Introduction
CHAPTER-1

INTRODUCTION

*Pseudomonas aeruginosa* is a remarkable ubiquitous bacterium which is seldom a member of normal microbial flora in humans. It occasionally migrates from its natural environment and successfully colonizes at specific sites in patients especially with impaired immunity or who have experienced severe illness, trauma, impaired mucociliary function (cystic fibrosis), epithelial breaches (burns, wound), mechanical ventilation, indwelling material (urinary catheter, orotracheal tube) or surgery (Bielecki et al., 2008). Due to its enormous versatility and adaptive physiology, *P. aeruginosa* can be regarded as a successful environmental bacterial genus which can adapt human body as one of its habitats and can become severely pathogenic. In humans, *Pseudomonas aeruginosa* becomes an opportunistic pathogen of high clinical relevance as it causes deleterious acute and chronic infections of lungs, ears, eyes, skin, burn wounds and urinary tract in nosocomial settings (Ehrlich et al., 2002; Tredget et al., 2004; Bendiak & Ratjen, 2009; Mena & Gerba, 2009).

In context to nosocomial infections, *P. aeruginosa* is the fourth most frequently isolated pathogen accounting for 10.1% of all hospital-acquired infections (Ha & Cho, 2006; Todar, 2008) and third most common pathogen of complicated urinary tract infections (UTIs). It is a dominant agent in 35% of catheter associated UTIs (CAUTIs) (Ronald, 2003). A recent epidemiological study has ranked *P. aeruginosa* as the third most common pathogen in the Asia-Pacific region causing 7.2% of total UTIs (Lu et al., 2012).

This organism is endowed with an arsenal of virulence factors allowing it to survive under hostile environment. Virulence of *P. aeruginosa* is multifactorial and combinatorial, and it varies considerably depending on bacterial physiology and strain involved (Lee et al., 2006). Plethora of factors contributing to pathogenesis of *P. aeruginosa* include cell associated factors such as lipopolysaccharide (LPS), alginate, pilus and non-pilus adhesins, flagellum and extracellular factors like protease, elastase, hemolysin, siderophores, rhamnolipid and exotoxins (Matheson et al., 2006; Yates et al., 2006; Veessenmeyer et al., 2009). In addition, another strategy for successful
induction of infection is its inherent tendency to form biofilms which protect it from the harsh environmental conditions generated by multiple factors such as antimicrobials, nutrient shortage and host immune system. Bacteria within biofilm exhibit up to 1000 fold increase in resistance to diverse antimicrobial agents (Davies, 2003). Taken together, biofilm lifestyle, high intrinsic resistance and its ability to develop resistance during antibiotic treatment makes infections with *P. aeruginosa* notoriously difficult to treat (Wagenlehner & Naber, 2006).

Plethora of virulence factors and biofilms play an indispensible role in the establishment and disease progression of infections caused by *P. aeruginosa*. However, majority of these factors are not expressed constitutively rather, regulated directly or indirectly in a cell density-dependent manner under the strict supervision of fool-proof mechanism known as ‘quorum sensing’ (Gilbert et al., 2009). In general term, quorum sensing refers to cell to cell communication mechanism in which accumulation of ‘signal’ molecules in the surrounding environment enables a single cell to sense the number of bacteria (cell density), so that the population as a whole can make a coordinated response (Fuqua et al., 1994). This often leads to autoinduction of signal leading to rapid increase of signal concentration in the nearby environment. At critical cell densities, these signal molecules bind to a regulatory protein which leads to expression of genes controlled by quorum sensing (Whitehead et al., 2001). In *P. aeruginosa*, QS response is a relevant element for their infective process as it uses QS to collectively release a suite of virulence factors that contribute to its disease-causing ability.

*P. aeruginosa* has evolved a sophisticated regulatory network of at least three distinct signal/receptor pairs that work in a hierarchical manner: two LuxI/LuxR-type QS circuits, *las* and *rhl*, that function in sequence to control the expression of virulence factors and a third, non-LuxI/LuxR-type *Pseudomonas* quinolone signalling (*pqs*) system. Both *las* and *rhl* system consist of an AHL synthase (LasI and RhlI) and a sensor-regulator (LasR and RhlR) which are homologue pairs of LuxIR. In the first circuit, LasI directs the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). 3-oxo-C12-HSL molecule docks with the transcriptional regulator LasR which, in turn, dimerizes and bind to target promoters to control gene expression...
(Pearson et al., 1994; Parsek et al., 1999; Gould et al., 2004; Bottomley et al., 2007). The LasR-3OC12HSL complex activates transcription of target genes including those encoding virulence determinants such as elastase ($\text{lasB}$), LasA protease ($\text{lasA}$), alkaline protease ($\text{aprA}$), exotoxin A ($\text{toxA}$) and $\text{lasI}$ itself (Storey et al., 1998; Winzer & Williams, 2001; Camara et al., 2002). Likewise, in the $\text{rhl}$ system, the Rhll direct the production of N-butanoylhomoserine lactone (C4-HSL). As C4-HSL accumulates to threshold levels, it binds to its transcriptional regulator RhIR to control the activity of target promoters (de Kievit & Iglewski, 2000; Winzer & Williams, 2001; Camara et al., 2002). The $\text{rhl}$ system controls the synthesis of rhamnolipids ($\text{rhlAB}$), elastase ($\text{lasB}$), stationary phase sigma factor ($\text{rpoS}$), hydrogen cyanide ($\text{hcnABC}$), pyocyanin, siderophores and cytotoxic lectins PA-IL and PA-IIL (Latifi et al., 1995, 1996; Winson et al., 1995; Schuster et al., 2003; Schuster & Greenberg, 2007). These two systems do not operate independently as the expression of $\text{rhl}$ system is positively regulated by $\text{las}$ system at both transcriptional and post-transcriptional levels (Latifi et al., 1996). However, in a recent report it has been suggested that $\text{rhl}$ system can also function independently of the $\text{las}$ system (Diggle et al., 2003).

In addition, $\text{P. aeruginosa}$ harbor a third non-Lux/I/LuxR signalling system which is based on the 4-quinolone family of synthetic antimicrobials. Quinolones molecules are structurally derived from the heterobicyclic aromatic compound quinoline. This name quinoline originated from the oily substance obtained after the alkaline distillation of quinine (Gerhardt, 1842). Several different animals, plants and microorganisms have been found to produce a wide variety of quinolone compounds. Some of these naturally occurring quinolones possess medicinal activities ranging from antimicrobial to antiallergenic and anticancer; while others have served as lead structures in the development of synthetic drugs, including fluoroquinolones (Heeb et al., 2010). $\text{P. aeruginosa}$ produces more than fifty 2-alkyl-4(1H)-quinolones, some of which exhibit antimicrobial activity. Out of these quinolones, only Pseudomonas quinolone signal (PQS), designated as 2-heptyl-3-hydroxy-4(1H)-quinolone, and 2-heptyl-4(1H)-quinolone (HHQ) have been assigned with signalling function (Pesci et al., 1999; McKnight et al., 2000; Diggle et al., 2003; Deziel et al., 2004). The biosynthesis of PQS depends upon number of
structural genes arranged in an operon, pqsABCDE and pqsH which is under the regulation of transcriptional regulator PqsR (Gallagher et al., 2002). Expression of pqsH and pqsR is activated by LasR–3OC12HSL, whereas RhlR-C4HSL exerts a negative effect on the quinolone system (Gallagher et al., 2002; Deziel et al., 2004; McGrath et al., 2004; Xiao et al., 2006b). PQS controls its own production by driving the expression of pqsABCDE operon via direct interaction with PqsR and further activates rhl and rhlR expression (Xiao et al., 2006a; Diggle et al., 2007). PQS binds to its cognate LysR-type receptor PqsR with high affinity (Wade et al., 2005; Xiao et al., 2006a). The pqs system controls approximately 141 genes, majority of which are co-regulated by acyl-HSL mediated QS (Deziel et al., 2005). Thus, the pqs circuit is intimately tied to the LasI/LasR and RhlI/RhlR QS systems and act as a regulatory link between the AHL dependent systems. PQS is trafficked via naturally produced membrane vesicles (MVs) within P. aeruginosa population. PQS is not only packaged into MVs but is also required for MVs formation in P. aeruginosa (Mashburn & Whiteley, 2005), thus implicating this signal molecule as a key player in MV formation (Mashburn-Warren et al., 2008).

Two acylhomoserine lactone (AHL) based QS systems together with the AQ-based pqs system create a sophisticated hierarchical network to control global gene expression in P. aeruginosa.

P. aeruginosa associated infections have become very difficult to treat by conventional antimicrobial therapies. Quorum sensing has emerged as a major controller of overall pathogenicity of P. aeruginosa in the battlefield inside host. Considering central role of quorum sensing in different infections by Pseudomonas aeruginosa, it is being targeted as a potential candidate to develop antivirulence strategies against P. aeruginosa. Few experimental studies have shown the contribution of las and rhl QS system in respiratory tract infections, burn wound infections, keratitis, acute pneumonia model and ascending urinary tract infection (Rumbaugh et al., 1999; Pearson et al., 2000; Erickson et al., 2002; Zhu et al., 2004; Kumar et al., 2009b). However, very few reports regarding detection and isolation of third signalling molecule PQS exist in literature. Although Collier et al. (2002) and Que et al. (2011) provided evidences of PQS production during human infections by directly detecting PQS in clinical samples obtained from cystic fibrosis and burn
wound patients infected with *P. aeruginosa*, but the role of PQS signal in the pathogenesis of infections is not very well defined. Complete understanding of complex quorum sensing mechanism has become essential to find out new means of controlling infections caused by *P. aeruginosa*. PQS is found to be necessary for *P. aeruginosa* virulence in *Caenorhabditis elegans* killing model (Gallagher et al., 2002). PQS also has been identified as a critical factor to shape the bacterial population structure and to assist in their adaptation to conditions of increased stress that may contribute to bacterial persistence during infectious diseases (Haussler & Becker, 2008). PQS adds a further level of complexity to the quorum sensing network, as it provides a link between the *las* and *rhl* systems. Hence, its possible involvement in maintaining chronic state in catheter-associated urinary tract infections cannot be ruled out. Since research on *pqs* system is in its nascent stage, its contribution in infections caused by *P. aeruginosa* needs to be determined. Therefore, the present study was planned with following objectives:

**OBJECTIVES:**

1. Development and standardization of high performance thin layer chromatography method to analyze and quantitate *Pseudomonas* quinolone signal (PQS) molecules produced by *Pseudomonas aeruginosa*.
2. Characterization of *Pseudomonas* quinolone signal molecules by nuclear magnetic resonance and mass spectrometry.
3. Isolation, production and characterization of membrane vesicles produced by standard strain MPAO1 and *pqs* mutant strains and their correlation with virulence of *Pseudomonas aeruginosa*.
4. *In vitro* evaluation of the effect of exogenous supplementation of PQS on virulence potential of *Pseudomonas aeruginosa*.
5. Effect of PQS on the sensitivity of *Pseudomonas aeruginosa* towards antibiotic and hydrogen peroxide induced stress conditions.
6. *In vivo* evaluation of contribution of *Pseudomonas* signalling system in the course of acute urinary tract infection by employing standard strain MPAO1 and *pqs* mutant strains of *Pseudomonas aeruginosa* in terms of
   - Detection of PQS in the tissue
   - Bacteriological count
Introduction

- Histopathological analysis
- Pathological index markers
- Virulence factors production
- Inflammatory cytokines

7. To study the influence of PQS molecules on innate components of immune response
   - Uptake and killing action of macrophages
   - Apoptosis of macrophages
   - Serum bactericidal action