in the aliphatic carbon chain and producing fragment ion identical to particular class.



Time	Height	Area	Area%
4.17	364121	30552.09	0.70
4.29	7604	236.62	0.01
4.48	1782456	131518.47	2.99
4.83	268306	17182.08	0.39
5.12	155253	9647.69	0.22
5.36	236042	14062.10	0.32
5.48	14504	718.63	0.02
5.98	750658	44381.46	1.01
6.46	905624	71579.06	1.63
6.60	4714976	311788.41	7.10
6.85	5443553	536352.81	12.21
7.08	700534	22865.39	0.52
7.15	7605016	614533.68	13.99
7.41	2312213	127838.57	2.91
7.53	6044506	380765.81	8.67
7.63	6173572	397815.91	9.06
7.73	5100439	358690.09	8.17
7.82	2876335	178665.48	4.07
8.01	6258325	601016.69	13.68
8.18	358197	16321.82	0.37
8.31	4938090	381483.88	8.69
8.52	1092758	70264.21	1.60
8.62	878721	73532.80	1.67

Fig3.9: Total ion chromatogram of P. aeruginosa Outer membrane Vesicles extract at 24 hrs of biofilm culture. Clarified add organic extracted OMVs of P. aeruginosa were subjected to LC-MS and analyzed the presence of AQs. OMV samples (24 h) was extracted by ethyl acetate, dried and reconstituted in methanol containing 0.1% TFA. The sample (20 uL) was injected in (A i) UPLC and subjected to online MS. Analytes between (ii) 3.5 min to 7.5 min were targeted for QS precursor ions using data from pure standards. All putative percussor ions for QS were validated by CID

AQS	($M+H^+$)	Retention time	Peak Area	Fragments ion
HHQ	244.07	6.60	311788	172 and 159
HNQ	272.10	7.63	397816	172 and 159
UHQ	300.13	8.52	70264	172 and 159
C7PQS	260.07	6.85	536353	188 and 175
C9PQS	288.10	8.01	601017	188 and 175
HQNO	260.07	7.15	614524	186, 172, and 159
NQNO	288.10	7.82	178665	186, 172, and 159

Table 7 : List of 7 class of AQs with Precursor mass and their retention time detected from *Pseudomonas* OMV using LC-MS and subsequently subjected to CID for the confirmation of the daughter ions.

Single step analysis of OMV QS metabolites by LDI –MS

After successfully detection validation of OMVs QS metabolites using LC-MS platform we afterwards targeted the single step detection of OMV associated metabolites. All AQs are easily ionized on LDI surfaces and exhibit discrete mono-protonated, sodiated and pottasiated precursor ions at picomolar sensitivity (Table 1). Application of trace DHB improves signal intensity, but not mandatory. All AQs are soluble in methanol and matrix free LDI-MS generates excellent signals. Untargeted LDI-MS of AQs in OMVs reveal group of 8 major AQs expressed in the OMV. These are the 2-alkyl-quinolones (2-heptyl-quinolone; HHQ), 3,4-dihydroxy-2-alkylquinolines (3,4-dihydroxy-2-heptylquinoline, PQS) and 2-alkyl-4-hydroxyquinolines N-oxide (2-Nonayl-4-hydroxyquinoline N-oxide, NQNO) classes respectively (**Fig 3.10 and Table 8**). The association with AQ within the OMV membrane is directly related to their relative expression and hydrophobicity. AQs with logP (log octanol-water partition coefficient) > 5.5 are present in OMV (Table XX). AQ like DHQ (log P 2.33+/- 0.27), 2-Heptyl-4-hydroxyquinoline N-oxide (logP 4.90+/- 0.90) and the 3-alkyl-2,3-dihydroxy-4-quinolones (logP <5) are present in the culture supernatant but not in the OMV.

Tandem mass spectrometry of representative AQs from respective classes of Quinolones

The CID of HHQ exhibit a signature product ion at m/z 159 (the quinolone ring) which is common in all AQs of this class including UHQ, NHQ, PHQ and DHQ (**Fig. 3.11 A**). This product ion is generated by cleavage between the □□and β carbon of the side chain of these molecules respectively. The other product ion resulting from the cleavage of HHQ side chain is m/z 172, produced by cleavage between the β and ɣ carbon. Further product ions are m/z 226, produced by neutral loss of water; m/z 216, produced by neutral loss of CO and m/z 200, produced by neutral loss of CO_2. Besides, another product ion at m/z 145 is formed due to loss of methyl group from the quinolone ring.The CID of C7PQS produces a major signature product ion at m/z 175, and m/z 188 which corresponds to quinolone ring with an OH group at R1 and produced by a similar cleavage between the □ and β carbon and β and ɣ carbon of the side chain as in HHQ (**Fig. 3.11B**). Neutral loss of this OH generates m/z 159, but this species is not consistent in PQS. Other ions are m/z 242, produced from the neutral loss of water; m/z 232, produced from neutral loss of CO and m/z 216, resulting from the neutral loss of CO_2. The 2-alkyl-4-hydroxyquinoline N-oxide class is present in form of NQNO (and trace amounts of UQNO, 2-Undecyl-4-hydroxyquinoline N-oxide). The precursor ion of NQNO is m/z 288 ($M+H^+$) which produces signature product ion at m/z 159 and m/z 172. Further product ions are m/z 244.18 by neutral loss of CO and m/z 270.29 by neutral loss of water **(Fig3.11C**).

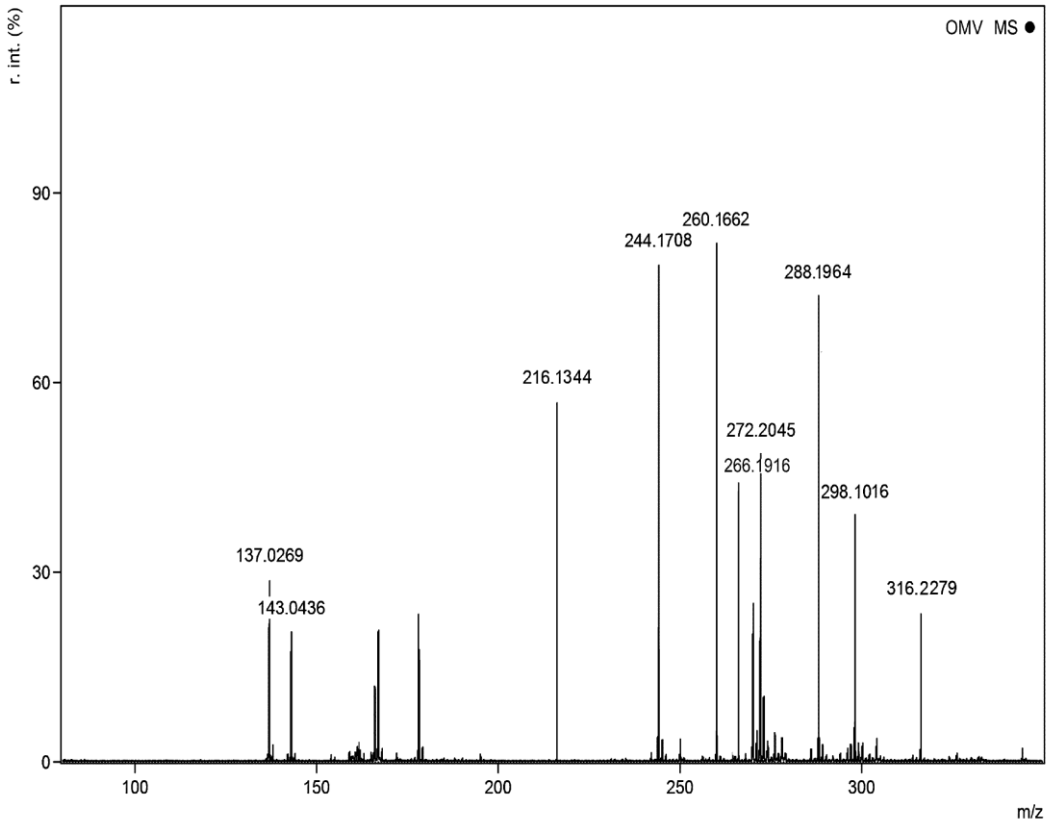


Fig. 3.10 Targeted QS metabolomics of OMVs from *P. aeruginosa* by ceramic LDI-MS in-situ platform. Clarified P. aeruginosa biofilm samples were subjected to ultrafiltration on 300 k. ceramic membranes and directly subjected to LDI-MS. Each QS precursor ion was further verified by CID. Spectral acquisition was accumulated for 3000 laser shots in the range m/z 100– 1500 m/z.

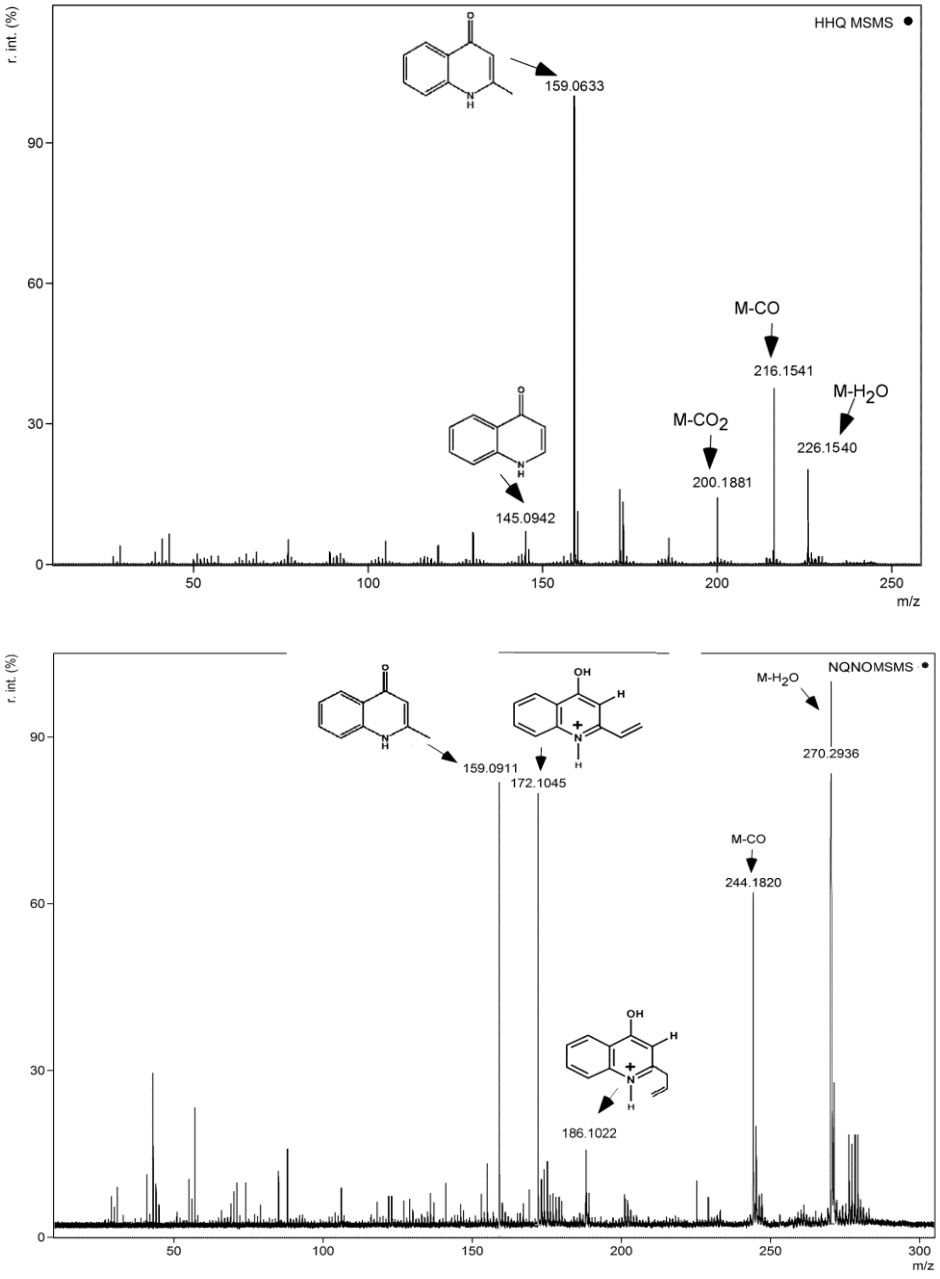
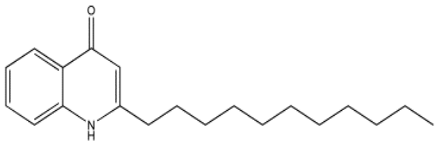
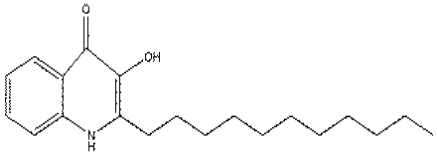
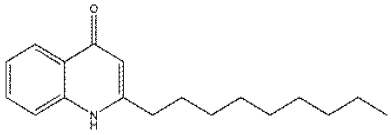
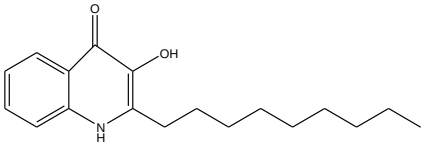
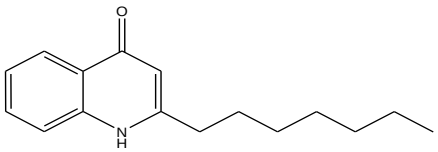
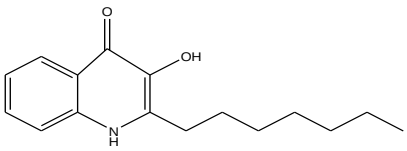


Fig3.11(A) LDI-MS/MS spectrum of the HHQ. Precursor ion m/z 244 (M+H+) produces signature product ion at m/z 159 (the quinolone ring) and m/z 172 resulting from the cleavage of HHQ side chain. Further product ions are m/z 226, produced by neutral loss of water; m/z 216 produced by neutral loss of CO and m/z 200, produced by neutral loss of CO2. Besides, another product ion at m/z 145 is formed due to loss of methyl group from the quinolone ring. **Fig3.11(B) LDI-MS/MS spectrum of the NQNO**. Precursor ion m/z 288 (M+H+) from the OMV, (M+H+) produces signature product ion at m/z 159 (the quinolone ring) and m/z 172. Further product ions are m/z 244.18 by neutral loss of CO and m/z 270.29 by neutral loss of water.

OMV Quorum Signalling Molecules	Structure	logP	Precursor Mass (Observed)	Product Mass (Observed)
			(M+H$^+$) *(M+Na$^+$)* *(M+K$^+$)*	
UHQ ($C_{20}H_{29}NO$)		8.40+/-0.77	300.23 *(322.21)* *(338.18)*	159.06, 282.22, 272.23, 256.24
C11PQS ($C_{20}H_{29}NO_2$)		8.17+/-0.77	316.22 *(338.20)* *(354.18)*	175.06, 188.09, 298.21, 288.23, 272.23
NHQ ($C_{18}H_{25}NO$)		7.34+/-0.77	272.20 *(294.18)* *(310.15)*	159.06, 254.19, 244.20, 228.21
C9PQS ($C_{18}H_{25}NO_2$)		7.10+/-0.77	288.19 *(310.17)* *(326.15)*	175.06, 188.09, 270.18, 260.20, 244.20
HHQ ($C_{16}H_{21}NO$)		6.28+/-0.7	244.17 *(266.15)* *(282.12)*	159.06, 226.15, 216.17, 200.18 145.09
C7 PQS ($C_{16}H_{21}NO_2$)		6.04+/-0.77	260.16 *(282.14)* *(298.12)*	175.06, 188.13, 242.15, 232.17, 216.17

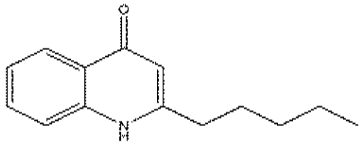
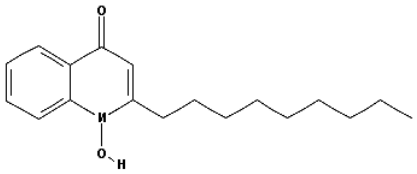
PHQ ($C_{14}H_{17}NO$)		5.21+/- 0.77	216.13 *(238.12)* *(254.09)*	159.06, 198.12, 188.14, 172.14
NQNO ($C_{18}H_{25}NO_2$)		5.96+/- 0.90	288.19 *(310.17)* *(326.15)*	159.09, 172.10, 186. 10, 244.18, 270.29

Table 8. List of OMV associated AQs analyzed by LDI-MS. Three distinct classes of AQs are present in *P. aeruginosa* OMV, distinguished by the presence of either a hydrogen or a hydroxyl group at the position 3 or a N-oxide group in position 1 of the quinolone ring. Each class exhibits at least one signature product ion and other products rationalized with their structure. This helps discriminate isomers (NQNO and C9PQS) with discrete product ions that are easily characterized by CID tandem mass spectra.

Temporal expression and Absolute Quantitation of 3-oxoC12-HSL - a pathogen specific determinant of PA biofilms

3OC12 HSL expression was consistent among all the isolated clinical strains and showed positive signal with high intensity. However, 3oxo C12 expression varies largely during biofilm formation stages. It has been proved genetically that expression of LasI gene is highest expressed in early biofilms but the expression drops as the biofilm matures. Here, using LC-MS technique the absolute concentration of 3oxo-C12-HSL from designated clinical strains has been achieved. Biofilm sample of 12h, 24h, 36h and 48h have been pooled and extracted with ethyl acetate and subjected to LC-MS. For calibration graph 3-oxo-C12 HSL standard of 3different concentration 25µM, 50µM and 100µM was run (**Fig3.12A**). Peak area of the precursor ion mass was determined. With the help of the peak area Calibration graph was plotted and R^2 was also obtained, which showed good linear response (R^2=0.9980) (**Fig3.12B**). Subsequently biofilm sample 12h, 24h, 36h and 48h was run and peak area was taken. Good peak shape was achieved for both the analyte and sample (**Fig 3.13**). Absolute concentration was determined with the help of MassLynx Software (Waters). Biofilm sample showed HSL concentration of 9umol in 12h early biofilm. The concentration reaches maximum of 643umol in 24h old biofilms and declined to 99umol in 36h and finally to 40umol in 48h biofilm (**Fig3.12B**).

Relative expression of C7PQS and 3-oxo-C12-HSL in *Pseudomonas* biofilm

Relative expression of two major class of *Pseudomonas* QS molecule i.e 3-oxo-C12-HSL and C7PQS was determined for both clinical and type strains in temporal based assay. Biofilm samples (12-48h) were extracted and subjected to LC-MS analysis. Temporal assay of *Pseudomonas* type strains showed that 3-oxo-C12 HSL dominates over PQS in the early phase of biofilm (12h), as the biofilm matures to 24h HSL starts decreasing in concentration and PQS dominates which continues up to late stationary phase (**Fig3.14A**). The data agreed to the earlier reports. Whereas in clinical strains the data showed that 3-oxo-C12 HSL concentration was relatively more than PQS upto 6h i.e only till initial attachment phase. At 12h PQS expression became high and was gradually increased to 24h after that it reaches to stationary phase at the end of 48h. 3-oxo-C12-HSL peak concentration reached at 24h and dropped significantly after 36h (**Fig3.14B**).

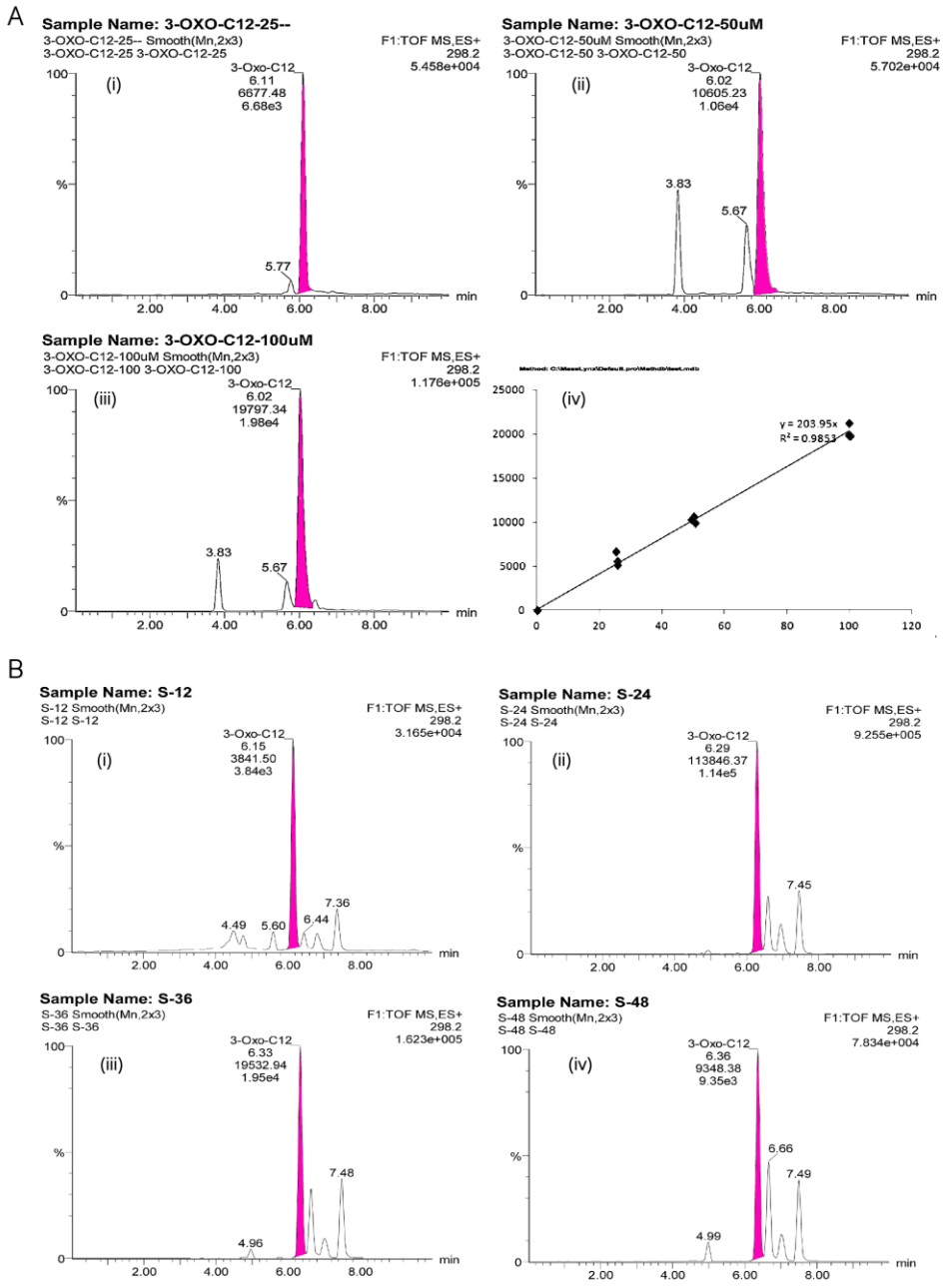


Fig3.12 A: Total Ion chromatogram (TIC) of 3-oxo-C12-HSL. HSL standards of (25-100)µM concentrations was injected into LC and acquired for 10min run in MS mode. HSL peak was eluted at 6 min (RT). Target Lynx Screen shot showing calibration curve of 3-oxo-C12 HSL.HSL standards showing linear response with three concentrations (25-100µM). **B: Total Ion chromatogram (TIC) showing 3oxoc12HSL from biofilm temporal assay**. Biofilm samples (12, 24, 36, 48)h old was extracted and analysed for detection of 3-oxo-C12-HSL. HSL detection was positive for all four time points and eluted at 6min same as standards.

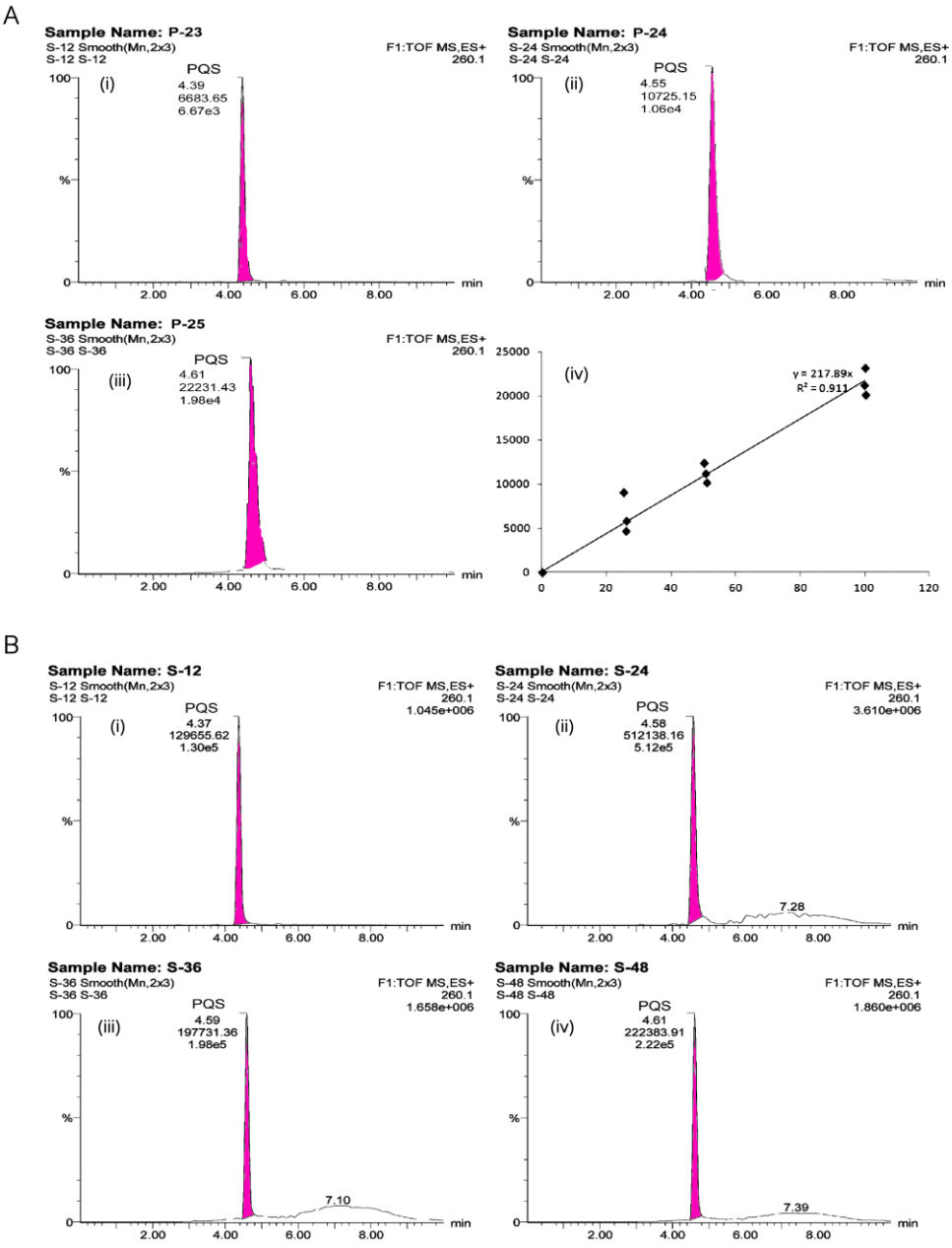


Fig 3.13 A. Total Ion chromatogram (TIC) of PQS: PQS standards of (25-100)µM concentrations was injected into LC and acquired for 10min run in MS mode. PQS peak was eluted at 4min (RT). Target Lynx Screen shot showing calibration curve of PQS. PQS standards showing linear response with three concentrations (25-100µM). **B: Total Ion chromatogram (TIC) showing PQS from biofilm temporal assay**. Biofilm samples (12h 24h, 36h, 48h) old was extracted and analysed for detection of PQS. PQS detection was positive for all four time points and eluted at 4 min similar to standards.

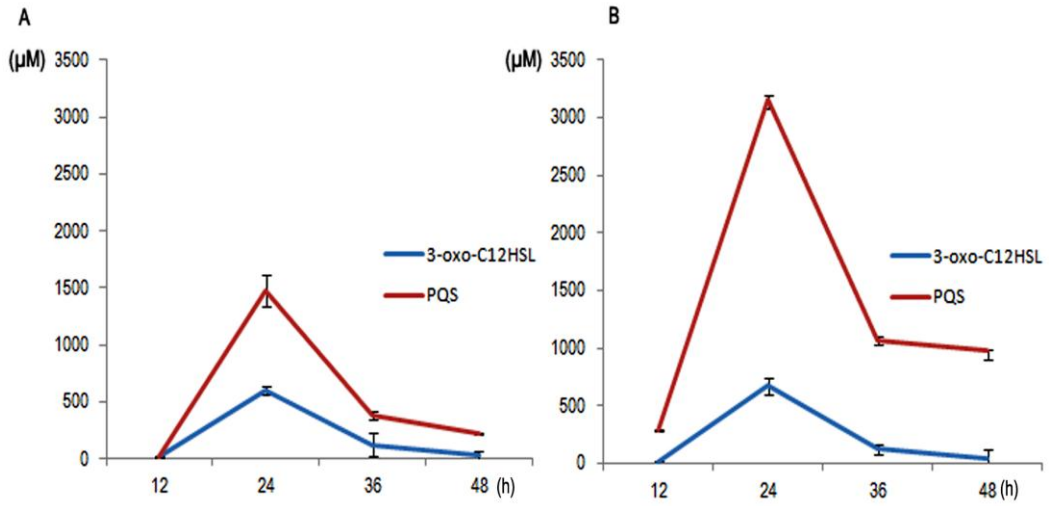


Fig 3.14: Relative expression of 3oxoC12 HSL and C7PQS in Pseudomonas biofilm A. ATCC PAO1 biofilms (12-48)h B. Clinical P. aeruginosa were extracted and analysed using LC-MS for relative quantitation of 3-oxo-C12-HSL and PQS. Absolute quantitation of 3-oxo-C12 HSL and PQS in (12-48h) biofilm samples was done using the linear equation obtained earlier. Relative expression of these two molecules was done using absolute concentration determined from the biofilm samples using Mass Lynx (Waters).

Detection of C7PQS and HQNO QS molecules isomers using high resolution Ion mobility mass spectrometry

Alkyl Quinolones (AQs) of *P. aeruginosa* exist in isomers. Among the Quinolone class the major PQS molecule i.e 2-Heptyl-3-hydroxy-4(1H)-quinolone (C7PQS) is in isomer form with the molecule i.e 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO). Precursor ion (M+H) for these two respective molecules is m/z 260.1. Therefore it is difficult to separate these two isomers using routine LC-MS or MALDI MS platform. After ethyl acetate extraction of OMVs sample, known to contain theses two isomers, samples were analysed on Synapt G2 HDM™S (Waters Corp., UK). Masslynx 4.1™ (Waters Corporation, Milford, MA, USA) was used to collect and process all the data. Ion Mobility Mass Spectrometer platform (IMS) study showed clearly two separated peaks for the same m/z 260.1 at different drift time(bins) spectra. 3D view of the spectra revealed that both the molecules PQS and HQNO peaks were isolated and showed up to 300 counts **(Fig3.15**).

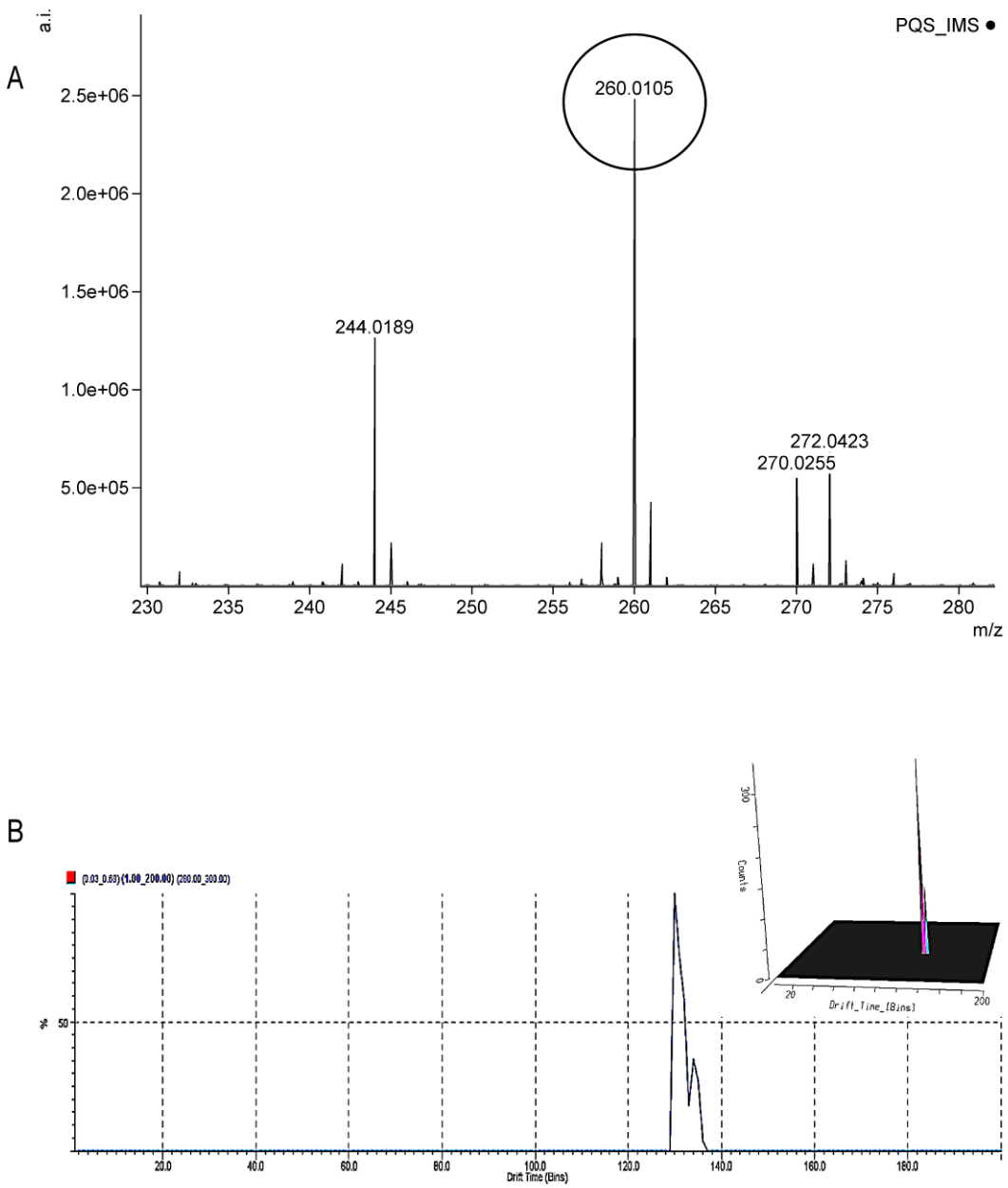


Fig 3.15 . Ion mobility spectra of HQNO and C7-PQS isomers in IMS enabled Quadrupole Time-of-flight Mass Spectrometry. (A) The two isomeric QS molecules were acquired in a single LCMS run of *P. aeruginosa* biofilm extract and the m/z 260.01 (circled) contained HQNO and C7-PQS. (B) High resolution IMS accurately resolved the two isomers on drift time and allowed (inset) structure based identification of the isomers (highlighted in purple and blue).

DISCUSSION

Biofilm formation is one of the important mechanisms enjoyed by *P. aeruginosa* to exacerbate infection in host. Thereby to understand the pathogenesis of *P. aeruginosa* infection simultaneous profiling of QS molecules which central to biofilm formation is very much needed[129]. The study started with the screening of several clinical *P. aeruginosa* biofilm forming strains. Almost all clinical strains procured, showed higher biofilm former than type strain (**Fig. 3.1**) especially when grown on in-dwelling device like ETT tubes etc. *P. aeruginosa* enjoys remarkable biofilm forming capabilities on innate surface like medical devises etc. The study also revealed that biofilm structure is different when grown under flow cells using peristaltic pump. High resolution Scanning Electron Microscopy deciphers that flow cell biofilm contain more water channels than static biofilm cells. Further the study was designed to understand the pathogenesis of the clinical strains with respect to its specific QS pattern molecules on Mass spectrometry based pattern. For this comparison was made between different Mass spectrometry techniques. First, two representative QS molecules fragmentation pattern was studied with the most commonly used ESI-MS and second with MALDI-MS. The fragmentation patterns of both molecules were different but the signature product ion of lactone ring (m/z102.0 for HSL) and (m/z 175.1 for PQS) was detected using both techniques. However due to in-source decay and high source voltage 20ev, the ESI MS could produce only fewer fragments. Here, MALDI- MS overcomes over ESI by producing additional M-H_2O, M-CO, M-CO_2 fragments which were common in all QS molecules (**Fig3.2**) and unique to only MALDI based ionization due to controlled collision induced dissociation (CID) energy. MALDI based metabolomics platforms very recently gathering interest in the area of small molecules metabolomics[130]. Therefore the study proposed also supported and provided a way where MALDI based metabolomics platform can be applied for Quorum Sensing metabolites analysis.

Further, to analyse the QS metabolites from biofilm extracts of PAO1, first earlier known Global LC-MS analysis was done. The LC profile (TIC) of biofilm extracts showed the presence of four major HSLs and 4 AQs of *Pseudomonas.* Retention time of the HSLs and Alkyl Quinolones matches with those from pure molecule LC profile with RT of 6 min for 3-oxo-C12-HSL and 2min for C7PQS. MS spectra also detected the entire precursor mass (M+H) of HSLs and AQs (**Fig 3.3**). This was performed for the validation of the project for further profiling of QS molecules with other available MS technique.

The proposed MALDI based metabolomics approached here opens a new way for fast profiling of QS metabolite which enjoys robust platform. However, MALDI suffers from poor resolution at < m/z 1500, due to matrix interference. Therefore MALDI has not been traditionally applied for metabolomics. In this study a novel method using SBA-15 as matrix free LDI surface has been proposed for QS based metabolomics. A comparative profile/analysis of MALDI vs LDI showed cleaner spectra in LDI with low signal to noise when analysed with pure HSL mix molecule (**Fig 3.4**). Thus it provided a platform that allows competitive advantage in analyzing small QS molecules. Whenever required the precursor ions subjected to Collision Induced Dissociation (CID) for their MS/MS signature. LDI-MS technique was then applied for global screening of QS molecules from PAO1 biofilm and it successfully competes with LC-MS for detection of all major QS molecules (**Fig3.5).** When the precursor mass of respective QS class i.e C4-HSL, C6-HSL , HHQ , NHQ subjected to MSMS all the above mention fragments were achieved within 20ppm error (**Fig 3.6**). This supported the potential of matrix free mass spectrometry for small molecules studies. Next LDI-MS applied as a tool to identify pathogenic specific QS molecule pattern. Here comparison was done between PAO1 Type strains and *P. aeruginosa* clinical strains using LDI-MS (**Fig3.7**). The in-house library was prepared for all the precursor masses (m)/z from MS of biofilm extract which revealed the detection of 3oxo-C12HSL, C7PQS and other AQs were more consistent and present in all clinical strains where as other HSL molecules like C4HSL, C6HSL was not detected in those clinical strains. From this study this, PQS and 3-oxo-C12 HSL association with *P. aeruginosa* pathogenicity can be concluded shown to be present consistently for all clinical strains.

Further this objective extended to analysis of Outer membrane vesicles (OMVs) associated Quinolones as also one of the mechanisms proposed for the pathogenesis of *P. aeruginosa.* AQs has been earlier reported to be associated with OMVs[79]. In this study, single step capture and analysis of global AQs has been done which showed all the major class of AQs to be associated exclusively with OMVs only using LC-MS and LDI-MS (**Fig 3.8, Fig 3.9 and Fig 3.10**). CID dissociation of the precursor mass showed all the fragments with signature ring (lactone/quinolone) (**Fig 3.11**). It has been hypothesized here that OMVs associated QS molecules are solely depend on their hydrophobicity co-efficient (log P) value, the more hydrophobic is the molecule, the more it chances it gets to packed as cargo inside OMVs.

Next the two pathogen specific QS metabolite 3oxo-C12HSL and PQS (positively detected in all clinical strains) has been taken to further quantitate its absolute concentration in temporal biofilm assays. Absolute quantitation of 3-oxo-C12-HSL in biofilm sample showed maximum concentration of 600µM at 24h and the concentration decreases gradually as the biofilm matures **(Fig3.12**). However the concentration of PQS showed constant expression in stationary phase. This also agreed according to previous reports. When relative quantitation was analyse for the *P. aeruginosa* clinical and non-clinical strains the graph showed higher expression of PQS than HSL in clinical when compared to non-clinical (**Fig 3.13-3.14**). From all the data it can now be concluded, First, PQS and 3oxo-C12 HSL are exclusive/necessary for *P. aeruginosa* pathogenesis. Second, Relative expression of PQS is higher in clinical strains which can play central role in pathogenesis of *P. aeruginosa*.

Further to add on more details of the PQS molecule, it is known that it has isomer with HQNO, the other major AQs of P. aeruginosa[106]. Thereby for identification of these two isomers using Mass spectrometry technique, high resolution Ion mobility mass spectrometry (IMS) based platform has been used to analyse OMVs samples. Here successful detection of the isomers has been achieved with two clear separated peaks along with the drift time detected from the sample (**Fig 3.15**). Thus the high resolution IMS technique allowed to detect most sensitive aspect of QS metabolites studies given the fact when two QS molecules having similar precursor mass.

Section 4

CHAPTER II

INTRODUCTION

P. aeruginosa Quorum signaling molecules can enter and employ cross kingdom signalling functions inside the host cells. Recent reports suggest that few of these class of molecules for example N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) and *Pseudomonas* quinolone signal (PQS) or 2-heptyl-4-quinolone (HHQ) have been shown to modulate host immune pathways [43, 59]. As soon as the bacterial population reaches quorum, the production of QS molecules accumulates which directs the synthesis of many virulence factors. In a very significant study Duon et al and group have reported that 3OC12 expression is highest in early biofilms phase and its concentration decreases gradually as the biofilm matures, whereas C4 HSL is constantly expressed throughout the formation and maturation of the biofilm[131, 132]. From the 1st chapter it was also observed that 3-oxo-C12 HSL reaches its peak concentration in log phase of biofilm which agreed in accordance with previous reports. Typical concentration of 3-oxo-C12 HSL was found to 600µM. 3OC12 HSL is a lipidic diffusible QS autoinducer of *P. aeruginosa* (molecular weight 297da), a key regulator molecule, involved in planktonic to biofilm transition and is highly associated with *Pseudomonas* virulence and pathogenicity[133]. Very recently, the QS metabolite 3OC12 has been shown to be recognised by mammalian epithelium and thereby suggested the interkingdom functioning of HSL inside mammalian cells[62]. The HSL has been shown to induce pro-inflammatory cytokines, chemokine and apoptosis[58, 59, 134]. This suggests that 3oxoc12 HSL actually launches inflammatory response in host cells. Prolonged inflammation could exacerbate host disease condition and ultimately end up in permanent organ failure. Various studies have employed the change in mammalian gene transcription after treatment with HSL but exact mechanism is not completely understood.

Further HSL has been reported to activate NF-κB signaling pathway by nuclear translocation of p65 which helps in induction of inflammatory genes mediators such as IL-8, cox-2 etc[58, 60]. Whereas there are contrasting reports where HSL inhibits LPS mediated activation of NF-κB activation[64, 135]. The contrasting report of 3-oxo-C12 mediated pro-inflammatory and anti-inflammatory activity by modulation of transcriptional activity of NF-κB may be dependent on particular cell types and via involvement of different receptors or HSL binding proteins which leads to different cell based signaling pathway[136].

Other than modulating host cell transcription, 3-oxo-C12 HSL also modulates host cells protein at translational level. Release of intracellular calcium ions has been shown to increase in the presence of HSL[56]. Along with this HSL is also responsible for distension of endoplasmic reticulum (ER) structures and phosphorylation of the eukaryotic translational initiation factor (eIF2α). Thus HSL has been shown to regulate overall ER stress[137]. The eIF2α phosphorylation has been shown to activate NF-κB via phosphorylation and inhibition of degradation of IkBα[138]. Thereby the data suggest despite of the transcriptional regulation 3-oxo-C12 can interfere with protein synthesis and thus providing ways where *P. aeruginosa* can exacerbate host immune response.

In addition to the involvement of NF-κB, recently it has been shown that 3-oxo-C12 binds and interact with a type of nuclear receptor i.e peroxisome proliferator-activated receptor (PPAR)[54, 139]. Among the family of PPARs, 3OC12 act as agonist of PPARß/d and antagonist of PPARϒ[54]. PPARs are ligand –dependent, a class of nuclear receptors (NRs) transcription factor whose activation affects genes controlling vital processes. Among NRs, PPAR has emerged as links between lipids, metabolic diseases and innate immunity and is widely known for its anti inflammatory roles[140]. In a recent study PPARϒ dependent enhance host immunity and clearing of *P. aeruginosa* has also been reported in macrophage cells[141]. 3-oxo-C12 HSL acts by inhibiting the DNA binding activity of PPARϒ. The reports showed the inhibition of pro-inflammatory effect of 3-oxo-C12 when exposed to lung cells when cells were treated with PPARϒ agonist[54]. Apart from these, PPARϒ has several inhibitory effects on inflammation including reduction of NF-κB transcriptional activities, reduction in the production of proinflammatory molecules in T lymphocytes, expression of anti-inflammatory mediators[55, 142]. PPARϒ inhibits NF-κB transcriptional activities by transrepression mechanisms is known as ‘cross coupling’ or ‘mutual receptor antagonism which involve undergo agonist dependent sumoylation and subsequent binding to nuclear receptor co-represor complex (NCoR)[143]. The co-repressor complex stabilizes promoter-bound NF-κB and trans-represses the DNA binding activity of NF-κB transcription factor and nuclear shuttling of RelA/p65, hence inhibits expression of inflammatory genes. Also it has been investigated that 3-oxo-C12 competes with PPARϒ ligand and thereby relieves the transrepression of NF-κB and can bind to promoter of inflammatory genes[58, 144]. Therefore, in this study we have proposed HSL mediated activation of NF-κB via PPAR dependent pathway.

During respiratory infection, lung epithelium forms primary host defence by serving as a mechanical barrier and also by secretion of various effectors agents which leads to killing of the invading pathogen. Production of antimicrobial peptide for example defensin, cathelicidins, lysozyme and other proteins with antimicrobial activity are the primary mechanism by which epithelium innate immunity protects itself against infection[145]. Antimicrobial peptides like beta defensins act as by membrane pore formation, cell lysis and other intracellular events. In case of *PA* lung infection the epithelium innate immunity plays significant role by upregulation of hBD-2 which is crucial for the host defence and barrier functions maintenance[99].

Among beta defensin classes, human beta defensins 2 is inducible and vital for protection against several infectious threats and has been shown to expressed/produced with PAMPs PRR interactions[146]. An abundance of reports strongly support the important role of defensins like hBD-2 against *Pseudomonas* in various infections either independently or in tandem with antibiotics[101]. hBD-2 promoter has binding sites for NF-κB and AP-1 transcription factor. Induction of hBD-2 involves activation of transcription factor either one of these or both[90, 147]. Further, with the facts that even the small molecular weight QS metabolite of *Pseudomonas aeruginosa* i.e 3-oxo-C12 are being capable of activation of NF-κB transcription factor and signal to the host immune response[58]. However 3-oxo-C12 HSL modulating or inducing hBD-2 via involvement of NF-κB is not known till date. From the first chapter successful isolation and detection of 3-oxo-C12 HSL was done. Subsequently, 3OC12 HSL concentration was also determined using temporal based assay starting from very early to late biofilm. So keeping all these points, further we have investigated the role of 3-oxo-C12 in regulating the expression of Beta defensin 2, mediated by Nf-kB signalling pathway via involvement of PPARϒ receptor on lung epithelial cells (A549). Finally, we have also studied the pathophysiology of A549 cells expressing human beta defensin2 to combat/fight against *Pseudomonas aeruginosa* epithelium infection and early biofilm formation. Although this signaling molecule is known to upregulate Nf-kB pathway but still till date, there are no reports of 3-oxo-C12 HSL inducing mammalian defensin via Nf-kB pathways. In addition biological relevance of HSL mediated hBD-2 upregulation for controlling *P. aeruginosa* infection on lung cells is also investigated.

Similar to 3-oxo-C12 HSL, other class of QS molecules of PA, PQS and HHQ have also been shown to modulate immune response in host cell[69, 148]. PQS and HHQ have been

shown to suppress immune system by downregulation of NF-κB signaling pathway[73]. PQS reduce NF-κB binding, thereby inhibiting expression of NF-κB targeted genes. PQS has also been reported to delay IkB degradation in monocytes. It acts by inhibiting cytokines release and monocytes, T cells, DCs proliferation[69, 70]. It has been thereby reported to impair macrophage activation which can help further in increasing the virulence of theses pathogen[72]. This suggests the potential of Quinolone class of QS molecules influencing host cells immune response.

Apart from these a major transcription factor hypoxia-inducible factor (HIF-1) has known to be an important key factor as regulator of immune response after pathogen interactions[149 150]. However pathogen inducing/activating HIF-1 pathway not clearly understood[151]. It has been reported that *Pseudomonas* cell free supernatant as well as PQS and HHQ significantly repressed/downregulate HIF-1 alpha expression in bronchial epithelial cell line and lung epithelial cells. The downregulation of HIF-1 by PQS was shown to act via 26S-proteasome proteolytic pathway[152]. Thereby manipulation of HIF-1α by *Pseudomonas* AQs can have major outcome of host response to the pathogens.

Antimicrobial peptide secreted from the epithelium as described earlier serves as important factor against microbial threats. Among all other human beta defensin family, the role of hBD-1 is crucial as it is constitutively expressed and is activate in hypoxic environment. hBD-1 promoter has HIF-1 binding sites and also recently it has been investigated that the epithelial HIF-1α or basal HIF-1α is required for the constitutive expression of hBD-1[150]. Constitutive expression of hBD-1 is required for the maintenance of epithelial immunity. However PQS or HHQ of PA regulating constitutive expression of hBD-1 is still not known. Therefore to investigate the role of *Pseudomonas* second class of QS molecule i.e PQS or AQs in constitutive expression of hBD-1 via modulating HIF-1 axis experiments has been performed. Pure PQS along with outer membrane vesicles derived all AQs has been studied for the regulating the constitutive hBD-1 expression in human lung epithelial cells.

RESULTS

3-oxo-C12 homoserine lactone increases endogenous hBD-2expression in A549 lung epithelial cells

To investigate the possible role of bacterial QS metabolite 3-oxo-C12 on endogenous hBD-2 expression, A549 lung cells were treated with 3-oxo-C12-HSL (0-150 µM) for 16h. Cell lysates were prepared and subjected to AU-PAGE western blot analysis using hBD-2 antibody. Immunoblot analysis showed induction of hBD-2 in HSL (150µM) treated samples compared to control. For positive control, TNF treated cells and recombinant hBD-2 peptide (2-10ng) was used (Fig5.1A). Simultaneously, direct hBD-2 protein expression on HSL induced cells also done using Immunocytochemistry (ICC) of control and HSL treated cells using same antibody. Immunocytochemistry results (ICC results also confirmed strong positive signal in induced cells compared to control (non induced) (Fi5.1B). Also hBD-2 is a secretory antimicrobial peptide so for quantitation of the induced peptide conditioned media was subjected to sandwich ELISA. Standard curve was obtained using standard rHBD2. ELISA results showed dose dependent induction hBD-2. HSL mediated induction of hBD-2 starts with 50µM and reaches maximum at 150 µM treated cells and again the level drops at 200µM due to cells toxicity to HSL (Fig5.1 C). This results concludes the early biofilm determinant QS molecule 3-oxo-C12 HSL (1st Chapter) is a potent inducer of innate immune effectors in host i.e beta defensin 2.

3oxoC12 HSL induces hBD2 transcription in A549 cells:

From the above experiments it is clear that 3-oxo-C12 HSL upregulates hBD-2 expression in lung cells. To further investigate whether the upregulation is due to increased mRNA copy of hBD-2, transcript level of hBD-2 was checked in HSL treated cells. A549 cells were treated with HSL (0-150µM) for (0-24h) and total RNA was extracted. Quantitative real time PCR was performed using hBD-2 and beta-actin primer as housekeeping gene. The data showed Dose dependent upregulation of hBD-2 mRNA with various concentration of 3oxo C12 HSL molecule (Fig5.2A). Subsequently temporal assay of hBD-2 mRNA regulation by HSL was also performed. Upregulation of hBD-2 mRNA was stimulated as soon as 4h after treatment and reached to the peak at 6h, remained constant from 12-24 h and then returned to baseline expression (Fig. 5.2B). The data showed 3-oxo-C12 induces approximately 3.4 fold induction in hBD-2 mRNA

expression when compared to control cells. The qPCR product was run on gel to confirm the specific amplified product size (Fig. 5.2C). The results confirmed that 3-oxo-C12 induction of hBD-2 peptide expression is due to increased hBD-2 transcripts level.

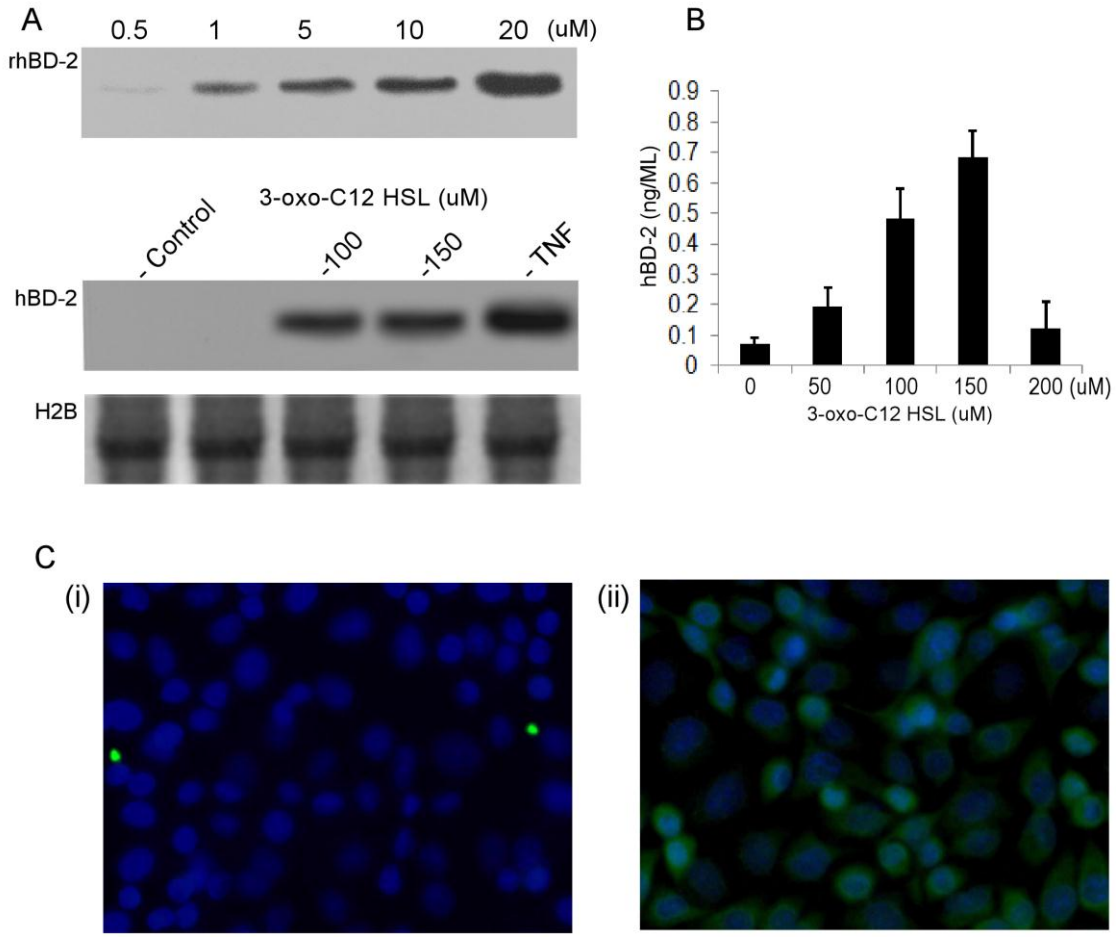


Fig 4.1: 3oxoC12-HSL induces expression and production of hBD2 in lung epithelial cells (Ai) Western blot analysis of recombinant hBD-2 peptide (0.5-20µM). (Aii) Western blot assay of hBD-2 from control A549 cells, HSL (100-150µM) and TNF (20ng/mL) treated cells. Whole cell lysate was prepared after 16h of treatment and subjected to AU PAGE western blot analysis. Histone 2B (H2B) was used as loading control. (B) hBD-2 ELISA assay using condition media from control and HSL treated cells. (C) Immunocytochemistry of A549 cont cells and HSL induced cells for 16h against hBD-2 antibody. For ELISA The result represents one of three independent experiments with similar result. ta represent the mean ± SD from at least three independent experiments

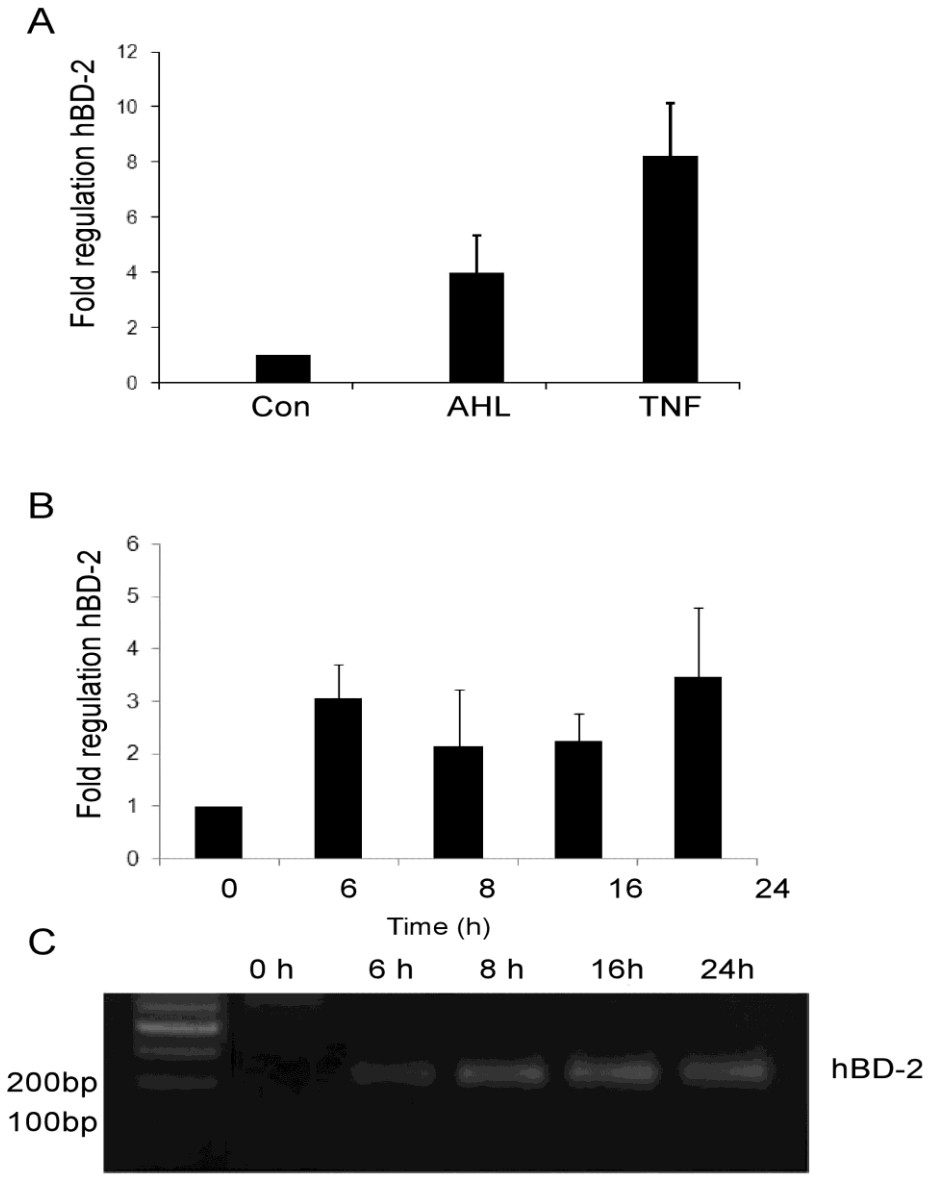


Fig 4.2: Effect of 3oxoC12 HSL on hBD2mRNA in lung epithelial cells. A. Total RNA was isolated from HSL (100μM) treated cells, TNF (10ng/mL) treated cells for 6h and quantitative Real Time PCR analysis was performed against hBD-2 and β-Actin primers. ta represent the mean ± SD from at least three independent experiments B. qPCR analysis of hBD-2 and Actin from HSL (100μM) treated and untreated cells for different time period (6, 8, 16 24h). The result represents one of three independent experiments with similar result. ta represent the mean ± SD from at least three independent experiments on separately prepared cultures. C. Representative gel pictures of hBD-2 amplified band from the above qPCR product on 2% Agarose gel.

NF-kB activity contributes to regulation of beta defensin2 by 3-oxo-C12 HSL

hBD-2 gene promoter contains consensus sequence for binding of transcription factor NF-κB and AP1. In lung epithelial cells induction of hBD-2 is primarily mediated by NF-κB. Here we have shown that HSL upregulates human beta defensin 2 via NF-κB pathway. Earlier there has been report where HSL have shown to induce nuclear translocation of NF-κB in lung fibroblast cells. Therefore, here we have hypothesized that HSL induction of hBD-2 involves NF-κB signalling pathway. To study and confirm the effect of 3-oxo-C12 HSL we performed EMSA gel shift assay using nuclear fraction after stimulation with HSL in a time bound manner. NF-κB proteins were analysed using RelA/p65 antibodies. HSL treated A549 cells showed enhanced binding of p65/RelA transcription factor and hence increased activation of NF-κB (**Fig.4.3A**). For further confirmation we subjected the nuclear extract for immunoblotting analysis using the NF-κB family antibody i.e RelA/p65, phospho p65, phospho IkBa and beta-actin antibody. Immunoblot analysis also confirmed the increased activation and expression of p65, and positive detection of phospho p65 and phospho IkBa proteins in the nuclear extract **(Fig4.3B)**. Stimulation of a549 cells with HSL showed p65 nuclear translocation and subsequently increased levels of phosphorylated p65 in the cells which was first observed in 15mins and reached peak in 30min after stimulation. Similarly phosphorylated IkB was also observed at 10 min after stimulation and was stable till 30 min (Fig. 4C). Thereby it indicates strong activation of NF-κB pathway by HSL and was confirmed with multiple transcriptional subunit activation. Activation of the pathway ultimately leads to upregulation of hBD-2 by binding of transcription factors to consensus sequence present in the hBD-2 promoter and upregulates hBD-2 production.

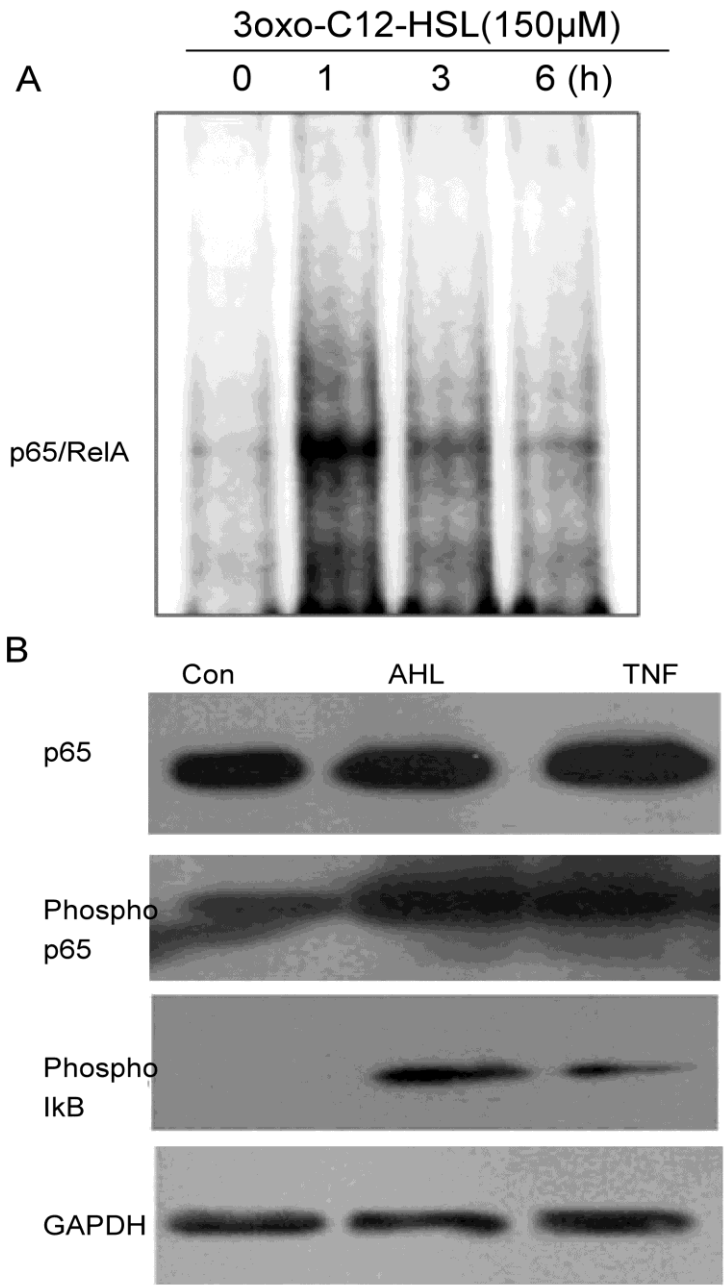


Fig 4.3: 3-oxo-C12 HAL upregulates hBD-2 expression via NF-κB signaling pathway. A. Human lung epithelial cells were incubated with HSL (100μM) for different time point (0-6h). EMSA was performed using nuclear lysates using RelA/p65 probe. Result represents one of three independent experiments with similar result. B. Cells were then incubated with HSL 100μM and TNF 10ng/mL for 1h. Immunoblot analysis of endogenous p65, phospho p65, phospho IκBα and Actin was performed from whole cell lysates respectively. The result represents one of three independent experiments with similar results.

NF-κB binding to hBD-2 promoter gene is required for HSL mediated induction of hBD2 in A549 cells. To investigate and further confirm the role of NF-κB in HSL mediated induction of hBD-2, the promoter of hBD-2 gene was cloned in promoter less luciferase pGL3 vector. Harder et al. has demonstrated that hBD-2 promoter of 2338 bp contains three NF-κB binding sites (positions-205 to-186, -596 to -572 and -2193-2184) (Harder, J.2000). hBD-2 promoter of 2338 bp was ligated to pGL3 promoter less vector (4383bp) (**Fig 4.4**).The construct was then used to transiently transfect A549 cells to check promoter activation. Along with the construct, A549 cells were co-transfected with pRL-TK vector (Renilla luciferase as transfection control) in serum free medium. After serum recovery cells were treated with HSL (0-150μM) for 6h. At the end of 6h cells were lysed and dual luciferase assay was done with control and HSL treated samples. The data showed clearly HSL induces hBD-2 promoter approximately 2fold when compared to promoter level in non-treated A549 cells (**Fig. 4.5**).

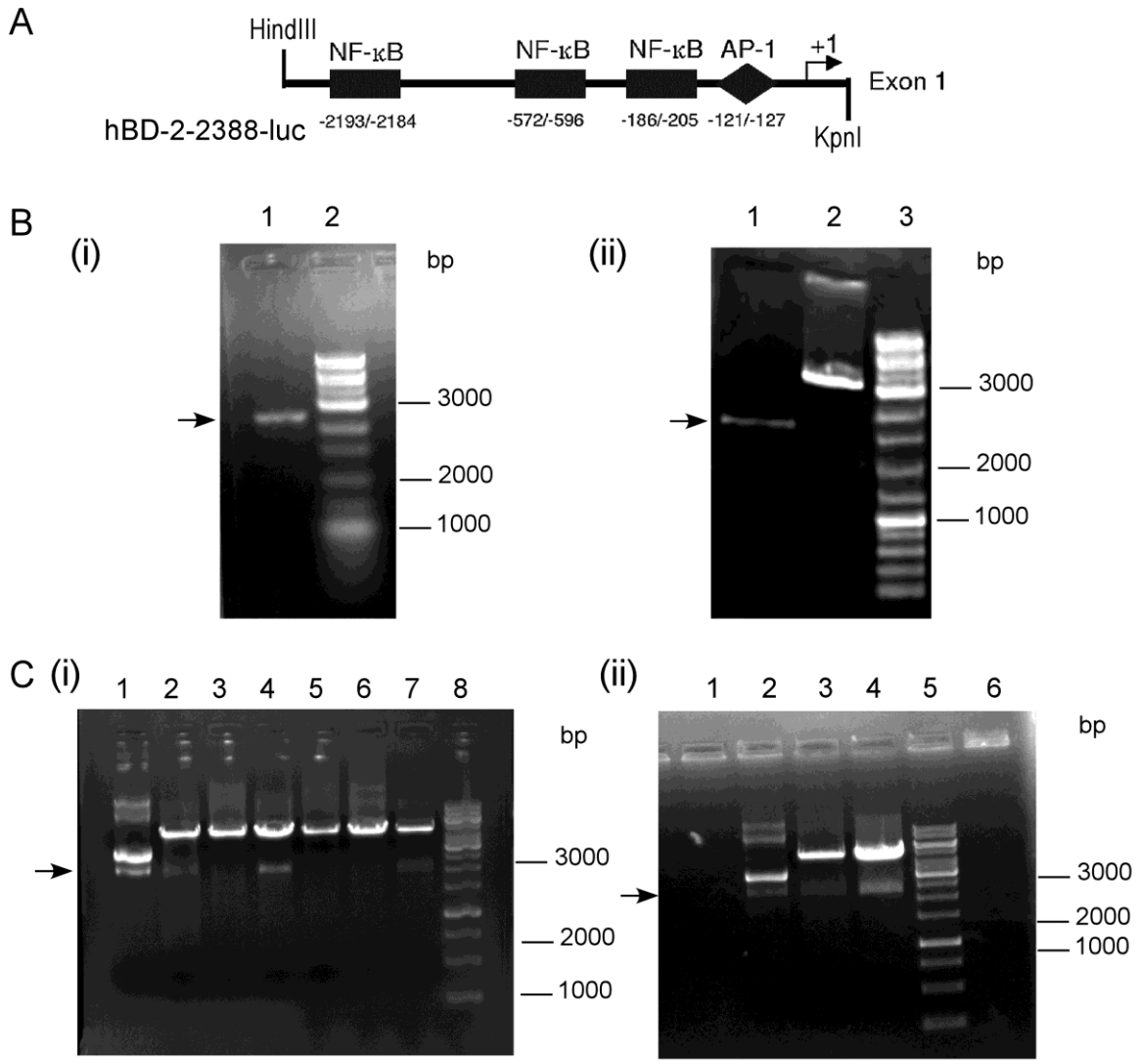


Fig4.4. Cloning and analyses of the hBD-2 promoter in pGL3 promoter less vector. (A). Schematic representation of the hBD-2 promoter (2388 bp) upstream of luciferase gene in pGL3 promoterless vector. (B). (i) Lane 1, PCR amplified product of hBD-2 promoter. (ii) Restriction digestion of the pGL3 vector 4818bp and insert hBD-2 promoter 2388bp; Lane 1 digested hBD-2 promoter, lane 2, digested pGL3 vector. (C). (i) Confirmation of clones by restriction digestion of the plasmid isolated from six colonies; lanes 2 - 7, clones digested with KpnI and HindIII; lane 8 undigested vector with insert (clones in lanes 2, 4 and 7 show inserts indicated in dark arrow); (iI) Restriction digestion of the transfected plasmid validating presence of construct inside the vector; lane 2 undigested vector and lanes 3 - 4 clones digested with KpnI and HindIII. rk arrows indicate the band(s) of interest.

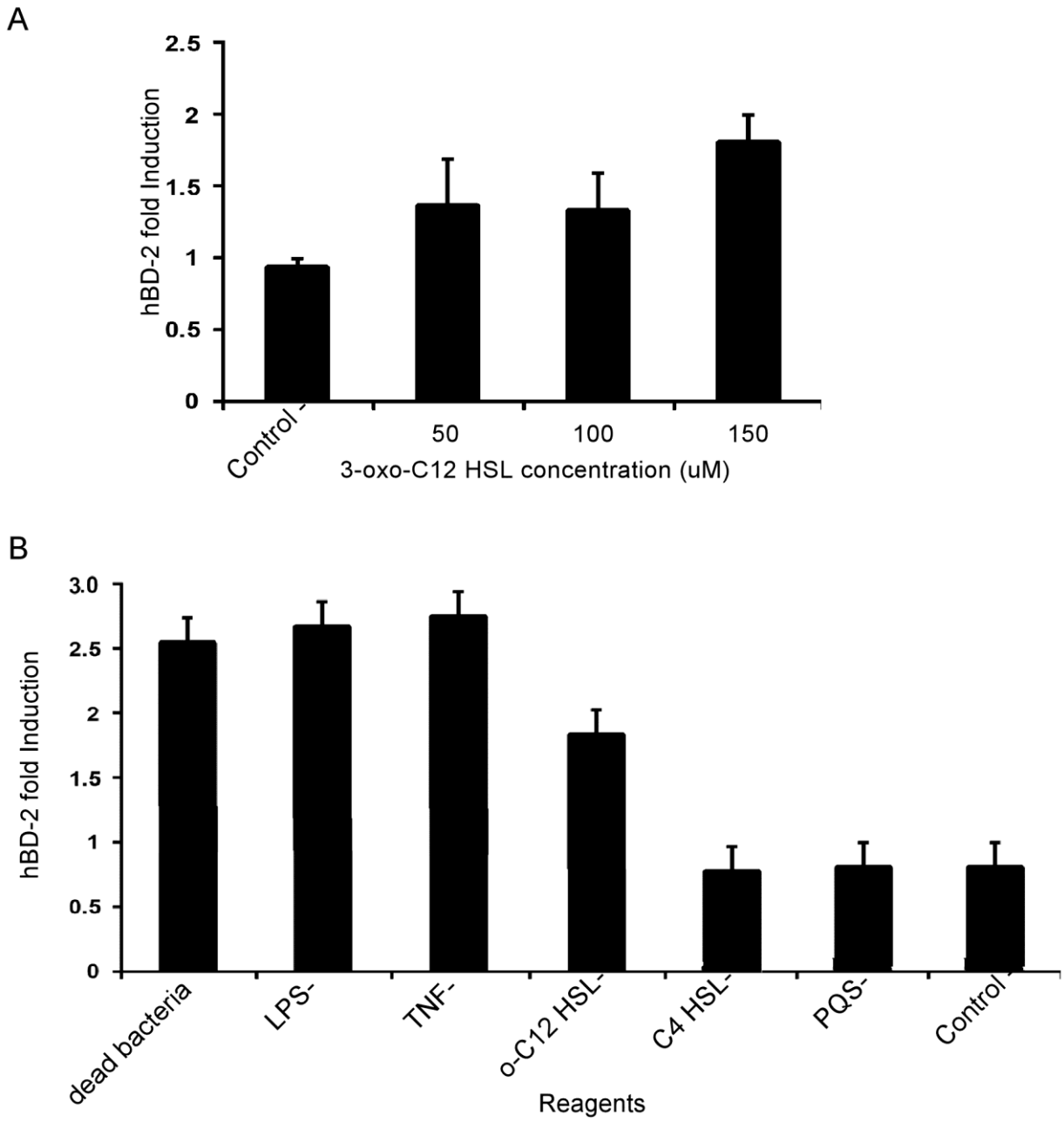


Fig4.5. Screening of hBD-2 promoter induction by *P. aeruginosa* PAMPs and QS molecules by cross kingdom signalling. Analyses of concentration dependent activation of hBD-2 promoter by 3-oxo-C12 HSL. A549 cells were transiently co-transfected with hBD-2 promoter containing pGL3 basic promoterless vector and pRL-TK vector (transfection control). Transfected cells were treated for 8 h with (A) increasing concentrations of 3-oxo-C12 HSL (0-150 µM) and screened by dual luciferase assays. (B) Identical sets were treated with heat killed *P. aeruginosa* (10^6/mL), LPS (20 µg/mL), TNF (10 ng/ML), 3-oxo-C12 HSL (150 µM), C4 HSL (200 µM) and PQS (100 µM). Sets were screened by dual luciferase assays. ta represents the mean ± SD from at least three independent experiments on independent cultures.

3-oxo-C12 HSL regulates synthesis of PPAR gamma in A549 cells

Jahoor et al has reported PPAR gamma as a putative receptor of 3-oxo-C12 HSL in mammalian cells and also it can affect binding of PPARs to PPREs of DNA. According to the reports 3oxo C12 HSL function as antagonist of PPAR gamma. Role of PPAR gamma in maintenance of innate immunity has also been well known. Also it has been now understood that HSL can also activates and upregulates NF-κB canonical pathway. However the missing link between PPAR gamma as an HSL receptor and NF-κB as a triggering pathway has not been reported earlier. Ligand bound PPAR gamma employs anti-inflammatory activity by trans-repression of NF-κB transcription factor which subsequently inhibits transcription of NF-κB dependent pro-inflammatory genes. To determine whether HSL can regulates PPAR gamma synthesis, A549 cells was incubated with different concentration of HSL (0-150µM), cells were lysed after 8h and western blot analysis was done using Anti PPAR gamma antibody. For housekeeping Beta Actin antibody was used for the same lysates. In contrast to the previous reports Immunoblot analysis showed increased expression at low concentration of 50µM and subsequently the expression decreased as the concentration of HSL was increased. (**Fig4.6)** To investigate whether the expression pattern of PPAR gamma induced by HSL is due to its synthesis, transcript level of PPAR gamma was also measured by qPCR in presence of HSL from low to high concentration. In contrast to western blot assay mRNA level of PPAR gamma showed reduced transcripts from 100µM and 150µM concentration whereas at 50µM concentration transcript level did not show any regulation (**Fig 4.6C**).

From the above data, it is clear that 3oxoc12 at low concentration binds to PPAR gamma and increases its synthesis but as soon as the concentration reaches >100µM, it inhibits its synthesis. 3-oxo-C12 bound PPAR gamma release the co-repressor complex of NF-κB and and activates the transcription of downstream NF-κB genes

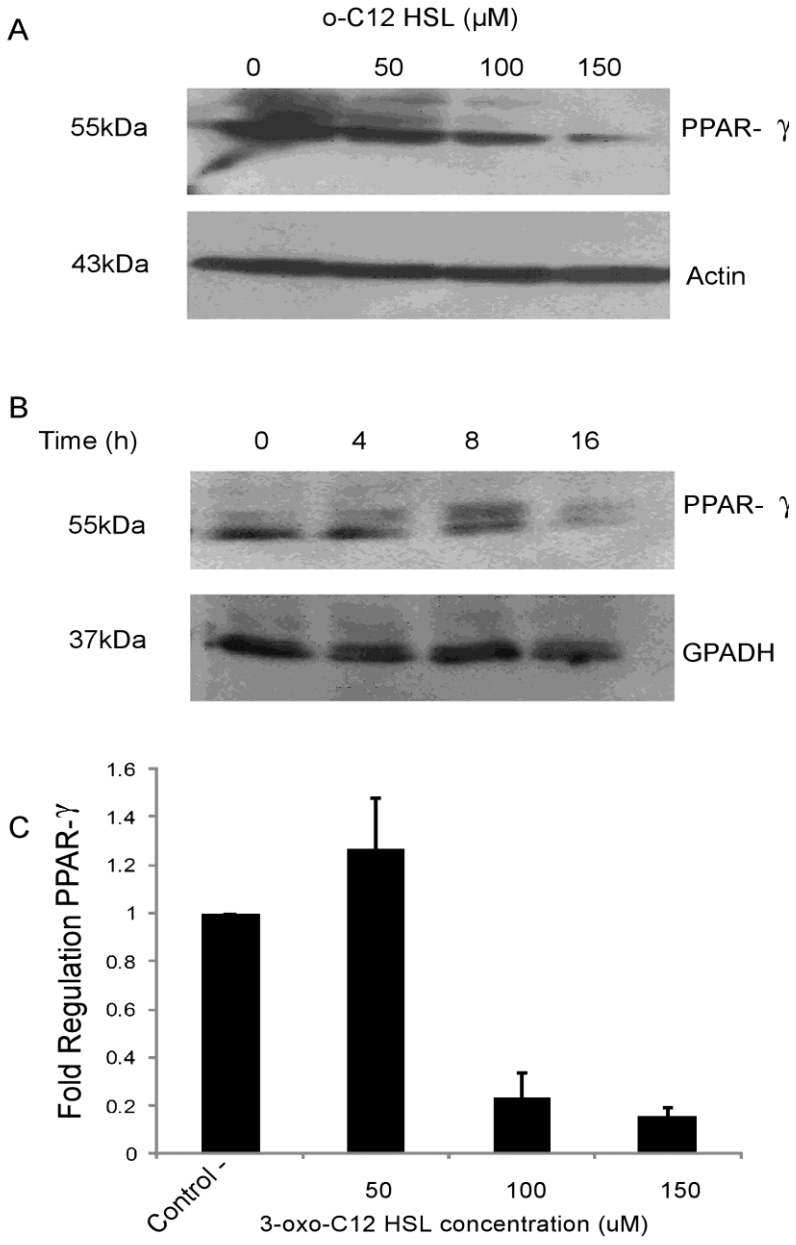


Fig 4.6: Temporal and concentration dependent assays of o-C12 HSL induction of PPARɤ in A549 lung epithelial cells. (A) Monolayers of A549 cells on were incubated with increasing concentrations of o-C12 HSL (0-150 µM) for 16 h and subjected to immunoblot analyses of PPARɤ. The concentration 150 µM o-C12 HSL was standardized and (B) temporally assayed for PPARɤ expression. The results represent one of three independent experiments to confirm the expression profiles. (C). Relative mRNA expression of PPAR gamma in o-C12 HSL treated (50 -150 µM) cells. ta represents the mean ± SD from three independent experiments.

3oxoC12-HSL induction of hBD2 protects lung epithelium from *Pseudomonas* infection.

Induction of NF-κB pathway on epithelial cells stimulates production of hBD-2. It was hypothesized for the first time that QS molecule; 3oxo C12-HSL not only induces production of hBD-2 but also in concentration equivalent to that, which has been shown to be potent antimicrobial. 3-oxo-C12 induced epithelial cells results in higher level of production of hBD-2 and hence showed more antimicrobial activity that control or non-induced cells when infected/co-culture with PA strains. Polarized epithelial cells in the apical compartment of Transwell were induced for 24h with HSL and subsequently activation was confirmed using IL-8 ELISA detection in the basolateral medium. Cells were then co-culture with ATCC *PA* strain for 6h. Following incubation the no. bacterial in C.F.U was enumerated from the apical cultures. Compared to control/untreated cells induced cells showed one log fold C.F.U was reduced **(Fig. 4.7 A&B**). Further to prove our hypothesis that hBD-2 induction is specifically reducing the bacterial number/load, we did hBD-2 knock down experiments. Cells were transfected with hBD-2siRNA and then induced with HSL for predetermined period and co-culture with same *PA* (**Fig4.7C**). The results clearly showed the antimicrobial activity of epithelial cell was reduced and no change in the C.F.U as compared to control was observed.

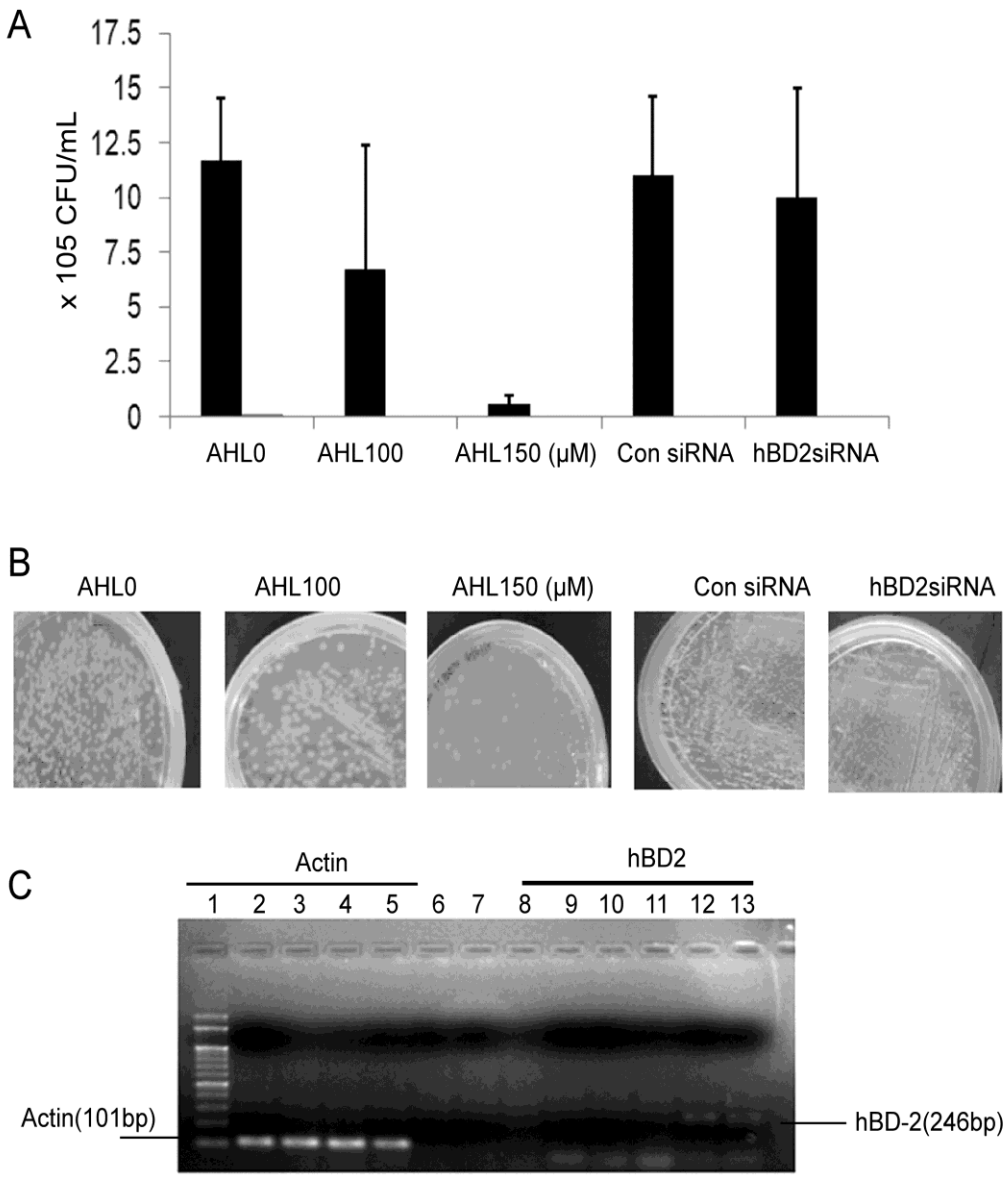


Fig 4.7: Upregulation of hBD2 by oxoC12 deters P. aeruginosa infection on lung epithelium. A. Overnight seeded A549 cells were treated with HSL (100-150μM) and subsequently transfected using hBD-2 siRNA and control siRNA. Cells were then co-culture with P. aeruginosa for 6h. Following co-culture cells were then washed with 1XPBS and plated on TSB agar plate and C.F.U was counted. ta represents the mean ± SD from at least three independent experiments. B. Representative images of the plates from the above experiment sets. The result represents three independent experiments with similar results. C. RT-PCR images of the cells when transfected with control siRNA, hBD-2 siRNA along with HSL (100-150μM) treatment for Actin and hBD-2.

Sub lethal dose of hBD-2 disturbs *Pseudomonas* early biofilm formation and HSL treated cells prevents *P. aeruginosa* infection and pathology in A549 cells

Further we analysed the activity of hBD-2 from induced basolateral medium and hBD-2 recombinant peptide on *P. aeruginosa in-vitro* biofilms. Conditioned media and pure peptide were incubated with 24h *P. aeruginosa* biofilms for 8 h. After incubation of the peptide or conditioned media with indicated period of time, sets showed significant inhibition of biofilms compared to control sets with profuse EPS producing biofilms (**Fig. 4.8 A & B**). *Live/dead*™ analysis of the bacterial cells showed 60% more killing than non treated cells/or cells treated with media alone. Next co-cultures of A549 with *P. aeruginosa* resulted in progressive loss of CellTracker™ signal after 8h and concomitant gain of propidium iodide till 12 h. In these sets, the A549 exhibited significant rounding and detachment within 6h of incubation leading to massive exfoliation of the monolayer and finally death (red cells) in remaining adhered cells (Fig 5.8C). In contrast, sets induced with HSL for overnight showed limited damage to monolayers, consistent CellTracker™ signal and impermeability to propidium iodide (Fig.5.8D). Since CellTracker™ is safely passed through several generations and indicates metabolically active cells, these observations indicated 3-oxo-C12-HSL protection against *P. aeruginosa* without any cytotoxicity to the A549. Finally the data indicates that HSL mediates activation of hBD-2 in a biologically significant concentration which can reduce /protects lung epithelia from invading *P. aeruginosa* infection.

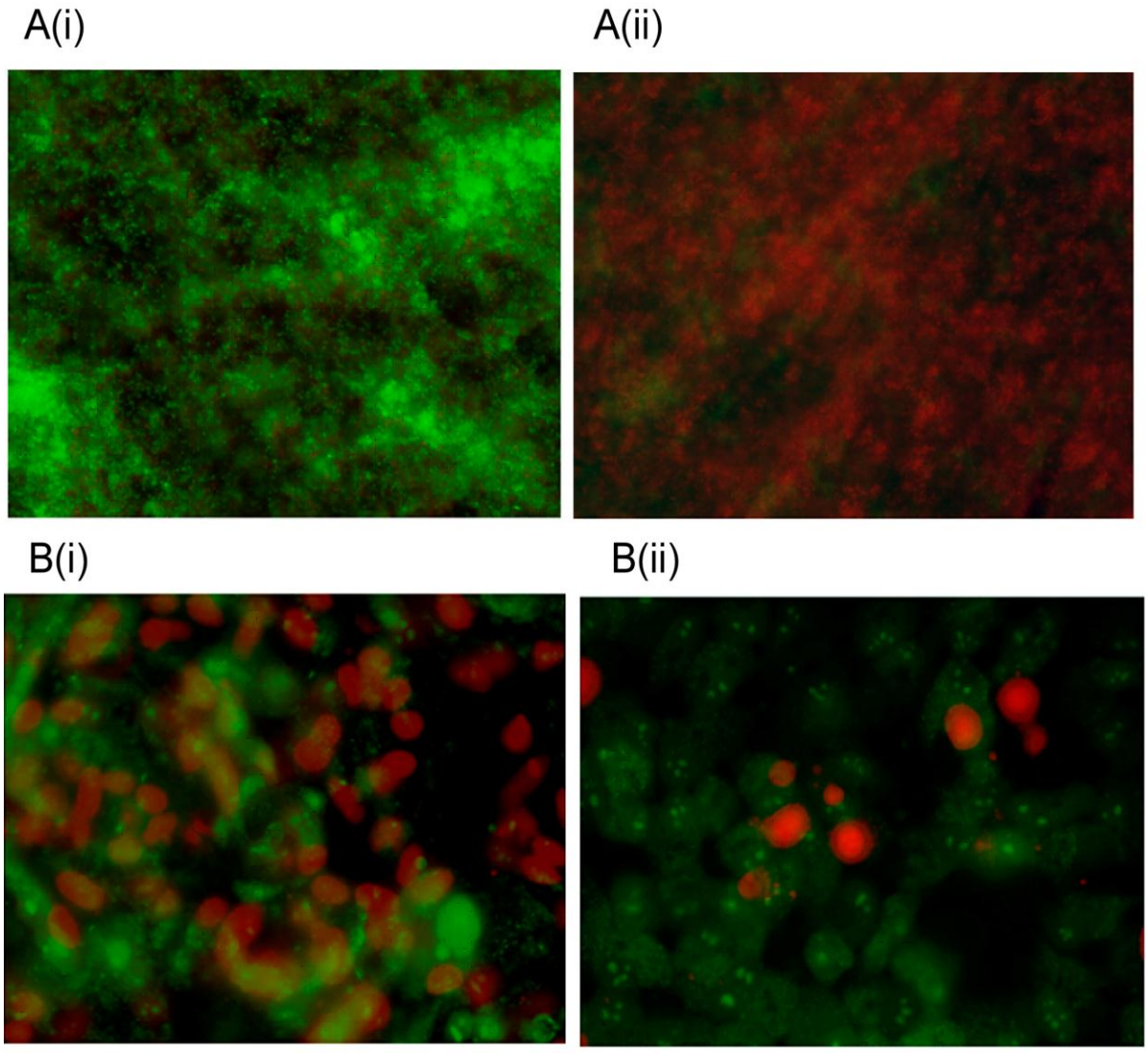


Fig 4.8 (A)Live dead analysis of *Pseudomonas* early biofilm 16h untreated (B) treated with sublethal dose of rhBD-2. Biofilms were stained with Syto9 and Propidium Iodide (PI).C. A549 cells when co-culture with *P. aeruginosa* for 8h D. HSL (150µM) induced A549 cells co-culture with *P. aeruginosa* for 8h. Cells were stained with CellTracker™ and PI.

3oxoC12- HSL induces hBD-2 mRNA in intestinal epithelium and corneal epithelium cells.

3-oxo-C12 induction of hBD-2 was detected in lung epithelial cells. Now as to confirm whether the induction of 3-oxo-C12 HSL can upregulates hBD-2 in other epithelium like intestine or eye where *Pseudomonas* infection has been reported like in case keratitis and *Pseudomonas* GI infection. HSL treatment (0 -150 µM) was done in human intestinal epithelium (HT-29 cells) as well as human corneal epithelium (HCEC cells). After treatment RNA was extracted and relative gene expression for hBD-2 was determined in control and treated set in dose dependent manner. HCEC showed maximum upregulation of hBD-2 by 3.3 fold when treated with 150µM HSL **(Fig. 4.9A**). Whereas HT-29 cells when treated with HSL showed upregulation of 2.4fold when treated with 150µM HSL **(Fig. 4.9B**)

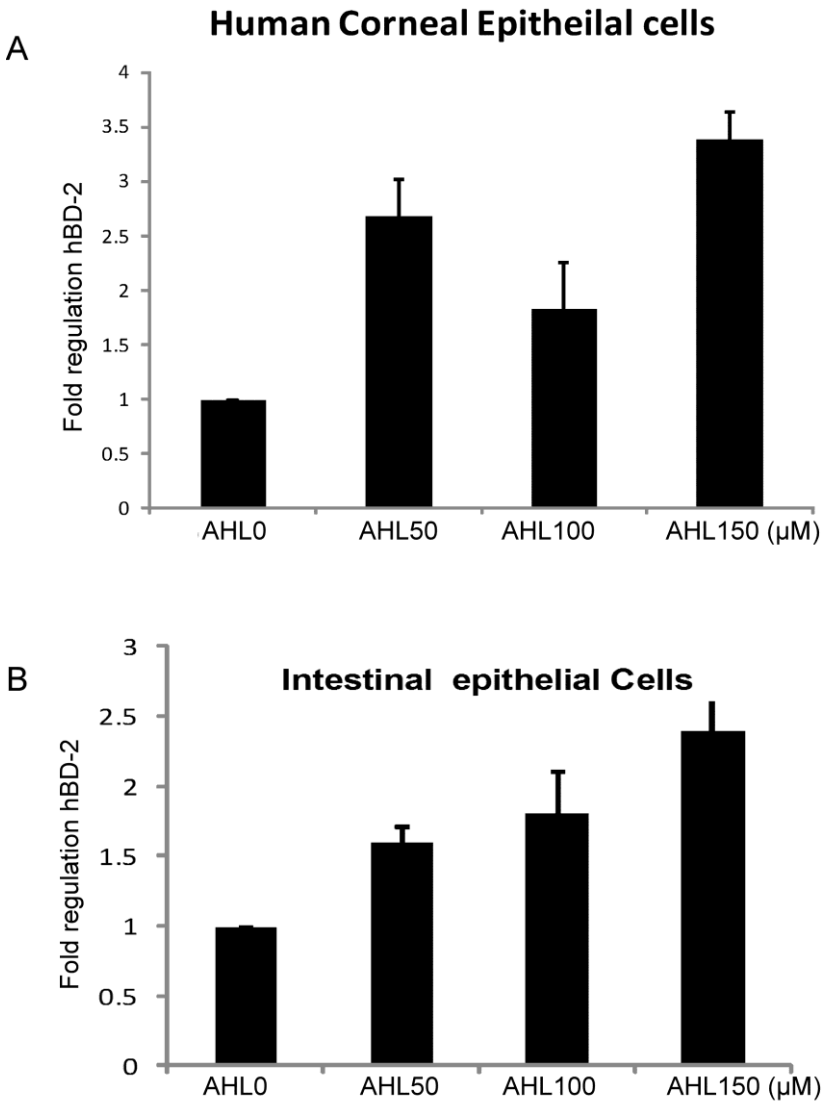


Fig 4.9. Fig 5.9. hBD2 mRNA regulation by 3oxo-C12-HSL in corneal epithelial (HCEC) and intestinal epithelial (HT29) cells. A. Total RNA was isolated from HSL (0-150(µM) treated Humar corneal epithelial cells (HCEC) and B.Huaman intestinal epithelial cells(HT-29). Quantitative Real Time PCR analysis was performed against hBD-2 and ß-Actin primers. ta represent the mean ± SD from at least three independent experiments

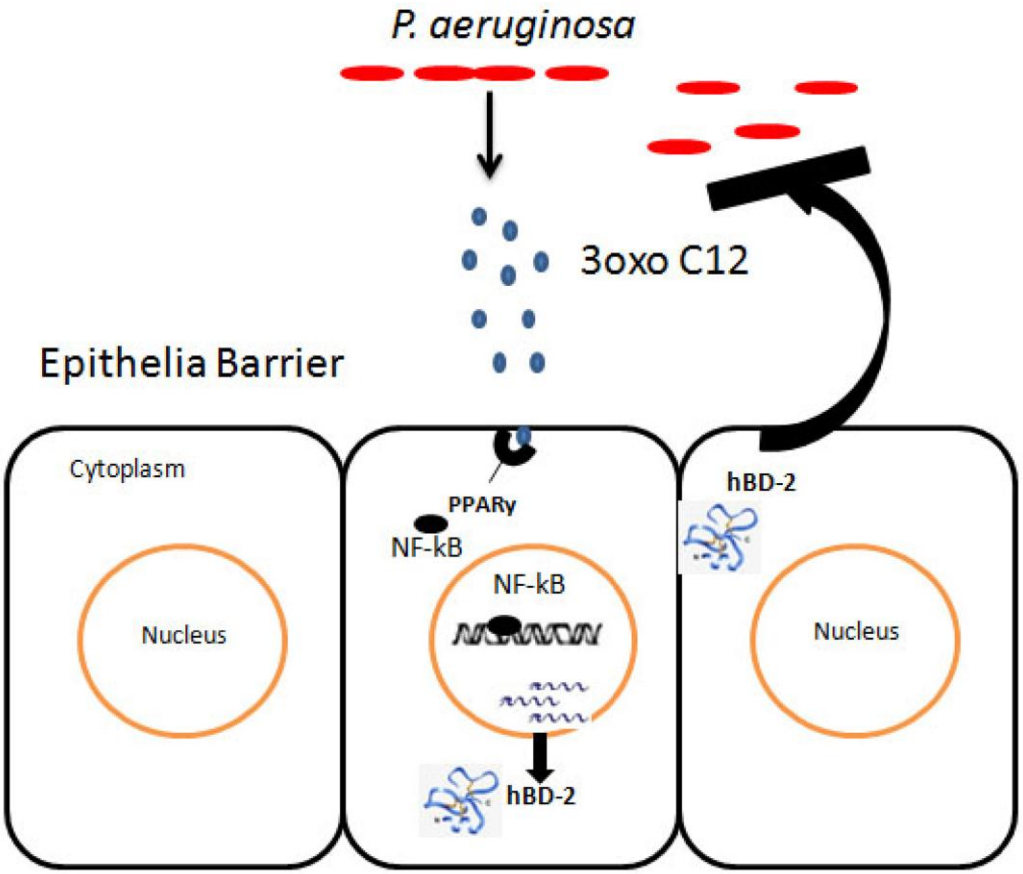


Fig 4.10: Proposed model for HSL mediated induction of epithelial innate immunity (hBD-2)

Pseudomonas Quinolone Signal (PQS) and Outer Membrane Vesicles (OMVs) associated quinolones down-regulates hBD-1 expression in lung epithelial A549 cells

In order to investigate the cross-kingdom signalling roles of the quinolone class of *Pseudomonas sp.* QS, on mammalian innate immune system, the Another major *Pseudomonas* QS molecule **Pseudomonas Quinolone Signal (PQS) was studied.** has also been known for cross kingdThe assays determined, whereas PQS had no influence in hBD-2 induction, this QS reduced the endogenous expression of hBD-1 in lung epithelial cells. A549 cells were treated with PQS (0-100µM) and purified OMV QS extract containing PQS f showed less immunofluorescence for hBD-1 in immunocytochemistry assays than control sets (**Fig4. 11A-C**). Similarly OMV extracted quinolones treated cells also showed lower levels of hBD-1 expression **(Fig4.11D**).

PQS downregulate hBD-1 transcription in lung epithelial A549 cells

As from the results it is clear that all class of Quinolones including PQS downregulate hBD-1 expression, thereby to check it on the transcription level, RNA from PQS treated (0-100µM) cells were extracted and relative gene expression was checked with hBD-1 primers and actin was kept as housekeeping control. Treated cells showed 1.7 fold dowregulation with 100µM of PQS and 1.9 fold dowregulation when treated with OMVs. The data thereby confirm with dowregulation of hBD-1 peptide expression in A549 cells (Fig4.12). These complimentary lines of evidences support that PQS and associated quinolones can downregulate innate immunity by lowering the constitutive expression of hBD-1.

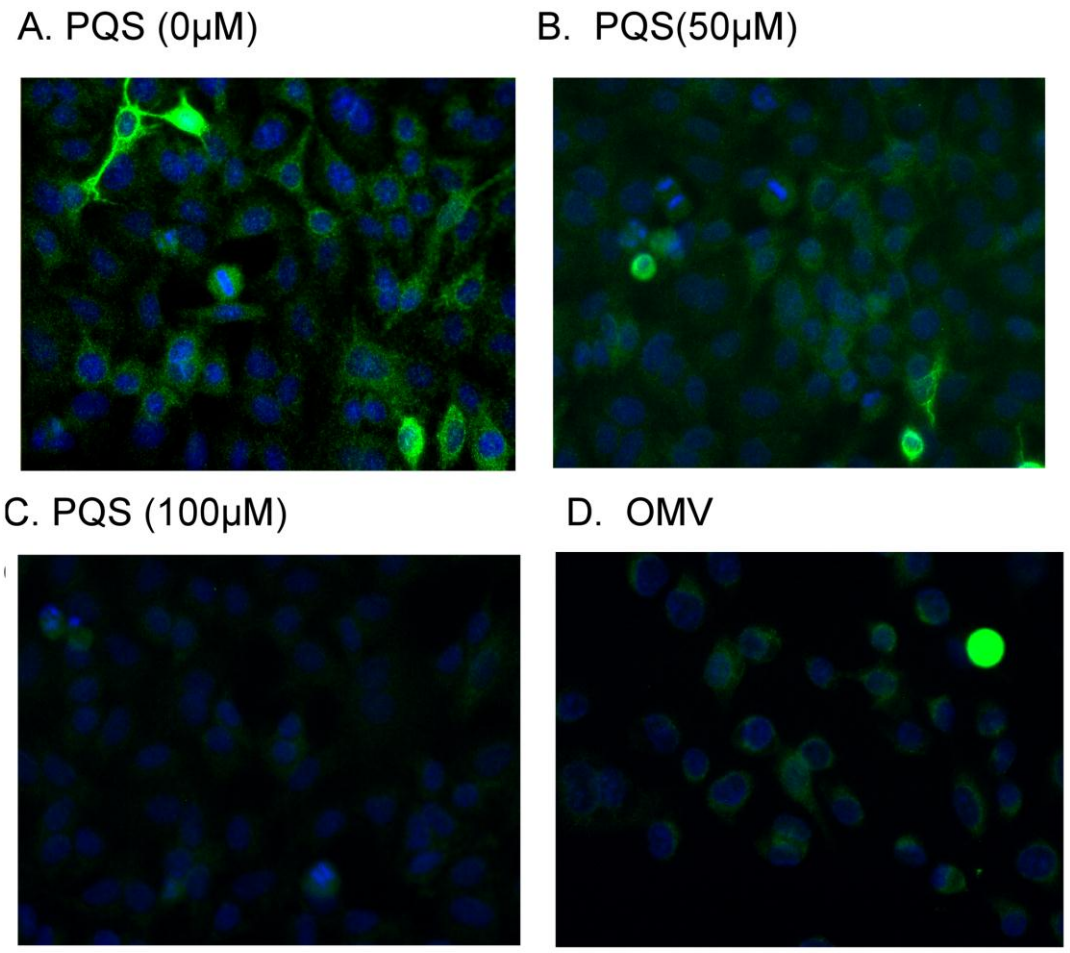


Fig 4.11: Regulation of constitutive human beta defensin1 (hBD1) expression by Pseudomonas Quinolone Signals and OMVs associated quinolones.A549 cells were treated with PQS (0-100μM) and purified OMV isolated from *P. aeruginosaP. aeruginosa* for 12h and immunocytochemistry was performed using hBD-2 antibody. Images were acuired using Olympus Fluroscence Microscope. The result represents one of the experiments with similar results obatined.

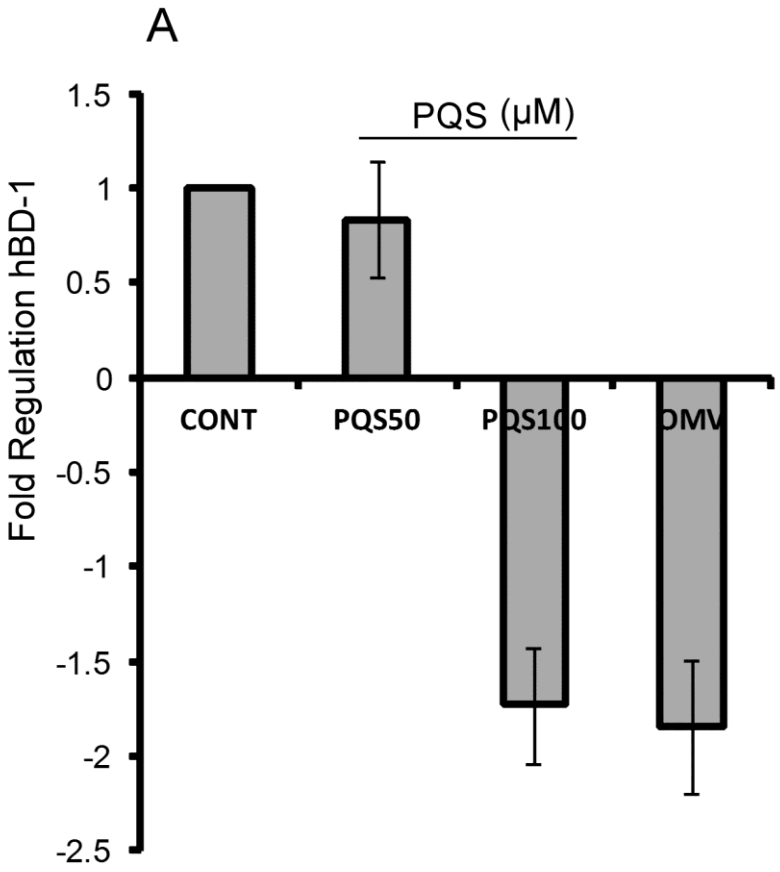


Fig 4.12 Effect of PQS and OMV associated quinolones in hBD1 transcription when treated with A549cells.A. Total RNA was isolated from PQS (0-100(μM) and OMV treated A549 cells and quantitative Real Time PCR analysis was performed against hBD-1 and ß-Actin primers. ta represent the mean ± SD from at least three independent experiments

PQS downregulates hBD-1 by repressing HIF-1 alpha transcription factor signalling

HIF-1 alpha is known to play an important role in maintenance of immunity. hBD-1 promoter has HIF-1 binding site in it. Further it is also investigated that HIF-1 alpha is required for the constitutive expression of DefB1. Thereby repression of HIF-1 alpha transcription factor can lead to dowregulation of hBD-1. There to check whether PQS suppression of hBD-1 is via HIF-1 or not, the investigation was done two different ways. First hypoxia was induced in A549 cells by treatment with cobalt chloride and then checked for hBD-1 expression, that whether HIF-1 over expression or hypoxic environment can induce hBD-1 mRNA expression. Second was checked for if over-expression of HIF-1alpha. Second PQS treated hypoxic cells can suppress the induction hBD-1. A549 cells were taken, induced for hypoxia and then treated with PQS. After treatment mRNA profile for hBD-1 gene was checked. The results showed clear induction of hBD-1 mRNA by cobalt chloride (100µM), where as PQS treated cells along with cobalt chloride suppressed the induction of hBD-1 mRNA. PQS alone did show down regulation as reported in earlier results **(Fig. 4.13).**

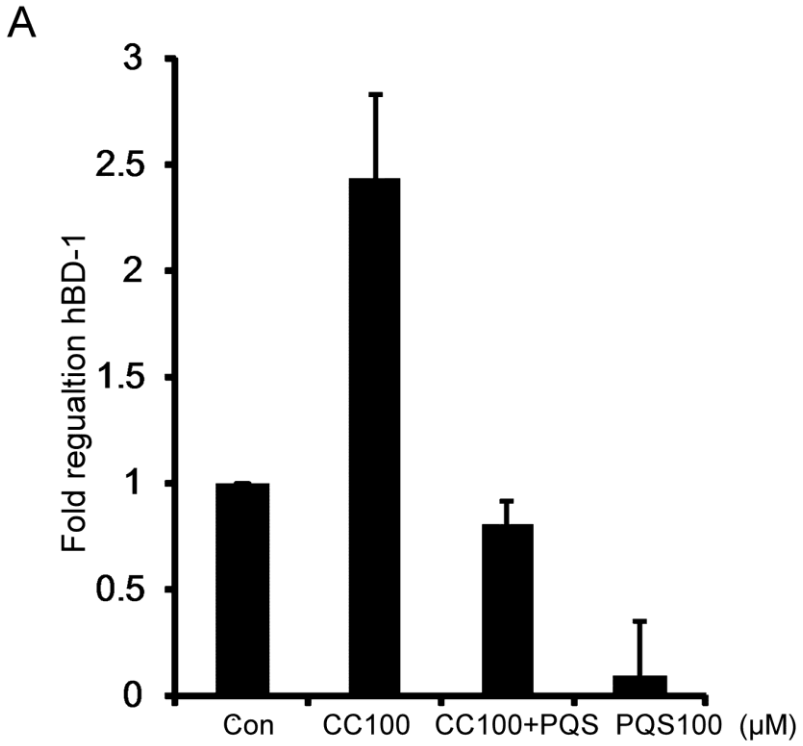


Fig4.13: Effect of PQS on hBD1 expression induced by hypoxia. Total RNA was isolated from cobalt chloride (100µM), cobalt chloride and PQS (100µM), only PQS (100µM) treated and control A549 cells and quantitative Real Time PCR analysis was performed against hBD-1 and ß-Actin primers. Data represent the mean ± SD from at least three independent experiments

PQS inhibits hypoxia mediated nuclear translocation of HIF-1alpha.

HIF 1 alpha is translocated to nucleus when gets activated in hypoxic environment. Cobalt chloride treated cells induces HIF-1 activation and translocation. Here A549 cells were treated with cobalt chloride 100µM for overnight, in another set cobalt chloride induced cells were treated with PQS. Cobalt chloride treated cell showed clear nuclear translocation of HIF-1 alpha compared to control cells **(Fig4.14A**). Whereas PQS treated cells along with cobalt chloride prevents any nuclear translocation and activation of HIF-1 alpha (**Fig4.14B**). Thereby the data suggest PQS downregulate and suppress HBD1 via HIF-1 alpha mediated pathway.

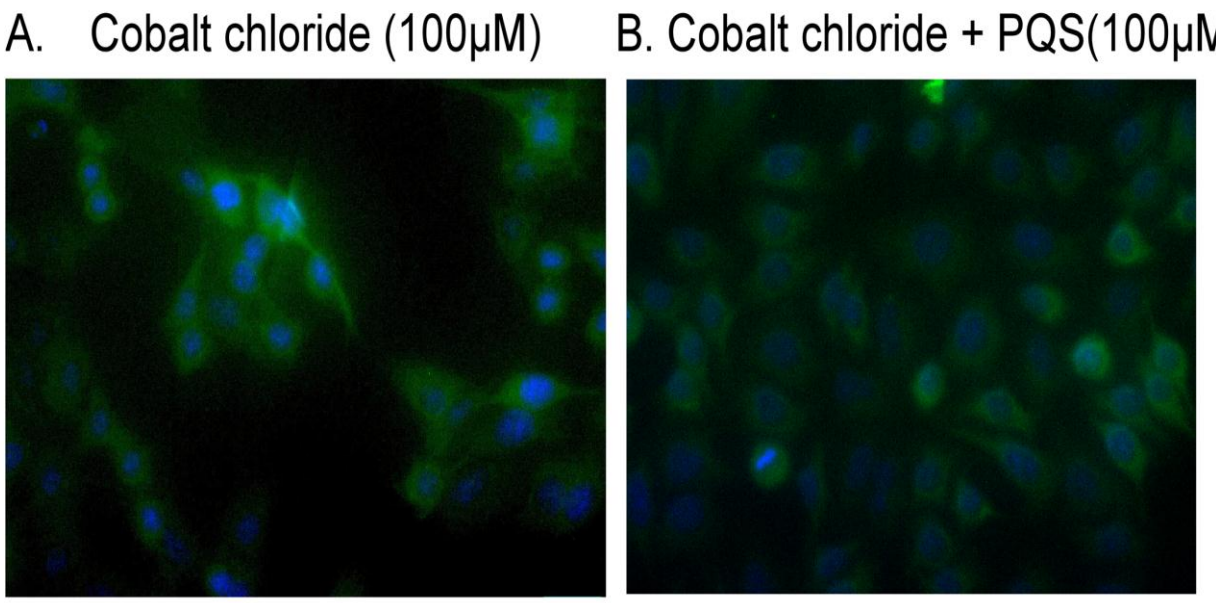


Fig 4.14: Effect of PQS on nuclear localization of Hif1alpha mediated by cobalt chloride induced hypoxia.A. A549 cells induced for hypoxia upon treatment with Cobalt chloride (100µM) B. Cobalt chloride treated cells induced with PQS (100µM) for 8h . Immunocytochemistry was performed using HIF-1a antibody using Olympus fluorescence microscope. The result represents one of the experiments with similar results obtained.

Targeted Metabolomics of QS molecules in endotracheal tube effluents from VAP patients by LDI-MS

QS molecules 3-oxo-C12 HSL and AQs have been earlier detected in Cystic fibrosis patients sputum infected with *P. aeruginosaP. aeruginosa* infection. However no studies have reported for VAP effluents analysis for screening of QS molecules from *P. aeruginosa* infected individuals. In this study, VAP effulent were collected from extubated ETT tubes from VAP patients with positive P. aeruginosa. Sample were then extracted twice with acidified ethyl acetate and subjected to High resolution LDI-MS mass spectrometry. LDI-MS showed the presence and detection of 3-oxo-C12-HSL, NHQ and PQS (**Fig4.15**) Precursor mass ($M+H^+$) was successfully obtained within 20ppm error. However signal to noise ratio was high because of the complexity of the sample, also contain other debris.

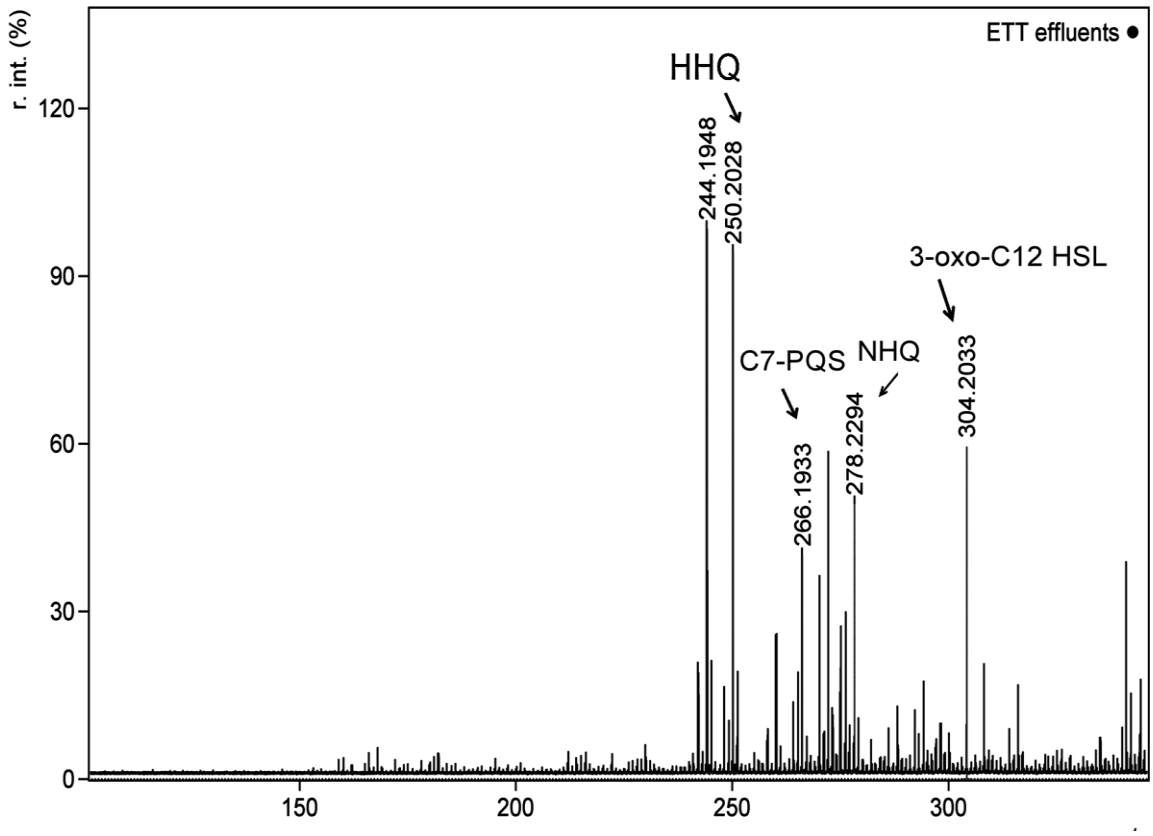


Fig 4.15: Targeted QS metabolomics of VAP ETT effluents/fluids from *P. aeruginosa* infected patients using LDI-MS. Ethyl acetate extracts of ETT effluents were mixed with Lithiated LDI surface and 1µL of the sample was spotted in MALDI plate and subjected to high resolution LDI-MS. Spectra was acquired three spots per sample and scanned for M+Li (m/z) precursor mass. The data represent at least one spectrum with similar results

***In-vivo* study of mouse beta defensin 3 regulation by 3oxoc12-HSL in mouse trachea.**

Swiss albino mice treated mice with HSL (150µM), TNF Non-pathogenic and Clinical strains were sacrificed and total RNA was extracted from trachea of control and experimental group. mBD3 is consider to be the homologue for hBD-2 in mouse. Thereby, qPCR for mBD3 was done, which showed significant upregulation in HSL treated groups and TNF as positive control. However non-pathogenic strain did not show any regulation for mBD-3. mBD-1 expression was found to be constitutive in all the cases except the pathogenic strain which showed downregulation for mBD1 (**Fig4.16)**.

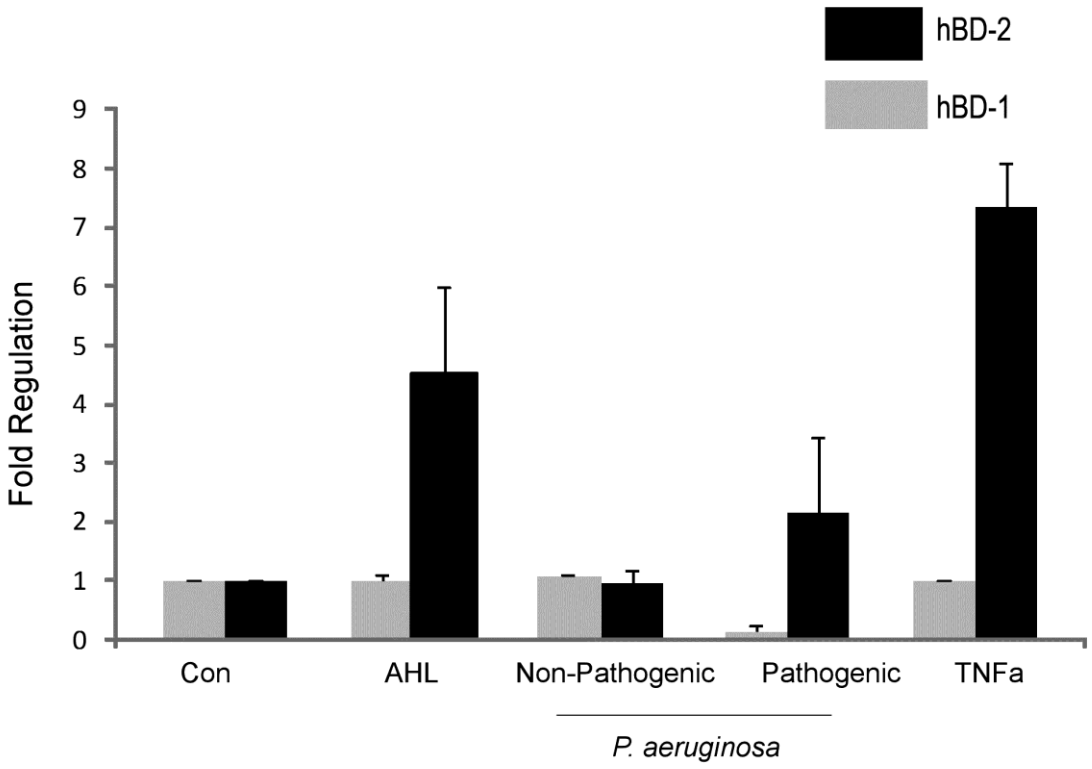


Fig 4.16: qPCR for mBD3 in HSL treated animals group and control animals group. Total RNA was isolated from control and HSL, TNF, *P. aeruginosa* treated animal group and quantitative Real Time PCR analysis was performed against mBD-1, mBD-3 and ß-Actin primers. ta represent the mean ± SD from at least three independent experiments

Discussion

Antimicrobial peptides constitute an important component of mammalian epithelial immunity. Whereas alpha defensins are found in polymorphonuclear leukocytes and paneth cells of small intestine, beta defensins are produced in all major epithelial cells. Among beta defensin family hBD-2 is remarkable in its way, that it is one of the strongest inducible AMP in response to various stimuli and thus helps maintaining host defence by eliciting strong antimicrobial response against gram negative pathogens. In this study it was identified that bacterial QS metabolite can regulate innate immune pathway. Earlier it has been reported that *Pseudomonas* QS 3-oxo-C12 HSL can modulate immune system in various cell lines and also in murine model[59]. However HSL modulating innate antimicrobial peptide or beta defensins in epithelial cells was not reported. In this study it was found out that when A549 lung epithelail cells were treated with *Pseudomonas* QS molecule 3-oxo-C12 upregulates endogenous expression of hBD-2 in dose dependent manner (**Fig 4.1**). Upregulation was confirmed with the help of western blot assays, ELISA and ICC. This suggests 3-oxo-C12 can upregulates hBD-2 expression in lung epithelial cells as well as hBD-2 secretion in the local environment as detected by ELISA. Further, **Fig 4.2** showed increased hBD-2 mRNA when A549 cells were treated with increasing concentration of HSL. Maximum upregulation was achieved when A549 cells were treated with 150µM HSL. Temporal based assays revealed hBD-2 mRNA upregulation started as soon as after 6h of treatment and the transcript is stable upto 24h. The data confirmed that hBD-2 mRNA upregulation was ultimately leading to increased protein expression in lung epithelial cells when treated with 3-oxo-C12 HSL. Further it was proposed the involvement of NF-κB signaling pathway as mechanisms for hBD-2 upregulation by 3-oxo-C12 HSL. hBD-2 promoter has binding sites for NF-κB transcription factor and is well known to gets activated in presences of stimulus which induced hBD-2 expression. Here 3-oxo-C12 HSL successfully activated NF-κB pathway which was confirmed with 3-oxo-C12 HSL binding with p65 in time dependent assay using EMSA. In support to this it was found out that the cells treated with HSL also showed increased phosphoP65 and phospho IkB expression when cells treated with HSL (**Fig 4.3**). To further confirm the activation of hBD-2 promoter containing NF-κB binding by HSL, the promoter region (2338bp) of hBD-2 was cloned in pGL3 basic vector (**Fig 4.4**) and by dual luciferase assay it was found out that cells upon treatment with HSL

showed approximately 1.8 fold upregulation (**Fig5.5**). All these data indicated activation and involvement of NF-κB pathway for HSL mediated induction of hBD-2.

Jahoor et al. reported a subset of nuclear receptor family PPAR as putative receptor for 3-oxo-C12 HSL, as soon as the molecule enters inside mammalian cells. They have reported one of the PPAR member family i.e PPARϒ act as a 3-oxo-C12-HSL potent receptor which can further activate multiple signaling cascades like pro-inflammatory genes etc. They have also reported that 3-oxo-C12 act as PPARϒ antagonist. Further there are earlier reports which have shown that 3-oxo-C12 competes with PPARϒ ligand and can inhibit its binding to the receptor[144]. Also PPARϒ itself plays crucial role in maintenance of epithelial innate immunity. It has been also reported that ligand bound PPARϒ trans-repress NF-κB by forming a co-repressor complex which further inactivates the NF-κB activation and transcription of NF-κB dependent pathways. Thereby concluding all these facts the hypothesis in this study proposed was HSL activating NF-κB pathway via PPAR dependent mechanism. It was shown here that HSL treated cells downregulation of PPARϒ endogenous protein in dose and time dependent manner (**Fig4.6A-B**). 150μHSL showed clear dowregulation of PPARϒ in A549 cells after incubating for 16h. In contrast to previous report the data showed slight upregulation of PPARϒ when cells were treated with 50μHSL. In addition to this PPARϒ transcript was also regulated in similar manner where slight up-regulation (20-30%) was observed with 50 μHSL but clear downregulation was seen when treated with 150μHSL (**Fig4.6C**). Hence, the study proved 3-oxo-C12 HSL repressed expression of PPARϒ which further can relieves downstream transrepression activity on NF-κB and switches on the NF-κB signaling pathway and ultimately leads to synthesis of hBD-2 expression. Next the antimicrobial activity of induced hBD-2 against *P. aeruginosa* was checked using co-culture assay (**Fig 4.8**). A549 cells when induced with HSL for overnight and co-cultured with *P. aeruginosa* one log fold reduce C.F.U than the control cells. Simultaneously to check the specific activity of hBD-2 activity, hBD-2 knock down assay was done using hBD-2 specific siRNA and cont siRNA. hBD-2 knock down cells when co-cultured with same MOI of *P. aeruginosa* showed same C.F.U as compared to control (**Fig 4.7**). This indicated HSL induced cells have the capability to clear out *P. aeruginosa* load on the epithelial cells. All these data strongly point out the ability of 3-oxo-C12 HSL to induce immunity by upregulation of hBD-2 in lung epithelium. **Fig 4.10** states the proposed

hypothetical model of HSL mediated upregulation of hBD-2 via involvement of PPARϒ and NF-κB signaling pathway.

In addition to this, *P. aeruginosa* infection is also commonly associated with eye epithelium in case of microbial keratitis or intestinal epithelium in case of dysbiosis. Thereby, further to check the potential of 3-oxo-C12 HSL as innate immune target, HSL treated human corneal epithelial cells (HCEC) and intestinal epithelial cells also showed upregulation of hBD-2 transcripts when compared to control in dose dependent manner. However detailed pathway of HSL mediated induction of hBD-2 is yet to be done in corneal and/or intestinal epithelium.

The other major class of *Pseudomonas* QS molecules i.e *Pseudomonas* Quinolone signal or PQS have also been shown to modulate immune system. reported that PQS suppress immunity by down regulation of NF-κB mediated signaling pathway. However, regulation of epithelial antimicrobial peptide is not known. The fact that hBD-1 is constitutive and first line defence of epithelial host immunity, the hypothesis here proposed was that whether PQS can regulate the constitutive expression of hBD-1 in lung epithelial cells. A549 cells when treated with PQS showed clear downregulation of constitutive hBD-1 peptide expression in dose dependent manner (**Fig 4.11**). Also as discussed in chapter 1 that all classes of Quinolone are associated OMVs of *P. aeruginosa*; thereby A549 cells were also treated with purified and extracted OMVs. The OMVs containing all class AQs also showed downregulation of hBD-1. In addition to the data also supported with reduced transcript level of hBD-1 when treated with different concentration of PQS (**Fig 4.12**). Hence the study clearly revealed PQS and other class of quinolones can suppress expression of hBD-1 which is because of reduced transcript level in A549 cells. The study revealed PQS to be a potent immunosuppressive agent. Further to understand the mechanism, it was proposed that PQS downregulation of hBD-1 is via involvement of HIF-1. The proposal was based on the two facts that hBD-1 promoter contains HIF-1α binding sites and PQS has been reported to degrade HIF-1α via proteosomal pathway. However PQS downregulation of hBD-1 via HIF-1α signaling pathway was not reported. So, first it was identified that HIF-1overexpression by cobalt chloride induce hypoxia leads to upregulation of hBD-1 in A549 cells. Whereas cobalt chloride when treated along with PQS or PQS alone treatment did not show any upregulation of hBD-1 transcript (**Fig4.13**). This also supported the fact that hypoxia can triggers upregulation of hBD-1. In addition to this the data showed PQS can inhibit the

activity of cobalt chloride induced hypoxia and thereby suppress the expression of hBD-1. To confirm the PQS mediated downregulation/inhibition of HIF-1α protein expression, A549 cells induced with cobalt chloride for hypoxia and then treated with PQS did not show any nuclear localization whereas only cobalt chloride treated cells showed successful nuclear localization of HIF-1α (**Fig 4.14**). Thereby, overall this can be employed that PQS and OMV associated quinolones can suppress epithelial immunity by downregulation of hBD-1 via HIF-1α mediated pathway. Overall observations made through this study suggested that during early *P. aeruginosa* infection HSL dominates and can induce immune system by inducing epithelial antimicrobial peptide hBD-2 expression. Whereas during chronic/late infection of *P. aeruginosa* PQS accumulate and dominates over HSL (also observed in 1st Chapter). As soon as the infection proceeds PQS signaling suppress the immune system and also lung epithelial immunity by downregulation of hBD-1. The mechanism hence can be enjoyed by the pathogen for persistence of the chronic infection.

Further, mouse beta defensin-3 or mBD-3 is considered as hBD-2 homologue. The validation of the project was finally confirmed *in-vivo* model by treatment of mice with HSL. 3-oxo-C12HSL treated mice results in significant upregulation of mBD-3 in trachea samples than control. **(Fig 4.16**). However regulation was observed in lung defensin expression. This may be due to route of HSL administration was via nasal drops and may didn't reach upto lung environment and/ or can also because of HSL degrading enzyme PON-2 present in the epithelium. Induced hBD-2 can in turn clearout/reduce bacterial load on the lung epithelium.

Pathology of QS molecules is now well known in case *Pseudomonas* infection where the molecules have been detected from bio fluids. However no systemic studies/profiling of these molecules is there with respect to disease specific or pathogen specific. Here from the VAP patients' extubated ETT effluents direct detection of QS molecules was achieved which includes 3oxo-C12HSL, NHQ and PQS (**Fig4.15**). This implies the significance of QS molecules as may be proposed biomarkers in VAP (*Pseudomonas* infection) after systemic routine analysis. Earlier Barr et al have reported the presence of Quinolones in CF sputum samples. Therefore this study can also propose/imply/ the direct involvement of QS molecules in disease.

Section 5

SUMMARY AND DISCUSSION

Coexistence of prokaryotes and eukaryotes subsist since billion of years in mutual shared environment. The co-evaluation of prokaryotes and eukaryotes (mammals) has driven the establishment of symbiosis, mutualism, commensalism and parasitic relationships. Mammalian epithelium plays a significant role in these associations[100]. Complex chemical communications interplay between colonizing prokaryotes and the epithelium and this cross kingdom communications is termed as interkingdom signalling[51]. The outcome of the association results in health or disease. The interkingdom signaling is potentially important for pathogens for establishment of their relationship with the host. Using rudimentary, but evolutionary conserved innate immune sensors and effectors, the epithelium can identify "Pathogen Associated Molecular Patterns" on bacteria and selectively eliminate infectious threat, yet promote symbiotic/commensal associations[100]. Bacteria as well as host can deliver signal to each other. For example epinephrine and norepinephrine produced by mammalian host activate indole mediated QS system of enterohemorrhagic *E. coli* (Clarke, 2005). In this work we have focussed here on the signaling induced by bacterial metabolite that regulates gene expression in mammalian cells. Both commensal and pathogenic bacteria can form biofilms on the epithelial surface; only the pathogens follow this with invasion. These biofilms use "quorum sensing" mediated by small, chemically distinct classes of messengers which modulate large gene sets within target bacteria; affecting as much as 6% of the gram negative bacteria *Pseudomonas aeruginosa* genome[41, 153]. This study delineates about the signaling mediated by QS metabolites in mammalian cells. We have focussed mainly on two major QS molecules of *Pseudomonas* i.e 3-oxo-C12HSL and *Pseudomonas* quinolone signal (PQS) and their capability to modulate innate immune signaling pathway inside host cell. Antimicrobial peptide constitutes an important part of innate immune response which exists in all classes of life. In human, two main antimicrobial peptide classes exist i.e defensins and cathelicidins. Among all antimicrobial peptide Defensins are unique in its way as it comprises of diverse group of members, expressed through large number of gene sets and most prominent in human. Defensin contributes to antimicrobial action of granulocytes, mucosal immunity in intestine and also in epithelial host defence[84]. Among defensins, whereas hBD-1 is constitutively expressed, beta defensin 2 is strongly inducible upon stimulus and also most potent antimicrobial peptide against Gram negative bacteria[146]. This study deals and reveals the uncharacterized pathway of early host microbe interaction and epithelial immunity via QS molecules. QS molecules are hallmark for *Pseudomonas* virulence and pathogenesis. Identification and

characterization of QS molecules is becoming a new strategy in order to determine the state of virulence in *Pseudomonas aeruginosa.*

In the first part of the project we have identified QS molecules from different *Pseudomonas* strains using novel mass spectrometry method i.e *matrix free*- Laser Desorption Ionization Mass Spectrometry (LDI-MS) on MALDI platform. The platform enjoys robust, without any matrix interference peaks that allows competitive advantage in analyzing small QS molecules. Using LDI-MS simultaneous profiling of all major QS molecules was obtained in *P. aeruginosa* type strains. Further we proposed lithiation of QS molecule which can improve the signal to noise ratio of by removing $M+H^+$, $M+Na^+$ and $M+K^+$ and yielding only $M+Li^+$ peaks. All HSLs and AQs molecules were easily lithiated and peak intensity was also improved. Application of mesoporous silica, used as matrix free LDI platform for biofilm metabolites analysis showed good reproducibility and sensitivity and thereby proved to be a potential alternative against regularly used MALDI-MS. Further, lithiation mechanisms proposed with extreme cationization properties seemed to be advantageous than other cationized species. The direct matrix free LDI with lithiated samples indicates strong interaction between material surface and analytes vital for charge transfer process. This indicates that the preformed ions as a major contributor to the major ionized molecules and proposed as a mechanism for LDI-MS process.

The potential of LDI surface was proved further with the screening of different clinical strains Vs Type (non- clinical) strains. LDI-MS technology successfully endorsed for specific QS metabolites which could differentiate between pathogens and non-pathogens. Further subsequent quantification of the samples using LC-MS based relative and absolute quantitation revealed that family of PQS molecules expression was quite higher than 3-oxo-C12-HSL in clinical strains than non-clinical/wild type strains. In addition, we could not detect any C4 -HSL in clinical strains. Therefore the data implies that in clinical strains PQS is over expressed than other QS molecules and thereby we can conclude that since it is being negatively regulated by $C4HSL^{154}$, we were unable to detect C4-HSL in theses strains. Overall the study supported application of LDI surface as global detection tool to identify pathogen specific metabolite signature. Further application of the study was implied on targeted metabolomics of outer membrane vesicles (OMVs) derived from the same pathogen. These nano-scale delivery vehicles contain discrete arrays of prokaryotic pathogenic determinants, including a family of small MW lipidic quorum

signaling alkyl-Quinolones (AQs) and also responsible for mediating cross kingdom signaling functions via AQs[71]. Accurate characterization of these OMV-AQs may reveal novel mechanism of diseases and *Pseudomonas aeruginosa* presents an ideal model. Untargeted LDI-MS of AQs in OMVs which reveal group of 8 major AQs expressed in the OMV and proved the elaborated application of the technique which can be employed for studying microvesicles analysis. Sometimes QS molecules exist in isomers forms for ex PQS and HQNO whose molecular weights are same and thereby difficult to identify using routine MALDI or ESI-MS. In order to achieve this we have targeted identification of OMVs extracted AQ isomers using Ion mobility mass spectrometry (IMS). Using high resolution IMS we could successfully able to identify two identical molecular weight metabolites which downstream can have different pathobiology. Thereby, in summary global QS metabolite study was achieved through high resolution mass spectrometry based platform. However limitations to this study remains with sample loss during extraction and lack of techniques present for fast in-vivo sampling. In addition to this targeting in-vivo sample using more sophisticated technique like MALDI-imaging etc can be one alternative to the approach.

Subsequent part of the project deals with the cross kingdom signaling function of 3-oxo-C12-HSL and PQS with respect to innate immunity in mammalian epithelium system. Host eradication/clearance of pathogens and pathogenic products are very crucial for maintenance of homeostasis and health. Continuous exposure of lung epithelium to microbes and microbial products can lead to chronic lung infection.

In this study we have specifically focussed first, on a potent QS autoinducer i.e 3-oxo-C12-HSL of *P. aeruginosa* and its role in cross kingdom signaling by inducing host innate immunity. The study aimed to find out the temporal expression of 3-oxo-C12-HSL from *PA* biofilms of clinical strains and to establish the early innate immune response of 3OC12HSL on lung epithelium. 3-oxo-C12-HSL expression and detection on temporal based assay established the fact that the molecule is expressed in the early biofilms and also its concentration decreases as the biofilms matures and slowly the expression is outcomes by group of other late expressing QS molecule (PQS etc). The Aim here is to study the first interaction of 3OC12-HSL from early biofilms in concentration dependent manner on mammalian A549 cells and particularly the effect of 3OC12-HSL on innate immune axis by regulation of antimicrobial peptide i.e hBD-2. This is the first report proposed for modulation of beta defensin by QS molecule. A549 lung epithelium when

induced by 3oxo-C12-HSL showed induction of hBD-2 in time and dose dependent manner. Upregulation of hBD-2 was via NF-κB dependent signaling pathway which was proved here by EMSA and western blot. In past 3-oxo-C12 has been known to upregulates NF-κB via activation of PERK and EIF-2alpha[155]. But HSL upregulation of NF-κB and subsequently crucial role of HSL in regulating the expression of hBD-2 was completely unknown earlier. hBD-2 promoter is well known to contain NF-κB sites, thereby we proposed here 3-oxo-C12 induction of hBD-2 in lung epithelium was due to binding of NF-κB transcription factors in the upstream of the promoter. Involvement of NF-κB dependent signaling pathways was confirmed by promoter reporter assay where activation of the hBD-2 promoter was observed upon inducing the cells with HSL.. 3-oxo-C12-HSL is a small lipidic molecule which can enters and functions inside the mammalian cells. As recently reported by Jahoor et al. that PPAR a class of nuclear receptor family as putative receptor of 3-oxo-C12 HSL in mammalian cells. They proposed 3-oxo-C12 act as a PPARϒ antagonist and PPARß/d agonist and thereby it can also regulates the transcriptional activity of PPAR. PPARϒ on the other hand has several anti-inflammatory activities and plays important in maintenance of epithelial innate immunity. PPARϒ plays a major role in transrepression of NF-κB associated genes. In presence of ligand it stabilizes the co-repressor complex which inhibits the activation of NF-κB, and prevents the recruitment of activator in response to inflammatory agents like LPS. Thereby, 3xoC12 acting as a ligand as well as antagonist of PPARϒ compete with its own ligand for ex rosiglitazone and inhibits the transrepression activity of NF-κB. Further it activates the inflammatory pathway. We have shown here the dose dependent and time dependent downregulation of PPARϒ endogenous protein expression by 3-oxo-C12 HSL which starts at typical concentration from 100µM-150µM in lung epithelial cells. In contrast to previous report we have seen a slight upregulation of the protein at 50µM. Similar profile was also obtained at transcripts level. Hence, this can be concluded that HSL at low concentration weakly activates the PPARϒ expression by acting as ligand and subsequently repressing the PPARϒ expression as soon as it starts accumulating in local environment and thereby acting as antagonist. In summary the overall mechanisms proposed for HSL mediated induction of hBD-2 is via involvement of PPARϒ and downstream activation of NF-κB pathway.

Epithelial defensins, well known for its antimicrobial activity is critical to fight against invading pathogens. Inducible Beta defensin 2 in lung epithelium is again an important

factor for the host to fight against lung infection(s)[146]. In the second part of the study we have proposed that HSL induced A549 lung epithelial cells expressing hBD-2 in turn protect the epithelium from *P. aeruginosa* early infection by reducing/clearing out the bacterial load. The hypothesis was proved when 3-oxo-C12 overnight induced cells was challenged with *P. aeruginosa* for 6h and plated subsequently; there was significant log fold difference in bacterial C.F.U in control than induced epithelium. This also established the fact that 3oxoc12 can actually help to boost/induce immunity which subsequently increases the epithelial immunity to combat/fight against infections.

To know the effect of hBD-2 and/or whether hBD-2 is directly playing role in protecting the lung epithelium we silenced hBD-2 gene by transfecting the cells with hBD-2 siRNA and thereby silencing the hBD-2 gene and further transcript. hBD-2 knock down A549 treated cells failed to reduce/clear bacterial C.FU count when challenged with *P. aeruginosa* in the same condition as done earlier. This demonstrated and supported the importance of inducible hBD-2 in lung environment. The overall data revealed one of the important cross kingdom signaling pathways mediated by 3oxoc12 HSL. Here, for the first time we have successfully showed the role of QS molecule in inducing innate immunity in the host. Using clinical *PA* biofilm model we have identified that not only the pathogen using classical PAMPs-PRR interaction can upregulates host defence but also its corresponding metabolites can equally take part and switches on the very central axis of immune system. Animal model of this study revealed clear upregulation of mBD-3 in trachea when mice were treated with HSL by nasal drop. This has strengthened the proposed hypothesis of HSL mediated induction of hBD-2.

Further through this study we have directed the future therapeutics targets via HSL molecule. In host immune compromised condition this can be employed to enhance immunity and can be further studied. Very recently the therapeutics role of 3oxo C12 has been known where tropical administration of the molecules has helped in epithelialisation of cutaneous wound in rats[156]. Thereby future aspects and role of the molecule in inducing innate immunity along with epithelial healing can act as a combinatorial therapy in case of damaged epithelium during chronic infection.

The other class of QS molecules i.e *Pseudomonas* quinolone signal (PQS) is also gaining interest into the field lately because of its capability to mediate cross kingdom signaling to human host. PQS is a late expressing biofilm gene and the molecule itself has also

been identified in CF sputum[157]. PQS accumulates and reaches threshold level during chronic infection of *Pseudomonas*[74]. In this study, using high resolution LDI-MS we have detected all class of AQs molecules including PQS and HHQ from clinical strains of *P. aeruginosa*. In addition to this relative quantitation of 3-oxo-C12-HSL Vs PQS was also done in temporal manner which revealed that the PQS expression was higher and more consistence than HSL in clinical strains when compared against type strains. In past several studies have proposed the crucial role of AQs in infection and virulence, where mutation in several PQS gene for example pqsE and pqsR resulted in less virulence factor production like pyocyanin and elastase[158]. A pqsE mutant also showed less lectin and rhamnolipids production. Other four genes mutation pqsH, pqsE, pqsC and pqsD resulted in lower nematode killing[159]. In addition to this burn wound mouse pqsA and pqsE mutant were less virulent compared to wild type[61, 112, 160]. All these indicate loss of virulence and pathogenicity in absence of pqs gene system. Therefore in our data, the clinical strains showing PQS overexpression can be correlated to the pathogenicity and virulence of the strains. Unlike HSL, PQS is highly hydrophobic in nature and thereby requires some trafficking system within the cells. Mashburn et al. demonstrated that PQS including other HHQ and HQNO are packaged and trafficked by outer-membrane vesicles (OMVs)[79]. *P. aeruginosa* OMVs is known to contain more than 90% of the PQS produced by the bacteria[161]. Other than AQs OMVs contain DNA, proteins, toxins which are involved in other downstream processes[76]. Packaging AQs into OMVs can be proposed as a mechanism for cross kingdom signaling by quinolones in mammalian cells. We have separated here soluble Vs insoluble AQs from clinical strains and detected higher expression of AQs in OMVs than only supernatant. Using single step capture of OMVs and LDI-MS technology we have detected all the major class of AQs along with PQS. Therefore the study suggested extended potential application of LDI surface for analysis of OMVs associated pathogenic factors. As well as the data indicates approximately all the AQs class of *P. aeruginosa* packed as cargo in OMVs for trafficking among the cells.

PQS as a small lipid soluble molecule can enter and function inside mammalian cells. Previous reports suggested that PQS can strongly modulate immune system by suppressing host immunity. As reported by Kim et al. HHQ and PQS suppress the production of innate cytokines in mouse monocytes/macrophage cells by downregualting/inhibiting NF-κB binding to its binding sites and thus downregulate

genes downstream of NF-κB pathway[72]. Hooi et al. also reported PQS act to IL-2 receptor (IL-2R) and function in T-cell signaling pathway. In addition to this as suggested by other reports that PQS can also inhibit LPS induced production of IL-12 and DCs induced T-cells proliferation[70]. Thereby all these findings revealed the overall host immune suppression activity by PQS. However till date innate immune modulation by PQS is still not known. hBD-1 as described earlier constitute first line host defence in epithelial immunity. It is constitutively expressed in epithelial cells and has potent antimicrobial activity. Basal expression of hBD-1 is crucial for maintenance of innate immunity and to fight against invading pathogens. Here in this study we have reported that PQS and OMVs associated quinolones can downregulate hBD-1 and suppress epithelial immunity. The finding is in support with earlier reports where PQS has been proposed to function as immunosuppressive agent. Further we have proposed that PQS downregualting hBD-1 is via HIF-1α signaling pathway. hBD-1 promoter has HIF-1 binding sites and also it has been investigated that HIF-1α is required for the basal expression of hBD-1[150]. Including this there are reports which suggest that PQS degrade HIF-1α via proteosomal pathway[152]. In our study we have shown that PQS inhibits hypoxia mediated nuclear localization of HIF-1α in lung epithelial cells. Along with PQS, OMVs associated quinolones also blocked HIF-1α expression and localization. Further A549 cells when induced with cobalt chloride for hypoxia showed high hBD-1 expression. This is also indicated that hypoxia can trigger hBD-1 expression.

Overall the data suggest that during late/chronic infection of *P. aeruginosa*, PQS accumulates in the environment and acts by degrading HIF-1 which is being induced by pathogen itself and further downregulate immunity by lowering the basal expression of hBD-1.

P. aeruginosa is a major cause of human lung infections and second most common pathogen associated with ventilator associated pneumonia (VAP). VAP causes high mortality rate worldwide and results into compromised and damaged epithelium of the host due to insertion of endotracheal tube (ETT) into the patients. Finally the role QS in human disease have been studied here in case of Ventilated associated pneumonia (VAP). In this context, first the biofilm formation on VAP patients' ETT tubes have been demonstrated which showed the presence of discrete structures with waves and ripples and has been proposed to rise in accordance with the flow of the ventilator intubated patients. Next high resolution metabolomics was performed with fresh ETT effluents

using LDI-MS technique. Metabolomics studies of VAP effluent showed successful detection of HSL and Quinolone. Detection of QS molecule has been earlier done in sputum sample. However this is the first report of detection of QS in VAP. This fact further proves the direct role of QS in disease and provides platform for mechanism to understand the exact state of *P. aeruginosa* infection in host.

Bibliography

1. Kersters K, Ludwig W, Vancanneyt M et al. Recent changes in the classification of the pseudomonads: An overview. *Systematic and applied microbiology* 1996; **19**: 465-77.
2. Yamamoto S, Kasai H, Arnold DL et al. Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. *Microbiology* 2000; **146 (Pt 10)**: 2385-94.
3. Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *Bmc Infect Dis* 2006; **6**.
4. Garbeva P, Veen JA, Elsas JD. Assessment of the diversity, and antagonism towards Rhizoctonia solani AG3, of *Pseudomonas* species in soil from different agricultural regimes. *FEMS microbiology ecology* 2004; **47**: 51-64.
5. Haas D, Keel C. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review of Phytopathology* 2003; **41**: 117-53.
6. Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. *Annual review of immunology* 2007; **25**: 697-743.
7. Nehme NT, Liegeois S, Kele B et al. A model of bacterial intestinal infections in Drosophila melanogaster. *PLoS pathogens* 2007; **3**: 1694-709.
8. Yu EN, Foster CS. Chronic postoperative endophthalmitis due to *Pseudomonas* oryzihabitans. *Am J Ophthalmol* 2002; **134**: 613-4.
9. Uy HS, Leuenberger EU, de Guzman BB et al. Chronic, postoperative *Pseudomonas* luteola endophthalmitis. *Ocul Immunol Inflamm* 2007; **15**: 359-61.
10. Campa M, Bendinelli M, Friedman H. *Pseudomonas aeruginosa as an opportunistic pathogen*: Springer Science & Business Media, 2012.
11. Lyczak JB, Cannon CL, Pier GB. Establishment of *Pseudomonas* aeruginosa infection: lessons from a versatile opportunist. *Microbes and infection* 2000; **2**: 1051-60.
12. Winsor GL, Lam DKW, Fleming L et al. *Pseudomonas* Genome tabase: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Research* 2011; **39**: D596-D600.
13. Rogers SS, van der Walle C, Waigh TA. Microrheology of bacterial biofilms in vitro: Staphylococcus aureus and *Pseudomonas* aeruginosa. *Langmuir : the ACS journal of surfaces and colloids* 2008; **24**: 13549-55.
14. Kuehn M, Lent K, Haas J et al. Fimbriation of *Pseudomonas* cepacia. *Infection and immunity* 1992; **60**: 2002-7.
15. Worlitzsch D, Tarran R, Ulrich M et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 2002; **109**: 317-25.
16. Christensen BE, Characklis WG. Physical and chemical properties of biofilms. *Biofilms* 1990: 93-130.
17. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; **284**: 1318.
18. Costerton JW, Lewandowski Z, Caldwell DE et al. Microbial biofilms. *Annual Reviews in Microbiology* 1995; **49**: 711-45.

19. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* 2004; **2**: 95-108.

20. Costerton JW, Lewandowski Z, Caldwell DE et al. Microbial biofilms. *Annual review of microbiology* 1995; **49**: 711-45.

21. Meluleni GJ, Grout M, Evans DJ et al. Mucoid *Pseudomonas*-Aeruginosa Growing in a Biofilm in-Vitro Are Killed by Opsonic Antibodies to the Mucoid Exopolysaccharide Capsule but Not by Antibodies Produced during Chronic Lung Infection in Cystic-Fibrosis Patients. *Journal of immunology* 1995; **155**: 2029-38.

22. Meers P, Neville M, Malinin V et al. Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas* aeruginosa lung infections. *The Journal of antimicrobial chemotherapy* 2008; **61**: 859-68.

23. Gnanadhas DP, Elango M, tey A et al. Chronic lung infection by *Pseudomonas* aeruginosa biofilm is cured by L-Methionine in combination with antibiotic therapy. *Sci Rep* 2015; **5**: 16043.

24. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *The American journal of medicine* 2002; **113 Suppl 1A**: 5S-13S.

25. Mittal R, Aggarwal S, Sharma S et al. Urinary tract infections caused by *Pseudomonas* aeruginosa: a minireview. *J Infect Public Health* 2009; **2**: 101-11.

26. Zegans ME, DiGiandomenico A, Ray K et al. Association of Biofilm Formation, Psl Exopolysaccharide Expression, and Clinical Outcomes in *Pseudomonas* aeruginosa Keratitis Analysis of Isolates in the Steroids for Corneal Ulcers Trial. *Jama Ophthalmol* 2016; **134**: 383-9.

27. Fernandes M, Vira D, Medikonda R et al. Extensively and pan-drug resistant *Pseudomonas* aeruginosa keratitis: clinical features, risk factors, and outcome. *Graef Arch Clin Exp* 2016; **254**: 315-22.

28. Glasson MJ, Stapleton F, Keay L et al. Differences in clinical parameters and tear film of tolerant and intolerant contact lens wearers. *Investigative ophthalmology & visual science* 2003; **44**: 5116-24.

29. Boisseau AM, Sarlangue J, Perel Y et al. Perineal Ecthyma Gangrenosum in Infancy and Early-Childhood - Septicemic and Nonsepticemic Forms. *J Am Acad Dermatol* 1992; **27**: 415-8.

30. Willcox MD. *Pseudomonas* aeruginosa infection and inflammation during contact lens wear: a review. *Optometry and vision science : official publication of the American Academy of Optometry* 2007; **84**: 273-8.

31. Garg P, Sharma S, Rao GN. Ciprofloxacin-resistant *Pseudomonas* keratitis. *Ophthalmology* 1999; **106**: 1319-23.

32. Morrow AL, Lagomarcino AJ, Schibler KR et al. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome* 2013; **1**: 13.

33. Kerckhoffs AP, Ben-Amor K, Samsom M et al. Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas* aeruginosa in irritable bowel syndrome. *J Med Microbiol* 2011; **60**: 236-45.

34. Percival SL, Emanuel C, Cutting KF et al. Microbiology of the skin and the role of biofilms in infection. *International wound journal* 2012; **9**: 14-32.

35. Li XZ, Barre N, Poole K. Influence of the MexA-MexB-oprM multidrug efflux system on expression of the MexC-MexD-oprJ and MexE-MexF-oprN multidrug efflux systems in *Pseudomonas* aeruginosa. *The Journal of antimicrobial chemotherapy* 2000; **46**: 885-93.

36. Strateva T, Yordanov D. *Pseudomonas* aeruginosa - a phenomenon of bacterial resistance. *Journal of Medical Microbiology* 2009; **58**: 1133-48.

37. Nordmann P, Naas T, Fortineau N et al. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of Staphylococcus aureus, Enterococcus spp. and *Pseudomonas* aeruginosa in 2010. *Curr Opin Microbiol* 2007; **10**: 436-40.

38. Breidenstein EBM, de la Fuente-Nunez C, Hancock REW. *Pseudomonas* aeruginosa: all roads lead to resistance. *Trends Microbiol* 2011; **19**: 419-26.

39. Ng W-L, Bassler BL. Bacterial quorum-sensing network architectures. *Annual review of genetics* 2009; **43**: 197-222.

40. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* 2005; **21**: 319-46.

41. Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. *Annual review of genetics* 2009; **43**: 197-222.

42. Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* 2007; **153**: 3923-38.

43. Diggle SP, Cornelis P, Williams P et al. 4-quinolone signalling in *Pseudomonas* aeruginosa: old molecules, new perspectives. *Int J Med Microbiol* 2006; **296**: 83-91.

44. Tait K, Williamson H, Atkinson S et al. Turnover of quorum sensing signal molecules modulates cross-kingdom signalling. *Environmental microbiology* 2009; **11**: 1792-802.

45. Alhede M, Bjarnsholt T, Givskov M et al. *Pseudomonas* aeruginosa biofilms: mechanisms of immune evasion. *Advances in applied microbiology* 2014; **86**: 1-40.

46. El-Shaer S, Shaaban M, Barwa R et al. Control of quorum sensing and virulence factors of *Pseudomonas* aeruginosa using phenylalanine arginyl beta-naphthylamide. *J Med Microbiol* 2016; **65**: 1194-204.

47. Skindersoe ME, Alhede M, Phipps R et al. Effects of antibiotics on quorum sensing in *Pseudomonas* aeruginosa. *Antimicrobial agents and chemotherapy* 2008; **52**: 3648-63.

48. Hoffmann N, Lee B, Hentzer M et al. Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas* aeruginosa and attenuates chronic P. aeruginosa lung infection in Cftr(-/-) mice. *Antimicrobial agents and chemotherapy* 2007; **51**: 3677-87.

49. Williams P, Camara M. Quorum sensing and environmental adaptation in *Pseudomonas* aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 2009; **12**: 182-91.

50. Schuster M, Greenberg EP. A network of networks: quorum-sensing gene regulation in *Pseudomonas* aeruginosa. *Int J Med Microbiol* 2006; **296**: 73-81.

51. Hughes DT, Sperandio V. Inter-kingdom signalling: communication between bacteria and their hosts. *Nature reviews Microbiology* 2008; **6**: 111-20.

52. Cooley M, Chhabra SR, Williams P. N-Acylhomoserine lactone-mediated quorum sensing: a twist in the tail and a blow for host immunity. *Chemistry & biology* 2008; **15**: 1141-7.

53. Bryan A, Watters C, Koenig L et al. Human transcriptome analysis reveals a potential role for active transport in the metabolism of *Pseudomonas* aeruginosa autoinducers. *Microbes and infection* 2010; **12**: 1042-50.

54. Jahoor A, Patel R, Bryan A et al. Peroxisome proliferator-activated receptors mediate host cell proinflammatory responses to *Pseudomonas* aeruginosa autoinducer. *Journal of bacteriology* 2008; **190**: 4408-15.

55. Kostadinova R, Wahli W, Michalik L. PPARs in diseases: control mechanisms of inflammation. *Current medicinal chemistry* 2005; **12**: 2995-3009.

56. Shiner EK, Terentyev D, Bryan A et al. *Pseudomonas* aeruginosa autoinducer modulates host cell responses through calcium signalling. *Cellular microbiology* 2006; **8**: 1601-10.

57. Li L, Hooi D, Chhabra SR et al. Bacterial N-acylhomoserine lactone-induced apoptosis in breast carcinoma cells correlated with down-modulation of STAT3. *Oncogene* 2004; **23**: 4894-902.

58. Smith RS, Fedyk ER, Springer TA et al. IL-8 production in human lung fibroblasts and epithelial cells activated by the *Pseudomonas* autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. *Journal of immunology* 2001; **167**: 366-74.

59. Smith RS, Harris SG, Phipps R et al. The *Pseudomonas* aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation in vivo. *Journal of bacteriology* 2002; **184**: 1132-9.

60. Smith RS, Kelly R, Iglewski BH et al. The *Pseudomonas* autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation. *Journal of immunology* 2002; **169**: 2636-42.

61. Diggle SP, Winzer K, Chhabra SR et al. The *Pseudomonas* aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* 2003; **50**: 29-43.

62. Williams SC, Patterson EK, Carty NL et al. *Pseudomonas* aeruginosa autoinducer enters and functions in mammalian cells. *Journal of bacteriology* 2004; **186**: 2281-7.

63. Chhabra SR, Harty C, Hooi DSW et al. Synthetic analogues of the bacterial signal (quorum sensing) molecule N-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. *J Med Chem* 2003; **46**: 97-104.

64. Telford G, Wheeler D, Williams P et al. The *Pseudomonas* aeruginosa quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infection and immunity* 1998; **66**: 36-42.

65. Ritchie AJ, Whittall C, Lazenby JJ et al. The immunomodulatory *Pseudomonas* aeruginosa signalling molecule N-(3-oxododecanoyl)-L-homoserine lactone enters mammalian cells in an unregulated fashion. *Immunology and cell biology* 2007; **85**: 596-602.

66. Ritchie AJ, Yam AOW, Tanabe KM et al. Modification of in vivo and in vitro T- and B-cell-mediated immune responses by the *Pseudomonas* aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infection and immunity* 2003; **71**: 4421-31.

67. Deziel E, Lepine F, Milot S et al. Analysis of *Pseudomonas* aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci U S A* 2004; **101**: 1339-44.

68. Diggle SP, Matthijs S, Wright VJ et al. The *Pseudomonas* aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chemistry & biology* 2007; **14**: 87-96.

69. Hooi DS, Bycroft BW, Chhabra SR et al. Differential immune modulatory activity of *Pseudomonas* aeruginosa quorum-sensing signal molecules. *Infection and immunity* 2004; **72**: 6463-70.

70. Skindersoe ME, Zeuthen LH, Brix S et al. *Pseudomonas* aeruginosa quorum-sensing signal molecules interfere with dendritic cell-induced T-cell proliferation. *FEMS immunology and medical microbiology* 2009; **55**: 335-45.

71. Dubern JF, Diggle SP. Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas* aeruginosa and other bacterial species. *Molecular bioSystems* 2008; **4**: 882-8.

72. Kim K, Kim SH, Lepine F et al. Global gene expression analysis on the target genes of PQS and HHQ in J774A.1 monocyte/macrophage cells. *Microb Pathog* 2010; **49**: 174-80.

73. Kim K, Kim YU, Koh BH et al. HHQ and PQS, two *Pseudomonas* aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway. *Immunology* 2010; **129**: 578-88.

74. Ortori CA, Dubern JF, Chhabra SR et al. Simultaneous quantitative profiling of N-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. *Analytical and bioanalytical chemistry* 2011; **399**: 839-50.

75. Mashburn-Warren LM, Whiteley M. Special delivery: vesicle trafficking in prokaryotes. *Mol Microbiol* 2006; **61**: 839-46.

76. Kadurugamuwa JL, Beveridge TJ. Virulence factors are released from *Pseudomonas* aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *Journal of bacteriology* 1995; **177**: 3998-4008.

77. Mashburn-Warren L, Howe J, Garidel P et al. Interaction of quorum signals with outer membrane lipids: insights into prokaryotic membrane vesicle formation. *Mol Microbiol* 2008; **69**: 491-502.

78. Tashiro Y, Uchiyama H, Nomura N. Multifunctional membrane vesicles in *Pseudomonas* aeruginosa. *Environmental microbiology* 2012; **14**: 1349-62.

79. Mashburn-Warren L, Howe J, Brandenburg K et al. Structural requirements of the *Pseudomonas* quinolone signal for membrane vesicle stimulation. *Journal of bacteriology* 2009; **191**: 3411-4.

80. Janeway CA, Jr. A trip through my life with an immunological theme. *Annu Rev Immunol* 2002; **20**: 1-28.

81. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002; **20**: 197-216.

82. Liu L, Wang LN, Jia HP et al. Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene* 1998; **222**: 237-44.

83. Hoover DM, Boulegue C, Yang D et al. The structure of human macrophage inflammatory protein-3alpha /CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human beta-defensins. *J Biol Chem* 2002; **277**: 37647-54.

84. Schroder JM. Clinical significance of epithelial peptide antibiotics. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 1999; **11**: 293-300.

85. Ghosh D, Porter E, Shen B et al. Paneth cell trypsin is the processing enzyme for human defensin-5. *Nature immunology* 2002; **3**: 583-90.

86. Meyer JE, Harder J, Gorogh T et al. hBD-2-gene expression in nasal mucosa. *Laryngo Rhino Otol* 2000; **79**: 400-3.

87. Harder J, Meyer-Hoffert U, Wehkamp K et al. Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. *J Invest Dermatol* 2004; **123**: 522-9.

88. Ghosh SK, Gerken TA, Schneider KM et al. Quantification of human beta-defensin-2 and -3 in body fluids: application for studies of innate immunity. *Clinical chemistry* 2007; **53**: 757-65.

89. Azzi L, Moretto P, Vinci R et al. Human beta2-defensin in oral lichen planus expresses the degree of inflammation. *Journal of biological regulators and homeostatic agents* 2017; **31**: 77-87.

90. Wehkamp J, Harder J, Wehkamp K et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infection and immunity* 2004; **72**: 5750-8.

91. Hazrati E, Galen B, Lu W et al. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. *Journal of immunology* 2006; **177**: 8658-66.

92. Madison MN, Kleshchenko YY, Nde PN et al. Human defensin alpha-1 causes Trypanosoma cruzi membrane pore formation and induces DNA fragmentation, which leads to trypanosome destruction. *Infection and immunity* 2007; **75**: 4780-91.

93. Tanaka T, Rahman MM, Battur B et al. Parasiticidal activity of human alpha-defensin-5 against Toxoplasma gondii. *In vitro cellular & developmental biology Animal* 2010; **46**: 560-5.

94. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nature reviews Immunology* 2003; **3**: 710-20.

95. Sela B. [Defensins: peptides of innate immunity serving as first-line antimicrobial defense]. *Harefuah* 2000; **139**: 112-6.

96. Rohrl J, Yang D, Oppenheim JJ et al. Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *Journal of immunology* 2010; **184**: 6688-94.

97. Rohrl J, Yang D, Oppenheim JJ et al. Specific binding and chemotactic activity of mBD4 and its functional orthologue hBD-2 to CCR6-expressing cells. *J Biol Chem* 2010; **285**: 7028-34.

98. Yang D, Biragyn A, Hoover DM et al. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* 2004; **22**: 181-215.

99. Shu Q, Shi Z, Zhao Z et al. Protection against *Pseudomonas* aeruginosa pneumonia and sepsis-induced lung injury by overexpression of beta-defensin-2 in rats. *Shock* 2006; **26**: 365-71.

100. Janeway Jr CA, Medzhitov R. Innate immune recognition. *Annual review of immunology* 2002; **20**: 197-216.

101. Harder J, Meyer-Hoffert U, Teran LM et al. Mucoid *Pseudomonas* aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *American journal of respiratory cell and molecular biology* 2000; **22**: 714-21.

102. Kravchenko VV, Ulevitch RJ, Kaufmann GF. Modulation of mammalian cell processes by bacterial quorum sensing molecules. *Methods in molecular biology* 2011; **692**: 133-45.

103. Kravchenko VV, Kaufmann GF, Mathison JC et al. N-(3-oxo-acyl) homoserine lactones signal cell activation through a mechanism distinct from the canonical pathogen-associated molecular pattern recognition receptor pathways. *Journal of biological chemistry* 2006; **281**: 28822-30.

104. Ochoa SA, Cruz-Cordova A, Rodea GE et al. Phenotypic characterization of multidrug-resistant *Pseudomonas* aeruginosa strains isolated from pediatric patients associated to biofilm formation. *Microbiological research* 2014.

105. Bairagya BB, Bhattacharya P, Bhattacharya SK et al. Genetic variation and haplotype structures of innate immunity genes in eastern India. *Infection, Genetics and Evolution* 2008; **8**: 360-6.

106. Lepine F, Milot S, Deziel E et al. Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas* aeruginosa. *Journal of the American Society for Mass Spectrometry* 2004; **15**: 862-9.

107. Kumar A, Sarkar SK, Ghosh D et al. Deletion of penicillin-binding protein 1b impairs biofilm formation and motility in Escherichia coli. *Research in microbiology* 2012; **163**: 254-7.

108. Lahiri P, Ghosh D. Single-Step Capture and Targeted Metabolomics of Alkyl-Quinolones in Outer Membrane Vesicles of *Pseudomonas* aeruginosa. *Methods in molecular biology* 2017; **1609**: 171-84.

109. Buchholtz C, Nielsen KF, Milton DL et al. Profiling of acylated homoserine lactones of Vibrio anguillarum in vitro and in vivo: Influence of growth conditions and serotype. *Systematic and applied microbiology* 2006; **29**: 433-45.

110. Gil-Perotin S, Ramirez P, Marti V et al. Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. *Crit Care* 2012; **16**: R93.

111. Hartmann M, Berditsch M, Hawecker J et al. mage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial agents and chemotherapy* 2010; **54**: 3132-42.

112. Huse H, Whiteley M. 4-Quinolones: smart phones of the microbial world. *Chemical reviews* 2011; **111**: 152-9.

113. Shaw PD, Ping G, ly SL et al. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proceedings of the National Academy of Sciences* 1997; **94**: 6036-41.

114. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. *Annual review of plant biology* 2010; **61**: 463-89.

115. Peterson DS. Matrix-free methods for laser desorption/ionization mass spectrometry. *Mass spectrometry reviews* 2007; **26**: 19-34.

116. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nature reviews Microbiology* 2015; **13**: 605-19.

117. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology* 2013; **200**: 373-83.

118. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual review of microbiology* 2010; **64**: 163-84.

119. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 2005; **437**: 422-5.

120. Collier DN, Anderson L, McKnight SL et al. A bacterial cell to cell signal in the lungs of cystic fibrosis patients. *FEMS microbiology letters* 2002; **215**: 41-6.

121. Gruber JD, Chen W, Parnham S et al. The role of 2,4-dihydroxyquinoline (DHQ) in *Pseudomonas* aeruginosa pathogenicity. *PeerJ* 2016; **4**: e1495.

122. Bala A, Chhibber S, Harjai K. *Pseudomonas* quinolone signalling system: a component of quorum sensing cascade is a crucial player in the acute urinary tract infection caused by *Pseudomonas* aeruginosa. *International journal of medical microbiology : IJMM* 2014; **304**: 1199-208.

123. Palmer GC, Schertzer JW, Mashburn-Warren L et al. Quantifying *Pseudomonas* aeruginosa quinolones and examining their interactions with lipids. *Methods in molecular biology* 2011; **692**: 207-17.

124. Diggle SP, Fletcher MP, Camara M et al. Detection of 2-alkyl-4-quinolones using biosensors. *Methods in molecular biology* 2011; **692**: 21-30.

125. Choi DS, Kim DK, Choi SJ et al. Proteomic analysis of outer membrane vesicles derived from *Pseudomonas* aeruginosa. *Proteomics* 2011; **11**: 3424-9.

126. Bala A, Gupta RK, Chhibber S et al. Detection and quantification of quinolone signalling molecule: a third quorum sensing molecule of *Pseudomonas* aeruginosa by high performance-thin layer chromatography. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 2013; **930**: 30-5.

127. Chutkan H, Macdonald I, Manning A et al. Quantitative and qualitative preparations of bacterial outer membrane vesicles. *Methods in molecular biology* 2013; **966**: 259-72.

128. Baig NF, Dunham SJ, Morales-Soto N et al. Multimodal chemical imaging of molecular messengers in emerging *Pseudomonas* aeruginosa bacterial communities. *The Analyst* 2015; **140**: 6544-52.

129. Njoroge J, Sperandio V. Jamming bacterial communication: new approaches for the treatment of infectious diseases. *EMBO molecular medicine* 2009; **1**: 201-10.

130. Wang JN, Zhou Y, Zhu TY et al. Prediction of acute cellular renal allograft rejection by urinary metabolomics using MALDI-FTMS. *J Proteome Res* 2008; **7**: 3597-601.

131. De Kievit TR, Gillis R, Marx S et al. Quorum-sensing genes in *Pseudomonas* aeruginosa biofilms: their role and expression patterns. *Appl Environ Microbiol* 2001; **67**: 1865-73.

132. Duan K, Surette MG. Environmental regulation of *Pseudomonas* aeruginosa PAO1 Las and Rhl quorum-sensing systems. *Journal of bacteriology* 2007; **189**: 4827-36.

133. Wu H, Song Z, Hentzer M et al. Detection of N-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas* aeruginosa. *Microbiology* 2000; **146 (Pt 10)**: 2481-93.

134. Tateda K, Ishii Y, Horikawa M et al. The *Pseudomonas* aeruginosa autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. *Infection and immunity* 2003; **71**: 5785-93.

135. Zhang J, Gong F, Li L et al. *Pseudomonas* aeruginosa quorum-sensing molecule N-(3-oxododecanoyl) homoserine lactone attenuates lipopolysaccharide-induced inflammation by activating the unfolded protein response. *Biomedical reports* 2014; **2**: 233-8.

136. Glucksam-Galnoy Y, Sananes R, Silberstein N et al. The bacterial quorum-sensing signal molecule N-3-oxo-dodecanoyl-L-homoserine lactone reciprocally modulates pro- and anti-inflammatory cytokines in activated macrophages. *Journal of immunology* 2013; **191**: 337-44.

137. Schwarzer C, Ravishankar B, Patanwala M et al. Thapsigargin blocks *Pseudomonas* aeruginosa homoserine lactone-induced apoptosis in airway epithelia. *American journal of physiology Cell physiology* 2014; **306**: C844-55.

138. Deng J, Lu PD, Zhang Y et al. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Molecular and cellular biology* 2004; **24**: 10161-8.

139. Shiner EK, Rumbaugh KP, Williams SC. Interkingdom signaling: Deciphering the language of acyl homoserine lactones. *FEMS microbiology reviews* 2005; **29**: 935-47.

140. Krasowski MD, Yasuda K, Hagey LR et al. Evolutionary selection across the nuclear hormone receptor superfamily with a focus on the NR1I subfamily (vitamin D, pregnane X, and constitutive androstane receptors). *Nuclear receptor* 2005; **3**: 2.

141. Bedi B, Yuan Z, Joo M et al. Enhanced Clearance of *Pseudomonas* aeruginosa by Peroxisome Proliferator-Activated Receptor Gamma. *Infection and immunity* 2016; **84**: 1975-85.

142. Standiford TJ, Keshamouni VG, Reddy RC. Peroxisome proliferator-activated receptor-{gamma} as a regulator of lung inflammation and repair. *Proceedings of the American Thoracic Society* 2005; **2**: 226-31.

143. Pascual G, Fong AL, Ogawa S et al. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 2005; **437**: 759-63.

144. Cooley MA, Whittall C, Rolph MS. *Pseudomonas* signal molecule 3-oxo-C12-homoserine lactone interferes with binding of rosiglitazone to human PPARgamma. *Microbes and infection* 2010; **12**: 231-7.

145. Martin TR, Frevert CW. Innate immunity in the lungs. *Proceedings of the American Thoracic Society* 2005; **2**: 403-11.

146. Schroder JM, Harder J. Human beta-defensin-2. *The international journal of biochemistry & cell biology* 1999; **31**: 645-51.

147. Wehkamp K, Schwichtenberg L, Schroder JM et al. *Pseudomonas* aeruginosa- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1. *J Invest Dermatol* 2006; **126**: 121-7.

148. Pritchard DI. Immune modulation by *Pseudomonas* aeruginosa quorum-sensing signal molecules. *Int J Med Microbiol* 2006; **296**: 111-6.

149. Nickel D, Busch M, Mayer D et al. Hypoxia Triggers the Expression of Human beta Defensin 2 and Antimicrobial Activity against Mycobacterium tuberculosis in Human Macrophages. *Journal of immunology* 2012; **188**: 4001-7.

150. Kelly CJ, Glover LE, Campbell EL et al. Fundamental role for HIF-1 alpha in constitutive expression of human beta defensin-1. *Mucosal Immunology* 2013; **6**: 1110-8.

151. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nature reviews Immunology* 2009; **9**: 609-17.

152. Legendre C, Reen FJ, Mooij MJ et al. *Pseudomonas* aeruginosa Alkyl quinolones repress hypoxia-inducible factor 1 (HIF-1) signaling through HIF-1alpha degradation. *Infection and immunity* 2012; **80**: 3985-92.

153. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. *Annual review of cell and developmental biology* 2005; **21**: 319-46.

154. Williams P, Camara M. Quorum sensing and environmental adaptation in *Pseudomonas* aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Current opinion in microbiology* 2009; **12**: 182-91.

155. Grabiner MA, Fu Z, Wu T et al. *Pseudomonas* aeruginosa quorum-sensing molecule homoserine lactone modulates inflammatory signaling through PERK and eI-F2alpha. *Journal of immunology* 2014; **193**: 1459-67.

156. Nakagami G, Minematsu T, Asada M et al. The *Pseudomonas* aeruginosa quorum-sensing signal N-(3-oxododecanoyl) homoserine lactone can accelerate cutaneous wound healing through myofibroblast differentiation in rats. *FEMS immunology and medical microbiology* 2011; **62**: 157-63.

157. Barr HL, Halliday N, Camara M et al. *Pseudomonas* aeruginosa quorum sensing molecules correlate with clinical status in cystic fibrosis. *The European respiratory journal* 2015; **46**: 1046-54.

158. Cao H, Krishnan G, Goumnerov B et al. A quorum sensing-associated virulence gene of *Pseudomonas* aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc Natl Acad Sci U S A* 2001; **98**: 14613-8.

159. Gallagher LA, McKnight SL, Kuznetsova MS et al. Functions required for extracellular quinolone signaling by *Pseudomonas* aeruginosa. *Journal of bacteriology* 2002; **184**: 6472-80.

160. Deziel E, Gopalan S, Tampakaki AP et al. The contribution of MvfR to *Pseudomonas* aeruginosa pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-L-homoserine lactones. *Mol Microbiol* 2005; **55**: 998-1014.

161. Mashburn-Warren LM, Whiteley M. Special delivery: vesicle trafficking in prokaryotes. *Molecular microbiology* 2006; **61**: 839-46.