RESULTS

Antibiotic sensitivity of the fifty one wild type clinical isolates was performed against commonly employed antibiotics belonging to different groups; third generation cephalosporins, aminoglycosides and quinolones. On checking the drug sensitivity of various *K. pneumoniae* isolates to individual antibiotics, it was observed that amikacin and ofloxacin were the most effective drugs followed by ciprofloxacin, gentamicin, netilmicin, cotrimoxazole, cefotaxime and ceftazidime. Maximum number of isolates were sensitive to amikacin (68.63%) and ofloxacin (62.75%) whereas maximum resistance (78.43%) was noted towards ceftazidime (Fig.6). 88.24% of the isolates were resistant to three or more antibiotics and thus were classified as multiple drug resistant (MDR). The observed resistance pattern in order of frequency is shown in Fig.7.

**Fig. 6:** Percentage sensitivity of wild type blood isolates of *K. pneumoniae* towards antibiotics.
Fig. 7: Drug resistance pattern of clinical isolates of *K. pneumoniae*

![Graph showing drug resistance pattern of clinical isolates of *K. pneumoniae*]

- Amikacin (A)
- Ciprofloxacin (C)
- Cefotaxime (CE)
- Gentamicin (G)
- Cotrimoxazole (Co)
- Netilimicin (N)
- Ceftazidime (CD)
- Ofloxacin (OF)
The minimum inhibitory concentration (MIC) of the three selected antibiotics namely ceftazidime, ofloxacin and amikacin was determined and the results are presented in Fig.8, 9 & 10 respectively. Concentrations of ceftazidime, ofloxacin and amikacin tested ranged from 10 μg/ml to 100 μg/ml, 0.8 μg/ml to 10 μg/ml and 10 μg/ml to 100 μg/ml, respectively. Results revealed that, for antibiotic ceftazidime 10 out of 51 isolates were sensitive to even 10 μg/ml concentration of this drug. 35 other isolates were resistant to all the concentrations of ceftazidime tested. Only six blood isolates gave MIC in the range of 10 μg/ml to 90 μg/ml (Fig.8). For ofloxacin, 24 blood isolates out of 51 were found to be sensitive to 0.8 μg/ml concentration of ofloxacin. Only 10 strains gave MIC ranging from 1 μg/ml to 7 μg/ml (Fig.9). For antibiotic amikacin, 31 blood isolates out of 51 were found to be sensitive to even 10 μg/ml concentration. 10 blood isolates gave MIC within the range of 10 μg/ml to 50 μg/ml (Fig.10). Standard strain, *K. pneumoniae* ATCC 43816 was found to be sensitive to both ofloxacin and amikacin at all the concentrations tested. However, for ceftazidime, a MIC value of 40 μg/ml was obtained.

**Fig. 8:** Minimum inhibitory concentration (MIC) of six wild type *K. pneumoniae* isolates which gave MIC in the range of 10-90 μg/ml of ceftazidime.

*Out of fifty one *K. pneumoniae* isolates, 35 were resistant to even 100 μg/ml concentration of ceftazidime.
Fig. 9: Minimum inhibitory concentration (MIC) of ten wild type *K. pneumoniae* isolates which gave MIC in the range of 1-7 μg/ml of ofloxacin

*Out of fifty one *K. pneumoniae* isolates, 17 were resistant to even 10 μg/ml concentration of ofloxacin.

Fig. 10: Minimum inhibitory concentration (MIC) of ten wild type *K. pneumoniae* isolates which gave MIC in the range of 10-50 μg/ml of amikacin

*Out of fifty one *K. pneumoniae* isolates, 10 were resistant to even 100 μg/ml concentration of amikacin.
Following antibiotic sensitivity and MIC determination, the virulence potential of wild type isolates was evaluated by intraperitoneal injection of relatively high dose of organisms \((10^{10} - 10^{11} \text{ cfu/ml})\) in mice. However, no mortality was observed with any of the wild type strains tested. Whereas, standard strain, \textit{K. pneumoniae} ATCC 43816, could establish septicemia in normal mice after intraperitoneal inoculation of \(10^4 \text{ cfu}\) of cells. This strain was selected for all future experiments as it gave excellent results in terms of bacterial colonization in different organs on intraperitoneal inoculation. The selected strain gave a MIC of 40 \(\mu\text{g/ml}\) for ceftazidime whereas it was sensitive to the other two selected antibiotics. The requirement of this study was to have a virulent strain with MIC values within the permissible limits against all the three selected antibiotics as OMP expression was to be checked on growth in presence of these antibiotics. For this reason, resistance to amikacin and ofloxacin was developed in the laboratory by repeated passages in liquid and solid media with increasing graded concentrations of respective antibiotic. \textit{Klebsiella pneumoniae} ATCC 43816, finally having MIC of 40 \(\mu\text{g/ml}\), 0.9 \(\mu\text{g/ml}\) and 20 \(\mu\text{g/ml}\) for ceftazidime, ofloxacin and amikacin respectively was selected.

**BACTERIAL DOSE STANDARDIZATION FOR INDUCTION OF SEPSIS IN MICE**

The results of the bacterial dose standardization studies are presented in Table-1 and Fig.11. A dose of 150 cfu, if given in fibrin clot, gave 50\% mortality spread over a period of 7 days. A higher dose of \(2 \times 10^5\) cfu resulted in 100\% mortality, during similar time period. Whereas, higher doses ranging from \(6 \times 10^3\) – \(6 \times 10^5\) cfu killed all animals within 3 days. Since, the aim of this study was to ascertain the \textit{in vivo} protective potential of different bacterial antigens in conjunction with antibiotic treatment, hence LD\textsubscript{100} dose \((2 \times 10^5\) cfu\) was selected for developing a sepsis model in which mortality was spread over one-week period (considered as sufficient time to produce actual disease process resembling human sepsis). No mortality was observed in mice, which were given sterile clots or selected dose of bacteria without entrapping it in fibrin clot.
Table-1: Mice dying at different post infection days on receiving different doses of bacteria (*K. pneumoniae* ATCC 43816)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Dose of bacteria in fibrin clot</th>
<th>0hr</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
<th>96hr</th>
<th>120hr</th>
<th>144hr</th>
<th>168hr</th>
<th>Mortality%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>6x10^6 cells</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>2.</td>
<td>6x10^3 cells</td>
<td>0/4</td>
<td>0/4</td>
<td>2/4</td>
<td>4/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>3.</td>
<td>2x10^3 cells</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2/4</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>4/4</td>
<td>100%</td>
</tr>
<tr>
<td>4.</td>
<td>1.5x10^2 cells</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
<td>1/4</td>
<td>2/4</td>
<td>50%</td>
</tr>
<tr>
<td>5.</td>
<td>Normal saline</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0%</td>
</tr>
<tr>
<td>6.</td>
<td>2x10^3 cells (without fibrin clot)</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0%</td>
</tr>
</tbody>
</table>

Fig. 11: Dose response relationship of *K. pneumoniae* ATCC 43816 with mortality in mice
Blood culture was positive within 24 hours in experimental mice which received either $2 \times 10^3$ cfu of *K. pneumoniae* in fibrin clot or un-entrapped bacteria. The bacterial counts in blood of mice receiving bacteria in the fibrin clot increased and reached a peak of 4.84 log units before the death of the animals. In non-clot group, blood counts became negative within 3 days after infection without any mortality.

Organ culture of liver and spleen revealed bacteria in the range of 7.39 – 8.25 and 6.8 – 7.4 log units on 2nd and 4th day respectively in the group of mice infected with *K. pneumoniae* entrapped in fibrin – thrombin clot (Fig.12). As there was no mortality in mice receiving bacteria without fibrin clot, mice were sacrificed and the organ culture of liver and spleen was done on 2nd and 4th day after infection. In this group the bacterial counts obtained in liver and spleen of mice were significantly less ($p<0.001$) on both the days as compared to group that received bacteria entrapped in fibrin clot. After two weeks the organs were found to be sterile.

**Fig. 12:** Comparative analysis of bacterial counts in liver and spleen of mice infected either with *K. pneumoniae* ($2 \times 10^3$ cfu) entrapped or un-entrapped in fibrin clot on different days.
Histopathological changes observed in both liver and spleen of mice receiving bacteria in fibrin clot were compared with the tissues from normal mice (Fig. 13a & 13b) as well as from mice receiving bacteria without fibrin clot. Mild inflammatory response characterized by degenerative changes in cells, mild congestion, oedema and presence of few inflammatory cells could be seen when animals were challenged with a dose of $2 \times 10^3$ cfu not entrapped in a fibrin clot (Fig. 14a & 14b). Whereas, severe inflammatory response, characterized by severe congestion, organ surface covered with inflammatory exudate, extensive area of necrosis with abscess formation, was observed in both liver and spleen when $2 \times 10^3$ cfu entrapped in fibrin clot were given (Fig. 15a & 15b).

**EFFECT OF ANTIBIOTICS ON CELL MORPHOLOGY OF *K. pneumoniae* ATCC 43816**

The morphology of *K. pneumoniae* was checked under light microscope (100 x, oil immersion) on growth in presence and absence of antibiotics, ceftazidime, ofloxacin and amikacin. Effect of minimum inhibitory concentration (MIC) as well as ½ MIC and ¼ MIC of each drug on cell morphology was evaluated. At all test concentrations (MIC, ½ MIC and ¼ MIC), alteration in the form of filamentation was observed with ceftazidime (Fig. 16) and ofloxacin (Fig. 17). However, with amikacin no change in the morphology was detected even at MIC level (Fig. 18). Exposure of *K. pneumoniae* to ceftazidime at all test concentrations yielded very long filaments till 3 hours of incubation and at 4.5 hours cell lysis was evident. Whereas, in case of ofloxacin treated cells, filamentation was not as pronounced as observed with ceftazidime treatment and lysis was not visible till 6 hours of incubation even at MIC level.
Fig. 13a: Photomicrograph showing transverse section (T.S.) of normal liver. Hepatic cell cords along with sinusoids and portal vein bounded by endothelial layer can be observed. Sinusoids have random distribution of kuppfer cells which are polyhedral in shape and are attached to walls of hepatocytes (H&E, X400)

Fig. 13b: Photomicrograph showing (T.S.) of normal spleen showing chords, sinuses, red pulp (RP) which has more of reddish stain of eosine and white pulp (WP) (H&E, X100)
Fig. 14a: Transverse section of liver showing mild inflammatory response. The ruptured blood cells and the ruptured hepatic cells can be observed in the portal vein. Sinusoids show decrease in their size. Some cell walls of the hepatic cells show necrosis and breakage. Some hepatic cells are without nuclei. The endothelial lining of hepatic cells is also disrupted (H&E, X400).

Fig. 14b: Transverse section showing mild inflammatory response. The blue pulp shows damaged nucleosides. Whereas red pulp shows few spaces due to necrosis of RBC's (H&E, X100).
Fig. 15a: Transverse section of liver showing severe inflammatory response. Severe damage and total area of necrosis can be seen. The identity of the cells is lost. There is contraction of hepatic cells. Number of kupffer cells has decreased. The cytoplasm does not show different inclusion as seen in normal hepatic cells. The portal vein is full of blood, which leads to abscess formation (H&E, X100)

Fig. 15b: Transverse section of spleen showing severe inflammatory response. Red pulp and white pulp shows severe damage as damaged RBCs can be observed. There is increase in the number of reticulocytes. The capillary is full of damaged RBC’s which lead to abscess formation in spleen (H&E, X100)
Fig. 16: Light microscopy micrographs of *K. pneumoniae* cells exposed to antibiotic ceftazidime: A, at MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (A1, A2, A3, A4 and A5 respectively); B, at 0.5 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (B1, B2, B3, B4 and B5 respectively); C, at 0.25 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (C1, C2, C3, C4 and C5 respectively)
Fig. 17: Light microscopy micrographs of *K. pneumoniae* cells exposed to antibiotic ofloxacin: A, at MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (A1, A2, A3, A4 and A5 respectively); B, at 0.5 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (B1, B2, B3, B4 and B5 respectively); C, at 0.25 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (C1, C2, C3, C4 and C5 respectively)
Fig. 18: Light microscopy micrographs of *K. pneumoniae* cells exposed to antibiotic amikacin: A, at MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (A1, A2, A3, A4 and A5 respectively); B, at 0.5 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (B1, B2, B3, B4 and B5 respectively); C, at 0.25 X MIC concentration for 0, 1.5, 3.0, 4.5 and 6.0 hours (C1, C2, C3, C4 and C5 respectively)
VIABLE COUNTS AND ENDOTOXIN RELEASE STUDIES AFTER ANTIBIOTIC EXPOSURE OF *K. pneumoniae*

**In vitro study**

To study viable counts and endotoxin release from *K. pneumoniae* on antibiotic exposure, ceftazidime, ofloxacin and amikacin at 0.5 x MIC were used. The viable counts taken at an interval of 1.5, 3.0, 4.5 and 6.0 hours are shown in Fig. 19. With ceftazidime, viable counts started declining after 1.5 hours and there was significant (p<0.001) decrease in viable counts after 6 hours. Similar trend was seen with antibiotic amikacin and there was complete killing of bacteria after 4.5 hours. While with antibiotic ofloxacin the viable counts increased with time and were comparable to that of control at all time intervals.

**Fig. 19:** *In vitro* viable count (log_{10} cfu/ml) of *K. pneumoniae* at different time intervals following addition of ceftazidime, ofloxacin and amikacin (0.5 x MIC) to the bacterial culture

Each antibiotic was added at time zero; No antibiotics were added to the control culture. Each value in the figure represents the mean of five individual samples. Bar = standard deviation
*In vitro* endotoxin release at different time intervals following addition of different antibiotics is shown in Fig. 20. As is evident from the graph, the amount of endotoxin released following exposure to amikacin was similar to that of control during all time intervals. With both ceftazidime and ofloxacin, endotoxin release was significantly higher (p<0.05) than the control and amikacin group at all time intervals. There was significant (p<0.01) rise in endotoxin level with ceftazidime and ofloxacin at 6 hours as compared to endotoxin release obtained with exposure to both control and amikacin group at 1.5 hours.

**Fig. 20:** *In vitro* endotoxin release at different time intervals following addition of antibiotics (0.5 x MIC) to the bacterial culture
In vivo study

In this experiment, mice received single intramuscular dose of respective antibiotic 24 hours after establishment of infection. Blood was collected by retro-orbital puncture after 1.5, 3.0, 4.5 and 6.0 hours of antibiotic administration for determining viable counts. Antibiotics were used in concentrations that would achieve high serum concentration necessary for a rapid bactericidal action. Fig. 21a shows mean bacterial counts achieved in the blood of mice on administration of respective antibiotic at different time intervals. After ceftazidime therapy, there was steady decrease in bacterial counts from $3.76 \log_{10} \text{cfu/ml}$ at 1.5 hours to $3.47 \log_{10} \text{cfu/ml}$ at 4.5 hours. After this there was significant reduction in the number of viable count at 6 hours ($1.4 \log_{10} \text{cfu/ml}$). A similar trend in viable counts was observed with ofloxacin and amikacin. The overall decrease in viable counts of the antibiotic treated groups when compared with control group at 6 hours was significant ($p<0.001$).

Fig. 21b shows the released endotoxin level in plasma of *K. pneumoniae* infected mice, treated with single intramuscular dose of respective antibiotic. Mice receiving normal saline instead of any antibiotic acted as control. With ceftazidime and ofloxacin, significant endotoxin release occurred between 3 to 4.5 hours exposure period, reaching a maximum of 1165 EU/ml and 590 EU/ml respectively, at 6 hours. Whereas, with amikacin there was no significant change in endotoxin level when compared with control group. Difference in the amount of endotoxin release caused by amikacin and that caused by ceftazidime and ofloxacin was significant ($p<0.01$).

Fig. 21c represents the amount of TNF-α released in the serum of *K. pneumoniae* infected mice following exposure to single intramuscular dose of
ceftazidime, ofloxacin and amikacin. With ceftazidime treatment maximum amount of TNF-α was released at 6 hours followed by ofloxacin and least with amikacin. When compared with control group (K. pneumoniae infected mice without any antibiotic treatment), TNF-α release caused by antibiotics ceftazidime and ofloxacin was significantly higher (p<0.001).

Fig. 21: Effect of ceftazidime, ofloxacin and amikacin on (a) Viable count (log₁₀ cfu/ml) of K. pneumoniae in blood, (b) Plasma endotoxin level (EU/ml) released from bacteria and (c) Serum TNF-α levels (ng/ml) in the K. pneumoniae infected mice at different time intervals.

(a)

![Graph showing viable count in blood over time with different antibiotics](image-url)
In all experiments each antibiotic was added at time zero (24 hours after establishment of infection in mice). No antibiotics were added to the control group. Each value in the figures represent the mean of five individual samples. Bar = standard deviation.
EXTRACTION AND PURIFICATION OF LIPOPOLYSACCHARIDE (LPS) FROM *K. pneumoniae* ATCC 43816

The lipopolysaccharide of *K. pneumoniae* was extracted by phenol-water extraction method and was purified further by column chromatography and sequential ultracentrifugation. The fractionation of LPS on sephadex G-50 column showed two peaks (Fig.22). Major thiobarbituric acid (TBA) reactive material (LPS) was detected in peak I whereas no such material was found in peak II. The peak I fractions were pooled, dialyzed against distilled water and lyophilized. This LPS preparation was designated as ‘crude LPS’, which was purified further by the method of Johnson and Perry (1976). In this, sequential ultracentrifugation of the crude LPS preparation was carried out to obtain gel like sediment. LPS preparation thus obtained was lyophilized and designated as ‘pure LPS’. Purified LPS was monitored for its carbohydrate, protein, KDO, DNA and RNA contents. The preparation showed negligible amount of nucleic acids and protein, thereby showing the purity of the preparation (Table-2).

<table>
<thead>
<tr>
<th>Carbohydrate content (µg/ml)</th>
<th>Protein content (µg/ml)</th>
<th>KDO content (µg/ml)</th>
<th>DNA % (wt/wt)</th>
<th>RNA % (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>45</td>
<td>90</td>
<td>900</td>
<td>0.5</td>
</tr>
</tbody>
</table>

FRACTIONATION OF PURIFIED LPS

Lipid A moiety of purified LPS was separated from polysaccharide portion by mild acid hydrolysis. O-polysaccharide of *K. pneumoniae* ATCC 43816 was loaded on a sephadex G-50 column and the elution profile of this water-soluble product is shown in Fig.23. Collected fractions were analyzed for the carbohydrate content by the phenol-sulphuric acid method. The high molecular weight O-polysaccharide was eluted in two poorly separated peaks, which were pooled, dialyzed against distilled water and lyophilized. O-polysaccharide, when analyzed for its lipid content showed negligible amount of lipid.
Fig. 22: Elution profile of *Klebsiella pneumoniae* ATCC 43816 lipopolysaccharide (LPS) on sephadex G-50 column

- Column size: 75cm x 1.5 cm
- Eluent: Tris-HCl buffer (pH 8.0, 0.1 M)
- Flow rate: 30 ml/h
- Fraction size: 3 ml
- Sample loaded: 0.5 ml
Fig. 23: Elution profile of water soluble product from the acetic acid hydrolyzed LPS of *K. pneumoniae* ATCC 43816 on sephadex G-50 column

Fractions were analyzed for carbohydrate content by the phenol sulphuric acid method.

| Column size | 75cm x 1.5 cm |
| Flow rate   | 30 ml/h       |
| Eluent      | Distilled water |
| *Fraction size | 3 ml         |
| Sample loaded | 0.5 ml       |
EXTRACTION AND PURIFICATION OF OUTER MEMBRANE PROTEINS (OMPs)

Outer membrane proteins (OMPs) of *Klebsiella pneumoniae* ATCC 43816 grown in presence and presence of antibiotics were extracted and designated as test OMPs and control OMPs, respectively. These were resolved on 12 percent SDS-polyacrylamide gel electrophoresis (Fig.24). On coomassie blue staining few additional bands were observed in the OMP profiles of *K. pneumoniae* grown in presence of ceftazidime and ofloxacin. Whereas, no new band was observed in the OMP profile of *K. pneumoniae* grown in presence of amikacin. With ceftazidime few high molecular weight bands above 97 kDa and low molecular weight bands of molecular weight 40 kDa and 22 kDa were detected under antibiotic stress. Few new high molecular weight bands above 97 kDa and low molecular weight bands of molecular weight 30 kDa and 22 kDa were also observed in OMP profile of *K. pneumoniae* grown in presence of antibiotic ofloxacin.

On immunoblotting, not all the additional bands were picked up with antisera. For antibiotic ceftazidime, two bands of molecular weight above 97 kDa and one band of molecular weight 40 kDa was detected with antisera (test antisera) raised against OMPs extracted from cells grown in presence of this antibiotic. These bands were not detectable with control antisera (Fig.25). For antibiotic amikacin no additional protein band in the OMP profile of the cells grown in presence of antibiotic was detected on immunoblotting (Fig.26). With antibiotic ofloxacin two bands of molecular weights 30 kDa and 22 kDa were recognized with antibodies raised against test OMPs but not with the sera raised against control OMPs (Fig.27).

To confirm that new proteins expressed in presence of antibiotics ceftazidime and ofloxacin were present on the surface of *K. pneumoniae*, the respective antisera raised against killed bacteria grown in presence of ceftazidime (TS-CD) or bacteria grown in presence of ofloxacin (TS-OF) were adsorbed with bacterial cells grown in absence of antibiotic. On immunoblotting adsorbed TS-CD reacted with a single new band of molecular weight 40 kDa in the test OMPs (Fig.28). This band was not visualized in control OMPs. Whereas, adsorbed TS-OF revealed a single new band of molecular weight 30 kDa (Fig.29) in test OMPs but not in control OMPs.
These immunoblot experiments confirmed that proteins of molecular weight 40 kDa (PR-CD) and 30 kDa (PR-OF) expressed by *K. pneumoniae* in presence of ceftazidime and ofloxacin, respectively were immunogenic as well as present on surface of the cells. These proteins were then purified by gel elution method of Hager and Burgess (1980). Purified proteins were analyzed for their carbohydrate, protein, KDO, DNA and RNA contents. Table-3 shows the chemical composition of the purified proteins. Both the purified proteins showed almost negligible amount of nucleic acids and KDO content.

Table-3: Chemical composition of proteins, PR-CD (40 kDa) and PR-OF (30 kDa) purified from total OMPs of *K. pneumoniae* grown in presence of ceftazidime and ofloxacin, respectively

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Preparation</th>
<th>Carbohydrate content (µg/ml)</th>
<th>Protein content (µg/ml)</th>
<th>KDO content (µg/ml)</th>
<th>DNA % (wt/wt)</th>
<th>RNA % (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Purified protein (PR-CD)</td>
<td>19</td>
<td>2500</td>
<td>30</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>2.</td>
<td>Purified protein (PR-OF)</td>
<td>20.5</td>
<td>3000</td>
<td>20</td>
<td>0.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Antisera were raised against the purified proteins. The respective antisera reacted in immunoblotting with the protein PR-CD and PR-OF as shown in Fig.30 and Fig.31 respectively.
Fig. 24: Photographic representation of electrophoretic pattern of outer membrane proteins (OMPs) (lane b, d, f) expressed in the absence of antibiotics ofloxacin, amikacin and ceftazidime respectively; (lane c, e, g) expressed in presence of antibiotics ofloxacin, amikacin and ceftazidime respectively and standard protein markers (lane a) in 12 percent SDS-polyacrylamide gel stained with coomassie blue.

Lane c: From top to bottom, the arrows represent the protein bands of molecular weight >97kDa (upper most two arrows), 30kDa and 22kDa respectively.

Lane g: From top to bottom, the arrows represent the protein bands of molecular weight >97kDa (upper most two arrows), 40kDa and 22kDa respectively.
Fig. 25: Immunoblot showing the presence of two proteins of molecular weight more than 97kDa and a 40kDa protein in the outer membrane proteins (OMPs) from the cells grown in presence of antibiotic ceftazidime (test OMPs) but not in the OMPs from the cells grown in absence of antibiotic (control OMPs).

Lane 1 and 3: OMPs from the cells grown in the absence of ceftazidime
Lane 2 and 4: OMPs from the cells grown in the presence of ceftazidime
Lane 1 and 2: Reacted with sera against control OMPs
Lane 3 and 4: Reacted with sera against test OMPs
Fig. 26: Immunoblot showing no difference in the outer membrane proteins (OMPs) from the cells grown in presence (test OMPs) and absence (control OMPs) of antibiotic amikacin.

Lane 1 and 3: OMP from the cells grown in the absence of amikacin
Lane 2 and 4: OMPs from the cells grown in the presence of amikacin
Lane 1 and 2: Reacted with sera against control OMPs
Lane 3 and 4: Reacted with sera against test OMPs
Fig. 27: Immunoblot showing the presence of two proteins of molecular weight more than 97kDa and two proteins of 30kDa and 22kDa in the outer membrane proteins (OMPs) from the cells grown in presence of antibiotic ofloxacin (test OMPs) but not in the OMPs from the cells grown in absence of antibiotic (control OMPs).

Lane 1 and 3: OMP from the cells grown in the absence of ofloxacin

Lane 2 and 4: OMPs from the cells grown in the presence of ofloxacin

Lane 1 and 2: Reacted with sera against control OMPs

Lane 3 and 4: Reacted with sera against test OMPs
Fig. 28: Immunoblot showing the presence of single new protein of 40kDa in the outer membrane proteins (OMPs) from the cells grown in presence of antibiotic ceftazidime (test OMPs) but not in the OMPs from the cells grown in absence of antibiotic (control OMPs).

Lane 1: OMP from the cells grown in the absence of ceftazidime

Lane 2: OMPs from the cells grown in the presence of ceftazidime

Lane 1 and 2: Reacted with antisera raised against killed cells grown in the presence of antibiotic ceftazidime and adsorbed with cells grown in absence of antibiotic.
Fig. 29: Immunoblot showing the presence of a single new protein of 30kDa in the outer membrane proteins (OMPs) from the cells grown in the presence of antibiotic ofloxacin (test OMPs) but not in the OMPs from the cells grown in absence of antibiotic (control OMPs).

- **Lane 1**: OMP from the cells grown in the absence of ofloxacin.
- **Lane 2**: OMPs from the cells grown in the presence of ofloxacin.

Lane 1 and 2: Reacted with antisera raised against killed cells grown in the presence of antibiotic ofloxacin and adsorbed with cells grown in absence of antibiotic.
Fig. 30: Immunoblot showing the reactivity of serum raised against the purified protein (PR-CD) with the OMPs from the cells grown in the presence of ceftazidime.

Fig. 31: Immunoblot showing the reactivity of serum raised against the purified protein (PR-OF) with the OMPs from the cells grown in the presence of ofloxacin.
PREPARATION AND CHARACTERIZATION OF *K. pneumoniae* CONJUGATES

In order to couple O-polysaccharide (O-PS) moiety of LPS with purified proteins (PR-CD) or (PR-OF) expressed by *K. pneumoniae* in presence of ceftazidime and ofloxacin, respectively, carbodiimide condensation reaction was carried out using adipic acid dihydrazide as spacer molecule in conjugate.

Following coupling procedure, the reaction mixture was subjected to pre-equilibrated sephadex G-100 column. Both the conjugates (O-PS – PR-CD) and (O-PS – PR-OF) were eluted in void volume fractions due to their high molecular weights (Fig.32 & 33). Collected fractions were screened for the presence of protein (absorbance at 280 nm) and polysaccharide content (Dubois, 1956). Void volume peak fractions containing both polysaccharide and protein were pooled and lyophilized. The total yield of coupling was recorded as 45.2% and 44% for conjugates O-PS – PR-CD and O-PS – PR-OF respectively. It was based on polysaccharide recovered in void volume peak of respective conjugates to the total polysaccharide loaded. Purified conjugates (O-PS – PR-CD and O-PS – PR-FO) showed a maximum polysaccharide / protein ratios of 1.58 and 1.9 respectively (Table-4).

### Table-4: Characterization of polysaccharide (PS) - protein (Pr) conjugates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Conjugate</th>
<th>Approximate yield (%)</th>
<th>Polysaccharide (μg/ml)</th>
<th>Protein (μg/ml)</th>
<th>Polysaccharide / protein ratio (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>O-PS-PR-CD</td>
<td>45.2</td>
<td>850</td>
<td>538</td>
<td>1.58</td>
</tr>
<tr>
<td>2.</td>
<td>O-PS-PR-OF</td>
<td>44.0</td>
<td>800</td>
<td>420</td>
<td>1.9</td>
</tr>
</tbody>
</table>
BIOLOGICAL STUDIES OF PURIFIED ANTIGENS

The toxic properties of \textit{Klebsiella pneumoniae} ATCC 43816 derived antigens (LPS, purified O-polysaccharide (O-PS), purified proteins PR-CD and PR-OF and conjugates of O-PS and protein) were studied. LPS was found to be non-toxic at 100 µg concentration in normal mice. However, it was found to be toxic at this dose in galactosamine sensitized mice. All other antigens tested were found to be non-toxic in normal as well as sensitized mice (Table-5).

\textbf{Table-5: Toxicity of \textit{Klebsiella pneumoniae} antigens}

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Preparation</th>
<th>Dose (µg/ml)</th>
<th>No. of mice killed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>1.</td>
<td>LPS</td>
<td>100</td>
<td>0/4</td>
</tr>
<tr>
<td>2.</td>
<td>Purified polysaccharide (O-PS)</td>
<td>100</td>
<td>0/4</td>
</tr>
<tr>
<td>3.</td>
<td>Purified protein (PR-CD)(^*)</td>
<td>100</td>
<td>0/4</td>
</tr>
<tr>
<td>4.</td>
<td>Purified protein (PR-OF)(^**)</td>
<td>100</td>
<td>0/4</td>
</tr>
<tr>
<td>5.</td>
<td>O-PS–PR-CD conjugate</td>
<td>100</td>
<td>0/4</td>
</tr>
<tr>
<td>6.</td>
<td>O-PS–PR-OF conjugate</td>
<td>100</td>
<td>0/4</td>
</tr>
</tbody>
</table>

\(^*\) (PR-CD) – protein expressed by \textit{K. pneumoniae} when grown in presence of ceftazidime  
\(^**\) (PR-OF) – protein expressed by \textit{K. pneumoniae} when grown in presence of ofloxacin

The ability of different preparations to evoke pyrogenic response in rabbits was determined. 100 µg each of LPS, purified polysaccharide (O-PS), purified proteins and O-PS – protein conjugates were tested separately by injecting intravenously through marginal ear vein in rabbits. The body temperature recorded at different time intervals showed that LPS was able to elicit pyrogenic response, whereas all other antigens were found to be non-pyrogenic (Table-6).
Table-6: Pyrogenic response to LPS, purified proteins and conjugates of polysaccharide and protein on intravenous injection in rabbits

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Preparation</th>
<th>Dose (μg/ml)</th>
<th>Sum of increase of temperature in two rabbits (°C)</th>
<th>Pyrogenicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (hours)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>LPS</td>
<td>100</td>
<td>2.1 2.3</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Purified polysaccharide (O-PS)</td>
<td>100</td>
<td>0.4 0.5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Purified protein (PR-CD)*</td>
<td>100</td>
<td>0.3 0.5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Purified protein (PR-OF)**</td>
<td>100</td>
<td>0.4 0.4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>O-PS–PR-CD conjugate</td>
<td>100</td>
<td>0.3 0.4</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>O-PS–PR-OF conjugate</td>
<td>100</td>
<td>0.5 0.4</td>
<td>-</td>
</tr>
</tbody>
</table>

* A preparation was considered to be pyrogenic if the sum of increase of temperature was > 1.15°C for 2 rabbits

(PR-CD) – protein expressed by K. pneumoniae when grown in presence of ceftazidime

(PR-OF) – protein expressed by K. pneumoniae when grown in presence of ofloxacin

LPS antigen of Klebsiella pneumoniae ATCC 43816 induced local skin reaction in rabbits showing second degree of induration with petechial hemorrhage. On the other hand O-polysaccharide, both the purified proteins and their conjugates did not induce any local skin reaction in rabbit after intradermal injection (Table-7).

Table-7: A comparison of biological properties of various antigens of Klebsiella pneumoniae ATCC 43816

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Preparation</th>
<th>Dose (μg/ml)</th>
<th>Pyrogenicity</th>
<th>Lethality for mice</th>
<th>Shwartzman reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LPS</td>
<td>100</td>
<td>+</td>
<td>-</td>
<td>++*</td>
</tr>
<tr>
<td>2</td>
<td>Purified polysaccharide (O-PS)</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Purified protein (PR-CD)*</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Purified protein (PR-OF)**</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>O-PS–PR-CD conjugate</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>O-PS–PR-OF conjugate</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Induration with petechial hemorrhage (Second degree)

(PR-CD) – protein expressed by K. pneumoniae when grown in presence of ceftazidime

(PR-OF) – protein expressed by K. pneumoniae when grown in presence of ofloxacin
HUMORAL IMMUNE RESPONSE

The immunogenic potential of polysaccharide (O-PS) or purified proteins (PR-CD, PR-OF) alone, mixtures of O-PS and protein and their conjugates was evaluated by injecting these preparations in mice and determining the level of IgG antibodies by enzyme linked immunosorbent assay (ELISA). Level of antibodies in pooled sera of animals immunized with different antigenic preparations was determined at 14th and 28th day of the first immunizing dose of respective antigen. Polysaccharide antigen and purified proteins alone evoked a weak immune response. However, conjugation of O-PS to purified protein significantly enhanced the immunogenicity of both the antigens.

The levels of O-PS specific IgG observed in the sera of animals immunized with O-PS + PR-CD and O-PS + PR-OF mixtures were comparable to the IgG levels observed on 14th and 28th day in animals immunized with O-PS alone (Fig.34a & 34b). However, conjugates O-PS–PR-CD (Fig.34a) and O-PS–PR-OF (Fig.34b) led to a significant increase (p<0.01) in O-PS specific IgG after first booster dose of the antigen.

Conjugation of O-PS to purified proteins also resulted in increased production of IgG specific to protein. The levels of protein specific IgG observed with conjugates O-PS–PR-CD (Fig.35a) and O-PS–PR-OF (Fig.35b) after first booster injection of antigen were significantly higher (p<0.001) when compared with protein specific IgG levels elicited by purified proteins alone or mixtures of O-PS and protein.
Fig. 34a: Polysaccharide specific IgG levels in pooled sera of mice immunized with O-PS alone, O-PS + PR-CD mixture and conjugate O-PS – PR-CD.

Fig. 34b: Polysaccharide specific IgG levels in pooled sera of mice immunized with O-PS alone, O-PS + PR-OF mixture and conjugate O-PS – PR-OF.

* Pool sera from four mice of different immunized groups were taken on 14th and 28th day of the first injection of the antigen; Bar=standard deviation.
Fig. 35a: Purified protein (PR-CD) specific IgG levels in pooled sera of mice immunized with PR-CD alone, O-PS + PR-CD mixture and conjugate O-PS – PR-CD

![Graph showing optical density (O.D.) at 405 nm for PR-CD alone, O-PS+PR-CD mixture, and O-PS - PR-CD conjugate on 14th and 28th days.]

* Pool sera from four mice of different immunized groups were taken on 14th and 28th day of the first injection of the antigen; Bar=standard deviation.

Fig. 35b: Purified protein (PR-OF) specific IgG levels in pooled sera of mice immunized with PR-OF alone, O-PS + PR-OF mixture and conjugate O-PS – PR-OF

![Graph showing optical density (O.D.) at 405 nm for PR-OF alone, O-PS+PR-OF mixture, and O-PS - PR-OF conjugate on 14th and 28th days.]

* Pool sera from four mice of different immunized groups were taken on 14th and 28th day of the first injection of the antigen; Bar=standard deviation.
The phagocytic function of peritoneal macrophages obtained from the immunized and control animals was compared. All the different antigenic preparations [LPS, purified proteins (PR-CD, PR-OF) alone, mixtures of O-PS and protein (O-PS + PR-CD, O-PS + PR-OF) and conjugates (O-PS–PR-CD, O-PS–PR-OF)] used in this study resulted in activation of peritoneal macrophages as increased uptake of bacteria by macrophages was observed. This resulted in reduction of relative survival rate of bacteria (Fig.36a & 36b). The maximum uptake of bacteria was observed with macrophages obtained from animals treated with either of the conjugate. However, survival values of bacteria with macrophages obtained from mice immunized with polysaccharide (O-PS) were comparable to that of control. Relative survival values obtained with macrophages from O-PS–PR-CD (Fig.36a) and O-PS–PR-OF (Fig.36b) conjugate immunized groups were least and varied between 55%-20% and 50%-18% respectively during phagocytic uptake assay.

Intracellular killing by peritoneal macrophages of mice immunized with different K. pneumoniae antigens was also studied in presence of normal mouse serum and the results were compared with intracellular killing by the macrophages obtained from normal mice (Fig.37a & 37b). Macrophages obtained from the mice immunized with conjugates (O-PS–OR-CD) (Fig.37a) and (O-PS–PR-OF) (Fig.37b) were found to be most efficient in killing bacteria. Significant differences were observed in the killing capacity of macrophages obtained from mice immunized with LPS, purified proteins and mixtures of O-PS and protein when compared to control group. However, results obtained with macrophages from polysaccharide treated mice were similar to control group in term of relative survival of bacteria.
Fig. 36: Kinetics of uptake of *K. pneumoniae* ATCC 43816 by peritoneal macrophages of normal and immunized mice in presence of normal mouse serum.

**a)**

![Graph a](image1)

**b)**

![Graph b](image2)

Bar = standard deviation
Fig. 37: Kinetics of intracellular killing of *K. pneumoniae* ATCC 43816 by peritoneal macrophages of normal and immunized mice in presence of normal mouse serum

(a) Relative survival values

(b) Relative survival values

Bar=standard deviation
ACTIVE PROTECTION STUDIES

In protection experiments, immunoprotective potential of different antigenic preparations (LPS, O-PS, purified proteins (PR-CD, PR-OF), mixtures of O-PS and protein and their conjugates) from *K. pneumoniae* ATCC 43816 was determined. The level of protection was assessed in terms of percentage survival of animals over a period of 14 days; quantitative bacterial determination in blood, liver, spleen and kidney; TNF-α levels in serum and pathological damage to different organs on 1\(^{st}\), 3\(^{rd}\) and 7\(^{th}\) day post infection.

Polysaccharide alone provided no protection in terms of percentage survival, whereas, intact lipopolysaccharide (LPS) provided 100% protection. However, both the purified proteins (PR-CD, PR-OF) and their mixtures with O-PS resulted in 50% protection. The percentage survival rates with their conjugates O-PS–PR-CD and O-PS–PR-OF were 75% and 62.5% respectively (Table-8).

Table-8: Protection provided by different antigens following challenge with 2x10^3 cfu/ml of *K. pneumoniae* ATCC 43816 entrapped in fibrin clot.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Immunizing antigen</th>
<th>Deaths</th>
<th>Survivals</th>
<th>Percentage survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (Normal saline)</td>
<td>8</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>Lipopolysaccharide (LPS)</td>
<td>0</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Polysaccharide (O-PS)</td>
<td>8</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>Purified Protein (PR-CD)</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>Purified Protein (PR-OF)</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>Mixture (O-PS + PR-CD)</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>Mixture (O-PS + PR-OF)</td>
<td>4</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>Conjugate (O-PS – PR-CD)</td>
<td>2</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td>9</td>
<td>Conjugate (O-PS – PR-OF)</td>
<td>3</td>
<td>5</td>
<td>62.5</td>
</tr>
</tbody>
</table>

- Each group consisted of eight animals.
- Mortality was noted for a period of 14 days.
- 100 µg of each antigen was used for immunization.
In polysaccharide immunized group, quantitative bacterial counts in blood on 1st and 3rd day following challenge with *K. pneumoniae* were similar to that of control group (Fig.38). Blood counts in the animals immunized with purified proteins PR-CD and PR-OF alone or mixtures of O-PS and respective protein decreased by more than one log cycle on 1st as well as 3rd day post bacterial challenge when compared with control group. Blood counts in the conjugate (O-PS–PR-CD and O-PS–PR-OF) immunized animals recorded on 1st and 3rd day after challenge were 2.1 and 2.6 log cycles and 2.0 and 2.5 log cycles respectively, which were comparatively less than the bacterial counts of 4.5 and 4.9 log cycles obtained in the blood of control group of animals on 1st and 3rd day respectively (Fig.38).

**Fig. 38:** Bacterial counts on 1st and 3rd post infection day in the blood of animals immunized with different antigens and challenged with *K. pneumoniae* ATCC 43816

* Blood for bacterial count determination was taken by retro-orbital puncture on 1st and 3rd day following infection with *K. pneumoniae.*
Similar to decrease in blood counts, a significant decrease in colonization of liver, spleen and kidney with *K. pneumoniae* ATCC 43816 was observed on 1\textsuperscript{st} and 3\textsuperscript{rd} post challenge day in mice immunized with different antigens. Whereas, quantitative bacterial counts in the polysaccharide immunized group were similar to that obtained in control group (Fig.39). Least bacterial counts in all the organs were obtained in the conjugate immunized animals which showed further decrease by 7\textsuperscript{th} day. However, counts could not be compared with control animals as there were no survivors on 7\textsuperscript{th} day post challenge in control group.

**Fig. 39: Bacterial counts in different organs on 1\textsuperscript{st} and 3\textsuperscript{rd} day following challenge of mice (immunized with different antigens) with *K. pneumoniae* ATCC 43816**

- Bar number 1,2,3 in each group represents bacterial counts in liver, spleen and kidney respectively on 1\textsuperscript{st} day following challenge with *K. pneumoniae*.
- Bar number 4,5,6 in each group represents bacterial counts in liver, spleen and kidney respectively on 3\textsuperscript{rd} day following challenge with *K. pneumoniae*.
A continued increase in the level of TNF-α in serum of all the immunized protected groups (groups immunized with purified proteins PR-CD, PR-OF, mixtures of O-PS and protein and their conjugates) was observed after 3rd post challenge day till 7th post challenge day (Fig. 40). Levels of TNF-α achieved in the serum of mice immunized with O-PS–PR-CD and O-PS–PR-OF conjugates were maximum and significantly higher (p<0.001) as compared to level obtained in the control group. Whereas, on 3rd day levels of TNF-α in polysaccharide immunized and control groups decreased by 42 and 60 units respectively as compared to their levels on 1st post challenge day (1 unit of TNF-α is equivalent to 0.05 ng/ml).

**Fig. 40:** TNF-α levels in the serum at different days when mice immunized with different antigens were challenged with *K. pneumoniae* ATCC 43816
Histopathological changes observed in different organs (liver, spleen, kidney and peritoneum) in all immunized animals (including control) showed mild inflammation and congestion on 1st day post challenge. By 3rd day (the peak day of infection), the inflammation had reached the moderate level in all the immunized infected groups (groups immunized with purified proteins PR-CD, PR-OF and mixtures of O-PS and protein) (Fig. 41a, 41b, 41c & 41d) except the groups immunized with conjugates, in which congestion and mild inflammation was seen (Fig. 42a, 42b, 42c & 42d). Moderate inflammation was characterized by marked congestion, organ surface covered with inflammatory exudate, focal collection of acute inflammatory cells, necrotic foci and mild microvesicular fatty changes. Severe inflammatory changes in all the organs were observed in control as well as polysaccharide immunized animals just before their death. Moderate inflammation in all the organs on 7th post infection day was detected in animals immunized with purified proteins alone or mixtures of O-PS and protein. In contrast, inflammation completely resolved by 7th post infection day in groups immunized with conjugates.

COMBINED THERAPEUTIC STUDIES

Animals following infection with 2x10^3 cfu/ml of *K. pneumoniae* ATCC 43816 entrapped in a fibrin clot were treated either with different concentrations of selected antibiotics alone or antisera raised against the conjugates or concomitantly with antisera and antibiotic. The animals were observed for survival thereafter for 14 days. Bacteriological counts in blood and organs (liver, spleen and kidney) in the treated groups were also compared with control group (untreated).

**Treatment with different concentrations of antibiotics alone**

Percentage survival values of mice treated with normal saline (control group) and different concentrations of antibiotics are shown in (Fig. 43a & 43b). Mice were treated with three different concentrations, 50 mg/kg/day, 75 mg/kg/day and 150 mg/kg/day of ceftazidime through intramuscular route (Fig. 43a). Lowest dose of this antibiotic gave 50% survival rate of the animals. However, treatment of mice with higher doses (75 mg/kg/day and 150 mg/kg/day) gave 62.5% and 100% survival rate respectively. For antibiotic ofloxacin a dose of 6.65 mg/kg/day and 13.3 mg/kg/day gave 75% and 100% survival respectively (Fig. 43b).
Fig. 41a: Photomicrograph shows transverse section of liver revealing degenerative hepatocytes with focal areas of congestion and infiltration by inflammatory cells (H&E, X100)

Fig. 41b: Photomicrograph shows transverse section of spleen revealing moderate sclerosis and focus of haematopoietic activity (H&E, X100)
Fig. 41c: Photomicrograph shows transverse section of kidney revealing areas of congestion in tubules, interstitium and glomerulus (H&E, X100)

Fig. 41d: Photomicrograph shows transverse section of peritoneum revealing moderate infiltration by chronic inflammatory cells with areas of granulation tissue (H&E, X100)
Fig. 42a: Photomicrograph shows transverse section of liver revealing mild congestion, dilation of sinusoids with slight kupffer cell hyperplasia (H&E, X100)

Fig. 42b: Photomicrograph shows transverse section of spleen revealing mild sclerosis with haematopoietic activity (H&E, X100)
Fig. 42c: Photomicrograph shows transverse section of kidney revealing mild congestion and infiltration by inflammatory cells (H&E, X100)

Fig. 42d: Photomicrograph shows transverse section of peritoneum revealing mild oedema, infiltration by acute and chronic inflammatory cells (H&E, X100)
Fig. 43a: Survival of *K. pneumoniae* ATCC 43816 infected mice following treatment with different concentrations of ceftazidime

Fig. 43b: Survival of *K. pneumoniae* ATCC 43816 infected mice following treatment with different concentrations of ofloxacin
Blood culture was found to be positive within 24 hours of implantation of fibrin clot. Fig.44a shows the comparative bacterial counts in the blood of mice at 24 and 48 hours after treatment with different concentrations of antibiotic ceftazidime. After 24 hours of ceftazidime treatment, bacterial counts in the blood of mice treated with 50 mg/kg/day or 75 mg/kg/day of antibiotic were not significantly different from the control group (without antibiotic treatment) (p>0.05). After 48 hours, similar results were obtained when doses of 50 mg/kg/day and 75 mg/kg/day of ceftazidime were used. Whereas, when a dose of 150 mg/kg/day of ceftazidime was used a significant decrease in bacterial count in the blood of treated mice was observed, after 48 hours. Result obtained after 24 hours of ofloxacin treatment (Fig.44b) showed insignificant decrease in the blood counts of bacteria with both the doses of antibiotic tested. However, after 48 hours of ofloxacin treatment (6.65 mg/kg/day), bacterial counts were significantly less (p<0.01) as compared to control. No viable bacteria after 48 hours in the blood of treated mice were detected when a dose of 13.3 mg/kg/day of ofloxacin was administered.

The bacterial load was quantitated in the liver, spleen and kidney after administration of different concentrations of antibiotics. Fig.45a shows the mean bacterial counts in liver, spleen and kidney of mice on 3rd day of ceftazidime treatment. On comparing the bacterial counts in liver of treated and untreated (control) mice, a decrease in number of viable bacteria in the liver of treated mice was observed at a dose of 50 mg/kg/day. This decrease was significant (p<0.01) at a dose of 75 mg/kg/day of ceftazidime. A similar trend of decrease in bacterial counts was observed when bacterial load was assessed in kidney. On the other hand, no significant difference in the number of viable bacteria in the spleen of treated (50 mg/kg/day of ceftazidime) and untreated (control) mice was detected (p>0.05). The decrease in viable counts was significant when 75 mg/kg/day of ceftazidime was used for treatment (p<0.05). With highest dose (150 mg/kg/day) of ceftazidime used in this study, bacterial counts in all the organs decreased appreciably in comparison to control group (Fig.45a). Mean bacterial counts obtained in the liver, spleen and kidney of mice treated with different doses of ofloxacin are presented in Fig.45b. On comparing the results obtained in the test and control group, no significant (p>0.05) decrease in the number of viable bacteria in liver, spleen and kidney was observed when 6.65 mg/kg/day ofloxacin was used for treatment. However, at a higher dose (13.3 mg/kg/day) of ofloxacin,
significant decrease (p<0.001) in viable bacterial counts could be detected in all the organs.

**Fig. 44a:** Viable bacteria in the blood of *K. pneumoniae* infected mice after 24 and 48 hours following treatment with different concentrations of ceftazidime

```
Bacterial count (log^ cfu/ml) in blood

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without antibiotic treatment)</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>50 mg/kg/day of ceftazidime</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>75 mg/kg/day of ceftazidime</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>150 mg/kg/day of ceftazidime</td>
<td>3.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
```

**Fig. 44b:** Viable bacteria in the blood of *K. pneumoniae* infected mice after 24 and 48 hours following treatment with different concentrations of ofloxacin

```
Bacterial count (log^ cfu/ml) in blood

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without antibiotic treatment)</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>6.65 mg/kg/day of ofloxacin</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>13.3 mg/kg/day of ofloxacin</td>
<td>3.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
```

* Each bar represents the mean of the bacterial counts obtained from four different animals.
Fig. 45a: Number of viable bacteria in the liver, spleen and kidney of *K. pneumoniae* infected mice on 3\textsuperscript{rd} day following treatment with different concentrations of ceftazidime.

*Each bar represents the mean of the bacterial count obtained from four different animals.*

Fig. 45b: Number of viable bacteria in the liver, spleen and kidney of *K. pneumoniae* infected mice on 3\textsuperscript{rd} day following treatment with different concentrations of ofloxacin.

*Each bar represents the mean of the bacterial count obtained from four different animals.*
Treatment with antisera of respective conjugates

Percentage survival values of mice treated with antisera raised against conjugates, O-PS–PR-CD and O-PS–PR-OF respectively are shown in Fig. 46. As is evident from the results, antisera alone was unable to provide any protection as there was 100% mortality within a week in both the groups treated with either of the antiserum. Bacteriological counts obtained in blood (Fig. 47) as well as organs (liver, spleen and kidney) (Fig. 48) of mice treated with sera of the respective conjugate were comparable to the control group (infected with \( K. pneumoniae \) and given normal saline thereafter).

Fig. 46: Percentage survival of \( K. pneumoniae \) ATCC 43816 infected mice with or without treatment with sera of the respective conjugates
Fig. 47: Viable bacteria in the blood of *K. pneumoniae* infected mice after 24 and 48 hours following treatment with antisera of respective conjugate.

![Bar chart showing bacterial count (log_{10} cfu/ml) in blood for control and treated groups over 24 and 48 hours.

Fig. 48: Number of viable bacteria in liver, spleen and kidney of *K. pneumoniae* infected mice on 3rd day following treatment with antisera of respective conjugate.

![Bar chart showing bacterial count (log_{10} cfu/g) of tissue for control and treated groups in liver, spleen, and kidney.]

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Treatment with combination of antisera and antibiotic

In these experiments, mice were treated with antisera of respective conjugate in combination with a fixed concentration of antibiotic, ceftazidime, or ofloxacin. 100 µl of conjugate sera was administered intravenously, 24 hours after challenge with *K. pneumoniae*. Antibiotic treatment was simultaneously started and continued for 7 days. The concentration of each antibiotic chosen for treatment was unable to provide complete protection against sepsis. When 100 µl antisera against O-PS–PR-CD conjugate was used in combination with 75 mg/kg/day of ceftazidime, 100% protection in mice in terms of percentage survival was achieved. Similar results were obtained when 6.65 mg/kg/day of ofloxacin was used to treat mice in combination with antisera raised against conjugate O-PS–PR-OF.

Blood culture in these animals was found to be positive within 24 hours of implantation of fibrin clot containing *K. pneumoniae*. Fig.49a shows the viable bacteria in blood of mice at 24 and 48 hours after combined treatment. Combination treatment with sera of O-PS–PR-CD conjugate and ceftazidime (75 mg/kg/day) resulted in decreased blood bacterial counts on both time intervals. Counts were appreciably less (p<0.01) when compared with bacterial counts in blood of mice treated with antisera and ceftazidime alone. Similar results were obtained when combination treatment with O-PS–PR-OF conjugate sera and ofloxacin (6.65 mg/kg/day) was used to treat mice (Fig.49b).

Mean bacterial counts obtained in the liver, spleen and kidney of mice treated with combination of O-PS–PR-CD sera and 75 mg/kg/day of ceftazidime (Fig.50a) and O-PS–PR-OF sera and 6.65 mg/kg/day of ofloxacin (Fig.50b) on 3<sup>rd</sup> day post treatment were significantly less (p<0.001) when compared with counts obtained in both the control groups.

TNF-α in the serum continued to rise till 7<sup>th</sup> post treatment day in the groups of animal treated with combination therapy (Fig.51). Levels of TNF-α achieved in these groups were maximum followed by the groups treated with individual antibiotics alone. However, TNF-α levels declined after 1<sup>st</sup> day in groups of animal treated with respective antisera alone. TNF-α levels on 7<sup>th</sup> day post treatment could not be detected in these groups as there was no survived animal.
Fig. 49a: Viable bacteria in the blood of *K. pneumoniae* infected mice after 24 and 48 hours following combined treatment with O-PS–PR-CD conjugate sera and ceftazidime

![Bar chart showing bacterial counts](chart_a)

*Each bar represents the mean of the bacterial counts obtained from four different animals.*

Fig. 49b: Viable bacteria in the blood of *K. pneumoniae* infected mice after 24 and 48 hours following combined treatment with O-PS–PR-OF conjugate sera and ofloxacin

![Bar chart showing bacterial counts](chart_b)
Fig. 50a: Number of viable bacteria in liver, spleen and kidney of *K. pneumoniae* infected mice on 3rd day following combined treatment with O-PS–PR-CD conjugate sera and ceftazidime

![Graph showing bacterial count in liver, spleen, and kidney of mice](image)

Fig. 50b: Number of viable bacteria in liver, spleen and kidney of *K. pneumoniae* infected mice on 3rd day following combined treatment with O-PS–PR-OF conjugate sera and ofloxacin

![Graph showing bacterial count in liver, spleen, and kidney of mice](image)
Histopathological changes observed in different organs (liver, spleen and kidney) in all the groups on 1st day following treatment showed mild inflammation and congestion. By 3rd day severe inflammatory changes were observed in the groups treated with O-PS-PR-CD and O-PS-PR-OF sera alone. These changes were of moderate level in groups treated with 75 mg/kg/day and 6.65 mg/kg/day of ceftazidime and ofloxacin respectively. Whereas, mild inflammation was observed in all the organs of mice treated with O-PS-PR-CD sera + 75 mg/kg/day of ceftazidime and O-PS-PR-OF sera + 6.65 mg/kg/day of ofloxacin (Fig.52a,52b,52c and 52d). On 7th post treatment day the inflammation was of moderate level in the groups treated with either of the antibiotic alone, whereas, complete resolution of inflammation was observed in the groups treated with respective sera plus antibiotic.
Fig. 52a: Photomicrograph shows transverse section of liver revealing mild congestion, dilation of sinusoids with slight kupffer cell hyperplasia (H&E, X400)

Fig. 52b: Photomicrograph shows transverse section of spleen revealing mild sclerosis with haematopoietic activity (H&E, X250)
Fig. 52c: Photomicrograph shows transverse section of kidney revealing mild congestion and infiltration by inflammatory cells (H&E, X100)

Fig. 52d: Photomicrograph shows transverse section of peritoneum revealing mild oedema, infiltration by acute and chronic inflammatory cells (H&E, X100)