SUMMARY AND CONCLUSIONS

The salient findings of the study entitled “Protective potential of antibiotic induced outer membrane proteins of *Klebsiella pneumoniae* conjugated to polysaccharide moiety of lipopolysaccharide antigen in murine sepsis model” are:

1. Antibiotic sensitivity of the wild type isolates to different drugs showed that majority of the isolates (88.24%) were resistant to more than three antibiotics and hence were classified as MDR. Amikacin followed by ofloxacin were found to be the most effective antibiotics as 68.63% and 62.75% isolates respectively were sensitive to these drugs. Ceftazidime however was found to be least effective.

2. MIC of the three selected antibiotics namely ceftazidime (third generation cephalosporin), ofloxacin (quinolone) and amikacin (aminoglycoside) was determined for all the wild type isolates. The range of concentration for antibiotics to be tested was selected on the basis of information available in literature related to the peak levels of antibiotic achievable in blood of patients following administration of antibiotic *in vivo*.

   a) With ceftazidime ten strains were sensitive to lower dose i.e. 10μg concentration of antibiotic, whereas thirty-five strains were resistant upto 100μg concentration of the drug. Six isolates showed MIC in the range of 10μg to 90μg/ml for ceftazidime.

   b) With ofloxacin, 47.05% strains (24 isolates out of 51) were sensitive with a MIC of 0.8μg/ml, whereas seventeen strains were resistant upto 10μg concentration. Ten strains gave MIC in the range of 1 to 7μg/ml.

   c) Amikacin was much more effective as 60.78% strains (31 isolates out of 51) were sensitive with a MIC of 10μg/ml, whereas ten strains were
resistant up to 100μg concentration of the drug. Ten strains gave MIC in the range of 10 to 50μg/ml.

3. *Klebsiella pneumoniae* ATCC 43816 when checked for antibiotic sensitivity, was found to be sensitive to all the drugs except ceftazidime. MIC of ceftazidime for this strain was 40μg/ml. Resistance in this strain towards amikacin and ofloxacin was developed in the laboratory by repeated passages in liquid and solid media with increasing graded concentrations of respective antibiotic. Selected strain, finally having MIC of 40μg/ml, 0.9μg/ml and 20μg/ml for ceftazidime, ofloxacin and amikacin respectively was selected.

4. All wild type isolates when injected intraperitoneally in mice in high doses (10^{10}-10^{11} cfu/ml) were found to be avirulent, whereas standard strain, *K. pneumoniae* ATCC 43816 was found to be virulent at relatively low dose (10^{6} cfu/ml). This strain, therefore was selected for further studies.

5. For the development of model of sepsis, mice when given *K. pneumoniae* ATCC 43816 (2x10^{3} cfu) as such, intraperitoneally was cleared from the system very early (within three days after infection) with no mortality. The bacterial doses ranging from 6x10^{3}–6x10^{6} cfu/ml were lethal within three days. Doses, 6x10^{3}–6x10^{6} cfu/ml when given entrapped in fibrin clot also produced early mortality. However, 2x10^{3} cfu and 150 cfu when given in fibrin clot resulted in 100% and 50% mortality respectively spread over one week's interval. The dose 2x10^{3} cfu of bacteria was selected for future work.

a) Blood culture was found to be positive within 24 hours in the experimental model when selected dose was given un-entrapped or entrapped in fibrin clot. However, in former group, blood counts became negative within three days after infection, whereas in the latter group peak in bacterial counts was reached on third day. Maximum localization of bacteria in liver and spleen was observed on 4th post infection day. A positive correlation
between bacterial number and inflammatory response in organs was also evident.

6. For studying the effect of selected antibiotics on morphology of *K. pneumoniae*, the dose of exposure for all the drugs was kept at MIC, ½ MIC and ¼ MIC. No change in morphology was seen with amikacin at all the concentrations tested. In contrast, with other two drugs (ceftazidime and ofloxacin), change to filamentation form was observed. Filamentation was more pronounced with ceftazidime as compared to ofloxacin.

7. *In vitro* viable bacterial counts and endotoxin release in medium (nutrient broth), following exposure of *K. pneumoniae* to ½ MIC level of each selected drug was measured. Decline in viable bacterial counts within 1.5 hours was seen after exposure to amikacin and ceftazidime, whereas no decrease was observed with ofloxacin with matching growth with controls (without any antibiotic). Endotoxin release was minimal with amikacin and maximal with other two drugs. No direct correlation between fall in viable bacterial counts and endotoxin release was observable.

8. Under *in vivo* condition, all the three antibiotics resulted in almost equal killing rates of bacteria in blood but the endotoxin release was significantly different. Minimum endotoxin release was with amikacin, whereas maximum was with ceftazidime followed by ofloxacin. The level of TNF-α also showed similar trend where levels of TNF-α were higher on exposure to ceftazidime followed by ofloxacin and amikacin.

9. The lipopolysaccharide of *K. pneumoniae* ATCC 43816 was purified by column chromatography. The results showed that peak I contained the LPS moiety. On further purification by ultracentrifugation the purified LPS showed presence of negligible amount of proteins, carbohydrates and nucleic acids.

10. The O-polysaccharide (O-PS) was fractionated and was resolved as two poorly separated peaks on sephadex G-50 column. In lyophilized preparation negligible amount of lipid was detected in this preparation.
11. The outer membrane proteins (OMPs) of *K. pneumoniae* were extracted following growth of bacteria in presence of all three antibiotics. The OMPs were resolved on SDS-PAGE gel and the results showed no alteration in the OMP profile of bacteria grown in presence of amikacin. However, proteins isolated from bacteria following growth in presence of ceftazidime and ofloxacin showed expression of new OMPs of high molecular weight (more than 97 kDa) as well as proteins with low molecular weight (22 kDa, 30 kDa and 40 kDa).

12. To confirm the immunogenicity of the newly expressed proteins, antisera was raised against the OMPs extracted from the cells grown in presence of ceftazidime and ofloxacin which were designated as OMPTS-CD and OMPTS-OF respectively. On immunoblotting, two bands of molecular weight above 97kDa and one band of molecular weight 40kDa were detected with OMPTS-CD in test OMPs but not in control OMPs. Whereas, with OMPTS-OF sera two bands of molecular weight above 97kDa and one band of 30kDa was detected in test OMPs.

13. Further, to confirm the surface location of these newly expressed proteins, antisera raised against killed whole bacterial cells grown in presence of ceftazidime (TS-CD) or ofloxacin (TS-OF) was adsorbed with bacterial cell grown in absence of antibiotic. On immunoblotting, a single new band expressing protein of molecular weight 40kDa and 30kDa was detected with adsorbed TS-CD and TS-OF respectively. These bands were not visualized in control OMPs.

14. These low molecular weight bands which are expressed by *K. pneumoniae* on surface in presence of ceftazidime and ofloxacin were designated as PR-CD (40kDa) and PR-OF (30kDa). These were purified by gel elution method. These purified proteins showed negligible amounts of carbohydrate, KDO and nucleic acids.

15. Both the purified proteins were separately conjugated to the O-polysaccharide (O-PS) moiety of LPS antigen by carbodiimide method. The conjugates thus prepared were run on sephadex G-100 column. Both
the conjugates were eluted in the void volume. The total yield of coupling for O-PS–PR-CD and O-PS–PR-OF conjugates was 45.2% and 44.0% respectively. Purified conjugates showed maximum polysaccharide / protein ratios of 1.58 and 1.9 for O-PS–PR-CD and O-PS–PR-OF respectively.

16. Assessment of lethality in normal and sensitized mice, pyrogenicity and schwartzman reaction was done with different preparations (LPS, O-PS, PR-CD, PR-OF, O-PS—PR-CD and O-PS—PR-OF). The results indicated that both the conjugates were devoid of any significant biological activity.

17. Development of specific immune response, in terms of IgG antibody levels, in pooled sera of the animals immunized with different antigenic preparations (individual purified proteins, O-PS, their mixture and conjugates) was evaluated by ELISA. O-PS and purified proteins (PR-CD, PR-OF) alone were found to be weak immunogens, whereas enhanced production of IgG antibodies specific to both O-PS as well as conjugated protein were observed in the sera of animals immunized with either of the conjugate after first booster dose of the antigen. Both the conjugates evoked similar levels of IgG antibodies.

18. Study of phagocytic uptake and killing of *K. pneumoniae* was done with peritoneal macrophages obtained from animals immunized with different antigenic preparations. Maximal recruitment of macrophages with enhanced phagocytic and killing activities was observable in animals immunized with both conjugates. These were equally efficient in enhancing phagocytosis and killing of bacteria.

19. Immunoprotective potential of both the conjugates was evaluated by actively immunizing the animals with these conjugates before giving challenge with *K. pneumoniae*. Both the conjugates provided significant higher protection in terms of percentage survival when compared with control group (unimmunized animals) as well as animals immunized with individual components of the conjugates. Although complete protection
could not be observed with these conjugates, there was significant decline in bacteriological load in blood and organs like liver, spleen and kidney. A positive correlation between decrease in viable counts and pathology in these organs was observed, since mild inflammatory change could be detected in animals immunized with conjugates in contrast to severe inflammation in control group.

20. There was prolonged production of TNF-α (till 7th day) in the serum of animals immunized with purified proteins (PR-CD, PR-OF) alone, mixtures of O-PS and purified protein and their conjugates. TNF-α levels detected in the conjugate immunized protected groups were significantly more as compared to control group (infected but not immunized).

21. Protective potential of antisera raised against respective conjugates alone and in combination with antibiotics was evaluated in *K. pneumoniae* infected mice, since it is more relevant in patients of sepsis.

a) Outcome of antibiotics administered alone:

Although, antibiotics were found to be effective and dose related response was observed, however higher doses of ceftazidime (75mg/kg/day) and ofloxacin (6.65mg/kg/day), when used for treating sepsis, were unable to provide complete protection.

b) Outcome of antisera administered alone:

Treatment with antisera raised against respective conjugates did not show any protection when single dose of antisera was administered intravenously. Bacteriological counts in the blood as well as in liver, spleen and kidney were comparable to the bacterial counts obtained in control animals.

c) Outcome of antibiotic and antisera administered in conjunction:

Simultaneous administration of antisera raised against either of the conjugate along with respective antibiotic resulted in 100% protection in mice following challenge with *K. pneumoniae*. There was significant
decline in bacterial counts in blood as well as organs of mice treated with combination therapy as compared to groups treated with respective antisera or antibiotic alone. A positive correlation between decline in bacterial counts and inflammation was observable on assessment of pathology in different organs.

Conclusion

The increasing problem of treating bacterial sepsis has focussed the attention of many scientists to look for adjunct therapies. In this direction, the results of the present study show that proteins expressed under antibiotic stress when conjugated to polysaccharide moiety of *K. pneumoniae* makes meaningful antigen. The antisera raised against relatively low molecular weight antigens when conjugated showed better resolution of infection following use in conjunction with antibiotics. This strategy would be meaningful as this approach has potential to affect bacteria as well as endotoxin component released in presence of antibiotics.