A COMPARATIVE STUDY OF IN VIVO EFFICACY OF CURRENTLY USED ANTIBIOTICS IN MURINE MODEL OF KLEBSIELLA PNEUMONIA

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ABSTRACT
A comparative in-vivo efficacy of commonly used antibiotics was evaluated in the murine model of Klebsiella pneumonia. Six groups of immunosuppressed (cyclophosphamide 80mg/kg i.p. on -4 and -1 day) female Wistar rats (n=6-12) were employed to induce pneumonia. Bacterial inoculum of strength 1x 10^7 CFU/ml of Klebsiella pneumoniae strain isolated from sputum in the Department of Microbiology, Kasturba Medical College, Mangalore was injected intratracheally. Gentamicin (0.032g/kg body weight/day), Levofloxacin (0.036g/kg body weight/12hrly), Cefepime (0.18 g/kg body weight/12hrly), Aztreonam (0.18g/kg body weight/8hrly) and Meropenem (0.09 mg/kg body weight/8hrly) were administered i.p. within 4 hours following bacterial inoculation and continued for 5 days. On the sixth day, rats were sacrificed and the pneumonic lung tissues were used for bacterial count and histopathological studies.

Levofloxacin significantly offered protection in Klebsiella pneumonia. Aztreonam seems to be a better choice to Cefepime and Gentamicin. Cefepime is found to be an effective antibiotic whereas Gentamicin is not effective against the local strains of K.pneumoniae. Thus in-vivo efficacy of antibiotic data may help to substantiate the choice of antibiotic against local strains of pneumopathogens. Also, these results may ensure more scientific authenticity than in-vitro bacteriological investigation reports.

Key words: In vivo efficacy of antibiotics, Klebsiella pneumoniae, Pneumonia.

INTRODUCTION
Prompt use of appropriate antibiotics is essential to optimize the outcome of nosocomial infections like Hospital acquired pneumonia. Any delay in the administration of antibiotic has been associated with greater hospital costs and prolonged hospital stay for patients. This has led to the development of a novel paradigm guiding the administration of empirical antimicrobial therapy for patients with serious infections like hospital acquired pneumonia. Antibiotics (in vitro laboratory tests for testing bacterial sensitivity to antibiotics) are often taken into account to define a rational selection of an empirical antimicrobial therapy for treating patients with HAP infections. However, they are not always reliable. Recent studies have indicated that an ‘in vivo’ in vitro paradox does exist and microbiological resistance determinations in vitro are not always predictive of treatment outcomes in vivo. It is seemingly possible that pathogen virulence changes with time and sensitivity to antibiotics also change. We have been observing the re-emergence of antibiotics once considered as useless against a pathogen. Over years, in-vivo efficacy of an antibiotic heavily relied on the original manufacture assessment data. Can we accept this for indefinite period? Is there a compelling need for reviewing in vivo efficacy profile of an antibiotic rather than depending solely on laboratory investigation? Essentially, in vivo antibiotic efficacy data generated frequently may render more accountability for the choice of an antibiotic. Eventually, this will make antibiotic choice more rational and may help to design cost effective treatment indeed. Pertinently, in-vivo bacterial susceptibility data needs to be re-examined at least for life threatening infections like pneumonia. Antibiotic in vivo efficacy is tested only once at the time of development and approval. Periodic in vivo antibiotic efficacy against local bacterial strains may provide much needed basis for especially understanding altered sensitivity seen at local bacterial level.

The present study is an attempt to compare the in vivo efficacy of currently used antibiotics against local strains of Klebsiella pneumoniae in a murine model of pneumonia. Hopefully, the generated preclinical chemotherapeutic in vivo efficacy data may help to alter empirical choice of antibiotic for achieving more clinical benefit.

MATERIALS AND METHODS
Animals
Six groups of female Wistar rats (n=6-12), weighing 100-150g, 60-90 days old, were employed for the study. Animals were housed individually in polypropylene cages and had access to commercial chow and water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee.

Immunosuppressive treatment
The rats were given cyclophosphamide (80mg/kg body wt) (German Remedies Ltd., Mumbai) intraperitoneally on -4 & -1 day before bacterial inoculation.

Bacterial strain
K. pneumoniae, isolated from sputum in the Department of Microbiology, Kasturba Medical College, Mangalore was used in the present study. The bacterial culture was preserved in small aliquots in brain heart infusion broth (Hi-Media, Mumbai) with 20% glycerol at -20°C. For daily use, bacterial culture was maintained on nutrient agar (Hi-Media, Mumbai) slope at 4°C.

Inoculum preparation
Stock cultures were thawed and 0.1ml was inoculated into 50 ml nutrient broth (Hi-Media, Mumbai) and incubated at 37°C for 24 hours. A small volume of this culture was added to 50 ml of fresh medium and grown to mid log phase (OD 0.1 at 550nm). The broth culture was then centrifuged at 10000g for 20min in a cold centrifuge. The cells were washed twice using phosphate buffered saline (PBS) (0.2 M, pH 7.2) and suspended in a final volume of 20ml of PBS to match with Mc Farland’s 0.5 standard. Viable bacterial count was determined by surface plating on nutrient agar (Hi-Media, Mumbai) slope at 4°C.

Induction of pneumonia
The rats were anaesthetized by injecting sodium pentobarbitone i.p. (40mg/kg body weight) (Sigma Fine Chemicals, St.Louis, Mo., USA) and placed on the board in supine position. The skin over the neck region was shaved and cleaned with methylated spirit. An incision (1 cm) was made just above the sternum to expose the trachea. A fixed dose of K. pneumoniae (1X10^7 CFU/50µl) was injected intra-tracheally with a syringe using a 25 gauge needle. Following inoculation, the animals were gently shaken for 15 seconds to equally distribute the inoculum in the lungs. The incision was sutured with an absorbable ethicon 3/0 thread and the antibiotic ointment Betadine was applied to the sutured cut.

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On the sixth post surgical day, the rats were sacrificed by cervical dislocation. The lungs were collected under aseptic conditions, gently blotted with sterile absorbent paper to remove blood, weighed and placed in 25 ml of ice cold saline.  

Drug treatment

Gentamicin (Nicholas Piramal India Ltd., Mumbai), Cefepime (Alkem Laboratories Ltd., Mumbai), Aztreonam (Aristo Pharmaceuticals Ltd., Raisen,M.P.), Meropenem (Alkem Laboratories Ltd., Mumbai) and Levofloxacin (Protec, Cipla Ltd., Jaipur) were administered intraperitoneally at a dose of 0.032g/kg body wt/day, 0.18g/kg body wt/12hrly, 0.18g/kg body wt/8hrly, 0.09g/kg body wt/8hrly and 0.036g/kg body wt/12hrly respectively for a duration of 5 days.

Quantitation of bacteria in pneumonic lung

For bacterial quantitation, lungs were homogenized in 5ml of sterile PBS at 4°C with a tissue homogenizer (Dalal & Co.,Chennai). The homogenates were then diluted in 10-folds, using sterile physiological saline. Fixed volumes (0.01ml) of dilutions were placed on blood agar (Hi-Media, Mumbai) and incubated at 37°C for 24 hours. The colonies were counted and concentration of K. pneumoniae in lung was calculated. Lung bacterial counts were calculated as total number of bacteria present in an entire lung specimen and reported as total bacterial count per set of lungs.  

Histopathological examination

The lung tissues removed were immediately fixed in 10% neutral buffered formalin. It was then hydrated in ascending series of alcohol (70-100%). The tissue was embedded in paraffin wax, sectioned and then stained with hematoxylin-eosin (Hi-Media, Mumbai). For evaluation, a section of each lung was assessed on a semi quantitative scale of 0 to 3 (Table 1). A total score indicative of the overall severity of lesions was determined by adding the individual score.  

Table 1: Semi quantitative scores for grading the severity of pathologic lesions of the lungs

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histological change</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No change</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Edema</td>
<td>Inflammatory cells in alveolar lumina</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>Inflammatory destruction of alveoli (lung abscess)</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>Mild inflammation in the wall (without luminal slough)</td>
<td>+3</td>
</tr>
<tr>
<td></td>
<td>No change</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild inflammation in the wall (without luminal slough)</td>
<td>+2</td>
</tr>
<tr>
<td>Bronchioles</td>
<td>Severe inflammation in the wall (with luminal slough)</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>Severe inflammation with luminal slough &amp; peribronchial inflammation</td>
<td>+3</td>
</tr>
</tbody>
</table>

Statistical analysis

The results were statistically analyzed using ANOVA followed by Tukey’s test (Graph Pad Software, Inc.,USA). P value of < 0.05 was considered significant.

RESULTS

Course of disease in control animals

By day 2, post infection, most of the experimental rats in the control group appeared acutely ill. Mucous secretions exuded from their eyes and most exhibited short and rapid breathing. As the infection progressed, their skin coats became shabby and considerable weight loss was obvious. Reduced activity was also observed. Within 5 to 6 days post infection, animals died spontaneously and apparently due to pneumonia.

Antibiotic protection & mortality

As shown in Table 2, the cumulative percentage mortality in the control group was 10. However, the rats died earlier starting from day 2 post infections, with progressive increase in mortality on day 3 and day 4 post infection. In the gentamicin treated group (n=12), the cumulative mortality was 8. On day 2 post infection, 4 animals died with subsequent mortality of 2 animals on the next consecutive days. In the levofloxacin treated group (n=12), cumulative mortality was 3; however no deaths were observed up to day 4 post infection. All the three died on day 5 post infection. In the cefepime, aztreonam and meropenem treated groups (n=6), cumulative mortality was 4, 2 and 4 respectively. However, the deaths were observed only from day 3 post infection, onwards.

Histopathological evaluation

General features

Histopathological analysis of the lung tissue revealed that the rats had moderate to severe multifocal bronchopneumonia characterized by a cellular infiltrate composed of lymphocytes and neutrophils. These cells were seen in the interalveolar septa and in the bronchiolar walls. The interalveolar septa showed moderate to severe congestion. The smaller vessels were also congested. The alveolar walls however were destroyed to a limited extent. The alveolar lumina showed a few red blood cells focally. Exudates were seen in the lumina of the bronchioles. (Figure 1-6)

Pneumonic tissue grading

The lung sections were graded and the results are shown in Table 3. Significant improvement in the histopathological grading was observed in the levofloxacin and aztreonam treated groups. In the gentamicin treated group, the grading was similar to or a little worse than the control indicating its relative inefficacy. The cefepime and meropenem, treated groups showed improved grades compared to the control; however statistical significance was not seen with these groups.

Pneumonic pulmonary bacterial count

As shown in Table 3, the gentamicin group (P < 0.05), cefepime & aztreonam groups (P < 0.01) and levofloxacin treated group (P < 0.001) caused significant reduction in the bacterial count whereas meropenem reduced the bacterial count compared to control insignificantly.

DISCUSSION

Hospital-acquired pneumonia (HAP) accounts for 15% of all nosocomial infections and affects 0.5 to 2.0% of hospitalized patients. The mortality rate for HAP exceeds 30%, although attributable mortality is lower. In particular, K. pneumoniae alone accounts for highest prevalence up to 43% of pneumonia caused by Gram-negative bacteria. A surveillance study from a neonatal ICU in Bangalore found that Klebsiella pneumoniae is the most commonly identified organism causing pneumonia. Therefore, the management of Klebsiella pneumoniae demands concerted efforts to save the life of the patients. A key component of the treatment for severe bacterial pneumonia is administration of an appropriate antibacterial regimen. The initial choice of agents is typically empiric, since the results of sputum and blood cultures are usually not available when therapy is started.

Table 2: Percentage of mortality observed during the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortality on 2nd post infection day</th>
<th>Mortality on 3rd post infection day</th>
<th>Mortality on 4th post infection day</th>
<th>Mortality on 5th post infection day</th>
<th>Total percentage of mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=12)</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>Nil</td>
<td>83.33%</td>
</tr>
<tr>
<td>Gentamicin (n=12)</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>nil</td>
<td>66.67%</td>
</tr>
<tr>
<td>Levofloxacin (n=12)</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>3</td>
<td>25%</td>
</tr>
<tr>
<td>Cefepime (n=6)</td>
<td>nil</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>66.67%</td>
</tr>
<tr>
<td>Aztreonam (n=6)</td>
<td>nil</td>
<td>1</td>
<td>nil</td>
<td>2</td>
<td>33.33%</td>
</tr>
<tr>
<td>Meropenem (n=6)</td>
<td>nil</td>
<td>2</td>
<td>nil</td>
<td>2</td>
<td>66.67%</td>
</tr>
</tbody>
</table>
Table 3: Summary of in-vivo efficacy of antibiotics in Klebsiella murine pneumonia

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number of animals</th>
<th>Histopathological grading (Mean ± SEM)</th>
<th>Bacterial count (log CFU/ml) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6+6</td>
<td>4.16 ± 0.4014</td>
<td>4.1 ± 0.4657</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>6+6</td>
<td>4.33 ± 0.33</td>
<td>2.6 ± 0.4257</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>6+6</td>
<td>2.5 ± 0.3416</td>
<td>1.15 ± 0.1726</td>
</tr>
<tr>
<td>Cefepime</td>
<td>6</td>
<td>3.33 ± 0.33</td>
<td>2.55 ± 0.2385</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>6</td>
<td>2.33 ± 0.33</td>
<td>1.86 ± 0.2204</td>
</tr>
<tr>
<td>Meropenem</td>
<td>6</td>
<td>3.33 ± 0.33</td>
<td>1.74 ± 0.1888</td>
</tr>
</tbody>
</table>
There are many ways to ensure antibiotic efficacy. One such feasible method is generating in vivo efficacy data of antibiotics against the local pathogen. Apparently, thus generated data bear significant impact on the clinician in the selection of antibiotics for pneumonia. It is needless to say that, in vivo efficacy data of antibiotics with local epidemiological and culture sensitivity laboratory data undoubtedly helps to rationalize chemotherapeutic regimens for hospital acquired pneumonia. In view of this, the current study was undertaken to provide in-vivo efficacy data of commonly used antibiotics against a local strain of *K. pneumoniae* in a murine model of pneumonia. Hopefully, this will ensure enhanced rate of cure. Also, it would reduce the development of bacterial resistance to antibiotics administered empirically.

Not withstanding the limitations of animal models, clinical evidence about the antibiotic efficacy will definitely help to streamline the selection of antibiotic. Significantly, this would relieve the clinical burden in the selection of antibiotic amidst a wide array of currently available chemotherapeutic agents.

The evaluation of in vivo efficacy of gentamicin, levofloxacin, cefepime, aztreonam and meropenem in the murine model of Klebsiella pneumonia is apparently well correlated with clinical outcome. The results of this study suggest that levofloxacin is more efficacious than gentamicin, cefepime, aztreonam and meropenem. Levofloxacin significantly reduced the mortality (*P* < 0.05) due to pneumonia. Comparatively, mortality was seen much later (post infection day 5) with levofloxacin indicating its higher efficacy. It also significantly reduced the pneumonic pulmonary bacterial count. (*P* < 0.001) (Table 3 and Figure 7) Further, levofloxacin predictably inhibited pneumonic pulmonary tissue damage as evidenced by the histopathological evaluation of the pneumonia lungs. (Figure 8)

Aztreonam seems to be a better choice to cefepime and gentamicin. Both aztreonam and meropenem showed greater reduction of bacterial count compared to cefepime. (Figure 7) However, mortality rates and histopathological evaluation scores were comparable between cefepime and meropenem. Aztreonam reduced the mortality rate much better than cefepime and meropenem. Also it was more successful in inhibiting the pulmonary tissue damage than cefepime and meropenem. (Figure 8)

Between aztreonam and meropenem, aztreonam appeared much more efficacious than meropenem as evidenced by the reduced mortality rate. Aztreonam offered better protection against tissue damage by *K. pneumoniae* compared to meropenem. (Table 3 and Figure 8) Nevertheless, this may remain incongruous in the clinical choice of antibiotics.

Evidently, the results of the present study when compared with clinical outcomes were astoundingly similar. As the study was conducted using one strain, further studies using different local strains is being evaluated.

In summary, the results indicate that levofloxacin is significantly efficacious than other antibiotics against pneumonia caused by *K. pneumoniae*. Aztreonam may be preferred to meropenem for pneumonia. Cefepime is found to be an effective antibiotic whereas gentamicin is not effective against local strains of *K. pneumoniae*. Evidently, this preclinical evaluation of in vivo efficacy of antibiotic may serve as a supporting basis for rational choice of chemotherapeutic agents, though expensive and time consuming.

**ACKNOWLEDGEMENTS**

The authors thank the Manipal Academy of Health Education, T. M. A. Pai research committee for financial support and Ms. Jayalakshmi, PhD scholar, MAHE, Mangalore-1 for technical assistance.

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