Asian Journal of Pharmaceutical and Clinical Research
Vol. 3, Issue 3, 2010 ISSN - 0974-2441

Research Article

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

PREETHI G PAI[1], ANOOP CH[1], MIRSHAD PV[1], GOPALKRISHNA BHAT[2], SHALINI M SHENOY[2], KV RAMESH[1]

[1]Department of Pharmacology, Kasturba Medical College, PB No. 53, Light house hill road, Mangalore-575001, India. E-mail: meddocpai2@yahoo.com

[2]Department of Microbiology, Kasturba Medical College, P.B. No. 53, Light house hill road, Mangalore-575001, India.

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.[1] Any delay in the administration of antibiotic has been associated with greater hospital costs and prolonged hospital stay for patients. [1-4] 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. Antibiograms (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.[5] It is seemingly possible that pathogen virulence changes with time and sensitivity to antibiotics also change.[6] We have been observing the re-emergence of antibiotics once considered as useless against a pathogen.[7] 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 days -4 & -1 day before bacterial inoculation. [8]

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 1000xg for 20minin 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).[9] An inoculum of size 1X10^7 CFU/50µl was used for induction of pneumonia.[9]

Induction of pneumonia

The rats were anaesthetized by injecting sodium pentobarbitone i. p. (40mg/kg body weight) (Sigma Fine Chemicals, St.Louis, Mo., USA) [10] 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 unabsorbable ethicon 3/0 thread and the antibiotic ointment Betadine was applied to the sutured cut. [11]

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. [11]

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[12] 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.[9]

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.[13]

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

Tissue	Histological change	Score
	No change	0
	Edema	+1
Alveoli	Inflammatory cells in alveolar lumina	+2
	Inflammatory destruction of alveoli (lung abscess)	+3
	No change	0
	Mild inflammation in the wall (without luminal slough)	+1
Bronchioles	Severe inflammation in the wall (with luminal slough)	+2
	Severe inflammation with luminal slough & peribronchial inflammation	+3

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 septae and in the bronchiolar walls. The interalveolar septae 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.

DISCUSSIONHospital-acquired pneumonia (HAP) accounts for 15% of all nosocomial infections[14] and affects 0.5 to 2.0% of hospitalized patients[15-16]. 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[17]. A surveillance study from a neonatal ICU in Bangalore found that *Klebsiella pneumoniae* is the most commonly identified organism causing pneumonia.[18] Therefore, the management of Klebsiella pneumonia 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

Group	Mortality on 2nd post infection day	Mortality on 3rd post infection day	Mortality on 4th post infection day	Mortality on 5th post infection day	Total percentage of mortality
Control (n=12)	4	4	2	Nil	83.33%
Gentamicin (n=12)	4	2	2	nil	66.67%
Levofloxacin (n=12)	Nil	nil	nil	3	25%
Cefepime (n=6)	Nil	2	1	1	66.67%
Aztreonam (n=6)	Nil	1	1	nil	33.33%
Meropenem (n=6)	Nil	2	nil	2	66.67%

Table 3: Summary of *in-vivo* efficacy of antibiotics in Klebsiella murine pneumonia

Antibiotic	Number of animals	Histopathological grading (Mean ± SEM)	Bacterial count (log CFU/ml) (Mean ± SEM)
Control	6+6	4.16 ± 0.4014	4.1 ± 0.4657
Gentamicin	6+6	4.33 ± 0.33	2.6 ± 0.4257
Levofloxacin	6+6	2.5 ± 0.3416	1.15 ± 0.1726
Cefepime	6	3.33 ± 0.33	2.55 ± 0.2385
Aztreonam	6	2.33 ± 0.33	1.86 ± 0.2204
Meropenem	6	3.33 ± 0.33	1.74 ± 0.1888

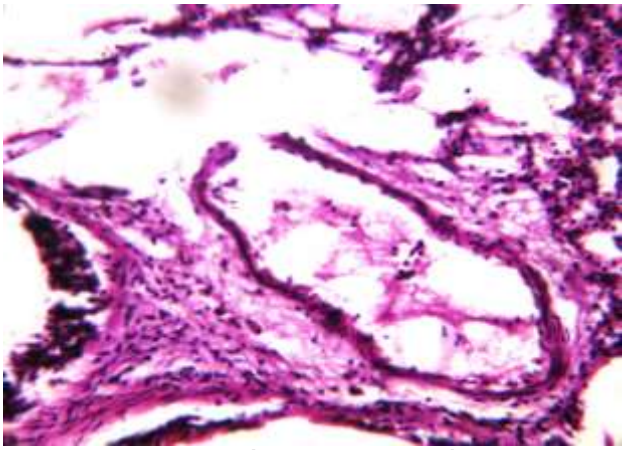

Figure 1: Bronchopneumonia -control group

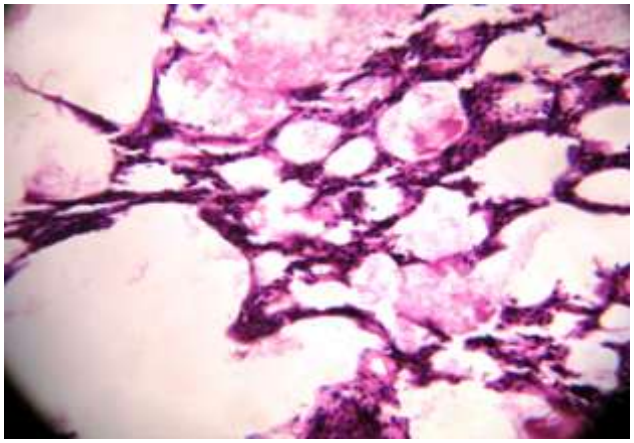

Figure 2: Bronchopneumonia –gentamicin treated group. Photomicrograph showing intensive intraalveolar exudates (green arrow). Inter alveolar septae showing lymphocytes and neutrophils (blue arrow) (scanner view, haematoxylin and eosin x40)

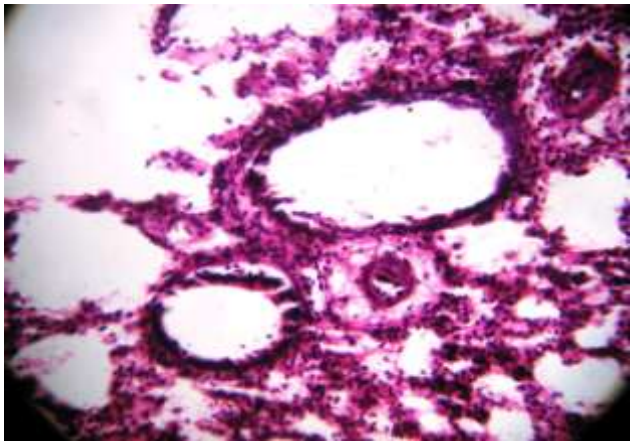

Figure 3: Bronchopneumonia –cefepime treated group. Photomicrograph showing inter alveolar septae with lymphocytes and neutrophils.(blue arrow) (scanner view, haematoxylin and eosin x40)

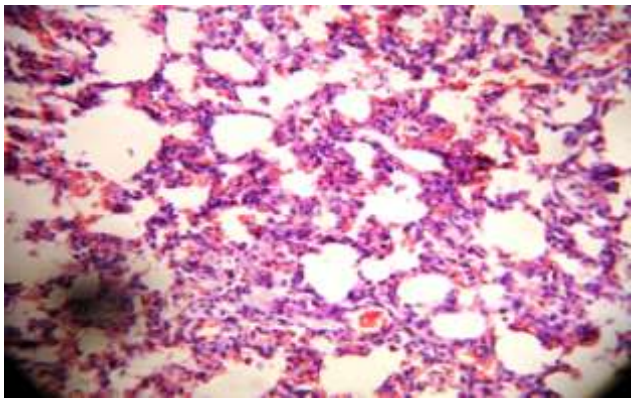

Figure 4: Bronchopneumonia –aztreonam treated group. Photomicrograph showing inter alveolar septae with lymphocytes and neutrophils.(green arrow) Severe congestion in between the alveoli is seen.(blue arrow). (scanner view, haematoxylin and eosin x40)

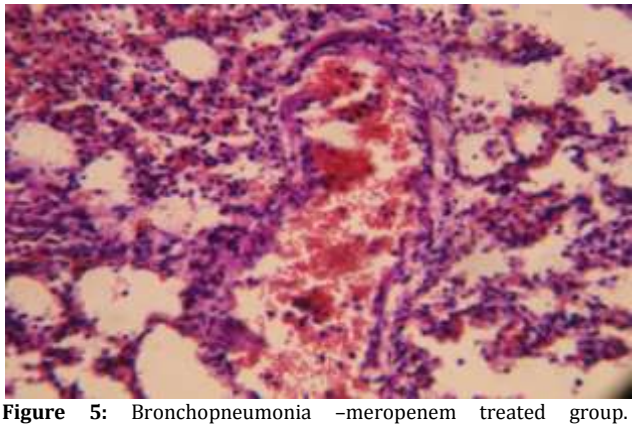

Figure 5: Bronchopneumonia –meropenem treated group. Photomicrograph showing intensive infiltrate and exudates in the bronchioles (green arrow). Interalveolar septae showing lymphocytes and neutrophils with congestion (blue arrow) (scanner view, haematoxylin and eosin x40)

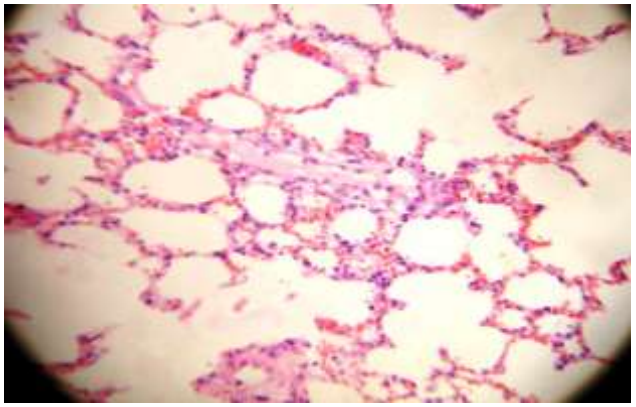

Figure 6: Bronchopneumonia –levofloxacin treated group. Photomicrograph showing inter alveolar septae with lymphocytes and neutrophils.(blue arrow) Severe congestion in between the alveoli is seen.(green arrow). Bronchioles are normal. (scanner view, haematoxylin and eosin x40)

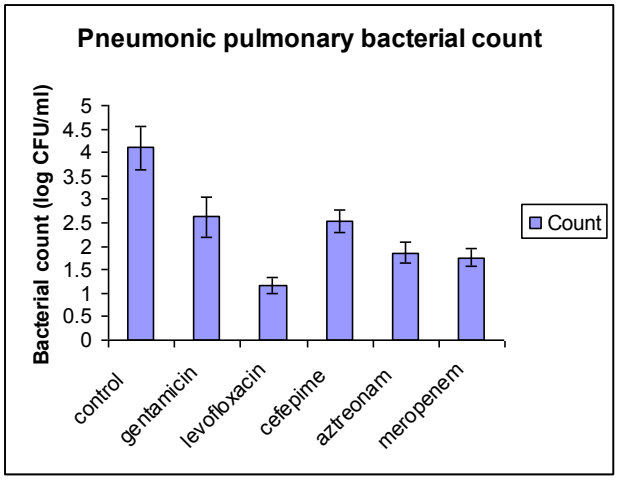

Figure 7: Effect of various antibiotics on pneumonic pulmonary bacterial count

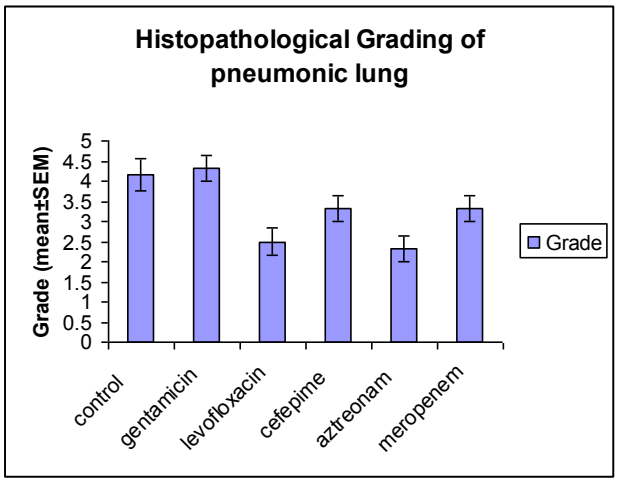

Figure 8: Assessment of efficacy of antibiotics based on the histopathological grading of pneumonic lungs

However empirical choice of antibiotics should always be guided by local epidemiological data regarding the susceptibility profile of the causative pathogen.[19] Traditionally, clinicians have used in vitro tests as a cornerstone in guiding the choice of an antibiotic. Over the past year, new studies have further chronicled the in vitro-in vivo paradox in lung infections.[5, 20, 21] There are several possible reasons for clinical success in the setting of in vitro resistance. During infection, microbes may be in an altered physiological state that may enhance their susceptibility to drugs. In addition, the host metabolism of antibiotics may in some instances potentiate their pharmacological activity. Anti-inflammatory effects of antibiotics may also play a role. Furthermore, susceptibility breakpoints may not adequately reflect clinical data outcomes.[5]

Data generated by the concerned manufacturing company regarding the in-vivo efficacy patterns dates back a few years. This has been followed over years on the basis of achieved clinical benefits. It is common knowledge that the resistance pattern of an antibiotic keeps fluctuating with time.[6] Is it valid even in the present scenario? The success of antibiotic therapy is undoubtedly dependent on bacterial susceptibility. Culture and sensitivity report although cover the uncertainty that cast shadow on the choice of antibiotic is not free from fall out. What guidelines should the clinician follow? Should he continue to believe in the in vivo efficacy report of the manufacturer which was prepared a few years ago using old strains? Are we heavily relying on laboratory reports on bacterial resistance? Do we use antibiotics which have retained their efficacy? Where is the evidence? Should we wait for clinical efficacy to be documented? Can we provide updated pre-clinical in vivo efficacy data which will help the clinician? Is this reliable?

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, [22] 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 pneumonic 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.

REFERENCES

1. Luna CM, Vujacich P, Niederman MS, Vay C, Gherardi C, Matera J et al. Impact of BAL data on the therapy and outcome of ventilator-associated pneumonia. Chest 1997; 111: 676-85.
2. Kollef MH, Sherman G, Ward S, Fraser VJ. Inadequate antimicrobial treatment of infections. A risk factor for hospital mortality among critically ill patients. Chest1999; 115: 462-74.

3. Iregui M, Ward S, Sherman G, Fraser VJ, Kollef MH. Clinical importance of delays in the initiation of appropriate antibiotic treatment for ventilator-associated pneumonia. Chest 2002; 122: 262-68.
4. Ibrahim EH, Sherman G, Ward S, Fraser VJ, Kollef MH. The influence of inadequate antimicrobial treatment of blood stream infections on patient outcomes in the ICU setting. Chest 2000; 118:146-55.
5. Bishai W. The in vivo-in vitro paradox in pneumococcal respiratory tract infections. Journal of Antimicrob. Chemother 2002; 49: 433-436.
6. Kaul S, Brahmadathan KN, Jagannati M, Sudarsanam TD, Pitchamuthu K, Abraham OC, JohnG. One year trends in the Gram-negative bacterial antibiotic susceptibility patterns in a medical intensive care unit in South India. Indian J Med Microbiol 2007; 25(3): 230-5.
7. Livermore DM. Bacterial resistance: origins, epidemiology and impact. CID 2003; 36(1): S11-23.
8. Candiani G, Abbondi M, Borgonovi M, Williams R. Experimental lobar pneumonia due to penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae* in immunocompetent and neutropenic rats: efficacy of Penicillin and Teicoplanin treatment. Journal of Antimicrob. Chemother1997; 39:199-207.
9. Domenico P, Johanson WJ Jr.,Straus DC. Lobar pneumonia in rats produced by clinical isolates of *Klebsiella pneumoniae.* Infect.Immun 1982; 37: 327-35.
10. Jochen M, Andeas WH, Segar K. Anaesthesia of experimental animals. In: Gerhard VH, Wolfgang HV editors. Drug discovery and evaluation. . New York: Springer; 1997. p.736.
11. Mimoz O, Leotard S, Jacolot A, Padoin C, Louchahi K, Petitjean O, et al. Efficacies of Imipenem, Meropenem, Cefepime and Ceftazidime in rats with experimental pneumonia due to a carbapenem-hydrolyzing β-Lactamase-producing strain of *Enterobacter Cloacae*. Antimicrob Agents Chemother 2000; 44: 885-90.
12. Paget GE, Barnds JM. Toxicity tests. In: Lawrence DR, Bacharach editors. Evaluation of drug activities- Pharmacometrics no. 1 London Academic Press, 1964: p.135.
13. Yadav V, Sharma S, Harjai K, Mohan H, Chhibber S. Induction and resolution of lobar pneumonia following intranasal instillation with *Klebsiella pneumoniae* in mice. Indian J Med Res 2003; *118:* 47-52.
14. Lynch JP. Hospital-acquired pneumonia Risk factors, microbiology and treatment Chest 2001;119:373S-384S
15. Mayhall CG. Nosocomial pneumonia: diagnosis and prevention. Infect Dis Clin North Am 1997; 11: 427-457.
16. Campbell GD, Meiderman MS, Broughton WA et.al. Hospital acquired pneumonia in adults: diagnosis, assessment of severity, initial antimicrobial therapy and preventative strategies: a consensus statement. Am J Respir Crit Care Med 1996;153: 1711-25.
17. Barlett JG, O'Keefe P, Tally FP, Louie TJ, Gorbach SL. Bacteriology of Hospital-acquired pneumonia. Arch Intern Med 1986; 146: 868-71.
18. Chandrashekhar MR, Rathish KC, Nagesha CN. Reservoirs of nosocomial pathogens in neonatal intensive care unit. J. Indian Med Assoc 1997;95:72-4,77.
19. American Thoracic Society document. Guidelines for the management of adults with hospital acquired, ventilator-associated and health care associated pneumonia. Am J Resp Crit Care Med 2005; 171: 388-416.
20. Ewig S, Ruiz M, Torres A, Marco F, Martinez JA, Sanchez M and Mensa J. Pneumonia acquired in the community through drug-resistant *Streptococcus pneumoniae.* Am J Respir Care Med 1999; 159: 1835-42.
21. Metlay JP, Hofmann J, Cetron MS, Fine MJ, Farley MM, Whitey C and Breiman RF. CID2000; 30: 520-28.
22. O'Grady F. Animal models in the assessment of antimicrobial agents. Journal of Antimicrob. Chemother 1976; 2:1-8.