Discussion
Urinary tract infections (UTIs) account for 30-40% of all nosocomial infections out of which 90% of these nosocomial UTIs are associated with urinary catheters. The major problems of the catheter associated UTIs (CUTIs) are morbidity, sizeable overall health care costs and mortality (Saint and Chenoweth, 2003). Gram-negative bacteria are responsible for 89% cases of CUTIs. Majority of these infections are caused by E. coli with a frequency of 26.6% followed by Enterococcus with a frequency of 22.0% (Wazait et al., 2003). Pseudomonas aeruginosa has emerged as a major opportunistic pathogen which accounts for about 14% of all hospital acquired UTIs (Jarvis and Martone, 1992). Reports are also available from India where P. aeruginosa has been demonstrated to be one of the commonest pathogen associated with complicated UTIs (Taneja et al., 2004). In patients undergoing long term indwelling bladder catheterization, P. aeruginosa form bacterial biofilms in which the organisms can grow to high densities (Hoiby et al., 2001). Biofilm cell forms become difficult to eradicate and hence become the cause of persistent and recurrent urinary tract infections leading to chronicity and morbidity (Stickler et al., 1998). Although epidemiological studies have correlated the association of P. aeruginosa with CUTIs, but understanding of pathogenesis of such infections is still not very clear.

Various epidemiological markers have been used to type P. aeruginosa strains on the basis of different properties. These include O-serotyping, pyocyanin typing, phage typing, antibiotic susceptibility typing and API 20 NE profile (Visca et al., 1991). The lipopolysaccharide (LPS) O antigen has been most commonly used for the classification of P. aeruginosa isolates from different clinical samples by various workers. In the present study, P. aeruginosa isolates were serotyped according to international antigenic typing system (IATS) by Laboratory of Healthcare Associated Infection (LHCAI), London. It was observed that majority of isolates belonged to
serotype O11 (33%) followed by O1II (23%), O6 (10%), O8 (8%), O1 (8%), O7/8 (6%), O3 (6%), O4 (4%) and O15 (2%). Based on differences of the B-band LPS, IATS has reported 17 different serotypes of *P. aeruginosa* (clinical as well as environmental strains) (Visca *et al.*, 1991). Nine of these were encountered in the present study. Only limited reports are available in relation to *P. aeruginosa* isolated from urinary tract infection patients. According to these, majority of *P. aeruginosa* strains isolated from UTIs belong to serotype O11 and O6 (Patzer *et al.*, 1986; Reali and Rosati, 1994). Besides these, serotypes O4, O12 and O5 were also found in high frequency in 121 uroisolates of *P. aeruginosa* by Visca *et al.*, (1991). In our study, serotype O11 has been found to be the most common serogroup causing CUTIs. These findings corroborate the earlier observations. Predominance of serogroup O11 was also observed by Farmer *et al.*, (1982) who reported that antibiotic resistance was more common in strains belonging to this serotype. They further reported that the strains belonging to this serogroup have greater ability to survive and grow in hospital environments, resist disinfection, colonize human tissue and cause diseases like keratitis, burn wound infections, respiratory tract infections and urinary tract infections (UTIs).

Incidence of UTI has been found to be more in patients with long-term indwelling bladder catheterization (Stamm and Cautinho, 1999). Some workers have also shown that previous microbial urethral colonization could be the cause of most UTIs where introduction of bacteria into the bladder takes place subsequently at the time of catheterization (Dickinson and Bisno, 1989; Beltran *et al.*, 1991). Besides disruption of the normal valvular function of urethra, catheters can also traumatize urethral and bladder mucosa, hence disrupting the normal mucopolysaccharide coating of the epithelium (Parsons, 1986). This damage of cellular structure renders it susceptible to attachment as well as entry of bacteria through surface
erosions (Bonadio et al., 2005; Bissett, 2005). Therefore, catheter serves as a direct conduit for pathogens which may be carried from the external meatus to the bladder when the catheter is introduced (Niel-Weise et al., 2005). In addition, internal and external surfaces of catheters have intrinsic irregularities providing convenient sites for organism's implantation as demonstrated by scanning electron microscopy (Locci et al., 1981). Following initial adherence, bacteria may exude or attract some products to further solidify attachment (Peters et al., 1982). Direct observations of bacteria growing in natural ecosystems, from soil (Bae et al., 1972; Hall-Stoodley et al., 2004), and water (Fletcher and Floodgate, 1973; Costerton et al., 1987) to the bovine rumen (Mc Cowan et al., 1978) and human urethra (Donlan and Costerton, 2002) have shown that these cells often grow in glycocalyx enclosed microcolonies which are adherent to surfaces. Costerton et al., (1981) related the pathogenesis of catheter associated UTIs to the production of biofilms by the infecting organisms in which bacterial population adhered to catheter surface through pili and/or exopolysaccharides. The organisms in biofilms are able to persist in host's tissues for longer durations and are able to cause continuous damage to the host (Boles et al., 2004). Once an opportunistic pathogen like P. aeruginosa enters the host, its ability to cause infection has been correlated with its tendency to form biofilms (Donlan and Costerton, 2002; Hall-Stoodley and Stoodley, 2005). Thus, in patients with indwelling catheter, P. aeruginosa has an innate propensity to stick to the surfaces of catheters, forming microcolonies which coalesce together to form biofilms (Donlan et al., 2001; Tambyah, 2004; Huang et al., 2004; Hampton, 2004; Trautner et al., 2005).

To study the pathogenesis of catheter associated infections, initially, formation of biofilms in vitro was studied from day 1 to day 7 on Foley's catheter and on the surface of microtitre plate. Scanning
electron microscopy (SEM) of catheter surface as well as scrapings from these catheter pieces revealed gradual increase in thickness of biofilms from day 1 to day 7. Biofilm cells were seen to be deeply embedded in slimy glycocalyx on 7th day. In vivo biofilm formation was reported by Nickel et al., (1985) where colonizing bacterial population was observed embedded in glycocalyx on the external and internal surfaces of Foley's catheter removed from patient. Ganderton et al., (1992) examined 50 indwelling Foley bladder catheters that had been indwelling for periods ranging from 3 to 83 days in patients for the presence of bacterial biofilms. Scanning electron microscopy revealed biofilm formation on the luminal surfaces of 44 of these catheters. These workers observed very thin to very thick biofilms embedded in a matrix. Stickler et al., (1998) compared nature of biofilms formed in urease producing and non-urease producing organisms. It was observed that urease producing organisms, *Proteus mirabilis*, *Proteus vulgaris* and *Providencia rettgeri* formed crystalline nature of biofilms whereas urease-negative bacteria, *Morganella morganii*, *Klebsiella pneumoniae* and *P. aeruginosa* produced non-crystalline biofilms on urethral catheter. Similar observation of biofilm formation in vivo by *Pseudomonas aeruginosa* on indwelling catheter in mice was made by Kurosaka et al., (2001). In their study, scanning electron microscopy revealed a thick biofilm formation on the surface of polyethylene tubing from day 2 onwards which gradually increased till day 14 whereas in the present study in vitro, maximal viable cell count of biofilm cells was observable on day 5 following which there was a gradual decrease on day 6 and day 7. Results of viable cell count experiment corroborated with the results of crystal violet staining assay since maximal count as well as maximum absorbance was observed on day 5 by both the methods which remained constant till day 7. Crystal violet staining of biofilms has also been employed by various workers for quantitative determination of biofilm formation.
(Favre-Bonte et al., 2003; Pruthi et al., 2003). Mathur et al., (2006) while comparing 3 techniques of biofilm formation by clinical isolates of Staphylococcus spp. indicated that tissue culture plate method (TCP) was the most sensitive, accurate and reproducible screening method for detection of biofilm formation. Comparison of 3 different techniques for detection of biofilm formation by P. aeruginosa employed in the present study brings out that quantitatively maximum amount of biofilm cells were formed on 5th day following which there was a gradual decrease. Friedman and Kolter, (2004) also observed rod shaped P. aeruginosa embedded in an extracellular matrix on glass and plastic surfaces in vitro.

The extracellular matrix of the glycocalyx has been the subject of detailed study by many workers (O'Toole et al., 2000; Mah et al., 2003; Allison, 2003). It has been stressed that matrix provides a barrier leading to enhanced resistance to host defense mechanisms as well as to antibiotics causing treatment failure and also promote adherence to epithelial cells (Walter et al., 2003; Costerton et al., 2003; Jackson et al., 2004; Bagge et al., 2004). Extracellular matrix of glycocalyx in the form of exopolysaccharide of biofilms of P. aeruginosa is mainly composed of alginate. Alginate is an acetylated polymer of beta- D- mannouronic acid and alpha-L- guluronic acids where two uronic acids are arranged in different ways to form block structures (Evans and Linker, 1973; Russell and Gausa, 1988).

In the present study, all the uroisolates in planktonic and biofilm cell forms were found to be alginate producers but quantitative variation from strain to strain was observable. Ciragil and Soyletir, (2004) compared alginate production in various clinical isolates of P. aeruginosa and found that uroisolates produced less amount of alginate. Biofilm cells were significantly producing more alginate. Davies et al., (1993) also reported that alginate production was significantly more in biofilm cells of P. aeruginosa as compared to
planktonic counterparts employing reporter gene technology. These workers compared β-galactosidase activity as marker of alginate biosynthesis in biofilm and planktonic cells of *P. aeruginosa* and observed threefold increase in reporter gene activity/mg of protein in biofilm population. In this study, fourier transform infrared spectroscopy of biofilm and planktonic cell preparations revealed higher carbohydrate content in biofilm cells. Interestingly, in the present study, in biofilm cells a fall in the quantity of alginate was observed 5th day onwards. No precise explanation for this change was obvious but in an earlier study of Boyd and Chakrabarty, (1994), *P. aeruginosa* was demonstrated to produce alginate lyase, an enzyme involved in degradation of alginate. Repeated pattern of cell death and lysis was shown to occur in biofilms of *P. aeruginosa* during the normal course of development.

In the present investigation, it was observed that with increase in age of biofilms of *P. aeruginosa* from day 1 to day 4, significant increase in adherence to UECs was observed. 4 day old biofilm cells were more adherent to UECs as compared to planktonic cells. No such study using uroepithelial cells is available in literature. However, in an unpublished data from our laboratory, direct relationship between the quantity of alginate produced and uroepithelial cell (UEC) adherence of biofilm cells of *P. aeruginosa* was observed. Biofilm cells of *P. aeruginosa* adhered to UECs to a greater extent as compared to planktonic cells (Kaur et al., 2001). Ramphal and Pier, (1985) and Doig et al., (1987) highlighted the importance of alginate in relation to respiratory tract infection and gastrointestinal tract infection where alginate was shown to promote adherence of this pathogen to human tracheal epithelial cells (TECs) and buccal epithelial cells (BECs).

Surface of the invading organism plays very important role in the initiation, evolution and establishment of infection. The understanding for the phenomenon is based on the fact that
hydrophobic interactions have bearing on the adhesive ability of the organism to different host cells including phagocytes and soft tissues (Garber et al., 1985). It was brought out that such interactions need to be studied in relation to colonization (Speert et al., 1987). This interaction also plays an important role in microbial attachment to implants, prosthesis and contact lenses. In Pseudomonas aeruginosa strains, hydrophobicity has also been correlated with susceptibility to phagocytosis and piliation (Speert et al., 1986). More hydrophobic strains were found to be heavily piliated which were more susceptible to phagocytosis. In the present study, all the strains were found to be hydrophobic. It was observed that majority of uroisolates (78%) were strongly hydrophobic whereas rest of strains (22%) showed low hydrophobicity. The method employed in the present study for measuring hydrophobicity was based on bacterial adherence to hydrocarbon (BATH) using p-xylene. In earlier studies, Tylewska et al., (1988) and Jankowski et al., (1997) studied hydrophobicity of P. aeruginosa strains isolated from soil, faeces and UTI patients. These workers reported that these strains exhibited moderate surface hydrophobicity. The method followed by these workers was salting out with ammonium sulfate and hydrophobic interaction chromatography. Surface structures of E. coli and P. aeruginosa like pili, outer membrane proteins (OMPs) and lectins have been shown to contribute to hydrophobicity (Saralaya et al., 2004). Precise role of the degree of hydrophobicity in relation to UTI caused by P. aeruginosa needs further studies. However, assessment of the ability of gram negative organisms like E. coli and P. aeruginosa to attach to red blood cells has been studied on the basis of their haemagglutination potential and is correlated with hydrophobic potential.

Following adherence to red blood cells (RBCs), P. aeruginosa causes lysis of the cells through elaboration of hemolysins. Strains of Pseudomonas aeruginosa produce two hemolytic substances, one is a
heat labile protein phospholipase C and the other is a heat stable rhamnolipid hemolysin (Berka and Vasil, 1982). Phospholipase C is a lecithinase which liberates phosphorylcholine from lecithin. Its potential as virulence factor of *P. aeruginosa* in lungs of pneumonia patients was speculated by Berka and Vasil, (1982). In the present study, all *P. aeruginosa* strains were producers of PLC but 68% of *P. aeruginosa* isolates were found to be elaborating high PLC assessed by using p-nitrophenylphosphoryl choline (PNPC) as substrate. The results of the present study corroborates with those of Janda and Bottone, (1981) who reported PLC production in 63% of *P. aeruginosa* isolates isolated from UTI and Berka and Vasil, (1982) who showed PLC production in 100% of isolates using plate assay. Woods et al., (1986) showed that PLC production by urinary tract isolates were significantly greater than that by lung, blood or wound isolates. Elevated levels of PLC were also shown to be produced by strains isolated from urine (Woods et al., 1997). These workers highlighted that mean levels of PLC produced by *P. aeruginosa* strains vary significantly depending on the site of infection. Correlation between PLC production by *P. aeruginosa* and overt infection or transient colonization in urinary tract infection is however not available in literature but a possible role is suggested by Woods et al., (1997).

*P. aeruginosa* possess two types of heat stable hemolysins, one is bound to the surface of this organism (cell bound hemolysin) and the other is secreted in to the host’s milieu (cell free hemolysin) (Terada et al., 1999). Heat stable hemolysin has been characterized in only one of the strain of *P. aeruginosa* isolated from a patient of urinary tract infection by Fujita et al., (1988). Hemolytic mechanism of this hemolysin was shown to be similar to some detergents like sodium salts of fatty acids. In the present study, all *P. aeruginosa* strains were hemolytic with 82% of the strains as high producers of cell bound hemolysin and 78% of the strains as high producers of cell free
hemolysin. No such study is available for comparisons in relation to *P. aeruginosa* strains. However, from our laboratory, it has been reported earlier that 18 strains of *P. aeruginosa* isolated from UTI patients were elaborating high levels of both cell bound and cell free hemolysin along with other virulence factors like alginate, pyochelin, pyoverdin, protease, elastase and PLC (Mittal *et al.*, 2006). Uropathogenic *E. coli*, strains have been found to be elaborating greater amounts of both cell free and cell bound hemolysin (Domingue *et al.*, 1988). Although, hemolysin production has been characterized as a definite virulence factor in case of *E. coli* causing nephropathogenecity but precise role of hemolysin in UTI caused by *P. aeruginosa* still remains unidentified. Provirulence activity of hemolysin is reported to be multifactorial which includes disruption of phagocytic function, direct toxicity to host tissues and release of iron from erythrocytes thus helping pathogens to survive in host’s milieu (Johnson *et al.*, 1993).

*P. aeruginosa* like other uropathogenic organisms such as *E. coli*, requires iron for growth and cell physiological functions (Ankenbauer *et al.*, 1985; Beare *et al.*, 2003). Inspite of abundance of iron present in animal tissue and human body, this element is unavailable to the organism due to its intracellular location and firm complex formation with iron binding transport proteins present in the body fluids (Neilands, 1981; Griffiths, 1987; Poole and McKay, 2003). To procure iron within the host’s milieu, *P. aeruginosa* synthesizes two types of siderophores namely pyochelin (Cox *et al.*, 1981; Mislin *et al.*, 2006) and pyoverdin (Demange *et al.*, 1990; Tummler and Cornelis, 2005). In our study, while screening for siderophore production, all the strains of *P. aeruginosa* were found to be producers of pyochelin and pyoverdin but 86% isolates were observed to be high producers of pyochelin whereas 78% strains were high producers of pyoverdin. Visca *et al.*, (1992) screened 121 uropathogenic strains of *P. aeruginosa* and suggested that identification of atleast one type of...
siderophore in supernatants of *P. aeruginosa* cultures points to an important role of these agents in the pathogenesis of UTIs caused by this organism. These workers further highlighted that defective variants of pyochelin and pyoverdin do occur but at very low frequency. High incidence of detection of siderophores in uroisolates of *P. aeruginosa* in the present study corroborates the importance of these in relation to UTIs.

Pyochelin is a structurally unique siderophore possessing phenolate (Visca *et al.*, 2002; Tseng *et al.*, 2006) and is comparable to enterochelin (phenolate compound) produced by *E. coli*, the commonest urinary pathogen (Williams and Carbonetti, 1986; Braun and Braun, 2002). However, enterochelin, described as 'Native or Basal' siderophore, is important for growth of *E. coli*, but it has not been associated with the virulence of this organism (Johnson, 1991; Harjai *et al.*, 1990). Pyoverdin is a fluorescent dihydroxyquinolene derivative containing hydroxamate and catecholate residues required for chelating ferric iron (Ankenbauer *et al.*, 1985; Schons *et al.*, 2005; Yoder and Kissalita, 2006) which is comparable to hydroxamate siderophore aerobactin produced by *E. coli* (Bagg and Neilands, 1987; Harjai *et al.*, 1996; Johnson, 2003). Aerobactin encoded by pcol V plasmids has been well characterized as an important virulence factor in *E. coli* by both epidemiological (Montogomerie *et al.*, 1984; Sharma *et al.*, 1991; Demir and Kaleli, 2004) as well as experimental studies (Harjai *et al.*, 1994; Williams and Schaeffer, 2004). Similarly, in case of *P. aeruginosa* while pyochelin is ubiquitous but only pyoverdin has been recognized as a virulence factor (Takase *et al.*, 2000). These siderophores are secreted into the surrounding medium and then actively taken up and complexed with the Fe$^{3+}$ ion by specific inner and outer membrane components making it available for transport across the bacterial membrane (Wooldridge and Williams, 1993). They, therefore, serve an essential purpose to provide nutrition and help in
establishment of these organisms in iron limiting milieu (Cox and Adams, 1985; Vasil and Ochsner, 1999).

In addition to iron mopping mechanisms, pathogen like *P. aeruginosa* are known to be endowed with invasive properties operative through extracellular enzymes like protease and elastase (Nicodeme *et al.*, 2005). In the present study, all the isolates were observed to be elaborating both these exoenzymes but 86% isolates showed high protease activity and 78% strains showed high elastase activity. Janda and Bottone (1981) reported poor production of elastase by uroisolates of *P. aeruginosa* with presence of 69% of elastase defective variants while Woods *et al.*, (1986) showed high production of elastase and protease in strains isolated from urinary tract infections in comparison to isolates from other infections like burn wounds infection, skin wound infection and acute pneumonia. Variations in amount of these extracellular products amongst different serotypes was observed by Visca *et al.*, (1992). Serotypes belonging to O1, O10, O11 and O17 were found to produce significantly high levels of total proteases and elastase than other O serotypes like O4 and O12. In the present study, high protease and elastase production in *P. aeruginosa* strains belonging to serotype O11 was observed. Role of protease in UTIs was highlighted by Ciragil and Soyletir, (2004) who observed higher levels of protease production by uropathogenic strains of *P. aeruginosa*. This matches with the observations of the present study where more than three fourth of the isolates were producing very high levels of this enzyme.

Expression of virulence factors and cell density within biofilms of gram-negative and gram-positive organisms have been reported to be monitored through quorum sensing signals mainly operative through autoinducers known as acylhomoserine lactones (AHLs) (Pearson *et al.*, 1995; Winson *et al.*, 1995; Miller and Bassler, 2001; Juhas *et al.*, 2005). *P. aeruginosa* possess two types of quorum-
sensing systems, \textit{las} (Gambello and Iglewski, 1991) and \textit{rhl} (Ochsner and Reiser, 1995). The \textit{las} system has been shown to regulate the expression of several virulence factors including elastase, alkaline protease, Las B, exotoxin A, pyocyanin, pyoverdin and hemolysin (Gambello \textit{et al.}, 1993; Passador \textit{et al.}, 1993; Pearson \textit{et al.}, 1997; Hentzer \textit{et al.}, 2002). Besides these, secretion pathway in \textit{P. aeruginosa} is also shown to be modulated by \textit{las} system (Chapon-Herve \textit{et al.}, 1997). The \textit{rhl} system is involved in modulating the expression of several virulence factors controlled by \textit{las} system (Zhu \textit{et al.}, 2002). Both these quorum-sensing systems have also been shown to regulate twitching motility (Glessner \textit{et al.}, 1999) and are involved in the differentiation of planktonic cells to biofilm cells (Davies \textit{et al.}, 1998). Results of screening of 50 isolates of \textit{P. aeruginosa} for production of quorum sensing signal molecules both qualitatively and quantitatively showed that 45 isolates were producing these signal molecules while 5 isolates were negative for these signals. However levels of these molecules varied from strain to strain in producer strains. These results are in agreement with those of Stickler \textit{et al.}, (1998) who also reported production of AHLs by \textit{P. aeruginosa} isolated from urethral catheters using cross feeding assay. These workers demonstrated production of AHL molecules in biofilms \textit{in vitro} as well as \textit{in vivo} in the patient's bladder.

Quorum sensing systems are reported to be required for the expression of virulence factors (Passador \textit{et al.}, 1993). Since all \textit{P. aeruginosa} isolates in the present study were positive for elaboration of varying combinations of virulence factors, it is not surprising that majority of these isolates were also positive for production of quorum sensing signal molecules. Quorum sensing producer strains showed high levels of alginate, pyochelin, pyoverdin, elastase, protease, PLC and hemolysin whereas quorum sensing non-producer strains showed low levels of these virulence factors. Amongst the 5 selected...
uroisolates, the strain (PA5) which was maximum producer of quorum sensing molecules was found to be elaborating maximum levels of all the virulence factors. Zhu et al., (2002) while analyzing ocular isolates of *P. aeruginosa* correlated production of signal molecules with the expression of phenotypic characters like protease production along with bacterial invasion and cytotoxic activity. High levels of AHLs were found to be corroborating with high protease activity and invasiveness. Quorum deficient strains are also reported amongst 200 isolates of *P. aeruginosa* from patients with urinary tract, lower respiratory tract and wound infections (Schaber et al., 2004). These quorum deficient strains produced variable levels of virulence factors like exotoxin A, pyocyanin, type III secretion system (TTSS) effector proteins, ExoS and ExoT. These isolates also produced a weak to moderate biofilms. Naturally occurring QS-deficient strains of *P. aeruginosa* were also shown to be causing infection but this ability was attributed to the presence of some additional factors (Schaber et al., 2004).

Based on the results of incidence of different virulence traits in 50 uroisolates screened in the present investigation, 5 strains elaborating maximal virulence factors were selected for biofilm formation *in vitro* for period extending upto 7 days. Comparison of virulence factors produced by these strains in biofilm cell form was made with their planktonic cell forms. These selected strains were further employed to assess their urovirulence potential *in vivo* in the mouse model of ascending UTI where both planktonic and biofilm cell forms were used.

A significant increase in the production of all the virulence factors was observed (with increase in days of biofilm generation) from day 1 to day 4 in comparison to those elaborated by planktonic cells. There is paucity of literature in relation to elaboration of virulence factors by biofilm cells of *P. aeruginosa* isolated from UTI patients.
However, reports are available in relation to other uropathogens. Ando et al., (2004) correlated biofilm-forming capacities of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from catheter related UTI cases with the presence of virulence determinants like α-hemolysin, β-hemolysin and fibronectin binding protein and their gene products, *hla, hlb, fnb A* respectively by PCR assay. In order to understand biofilm formation and pathogenicity of MRSA infection in the urinary tract, it was highlighted that during cell to cell interactions in biofilm formation, the level of hemolysin correlated with the level of biofilm formation (Caiazza and O'Toole 2003). Similarly, ability of *E. faecalis* isolates to form biofilms in vitro was considered as a marker of virulence trait that enhanced the ability of this pathogen to cause catheter related infections (Sandoe et al., 2003). Potential relationship between biofilm formation and genes encoding virulence factors like hemolysin, enterococcal surface protein and gelatinase in *E. faecalis* isolates from catheter-related UTIs was also highlighted by Seno et al., (2005). In the present study also the 5 selected uroisolates in the biofilm cell form were elaborating quantitatively greater amounts of almost all the virulence factors. On further incubation beyond 4 days, a decrease in elaboration of all the virulence factors was observed. Studies with more strains of *P. aeruginosa* isolated from UTI patients are thus warranted to prove potential relationship between biofilm formation and expression of different virulence genes.

In relation to urinary tract, the organism needs to survive and even grow in the complex medium of the urine. The invading pathogen, while in the milieu provided by the urine, is subject to exposure to wide range of environmental factors varying under different physiological and pathological conditions (Smith et al., 1983). Depending on variations in the diet of the individual, pH of human urine has been shown to vary between 5.0 and 8.0 (Kunin and
Kidney urine was shown to have lower pH than bladder urine because of the dilution effect in bladder (Ross and Neely, 1983). In case, infection and inflammation is established, further lowering of pH specially during prolonged catheterization was observed (Todt et al., 1992). In the present study, effect of pH range of 5.0 to 8.0 on elaboration of virulence factors by \textit{P. aeruginosa} grown in planktonic and biofilm mode \textit{in vitro} has been assessed. Since urine is a complex medium whose composition keep varying from batch to batch, therefore in order to assess the effect of pH alone, a basal medium that is nutrient broth was used in the present study. With the increase in pH from 5.0 to 8.0, significant increase in alginate production was observed in all the strains in this study. Heyde et al., (1987) while analyzing functions of porins (Omp, Omp C, pHoE) of \textit{E.coli} at varying pH observed a direct relationship between pore diameters of porins and pH variation from 4.0 to 9.4. They observed induction of large sized channels at high pH (8.1 to 9.4) and suggested that there was greater rate of influx of glucose and maltose at this pH, as compared to rate of influx observed at lower pH (4.0 and 5.0). These workers thus suggested that increase in porin size can lead to plentiful of carbon which can be associated with increased alginate synthesis. Effect of porins though was not looked into in the present investigation but it is possible that increase in porin size could have contributed to observed increased alginate synthesis at pH 8. Increase in exoenzyme production namely alkaline protease and elastase by planktonic and biofilm cells was also observed with increase in pH to 8.0 in the present study. Morihara et al., (1984) reported remarkable instability of \textit{P. aeruginosa} proteinases at pH 4.0 to 4.5. This may lower the level of detectable protease at low pH as observed in the present study. On the contrary significantly enhanced production of both pyochelin and pyoverdin as well as hemolysin and PLC was observed at low pH of 5 for all the strains. Sriyoschati and Cox, (1986)
also observed maximal transfer of iron by siderophores of planktonic cells of *P. aeruginosa* from transferrin at pH between 5.0 and 6.0. Recently it has been shown that siderophore pyoverdin secreted by *P. aeruginosa* acts as a signaling molecule and pyoverdin signaling system controls production of protease, exotoxin A and pyoverdin itself. This provides a link between exoenzyme production and iron mopping mechanisms. Since outer membrane receptor is involved in pyoverdin signaling system, it remains to be seen whether changes in pH or other environmental factors effect pyoverdin mediated signaling system or not. This change can also have further effect on other exoenzymes of *P. aeruginosa*. These observations can have special relevance in *in vivo* situations where during the process of inflammation, induced by microcolonies, lowering of pH occurs (Todt et al., 1992).

Epidemiological studies regarding demonstration of virulence factors in the uroisolates of *Pseudomonas aeruginosa* (Woods et al., 1986; Visca et al., 1992) can only point towards indirect association between virulence factors and the disease process. To precisely understand their role in pathogenesis of the urinary tract infections, study of interaction of infecting pathogen expressing these factors within the existing host’s environment is important. For this availability of an acceptable animal model preferably involving either minimal or no manipulation of the urinary tract is the foremost requirement (Larsson et al., 1980). Choice of animal species should be primarily based on the fact that it shares important aspects of the urinary tract which are of relevance in the pathogenesis of disease in humans. Although experimental pyelonephritis is relatively easy to establish in rats because of presence of spontaneous vesicoureteric reflux (VUR) but the expression of receptors on rat uroepithelial cells do no match with those on human cells (Korhonen et al., 1981). At the kidney level also, the globosides isolated from the rat kidney contain
Gal α (1-3) Gal β linkage instead of the critical Gal α(1-4) Gal β linkage present in humans (Lyerla et al., 1986). Mouse renal tissue, in contrast, has been found to contain an appreciable concentration of Gal α (1-4) Gal β containing glycolipid matching with those present in humans. Besides availability of the receptors, Mural et al., (2002) recognized marked genetic similarity between mice and humans relatively recently. Pak et al., (2001) showed human and mouse THP to be matching on the basis of SDS PAGE analysis. The findings of experimental studies in such animal model can, therefore, be extrapolated more meaningfully to human clinical situations. For more than one reason, thus, mouse is considered to be a more desirable animal for establishing experimental UTI with E. coli. In addition, P fimbriae of E. coli resemble lectin PAI-L of P. aeruginosa in both sugar specificity and binding to human P blood group system antigens (Lerrer and Garber, 2001). Suitability of mouse model, therefore, has also been found to hold true for establishing UTI with P. aeruginosa where planktonic cells of this pathogen have been employed previously in our laboratory (Yadav et al., 2000).

In the present investigation, acute UTI was established through ascending route in mice with planktonic as well as biofilm cells of 5 selected uroisolates of P. aeruginosa including one standard strain. All the uroisolates were able to cause acute UTI but average bladder, urine and renal counts varied from strain to strain. In acute infection, peak bladder bacterial counts were observed at 10th postinfection hour with both planktonic and biofilm cells which gradually declined indicating clearance of bladder infection from these animals. Maximal urine counts were observed on 3rd postinfection day with both planktonic and biofilm cells of P. aeruginosa. Peak renal bacterial load was observed on 3rd and 5th postinfection day respectively following infection induced with planktonic and biofilm cells. This indicated that biofilm cells of P. aeruginosa have the ability to persist for longer
durations in the renal tissue as compared to their planktonic counterparts. Evaluation of bladder pathology of mice infected with planktonic cells showed mild to moderate inflammation in mucosal and submucosal regions. On the other hand moderate to severe pathology along with shedding of epithelial cells was observable in bladder tissue of mice infected with biofilm cells. Renal tissue of mice infected with planktonic cells also revealed mild to moderate cortical and medullary inflammation. Severe inflammation along with destruction of tubules and vascular changes was seen in renal tissue of biofilm cell instilled mice. In relatively earlier study, Nischi and Tsuchiya, (1978) established UTI in mice by transurethral inoculation of planktonic cells of *P. aeruginosa* in the bladder. In their study, urethral obstruction in animals for 6 hours was given after bladder inoculation whereas in our study no urethral obstruction was given, instead the catheter was withdrawn slowly after 10 minutes from urethra. These workers observed maximal renal bacterial counts upto $10^6$-$10^7$ CFU on 3rd postinfection day. The organisms were recoverable from the kidneys of 95% of the mice and the gross lesions in the kidneys were observed in 77% of the mice, 1 week after inoculation. Montgomerie *et al.*, (1980) also reported maximal bladder load on 1st postinfection day and maximal renal bacterial load on 3rd postinfection day. In their study also, UTI was induced in mice following instillation of *P. aeruginosa* into the bladder through urethral catheter. Bacteria were recoverable from 50% of the kidneys at 1-week interval and number of infected kidneys decreased over 4 weeks. Pathology evaluation revealed PMN infiltration in the pelvic mucosa, submucosa or in the pelvic space indicating pyelitis. These workers highlighted that the natural history of infection in the bladder and kidneys of the mice is similar to the natural uncomplicated UTIs seen clinically. In another study, Montogomerie *et al.*, (1981) when injected *P. aeruginosa* into bladder tissue directly, the involvement and
damage was more extensive with demonstrable microulceration in the bladder coupled with bacteremia. Tanaka et al., (1982) induced ascending UTI in mice with elastase and protease producing strain of \textit{P. aeruginosa} by intracystic challenge ($10^{10}$ CFU). However, due to large amount of inoculum employed in this model, animals developed severe necrosis leading to systemic infection and death. Nakao et al., (1985) performed SEM on the bladder and kidney tissues of mice instilled with \textit{P. aeruginosa} through urethral catheter. These workers showed no demonstrable organisms in bladder tissue after 48th postinfection hour but observed morphological aberrations such as elongation, bulge and spheroplast formation. In literature, very few experimental studies are available where UTI has been induced in mice with biofilm cells of \textit{P. aeruginosa}. Only recently Kadurugamuwa et al., (2005) developed a mouse model of catheter associated UTI induced with \textit{P. aeruginosa} where importance of biofilm formation \textit{in vivo} has been addressed to. Interestingly in this model, they have reported a methodology which allows spatial information to be monitored sequentially throughout the disease process including treatment efficacy and relapse, all without exogenous sampling. This is not possible with conventional methods. These workers employed bioluminescently engineered \textit{P. aeruginosa} cells and performed biophotonic imaging to monitor infection. In their model, maximal bladder counts were observed on 1st postinfection day and peak renal counts on 3rd postinfection day. It was stressed that in future investigations the model employing bioluminescent engineered bacteria can give relevant desired information regarding different pathogenetic aspects of UTI caused by both cell forms of \textit{P. aeruginosa}. However in this reported model, it is not possible to evaluate precise extent of pathology in renal and bladder tissues which forms one of the most important criteria for defining the extent of UTI (Garg et al., 1987; Tardif et al., 1994). Present investigation is,
therefore, more relevant where assessment of pathology brings out that severity of lesions in renal and bladder tissue induced with biofilm cells are significantly more as compared to planktonic cells.

Inability of host’s defense system to eliminate invading pathogen can lead to persistence of infection and progression of acute renal infection to chronic form of the disease (Tebloeva et al., 1982; Miller et al., 1986; Shulutko et al., 1993; Goluszko et al., 1997; Sidorova et al., 2003; Tamaki et al., 2006). Chronic pyelonephritis has been defined histologically as a destructive inflammatory process involving both the pelvocalyceal system and the renal parenchyma (Labat et al., 2005).

To study pathogenesis of chronic pyelonephritis caused by, opportunistic pathogen like *P. aeruginosa*, an experimental model mimicking natural course of infection has been standardized in the present study. Chronic infection was inducible in mice with both planktonic and biofilm cells of *P. aeruginosa* based on the assessment of renal bacterial load, neutrophil recruitment, MDA production and tissue pathology. After each successive dose of infection after 1st, 15th and 120th day, biofilm cells persisted for longer interval of time in renal tissue of mice as compared to their planktonic counterparts indicating that preformed biofilm cells of *P. aeruginosa* may help in its persistence in the urinary tract for relatively longer period of time. Evaluation of pathology of renal tissue also revealed that severity of lesions were more with four day old biofilm cells as compared to planktonic cell instilled animals at all time intervals. Kidneys were found to be sterile with both the cell forms at 150th postinfection day. Guze et al., (1973) demonstrated opsonizing antibodies in case of *E. coli* induced chronic pyelonephritis at 120th postinfection day showing sterile kidneys. Absence of organisms in renal, bladder and urine, observed in the present study, could also be explained on the basis of presence of opsonizing antibodies which could have helped in clearance of bacteria. This needs confirmation in future investigations.
In one available report of foreign body associated chronic UTI in rats induced with *P. aeruginosa*, Kur osaka et al., (2001) used a spiral polyethylene tubing (PT) which was placed transurethrally into the bladder without surgical manipulation. This was followed by inoculation with planktonic cells of *P. aeruginosa*. Persistence of organisms in the kidneys and bladder was significantly enhanced due to development of thick biofilm on the surface of PT suggesting continuous introduction of bacteria from the biofilm adhering to the PT through ascending route. Kidneys were shown to have chronic renal inflammation which was continuous and sporadic in the pelvis as well as in adjacent tissues. In contrast, bacteria were eliminated rapidly from the urinary tract in the animals without the PT. However, this model involves placement of foreign body into the bladder which is likely to disrupt the mucopolysaccharide coating, interfering with natural resistance to infections. In comparison, an unmanipulated chronic model of pyelonephritis was employed in the present study. Further the experimental animals were observed only for 14 days by these workers thus missing the evolution of true form of chronic pyelonephritis. The observation period of the present study, however, was 150th postinfection day, time at which renal scarring was also observable, hence offering distinct advantage. In earlier study from our laboratory, chronic renal ascending infection was reported to be inducible with uropathogenic *E. coli* where same approach was used (Gupta et al., 1995). Chronic UTI model reported in the present study, hence, is simple and relevant which can be further employed for studying pathogenesis of chronic form of renal infection caused by *P. aeruginosa* without placement of any foreign body.

Recently it has been proposed that quorum sensing signals (QS) operative through autoinducers like acylhomoserine lactones (AHLs) may play an important role in pathogenesis of biofilm induced infections of *P. aeruginosa* (Juhas et al., 2005). In the present
Investigation, an attempt was made to study the role of quorum sensing signals in vivo using mouse model of experimental UTI induced with a reference laboratory parent strain, PAO1, quorum sensing mutants and QS negative uroisolates. High renal bacterial counts were observed in the mice till 7th postinfection day infected with parent strain PAO1, possessing both rhl and las quorum sensing systems whereas significant decrease in renal bacterial load in kidneys of mice infected with mutant strains as well as quorum sensing deficient uroisolate of P. aeruginosa was observed after 3rd postinfection day. In addition, significant increase in renal MDA level was observed following infection with parent strain in comparison to mutants and uroisolate. These findings suggest that both las and rhl quorum sensing systems are important for virulence of P. aeruginosa in the pyelonephritis model during the acute phase. In different infection models like that of pulmonary infection, mouse burn wound and keratitis, quorum-sensing signals have been shown to contribute to the virulence of P. aeruginosa. In P. aeruginosa induced acute pneumonia (Pearson et al., 2000) and chronic lung infection mouse model (Wu et al., 2001), it was observed that lasl rhl double mutant was avirulent whereas respective single mutants had reduced virulence as compared to wild type strain. Similarly in a mouse burn model of P. aeruginosa infection quorum-sensing systems were shown to play an important role in the horizontal spread of P. aeruginosa within burned skin (Rumbaugh et al., 1999). No such study highlighting the role of these signal molecules in the pathogenesis of urinary tract infection is available. In relation to UTI, only one report available in literature demonstrates production of quorum sensing signal molecules by biofilms on the surface of catheters both in physical model of bladder (in vitro) as well as in patient's bladder (in vivo) (Stickler et al., 1998). The results of these workers indicated that quorum sensing signals are produced in vivo in the urinary tract...
following infection with *P. aeruginosa* in catheterized patients. The present study is unique in highlighting the importance of these molecules in experimental animal model of urinary tract infection.

Results of renal pathology scoring provided additional evidence in support of contribution of quorum sensing systems to the virulence of *P. aeruginosa* in this model. Severe inflammation coupled with destruction of tubules was observed in kidneys of mice infected with quorum sensing producer strain. On the contrary, significant decrease in renal severity scores was observed with both the mutants as well as quorum sensing negative uroisolates. Renal tissue of mice infected with these strains showed mild inflammation with severity scores of 1-2. Importance of quorum sensing signals in the pathogenesis of respiratory tract infections was highlighted in model of acute pneumonia in mice (Pearson *et al.*, 2000) and rats (Wu *et al.*, 2001) where lung tissue pathology was studied. Animals infected with PAO1 strain showed severe confluent pneumonia as compared to mild focal pneumonia in lung tissue of mice infected with quorum sensing deficient mutant strains.

In the present study, level of MPO in renal tissue was taken as an index for tissue neutrophil recruitment. Significant decrease in MPO level was observed when mutant strains and quorum sensing deficient uroisolates were used to induce infection. This observation indicated that infection with quorum deficient strains resulted in mild infiltration of neutrophils in kidney tissue compared with the parent strain. This may be attributed to lack of functional *las* system in the mutant strains. Other workers have reported immunomodulatory effect of *las* and *rhl* system in host (Telford *et al.*, 1998). Explanation put forth is that *las* molecule is a potent inducer of neutrophil chemokine IL-8 and inflammatory mediator Cox-2 enzyme, both of which serves as neutrophil attractants and are shown to be associated
with pathophysiological process of inflammation and edema (Dimango et al., 1995).

Results of the present study bring out that quorum-sensing signals play significant role in evolution of *P. aeruginosa* induced pyelonephritis. This was demonstrated by reduced ability of mutants and quorum deficient clinical strain to colonize and cause mild pathological alterations and also by decreased neutrophil influx and decreased MDA production in renal tissue of experimental animals as compared to parent strain. Our study indicates that both *las* and *rhl* quorum sensing systems are important for the virulence of *P. aeruginosa* in development of pyelonephritis. Based on the results of available literature and the present study, it can be speculated that AHLs can serve as potential target molecules for developing effective preventive strategy against urinary tract infection caused by biofilms of *P. aeruginosa*. Further studies employing purified molecules of AHLs are warranted which can throw more light on contribution of quorum sensing signals in the pathogenesis of *P. aeruginosa* induced pyelonephritis.

Quorum sensing signals have been reported to play an important role in helping *P. aeruginosa* to adapt to environmental stresses prevalent in the host milieu. Stress conditions have been reported to exist in the urinary tract. Invading pathogen has to adapt to fluctuations in osmolarity levels of urine in order to establish in urinary tract (Kaye, 1968). Although uropathogens grow very well in human urine but their multiplication has been shown to be affected by osmolarity of this medium (Kunin and Chambers, 1989; Kunin et al., 1992). Osmolarity of the normal human urine ranges from 100-200 mOsmol (Schmidt et al., 1983; Suzuki et al., 1987; Loeb et al., 1989). However, the osmolarity of the urine in the kidneys exceed the osmolarity of urine held in the bladder (Ross and Neely, 1983). Fluctuating osmolarity thus distinguishes the urinary tract from most
other mammalian systems (Culham et al., 2001). Osmolarity values may also vary under different physiological and pathological conditions (Schwan et al., 2002). The important clinical conditions in which hyposmolarity is encountered are in patients suffering from renal tubular defects, chronic renal failure, in patients suffering from diabetes insipidus and in patients taking diuretic therapy as well as taking excess intake of water. On the other hand, hyperosmolarity is encountered during dehydration, high glucosuria, excessive salt intake, acute renal failure specially pre renal failure and excretion of high molecular weight proteins like myeloma protein in the urine (Schmidt et al., 1983; Chambers and Kunin, 1985). In the present study, to observe the effect of osmolarity on elaboration of virulence factors by biofilm and planktonic cells of *P. aeruginosa*, organisms were grown in M9 medium having different osmolarity values ranging from 200 to 350 mOsmol. It was observed that with increase in osmolarity of medium from 200 to 300 mOsmol there was significant increase in elaboration of all the virulence factors. However, with further increase in osmolarity of medium to 350 mOsmol, there was significant decrease in production of all the virulence traits. In addition, significant decrease in uptake and intracellular killing of organisms grown in 300 mOsmol M9 medium by murine peritoneal macrophages was observed. There is paucity of literature regarding role of osmolarity on virulence of uropathogenic *P. aeruginosa* but limited reports in relation to other uropathogens like *E. coli* and *S. saprophyticus* are available. Effect of different osmolarities on phagocytosis and killing of *E. coli* and *S. saprophyticus* by PMNs *in vitro* was studied by Gargan et al., (1983). Impaired phagocytosis and killing of *E.coli* by human PMNs in urine with high osmolality and low pH *in vitro* was observed by these workers. Osmolarity was shown to be main modulating factor affecting phagocytosis which is a pivotal function required for clearance of bacteria from the urinary tract.
These workers suggested that raising the urinary pH and reducing the osmolality may help in restoring normal bactericidal activity of PMNs leading to clearance of organisms. Culham et al., (2001) studied the osmotic stress response and virulence in pyelonephritic isolates of E. coli. Osmotolerance of E. coli was reported due to the presence of osmoprotective agents like glycine betaine and proline betaine. Loss of type I pilus expression when E. coli was grown in hyperosmolar medium (containing 400mM NaCl) was reported by Schwan et al., (2002). It was also reported that combination of low pH and moderate to high osmolarity can have profound synergistic effect on the expression of the Type I pili on the surfaces of the bacteria. These studies bring out that alteration of urine pH and osmolarity may be highly relevant to treatment of urinary tract infections since optimal functions of PMNs in vivo could be restored.

Reports on effect of osmolarity on elaboration of some of the virulence factors by P. aeruginosa isolated from respiratory tract infections are, however, available in literature. In P. aeruginosa strains isolated from cystic fibrosis patients, workers have reported increased production of alginate by this pathogen under hyperosmolar conditions. These workers employed molecular biology techniques for the studies (Berry et al., 1989; Zielinski et al., 1992). Genes involved in alginate expression were reported to enhance neuraminidase production by P. aeruginosa under hyperosmolar conditions facilitating adherence of this pathogen to respiratory tract (Cacalano et al., 1992). Ability of P. aeruginosa to tolerate environmental stresses like osmotic stress has been attributed to the presence of sigma factor Rpo S. Suh et al., (1999) reported that Rpo S mutant strains of P. aeruginosa were hypersensitive to increased osmolarity which produced lower amounts of elastase, protease and exotoxin A. These workers further observed that in an alginate overproducing CF isolate of P. aeruginosa, Rpo S mutation almost completely abolished the
production of alginate when the bacterium was grown in liquid medium. On solid medium, Rpo S mutant produced 70% less alginate than the wild-type strain. Role of Rpo S on the ability of *P. aeruginosa* to resist environmental responses and in elaboration of virulence factors was suggested by these workers. Further studies are warranted in order to elucidate the role of osmolarity on the induction and evolution of urinary tract infections caused by *P. aeruginosa*.

Urinary tract imposes multiple physiochemical stresses on colonizing bacteria. Therefore, employment of urinary tract infection model is useful for studying the relationship between virulence of the invading bacterium and the stress which it encounters. Opportunistic pathogen like *P. aeruginosa* has to survive major environmental stresses during causation of UTI which are in the form of high osmolarity, low pH, iron depletion, presence of THP and MSPs present in the milieu of urine (Schwan *et al.*, 2002). These factors may also play role in the pathogenesis and final evolution of disease process (Suh *et al.*, 1999). In the present study, different stress factors in varying concentrations were employed for monitoring the growth of *P. aeruginosa* *in vitro*. Concentrations which allowed maximal expression of virulence factors were selected. Organisms grown in presence of these factors were then employed to induce UTI in mice. Lodgement of organism alongwith influx of PMNs, indicators of tissue damage and pathology was analyzed. Earlier work from our laboratory has shown that constituents of urine can modulate elaboration of virulence factors by uropathogenic *E. coli* (Harjai *et al.*, 1996; Kumar *et al.*, 1997). However there has been no report as to how the growth of opportunistic pathogen like *P. aeruginosa* exposed to variations in different environmental conditions of urine will affect its *in vivo* colonizing ability.

In the present investigation, *P. aeruginosa* (planktonic and biofilm cells) grown in 300 m Osmol M9 medium when used for
infecting mice were found to be more virulent in the urinary tract as indicated by significantly higher neutrophil recruitment, bacterial load, MDA production and renal as well as bladder pathology in comparison to control. Culham et al., (1998) highlighted that in addition to directly influencing bacterial growth in the urinary tract, osmoregulatory mechanisms may indirectly influence urinary tract infection by affecting the expression of virulence determinants. Later in 2001, these workers reported presence of additional osmoregulatory systems in *E. coli* K-12 which may facilitate growth of pyelonephritic isolates in human urine and also assist in colonization of mammalian urinary tract. Number of bacteria recovered from urine and bladder of mice inoculated with osmotolerant organisms were significantly higher than those recovered after inoculation with faecal isolate. Mutations in osmoregulatory transporter reduced recovery of *E. coli*. In case of *P. aeruginosa*, the sigma factor, Rpo S, has been shown to play an important role during exposure of this organism to various environmental stresses including osmotic stress. Suh et al., (1999) also suggested importance of Rpo S in the pathogenesis of *P. aeruginosa* induced respiratory infections. There is strong possibility that similar mechanism may be operative in the urinary tract affecting the evolution of infection caused by *P. aeruginosa* although this needs further confirmation.

Complex urinary medium has been shown to have variable amounts of ions especially iron which is known to play an important role. Low iron solubility, together with the process of withholding iron from invading organism by the host through iron complexing with proteins such as transferrin and lactoferrin is an important strategy in host defense hence limiting iron conditions prevail in the milieu of urinary tract (Shand et al., 1985; Takase et al., 2000). Thus acquisition of iron is important for the infection and survival of *P. aeruginosa* in their hosts (Vasil and Ochsner, 1999; Beare et al.,
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2003). In the present study effort was made to create iron limiting conditions in a defined medium by using different concentrations of dipyridyl ranging from 100 to 300 μM and to create iron replete conditions using different concentrations of FeSO₄ ranging from 0.5 to 5 μg/ml in the medium. Planktonic and biofilm cells of *P. aeruginosa* were generated in medium having iron content ranging from 0.3 to 10 mg/l and production of virulence traits by this pathogen under these conditions was assessed. Maximum production of all the virulence determinants was observed at iron concentration of 0.03 mg/l whereas least production was observed at iron levels of 6 mg/l. In addition decreased phagocytosis (both uptake and intracellular killing) of planktonic and biofilm cells of *P. aeruginosa* grown in presence of 5 μg/ml of FeSO₄ was observed. In an earlier study, Bjorn et al., (1979) reported increased production of toxin A, protease, elastase and protease under iron limiting conditions and decreased production of these virulence traits following growth of *P. aeruginosa* in iron rich medium. The results of the present study are in agreement with those of Kim et al., (2003) who also observed increased production of elastase, pyochelin and pyoverdin by planktonic cells of *P. aeruginosa* grown under iron limiting conditions (0.6 mg/l) and decreased production of these virulence factors in iron-rich medium (7mg/l). Explanation given by these workers was that iron deficiency triggered oxygen limitation which resulted in drastic increase in secretion of proteins like elastase. Increased elastase synthesis has been directly linked with iron acquisition ability of *P. aeruginosa* under iron limited conditions. These workers also observed alginate capsule on the surface of *P. aeruginosa* grown in iron-deficient medium. No capsule was demonstrable when it was grown in iron-rich conditions using transmission electron microscopy (TEM) indicating that iron-deficiency triggered the formation of alginate in this organism. Sabra et al., (2002) also observed increased alginate concentration in supernatants
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of *P. aeruginosa* cultures grown in iron-limited medium and negligible amount of alginate in *P. aeruginosa* strains grown in iron-rich medium. These workers attributed enhanced formation of alginate in iron-limiting medium to iron-regulation known to be activated under iron deprivation (Vasil and Ochsner, 1999). However, these workers employed only one standard strain of *P. aeruginosa* (PAO1). In this regard, the present study is more relevant as uroisolates of *P. aeruginosa* along with standard strain were also employed. Workers have reported that formation of biofilms by *P. aeruginosa* depend on the concentration of iron. Musk *et al.*, (2005) reported inhibition of biofilm formation on microtitre plate surface, glass surface and flow chamber by cystic fibrosis isolates and standard strain of *P. aeruginosa* in the presence of iron salts like ferric ammonium citrate (FAC), ferric chloride, ferric sulphate and ferrous sulphate. In addition biofilms that were pregrown for 5 days in flow chamber without iron supplementation to the media and then subjected to 200 μM FAC for 5 additional days were completely cleared as observed by fluorescence microscopy indicating disruption of pre-existing biofilms. Inhibition of biofilm production was shown to be likely through the repression of various genes involved in iron scavenging and quorom sensing. Singh *et al.*, (2002) also showed that in virtual absence of iron *P. aeruginosa* cannot form biofilms. Decrease in elaboration of virulence factors observed in this study may be due to disruption of biofilms for which further studies are required. Results of the present study supplemented by the existing literature indicate that levels of iron dictate virulence of *P. aeruginosa* and are thus critical for its pathogenicity.

Since available iron in the urine under normal circumstances is in bound form, the organisms are recognized to elaborate iron chelating compounds broadly belonging to phenolate and hydroxamate group of siderophores *in vivo* (Neilands, 1984; Crosa,
1989; Johnson et al., 1992; Beare et al., 2003; Cobessi et al., 2005; Tseng et al., 2006). These siderophores make iron available to the invading organism and help them to cause infection as well as affect their survival in the host (Bullen, 1985; Vasil and Ochsner, 1999). In relation to urinary tract infection, one approach to study iron dependent virulence of organisms has been to check the intensity of infection in experimental animals whose iron status has been altered (Barry and Reeve, 1977; Becroft et al., 1977; Weinberg, 1978; Bullen et al., 1991). In this context, Hart et al., (1982) observed that iron deficient rats were significantly less susceptible to \( P. \text{mirabilis} \) induced experimental pyelonephritis. In another study of Sharma et al., (1987), with uropathogenic \( E. \text{coli} \), urovirulence of the organism was shown to be enhanced under the influence of available host's iron where relatively sensitive marker like brush border enzyme assay was employed as the assessment criteria. Besides iron saturation of the host \( \text{in vitro} \) cultivation of organisms to check the degree of virulence of bacterium \( \text{in vivo} \) was also stressed upon. Another approach has been to grow organisms \( \text{in vitro} \) in iron deplete or replete medium before inducing infection. Sharma et al., (1995) showed enhanced mouse renal pathogenicity with \( E. \text{coli} \) when organisms given repeated passages in the urine, supposedly providing iron limiting conditions \( \text{in vitro} \) were used for inducing infection. This was deduced on the basis that urine grown organisms showed increased production of siderophores with successive passages. In relation to \( P. \text{aeruginosa} \) some \( \text{in vitro} \) studies are available where iron has been shown to regulate production of toxin A (Bjorn et al., 1978), alkaline protease (Bjorn et al., 1979), elastase (Bjorn et al., 1979; Kim et al., 2003) and siderophores (Kim et al., 2003), the recognized virulence factors of this opportunistic organism. Iron concentration of the culture medium employed for growth of \( P. \text{aeruginosa} \) was also shown to have the potential to influence pathogenicity of this organism in corneal (Woods
et al., 1982) as well as in the acute respiratory tract infection model (Sokol and Woods, 1984). In the present study, planktonic and biofilm cells of *P. aeruginosa*, generated in iron-limited and iron-rich medium were used for inducing infection in mice. Significant increase in bacteriological counts, neutrophil influx, pathological lesions and tissue damage were observable in mice infected with *P. aeruginosa* grown in iron limiting conditions. On the contrary, all these parameters were decreased in mice infected with planktonic and biofilm cells of *P. aeruginosa* grown in iron-rich medium. These in vivo results may be attributed to enhanced production of siderophores pyoverdin and pyochelin by *P. aeruginosa* along with other extracellular virulence factors like elastase, protease, phospholipase C and hemolysin in vitro in iron deplete conditions. In relatively early study of Sokol and Woods, (1984), it was stressed that availability of iron in growth medium, in vitro, used to culture bacteria before inoculation of animals to cause respiratory tract infection has the capacity to alter virulence of *P. aeruginosa* in vivo. Its relevance was especially based on the fact that extracellular products of this organism are regulated by iron. However, no matching studies in relation to UTI induced with *P. aeruginosa* are available. The present study thus provides the basis for the use of iron limited and iron rich medium for the growth of pathogens before inducing infection for studying the iron dependent virulence in animals since iron affects the virulence of this organism.

In the urinary tract, the invading organism is required to have the ability to survive the flushing action of the urine and to be able to grow in order to establish infection. Complex urine provides a medium which has copious amounts of mucus. The urinary mucus predominantly has Tamm-Horsfall protein (THP) which is a polymeric glycoprotein produced in thick ascending limb of loop of Henle in renal tissue (Kokot and Dulawa, 2000). Majority of THP is in the form of
secreted protein in urine but it also exists in membrane bound form especially at the renal distal nephron cell surface (Serafini-cesssi et al., 2003). THP has been shown to have the potential to alter pathogenesis of UTIs caused by type I fimbriated E. coli either by flushing them out (Saemann et al. 2005) or by mediating their adhesion to host’s target cell (Hawthorn et al., 1991). Since during initial colonization the mucous lining is encountered by infecting bacteria therefore, it is likely that this interaction could be an important signaling mechanism for the regulation of specific virulence genes present in the organism (Wang et al., 1996). Whether interaction of invading pathogen with THP has any influence on the elaboration of the organism’s virulence factors is yet not defined. Concentration of THP has been reported to be crucial in deciding the ultimate role played by this protein (Duncan, 1988). Significant enhancement in elaboration of alginate, protease, elastase, PLC, siderophores and hemolysin in presence of 50 μg/ml of THP was observed, in the present study. With increase in concentration of THP from 10 μg/ml to 50 μg/ml, gradual rise in elaboration of all the virulence factors was observed as compared to control (i.e. in absence of THP). However with further increase in concentration of THP from 50 μg/ml to 70 μg/ml there was significant fall in production of all the virulence traits by biofilm cells of P. aeruginosa. Decreased uptake and intracellular killing of THP (50 μg/ml) coated planktonic and biofilm cells of P. aeruginosa by murine peritoneal macrophages were also observed. In an earlier study, Kuriyama and Silverblatt, (1986) showed that dissolved THP formed a pseudocapsule around E. coli bearing type I fimbriae resulting in less susceptibility to phagocytosis and killing. Concentration dependent differential effect of THP has also been demonstrated on adherence of E. coli to transitional epithelial cells (Duncan, 1988). It was observed that although high concentrations of THP almost completely inhibited adherence of type I piliated E. coli to
transitional cells but low concentrations actually increased adherence. Although Lerrer and Garber, (2001) identified PA-IL and PA-IIL as the lectins of \textit{P. aeruginosa}, which resemble P-fimbriae and type I fimbriae of \textit{E.coli} respectively, however, their role in adhesion of \textit{P. aeruginosa} to THP still remains to be elucidated. This will have direct bearing on the course of UTI since interactions between bacteria and host cell surfaces are bound to get modified once the pathogen bind to soluble host proteins. Expression of three genetic loci encoding potent virulence factors of \textit{P. aeruginosa} have been demonstrated following interaction of this pathogen with respiratory mucus derived from cystic fibrosis patient (Wang \textit{et al.}, 1996, Lory \textit{et al.}, 1996). Respiratory mucus inducible genes (mig A) have been shown to regulate expression of virulence traits in \textit{P. aeruginosa} including siderophore production, biosynthesis of lipopolysaccharides and alginate (Yang \textit{et al.}, 2000). Human tracheobronchial mucin (HTBM) is also known to have some similarity with regard to sugar moieties to THP. Sugars commonly present in both the mucins include D-galactose, N-acetylglucosamine, N-acetyl galactosamine, N-acetyl neuraminic acid and L-fucose (Williams \textit{et al.}, 1984; Vishwanath and Ramphal, 1985). Mucins are present on all the mucosal surfaces, hence there can be a commonality as far as true role of mucus is concerned. The changes observed following interaction of \textit{P. aeruginosa} with respiratory mucus could be applicable to its interaction with uromucoid containing THP.

In order to correlate the \textit{in vitro} findings of interaction of THP with \textit{P. aeruginosa} to \textit{in vivo} establishment, an ascending urinary tract infection model in mice was employed. It was observed that THP coated \textit{P. aeruginosa} cells were more virulent \textit{in vivo} in UTI model, showing higher level of destruction in kidney as well as in bladder tissue in comparison to uncoated organisms. These results therefore bring out that THP coating provide better opportunity to this pathogen
for survival in vivo. This may be due to the capacity of P. aeruginosa to resist phagocytosis after getting coated with THP. Decreased susceptibility of THP coated organism to phagocytosis has been reported for type 1 fimbriated E. coli by Kuriyama and Silverblatt, (1986) affecting ultimate disposal of this pathogen from the urinary tract. These findings suggest different role of THP with regard to different uropathogens. Hawthorn et al., (1991) while comparing adhesion of three uropathogens to THP coated renal tubular cells in vitro, also stressed that THP may not help to remove all uropathogens from urinary tract. It may help in renal colonization of some uropathogens like P. mirabilis and P. aeruginosa. In the mileu of the kidney where THP is available in abundance the observations reported above, are relevant. Once P. aeruginosa reaches renal parenchyma, this ability may help this organism to colonize, get established and persist. Due to non-availability of in vivo studies, direct comparison is not possible. Further in vivo studies using THP knock out mice for establishment of P. aeruginosa in urinary tract will be of special interest to confirm these observations.

Urinary tract infections, an example of mucosal infections, are recognized to be capable of triggering systemic inflammatory response which is accompanied by polymorphonuclear neutrophil (PMN) recruitment at the site of infection (Kunin, 1987; Shahin et al., 1987). Neutrophils which leave the bloodstream, migrate to the tissue, crossing the epithelial barrier and finally reaching into the lumen. In the context of UTIs, therefore, study of presence of neutrophils in the renal tissue, bladder tissue and urine carries importance. In the present study, accumulation of neutrophils in the urine (assessed on the basis of cell count assay) was observed as early as 2-hour postinfection period showing decrease on 1st day in case of both planktonic and biofilm cell infected mice. Rise in neutrophil influx was again observed on the 3rd postinfection day in case of planktonic cell
instilled mice and on 5th postinfection day in case of biofilm cell infected mice. Evaluation of kidney and bladder tissue of experimental animals (based on MPO estimation) confirmed to the same pattern. Study of pathology revealed that neutrophil infiltration was more in the renal pelvis which extended into the medulla and cortex. PMNs, however, were seen scattered throughout the kidney tissue. On the whole, density of neutrophils in bladder tissue was less than that observed in kidney tissue. On one hand, these cells are essential for clearance of bacteria from urinary tract, on the other hand neutrophils have been implicated in tissue damage leading to renal scarring (Haroaka et al., 1999). In case neutrophils are trapped and tissue is destroyed, the kidney pathology has been reported to be progressing to the stage of chronicity and renal scarring. Higher renal neutrophil influx was observed during chronic UTI as compared to acute UTI. Thus the observations of present study are in accordance with findings of Hang et al., (2000) who studied neutrophil influx in E. coli induced infections.

The importance of neutrophils coming in response to epithelial cell chemokines like IL-8 leading to clearance of bacteria (E. coli) from the local site of infection in UTI has been highlighted by Agace et al., (1993). IL-8 has also been detected in urine of patients especially during acute E. coli induced pyelonephritis in patients suffering from acute UTI (Ko and McFadden, 1990; Jantausch et al., 2000). Increase in urine IL-8 concentration is also found to correlate with urine neutrophil numbers in patients (Svensson et al., 2005). Studies in IL-8 receptor knockout mice have confirmed that molecular deficiency of IL-8 may be responsible for progression of disease from acute to chronic pyelonephritis (Godaly et al., 2000). In an experimental study, an association between neutrophil migration and IL-8 production has been shown in rabbits (Colditz et al., 1990). In experimental infections carried out in mice, it is accepted since long that MIP-2 is the
counterpart of human IL-8 and plays a central role in the influx of PMNs (Wolpe et al., 1989). Hang et al., (1999) found time dependent MIP-2 production in urine, bladder and kidney of BALB/C mice with *E. coli* induced UTI. Further with immunohistochemistry technique, it was demonstrated that renal pelvic mucosal cells were the local site of MIP-2 production. This was confirmed by treating the mice with anti-MIP2 antibodies. These animals showed reduced neutrophil numbers in urine and normal neutrophil numbers in bladder tissue indicating the importance of MIP-2 in neutrophil migration across the epithelium in *E. coli* induced pyelonephritis. In the present study, similar pattern of MIP-2 production was observed in urine, bladder and renal tissue in *P. aeruginosa* induced UTI. MIP-2 was detectable in urine, bladder and renal tissue at 2nd postinfection hour showing peak at 6th hour and coming to basal levels at 24th hour. The results of the present study thus brings out that in case of *P. aeruginosa* induced UTIs also, the basic pattern of neutrophil influx as well as elaboration of MIP-2 (IL-8 counterpart) corroborates with each other and is very similar to that observed in *E. coli* induced infections. This regulatory chemokine thus not only has the potential to influence the establishment of acute infection but has a role to play in the evolution of chronic form of disease with far reaching consequences. Since indwelling catheterization having inbuilt hazard of biofilm cell formation with an organism like *P. aeruginosa* can cause persistent infection (Fux, 2005) there is a need to study the precise role of MIP-2 levels during acute as well as chronic UTIs. For this, the model proposed in the present study especially in IL-8 knockout mice can serve useful purpose.

During the process of establishment of infection, in urinary tract, at the site of inflammation different virulence determinants of invading organism interact with the host factors. Earlier Nathan, (1987) reviewed the subject and stated that macrophages of tissues as well as those coming from circulation congregate in most acute and
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chronic inflammatory reactions. In this regard, macrophages constitute important line of defense against most bacterial pathogens which pour their secretory products at the site of infection containing cocktail of biomolecules (Aderem and Underhill, 1999; Muller, 2003). Macrophages respond to antigenic stimuli and secrete a range of over 100 substances, which vary in their biological activities affecting induction of cell growth, cell death as well as multiple metabolic functions. This cocktail of secretory products of macrophages include peptide hormones, complement components, enzymes, bioactive oligopeptides, lipids, reactive oxygen species, reactive nitrogen intermediates and other biological substances (Nathan, 1987). The principal constituents of macrophage secretory products (MSPs) are cytokines. In the present study, cytokine levels, reactive nitrogen intermediates and protein content were found to be more in supernatants collected from macrophages stimulated with biofilm cells as compared to the supernatants collected from planktonic cell stimulated macrophages. In addition quantitative variation in the amount of different products in the MSPs was observable showing strain to strain variation.

In literature, studies are available where in vitro binding of cytokines like TNF-α, the principal constituent of MSPs, with pathogen has been shown to result in enhancement of growth of E. coli, P. aeruginosa, S. aureus and Acinetobacter in vitro (Porat et al., 1991; Meduri et al., 1999; Kanagat et al., 1999). Cytokines thus can serve as growth factors for bacterial pathogens as reported by Wilson et al., (1998) who employed different intra as well as extracellular gram negative bacteria but did not include P. aeruginosa. Recently Lee et al., (2003) showed growth enhancing effect of TNF-α on E. coli which was not demonstrable in case of P. aeruginosa. This growth promoting effect of TNF-α on E. coli appeared to be highly specific, as it occurred in a concentration dependent manner and was blocked by anti-TNF-α
antibodies. These workers observed that low concentrations of TNF-α increased bacterial growth which decreased at intermediate levels but again enhanced at higher concentrations of TNF-α. It was suggested that ability to adapt to a cytokine rich milieu may give the organism a fitness advantage over its competitors. In the present investigation, significant enhancement in growth of both planktonic and biofilm cells of \textit{P. aeruginosa} in presence of MSPs is in contrast to the observations of Lee \textit{et al.}, (2003) but substantiates the work of others who also found growth enhancement of this pathogen in presence of proinflammatory cytokines. The bacterial growth enhancing effect of MSPs indirectly, is possibly operative through cytokines. In addition to growth, in the present study, effect of MSPs on the production of virulence factors by planktonic and biofilm cells of \textit{P. aeruginosa} revealed enhanced elaboration of all the virulence determinants at 30\% concentration of MSPs which declined at the concentration of 50 and 70\%. On the whole, biofilm cells of \textit{P. aeruginosa} produced significantly higher levels of all these virulence traits following interaction with MSPs as compared to their planktonic counterparts. Due to paucity of literature no direct comparisons are possible. However, in available reports, effect of MSPs has been studied in relation to host cells like mesengial cells and other renal cells by Sharma \textit{et al.}, (1996) who reported mesenglial cell proliferation and matrix synthesis at 3 hour postinteraction of \textit{E. coli} with macrophages. This effect had been shown earlier to be concentration dependent where in the range tested was from 30-80\% with maximal effect observable at 30\% concentration of MSPs (Mattana and Singhal, 1993).

Once the invading pathogen has acquired the ability to respond to cytokine like TNF-α possibly through receptors as reported in case of \textit{E. coli}, further there is a need to understand as to how this can help the virulence potential of organism. In the cocktail of MSPs prepared
following interaction with *P. aeruginosa*, maximal amount of TNF-α was present. Luo *et al.,* (1993) reported enhanced invasion of HeLa cells by *Shigella flexneri* following binding of TNF-α on surface of this pathogen. The binding of cytokines, including TNF-α, to the bacterial cells has also been studied by Meduri *et al.,* (1999) who reported that these molecules are internalized and bacteria breakdown cytokines to biologically active fragments with a capacity to get transported across the bacterial cell membrane. These then act on transcription and translation of specific genes leading to alteration in virulence properties of organism. The *in vitro* observations where the presence of proinflammatory cytokines is able to help the invading organism in terms of growth as well as enhancement of virulence traits is, however, requires *in vivo* studies. In relation to pneumonia, another example of mucosal infections, Lee *et al.,* (2003) by employing TNF-α knockout mice reported that host’s response plays more decisive role in the elimination and clearance of the invading pathogen. In the present investigation to study the effect of MSPs *in vivo* in relation to UTIs, ascending mouse model has served a useful purpose. When UTI was established with planktonic and biofilm cell forms of *P. aeruginosa* grown in presence of MSPs (30%), the extent of pyelonephritis was enhanced, more so in case of biofilm cells. Out of the two cell forms, biofilm cells again had an edge over the planktonic cells with respect to *in vivo* virulence.

Following infection, when phagocytes (macrophages and PMNs) get accumulated at the site of infection, these cells are known to produce free radicals like reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) (Nathan and Xie, 1994; Persson *et al.,* 2001). Activated macrophages synthesize NO synthase (NOS), whose activity produces RNI like nitric oxide (NO) by the metabolic conversions of L-arginine into L-citruline (Fang, 1997). These are shown to play dual role which is defensive as well as offensive. When
stimulated in appropriate concentration, these may help in the clearance and ultimate disposal of microorganisms (Wheeler et al., 1997; Hampton et al., 1998; Poljakovic and Persson, 2003). However, when produced in large amounts, these free radicals may contribute to the tissue damage thus helping in formation of nidus in the kidney. These uropathogens can multiply there and may even disseminate in rest of the body (Harlan, 1987; Goode and Webster, 1993). Although expression of inducible nitric oxide synthase (iNOS) following inflammation or infection (Smith et al., 1994) acts as a vital component of the host immune response against virulent pathogens, but production of NO may lead to reaction with the super oxide anion to form peroxynitrite anion which is a highly toxic molecule leading to DNA damage and protein modification (Jablonska et al., 2005). In rodent macrophages and neutrophils, inducible nitric oxide synthase (iNOS) has been reported to produce large quantities of nitric oxide (NO) that can modulate immune, inflammatory and cardiovascular responses (Nathan and Xie, 1994; Kolodziejska et al., 2005; Rumbo et al., 2005). In models of immunological renal diseases like nephrotoxic nephritis (Cattell et al., 1990) and membranous nephropathy (Cattell et al., 1991; Cook and Sullivan, 1991), infiltration of macrophages in glomeruli and production of L-arginine derived nitric oxide (NO) has also been demonstrated. These workers reported implication of this metabolite in glomerular injury as it alters intrinsic glomerular cells and has hemodynamic effects (Cook and Sullivan, 1991; Koroliczuk et al., 2001).

In the present study, during acute UTI, peak renal bacterial counts as well as maximum production of RNI were observed on 3rd postinfection day in animals infected with planktonic cells of P. aeruginosa. Thereafter a constant decrease in both these parameters was observed till 7th postinfection day. Generation of RNI seen in urinary leukocytes from patients has been linked to bacterial killing
Phagocytosed bacteria were shown to be killed by the formation of peroxynitrite, a reaction product of nitrite. This has also been associated with spontaneous resolution of acute UTIs (Maskell and Pead, 1982; Katoh et al., 1991). Wheeler et al., (1997) suggested that extended elevation of inducible nitric oxide synthase estimated in neutrophil enriched fraction from urine during UTIs may have both antimicrobial and proinflammatory functions. This activity did not decrease until 6-10 days of antibiotic treatment. During the course of acute infection, in the present study, renal severity scores varied from +2 to +4 on 3rd postinfection day in planktonic cell instilled mice with predominant neutrophil infiltration. This indicated that maximum RNI production caused clearance of bacteria and was also responsible for causing tissue damage during acute UTI induced with planktonic cells of *P. aeruginosa*.

In mice infected with biofilm cells, RNI levels were higher as compared to their planktonic counterpart infected experimental animals during acute UTI. However, bacterial cells persisted till 7th postinfection day in biofilm cell instilled mice despite of peak production of RNI on the 3rd postinfection day. This indicated that biofilm cells of *P. aeruginosa* may be resistant to RNI mediated killing. In addition severity of lesions in renal tissue of mice infected with biofilm cells was significantly more on 3rd postinfection day with severity score varying from +5 to +6. This possibly could also be due to higher tissue destruction by higher levels of RNI production. Presence of renal injury, thus, may be attributed to bacterial colonization as well as inflammatory reaction contributed due to the presence of inflammatory cells equipped with the arsenal of bactericidal molecules like RNI. Formation of peroxynitrites following reaction of RNI with superoxide anion may cause tissue damage which can be measured through estimation of lipid peroxidation (Gross and Wolin, 1995; Ciragil et al., 2005).
Available experimental data from literature confirms that products of lipid-peroxidation act as markers for determining extent of tissue damage. To measure this, a number of workers have quantitated malondialdehyde (MDA) levels for estimating intensity of lipid peroxidation in tissues (Laurent and Ardaollou, 1986; Higdon et al., 2000; Rosemary and Shi-Hua, 2000; Anjaneyulu and Chopra, 2004; Gonenc et al., 2006; Kedziora-Kornatowska et al., 2006; Ogetman, 2006). Rise in MDA levels has been correlated with ischemia (McCord, 1985), chronic renal failure (Kishore et al., 1983), tubulointerstitial injury (Kaisiske et al., 1989) and reflux nephropathy (Okur et al., 2003). In the present study also, quantitation of MDA was done to measure lipid peroxidation following infection with *P. aeruginosa* and has been used as an index of bladder and renal tissue damage as MDA estimation offers speed, reliability and sensitivity. In addition, workers have also suggested that this method can also be employed directly to complex tissues and since MDA is a stable product of oxidative attack of reactive oxygen species on unsaturated fatty acids, an essential constituent of cell membrane, it has a distinct advantage. Following infection with planktonic and biofilm cells of *P. aeruginosa*, in the present study, gradual increase in MDA production was observed in excreted urine, bladder tissue as well as renal tissue of mice observable from 2 hours to 7th postinfection day. This indicated tissue damage during the course of acute UTIs. Renal tissue pathology scores during acute as well as chronic form of infection caused by planktonic and biofilm cell forms on the whole correlated with the levels of MDA. Activated lipid peroxidation on cell membranes leading to structural and functional instability, contributed to membrane pathology (Pavlova et al., 2005) and could be responsible for the observations of the present study. Enhanced production of MDA by rodent monocytes following *in vitro* stimulation with antibiotic susceptible strains of *P. aeruginosa* correlated with exaggerated
inflammatory response and progression of death in rat peritonitis model (Giamarellos-Bourboulis et al., 2004). The results of present study together with the existing literature bring out that UTIs are accompanied by lipid peroxidation of involved tissues which correlate with MDA levels in urine.

Urinary tract infections cause oxidative stress leading to insufficiency of antioxidant enzymes. Oxidative stress is characterized as imbalance between the content of free radicals and antioxidants in favor of radicals (McCord, 1993; Roth, 1997; Misiaszek et al., 2004). Role of oxidant molecules like superoxide anions is well established in tubulointerstitial damage (Rodrigo and Rivera, 2002; Nangaku, 2006). Cellular injury or organ dysfunctions caused by oxidative stress occur when ROS accumulate in excess and damage host defense mechanisms (Bus and Gibson, 1982; Riley, 1994; Mochida et al., 1997; Rodrigo and Bosco, 2006). Different oxygen radicals are generated in response to inflammatory stimuli by phagocytes (Headlam and Davies, 2003; Sheppard et al., 2005; Oda et al., 2006; Roshchupkin et al., 2006). These relate to endothelial dysfunction as the endothelium is a source and target of oxidants and participate in the inflammatory response (Sahnoun et al., 1997; Cadenas, 2004). First line of defense against superoxide toxicity is provided by the enzyme superoxide dismutase (SOD) which catalyze the breakdown of superoxide anion (O$_2^-$) and provide antioxidant stress mechanism (Zbarskii and Peskin, 1982; Horton, 2003; Cook et al., 2004). These anti-oxidative stress mechanisms are localized in tissues and inside the cells where ROS is generated. In this context, number of workers has demonstrated that administration of antioxidants like vitamin C and melatonin reduced renal scarring during pyelonephritis and helped in resolving infection (Matsumato et al., 1992; Mochida et al., 1998; Zhelekhovs'kyi et al., 2001; Zhumurov et al., 2004; Imamoglu et al., 2006). In the present investigation production of ROS in acute
UTI model induced with planktonic and biofilm cells of *P. aeruginosa* was studied in urine, bladder and renal tissue in terms of concentration of SOD. Since fall in the SOD levels was observed following infection with *P. aeruginosa*, it indicated generation of ROS. Pavlova *et al.*, (2005) found that higher concentration of ROS in the lower UTI was due to bacterial as well as leukocyte accumulation and was maximal in patients with chronic renal failure. Reports are available where ROS has been shown to play an important role in pathophysiology of pyelonephritis. Mundi *et al.*, (1991) reported that extracellular release of ROS from neutrophils following interaction with *E. coli* was a cause of renal scarring. In earlier studies from our laboratory, Gupta *et al.*, (1995) demonstrated the role of ROS in the pathogenesis of pyelonephritis in BALB/c mice. It was shown that ROS are generated at the initiation of infection and the levels increased progressively during the course of infection which was also observed in the present study. In later studies, Gupta *et al.*, (1996) showed a clear correlation between extent of lipid peroxidation, ROS generation and subsequent DNA damage in kidneys during the course of infection. Increase in production of ROS following stimulation of human PMNs by uropathogenic *Pseudomonas aeruginosa* in *vitro* has been reported by Oh-Oka *et al.*, (2001). These workers suggested that bactericidal mechanisms involve ROS participation and is myeloperoxidase (MPO) dependant process. In glomerulonephritis model, the oxidant-antioxidant imbalance was shown to play an important role in the development of pathogenetic changes (Wojcicka *et al.*, 2004). Aggravation of oxidative stress in diabetic patients (Gul *et al.*, 2005) and pregnant females (Ciragil *et al.*, 2005) with urinary tract infections has also been reported where increased lipid peroxidation and decreased levels of SOD as well as catalase was demonstrated in urine of these patients. It is therefore concluded that establishment of UTI caused oxidative stress, increased lipid peroxidation levels.
coupled with insufficiency of antioxidant enzymes. Since UTI is an ongoing infectious process involving interplay of bacteria and host defense mechanisms, therefore, detection of ROS and MDA in urine can be of immense clinical relevance and may have practical applications especially in relation to diagnosis of upper UTI for which assessment methods are limited. Future studies with an aim to define precise underlying mechanisms can also help in developing effective antioxidant therapy with far reaching consequences.

UTIs activate both mucosal and systemic inflammatory responses in which cytokines play a pivotal role (Godaly et al., 2001; Svanborg et al., 2006). Cytokines both proinflammatory and anti-inflammatory have been reported to be produced largely by macrophages. In addition, wide variety of cells including lymphocytes, endothelial cells, pulmonary epithelial cells and urinary tract epithelial cells produce these cytokines in response to gram negative bacteria (Agace et al., 1993) or their products like LPS and fimbriae (Jirik et al., 1989; Hedges et al., 1990; Loppnow et al., 1990). TNF-α, a proinflammatory cytokine, has been reported to play an important role in urinary tract infections. Increased levels of this cytokine and its receptors were observed in urine and serum samples of patients having acute pyelonephritis (Kassir et al., 2001). In the present study maximum production of TNF-α was observed at 4th postinfection hour followed by significant decrease on 1st postinfection day in urine, bladder and kidney of mice infected with planktonic and biofilm cell forms of \textit{P. aeruginosa}. Rise in TNF-α was again observed on 3rd postinfection day in case of planktonic cell infected mice and on 5th postinfection day in case of biofilm cell infected mice. Biofilm cells were able to induce significantly higher levels of TNF-α in mice as compared to planktonic cells. The pattern of local production of cytokines was different in case of \textit{E. coli} induced pyelonephritis in BALB/c mouse model as observed by Rugo et al., (1992). These
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workers were unable to detect TNF-α protein in the urine or serum of BALB/c mouse as early as 2 hour postinfection time period although mRNA encoding TNF-α was detectable by PCR in the kidney on 12th hour as well as on 1, 2 and 3 days after bacterial challenge. This pattern was observed only in renal tissue suggesting a localized response. It did not involve extra-renal lymphoid organs like spleen and lymph nodes.

TNF-α also stimulates production of other proinflammatory cytokines like IL-1. This was reviewed by Dinarello and Wolff, (1993). These workers brought out that the interleukin-1 family consists of three structurally related polypeptides. The first two are interleukin-1α and interleukin-1β, each of which has a broad spectrum of both beneficial and harmful biologic actions, and the third is interleukin-1-receptor antagonist, which inhibit the activities of interleukin-1. Out of these, IL-1β which is primarily produced by monocytes and macrophages has been reported to play significant role in infections. It is one of the early produced potent multifunctional cytokines that play a central role in inflammation and immunity (Martins et al., 1994). The well worked out role of IL-1 has even been made use of in patient care where direct administration of IL-1 or its receptor blockage has been the target (Smith et al., 1990). Higher levels of this cytokine have been observed in bronchoalveolar lavage fluid (BALF) of cystic fibrosis patients as compared to healthy controls by a number of workers (Bonafield et al., 1995; Schuster et al., 1995). Martins et al., (1994) reported higher levels of IL-1β in urine of patients having bacterial cystitis and bladder tumours but not in individuals having interstitial cystitis. Candela et al., (1998) also observed elevated levels of this cytokine in urine of patients with bacterial cystitis and microbial hematuria. In the available study of Khalil et al., (2000), related to E. coli induced pyelonephritis, carried out in IL-6 knock out and normal mice, mRNA expression of IL-1β along with other cytokines has been found to be positive. Kinetics of IL-1 production (both IL-1α and IL-
1β) was studied by Rudner et al., (2000) in *P. aeruginosa* induced corneal infection (an example of superficial infection). These workers observed peak expression of IL-1α and IL-1β between 1st and 3rd day which returned to baseline preinfection levels on 7th postinfection day. It was suggested that prolonged elevation of IL-1 expression contributed to corneal destruction operative through continued influx of neutrophils coming in response to increased production of MIP-2 whereas timely downregulation of IL-1 helped in bacterial clearance.

In the present study, peak production of IL-1β in renal tissue was observed on 3rd postinfection day in mice infected with planktonic cells and on 5th postinfection day in biofilm cell instilled experimental animals. Relatively recently, it has been reported specially in relation to chronic kidney diseases that both TNF-α and IL-1 play potent proinflammatory role in the evolution of several acute as well as chronic kidney diseases (Suh et al., 2005). They concluded that monitoring of these, based on genetic susceptibility, specially operative through receptors could be a significant factor for the development of end stage renal failure. Acute pyelonephritis leading on to the chronic form of the disease is one of the important recognized cause for the chronic renal failure. In contrast, Sadeghi et al., (2005) have observed higher levels of IL-1 in urine of healthy females as compared to that of healthy males. They suggested that enhanced levels of this cytokine may provide protection against diseases like pyelonephritis. In a very recently published study, Manchanda et al., (2006) have also suggested that IL-1 gene cluster influenced the risk of development of end stage renal diseases (ESRD).

It thus appear that timely downregulation of IL-1β can help in the bacterial clearance but require delicate balance between protective and destructive responses operative through this cytokine. Martins et al., (1994) standardized measurement of this cytokine in human urine and stated that it can serve as clinically relevant marker for diagnosis of kidney diseases. The detection of TNF-α and IL-1β in the urine of pyelonephritic mice, in the present study following infection with *P.*
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*aeruginosa* requires to be looked into with an aim for its diagnostic potential for diagnosis of upper UTIs. Baseline information available from the present investigation can thus be gainfully employed for future studies where profile of TNF-α and IL-1β induced by different uropathogens can be studied critically following infection.

The interplay between the proinflammatory and anti-inflammatory mechanisms/mediators is essential in fight of the host against the invading pathogen in limiting its effect and resolving inflammatory process. In this context, IL-10, an anti-inflammatory cytokine, appear relatively late following induction of immune responses. This cytokine has been reported to be produced by subpopulations of helper T cells (Fiorentino *et al.*, 1989; Vieria *et al.*, 1991), B cells (O’Garra *et al.*, 1992) and macrophages/monocytes (Fiorentino *et al.*, 1991). In the present study, IL-10 production started to appear in kidneys on 3rd postinfection day and reached a peak on 7th postinfection day in case of both planktonic and biofilm cell infected mice. Thereafter gradual decrease in production of IL-10 was observed till 14th postinfection day. Maximal production of IL-10 was accompanied with decrease in renal bacterial load as well as reduced neutrophil recruitment. In models of *P. aeruginosa* infection, IL-10 has been reported to be an important regulator of host response with a complex role. This cytokine has been demonstrated to inhibit neutrophil and macrophage phagocytic as well as bactericidal activity *in vitro* (Laichalk *et al.*, 1996). In addition, IL-10 deficiency was found to exacerbate lung damage whereas treatment with IL-10 was associated with attenuation of excessive inflammation in mouse model of chronic endobronchial *P. aeruginosa* infection (Chmiel *et al.*, 1999). These workers suggested use of IL-10 as a potential therapeutic agent for treatment of cystic fibrosis patient. Chmiel *et al.*, (2002) demonstrated prolonged inflammation, persistence of PMNs and tissue damage in IL-10 deficient mice as compared to wild type mice following infection with *P. aeruginosa*. In the present study, significantly lower levels of IL-10 corroborated with higher tissue damage.
damage in mice infected with biofilm cells as compared to planktonic cell instilled mice. The findings of present study as well as available information from literature brings out that IL-10 has a potential for dampening inflammation resulting in resolution of urinary tract infections. This needs to be addressed to in future investigations especially with different strains of *P. aeruginosa* and other uropathogens since production of IL-10 has been associated with downregulation of proinflammatory cytokines like IL-1α, IL-1β, IL-6, IL-8 and TNF-α (Fiorentino *et al.*, 1991).

Scanty available information regarding pathogenesis of catheter associated UTIs caused by biofilms of *P. aeruginosa* in literature is an important bottleneck. This study gives information about the pathogenesis of UTIs in an ascending model of experimental UTI in mice. Since infectious process is a complex phenomenon in which both the host factors and the bacterial virulence characteristics are known to contribute, the present study has identified the major contribution of biofilm formation in the pathogenesis of catheter associated UTIs in terms of higher production of virulence traits *in vitro* as well as higher colonization, higher severity scores, induction of innate immune response in terms of PMN recruitment, production of cytokines and generation of free radicals *in vivo*. It further brings out that environmental and host factors present in the milieu of urinary tract like pH, osmolarity, iron levels, macrophage secretory products and presence of Tamm-Horsfall protein in the urinary mucus, have the potential to alter the course of this infection. The present study is also unique in highlighting the significant role of quorum sensing molecules in the evolution of urinary tract infections. Acyl homoserine lactones (AHLs) can be speculated to serve as potential target molecules for inhibition of biofilm formation. All this information can further help in developing effective preventive strategies against biofilms of *P. aeruginosa* formed on urethral catheters which are a major cause of recurrence, persistence and chronicity.