INTRODUCTION

Inspite of available advances in supportive care and potent antimicrobial treatment, sepsis continues to be a leading cause of death among hospitalized patients (Bone et al., 1991; Guerina, 1998; Roy et al., 2002). Bacterial infections are the most common cause of septic shock whereas fungi, viruses and protozoa are less commonly responsible for the syndrome (Southwick, 2003). The most common causative gram-positive organisms associated with sepsis are *Staphylococcus aureus* and *Streptococcus pneumoniae* and the most common gram-negative organisms are *Klebsiella pneumoniae*, *E.coli*, *Pseudomonas* and *Enterobacter* species (Bernard et al., 2001). Recently, the researchers have discovered a shift in the organisms responsible for causing septicemia from primarily gram-positive bacteria to gram-negative organisms (Roy et al., 2002; Mahapatra et al., 2002). Among these, *Klebsiella pneumoniae* continues to be a nightmare for neonatologists, microbiologists and hospital administrators as it is the predominant organism associated specially with neonatal septicemia (Basu et al., 2001). It is also responsible for sepsis in patients admitted in intensive care units (Karabinis et al., 2004).

Keeping in mind the high morbidity and mortality associated with sepsis, proper and timely choice of empiric antibiotic therapy is of utmost importance (Roy et al., 2002). Penicillin G or ampicillin together with an aminoglycoside are often selected for suspected early onset of sepsis (Isaacs and Royle, 1999). For late cases however, flucloxacillin and gentamicin (an aminoglycoside) are the drugs of choice (Isaacs, 2001). Empiric treatment with third generation cephalosporins can be used during an outbreak (Obiamiwe and Berkowitz, 2004).

Antibiotics used for treatment of infections operate by various mechanisms. These include inhibition of synthesis of bacterial cell wall, effect on permeability of cell membrane, inhibition of protein synthesis or influence on
nucleic acid metabolism. Apart from these mechanisms, antibiotics affect bacteria in other ways as well. One of the effects of antibiotic usage is the change in morphology of bacteria. Morphology change is induced in gram-negative bacteria by β-lactams (Dofferhoff et al., 1991; Jackson and Kropp, 1992; Prins et al., 1994; Prins, 1995; Horri et al., 1999) and quinolone group of antibiotics (Elliott et al., 1987; Sonstein and Burnham, 1993). However, aminoglycosides are not recognized to show this effect (Crosby et al., 1994). These surface changes can influence the strength of the attractive and repulsive forces responsible for bacterial surface interactions with molecules and cells in environment (Schifferli and Beachey, 1988) which are important in early stages of pathogenesis of infections caused by bacteria. The structural changes brought about by antibiotics augment the susceptibility of bacterial pathogens to humoral and cellular defenses as well (Zak and Kradolfer, 1979).

Apart from morphological changes, exposure of bacteria to antibiotics unmask / induce expression of new outer membrane protein (OMP) epitopes (Kadurugamuwa et al., 1985; Lun et al., 1994; 1997). One family of OMPs, the porins, are present in large amounts in the outer membrane and form water-filled channels that permit the diffusion of small hydrophilic solutes across the outer membrane (Domenech–Sanchez et al., 1999). Alterations in these porins have been linked to antibiotic resistance among bacteria. Co-resistance to β-lactam and floroquinolone group of antibiotics in K. pneumoniae is due to decrease in the permeability of the outer membrane to both classes of agents because of alterations in porins (Martinez-Martinez, 2002). It has been observed that there is alteration / re-arrangement in the OMP (particularly porins) expression under antibiotic pressure (Lun et al., 1994; 1997; Domenech and Sanchez, 1999; Chevalier et al., 1999; Emmanuella-De et al., 2001) as well as under various other stresses (Schmid et al., 1991; Werts et al., 1992; Sahu et al., 1994; Chhibber and Bhardwaj, 2004). The role of these antibiotic induced proteins when presented to immune system alone or in conjugated form remains to be determined.
Administration of antibiotics not only has the potential to bring about alterations in the structure and OMP profile of the organisms, but it also leads to the release of important biologically active molecules from them. One of these is the endotoxin. Endotoxin [lipopolysaccharide (LPS)] is a constitutive component of the outer membrane of gram-negative bacteria. It is released from the cell walls of gram-negative bacteria, during multiplication as well as following death. Release of this molecule has also been reported as a consequence of antibacterial action \textit{in vitro} (Goto and Nakamura, 1980; Cohen and McConnell, 1986) and \textit{in vivo} (Rokke et al., 1988). Several studies demonstrate that the rate at which endotoxin is released from the organisms, vary with class of an antimicrobial agent (Jackson and Kropp, 1992; Bingen et al, 1992; Crosby et al, 1994; Hurley et al, 1991; Horri et al, 1998a) and type of the bacterial strain studied (Prins et al, 1994). Cephalosporins and quinolone group of antibiotics are specially known to release large amount of endotoxin (Cohen and McConnell, 1986; Vanden Berg et al, 1992) whereas aminoglycosides cause less endotoxin release (Kusser and Ishiguro, 1988, Prins et al, 1994). Endotoxin release has been found to be a contributing factor to lethality in experimental gram-negative sepsis (Morrison and Bucklin 1996) as well as in clinical observations (Holzheimer, 2001).

Recently there has been good understanding about the basic mechanism through which biological action of endotoxin operates. The recognized biological action of endotoxin is the activation of macrophages, monocytes, endothelial cells and fibroblasts. It leads to production of potent inflammatory mediators which include TNF-\(\alpha\), IL-1\(\beta\), IL-6 and nitric oxide (Morrison et al., 1994). Some mediators especially TNF-\(\alpha\), interleukin (IL-1, IL-6) and other humoral factors are instrumental in the development of septic shock leading to death (Gardlund et al., 1995; Son et al., 2002). The mortality associated with sepsis, therefore is not preventable by the use of antibiotics alone. Available information in relation to drug therapy as well as emergence of ESBL-producing multiresistant strains require development of an adjunct supportive therapy. This could be achieved by developing effective immunoprophylaxis or immunotherapy given alone or in
combination with antibiotics (Cross et al., 1999). This has recently been suggested by Mahapatra et al. (2002) as well. These workers further have stressed the need to give due consideration to ever changing profile of organisms responsible for causing sepsis.

The most widely used vaccines against Klebsiella infections have relied upon capsular polysaccharide (CPS) where cocktail preparation approach has also been tried (Podschun and Ullmann, 1998). Experimentally, 24 valent Klebsiella CPS vaccine inspite of generating an excellent antibody response, has only been partially successful (Campbell et al., 1996; Donta et al., 1996; Yoshida et al., 2000). The fact that O-specific antibodies can penetrate the capsule of strains belonging to serotype K2 of Klebsiella (Meno and Amano, 1990; Tomas et al., 1991; Jong et al., 1995) has led to the exploration of protective potential of lipopolysaccharide (LPS) in immunoprophylaxis and immunotherapy (Yokochi et al., 1995). Protection with LPS molecule against gram-negative infections has also been obtained in few earlier studies (Rani et al., 1990; Chatley et al., 1996). Although LPS is highly toxic, but this molecule does have the potential for use in immunotherapy for prevention. Immunotherapy with antibodies against endotoxin have been shown to improve survival in animals (Baumgartner et al., 1985; Trautmann et al., 1988; Chhibber and Bajaj, 1995; Rukavina et al., 1997). Alternative concept regarding the use of LPS for immunotherapy is emerging, where emphasis is on splitting the LPS molecule and excluding the lipid A moiety of LPS. The polysaccharide (O-PS) moiety is then tagged to a carrier protein. Several OMP preparations are being tried as potential carrier molecules (Alcantar-Curiel et al., 2000). The efficacy of outer membrane proteins (OMPs) conjugated to either detoxified LPS or O-PS portion of LPS in protection has been explored by different workers in Klebsiella induced infections (Chhibber and Bajaj, 1995; Favre-Bonte et al., 1999; Cross et al., 2004). The potential of antibiotic induced OMPs as carriers in such conjugates will be of more clinical relevance since these OMPs are expressed in vivo during treatment with antibiotics. These conjugates have been shown to restore immunogenicity that is similar or even better than that of native LPS without restoring toxicity inherent in LPS (Cryz et al., 1983; Verhuel et al., 1993).
The present study therefore, was planned with following aims and objectives:

1. To obtain blood isolates of *Klebsiella pneumoniae* from cases of septicemia as well as standard strains of *K. pneumoniae* and to check their antibiogram.

2. a) Selection of three different antibiotics representing third generation cephalosporins, aminoglycosides and quinolones.

   b) Selection of strain for further studies, to be made on the basis of MIC levels for these antibiotics and virulence potential of the strain *in vivo*.

3. Selected strain to be exposed to three antibiotics and studies to be made in relation to:

   a) Change in the morphology of bacteria *in vitro*
   
   b) Endotoxin release *in vitro* and *in vivo*
   
   c) TNF-α release *in vivo*
   
   d) Expression and identification of new outer-membrane proteins in presence of antibiotics *in vitro*

4. Conjugation of newer proteins (expressed in presence of antibiotics) to polysaccharide moiety of LPS, to be made, based on following approach:

   a) Extraction and purification of LPS

   b) Detoxification of LPS by separating lipid A portion from its polysaccharide moiety.

   c) Chemical coupling of polysaccharide moiety to purified proteins by a method that utilizes spacer molecule


6. Protective efficacy (active immunization) of polysaccharide-protein conjugate prepared to be studied in *K. pneumoniae* induced sepsis model in terms of:
7. Protective efficacy of hyper immune sera (passive immunization) administered alone and in conjunction with antibiotics to be assessed in terms of:
   a) Percentage survival of animals
   b) Bacterial counts in blood
   c) TNF-α levels in serum
   d) Bacterial load and assessment of pathology in organs