Histological and Bacteriological Studies on the Effect of Some Antibiotics on Klebsiella pneumoniae-infected rats

1Sahar K.A. Darwish and 2 Hayam M. Hamouda

1Histology Department
2Microbiology Department, National Organization of Drug Control and Research (NODCAR).

ABSTRACT

Combination antimicrobial therapy is often necessary to treat infections caused by Gram-negative resistant bacteria. In vitro minimum inhibitory concentration (MIC) of 20 clinical isolates of Klebsiella pneumoniae against six tested antibiotics ranged from 0.5 to >256 µg/ml. Checkerboard tests yielded synergistic fractional inhibitory concentration (FIC) index ≤ 0.5 for 40% of the strains when using Cefepime with Levofloxacin, Azithromycin and Amikacin. Combinations of β-lactam and quinolone yielded synergetic and additive activities (FIC index ≥ 0.5), but no antagonistic activity (FIC > 4.0) has been yielded among the tested combination of antibiotics. In vivo efficacies of either single or combined antibiotics therapy against resistant K. pneumoniae were tested. Amikacin, Cefepime, Azithromycin and Levofloxacin were tested in groups on female rats infected with isolated K. pneumoniae. Lung tissues from the “infected non-treated” and “infected treated” groups were histologically examined; the severity of inflammation, and damage of the pulmonary tissues produced by the K. pneumoniae, were compared with those of the treated groups. The obtained results indicated that the best results were achieved in Amikacin combined with Cefepime, Azithromycin combined with levofloxacin, respectively. Meanwhile, in mono-therapy, using Cefepime, Amikacin, Levofloxacin or Azithromycin, reduction in number of inflammatory foci, edema and lung damage, respectively were shown.

Key words: Antibiotics, imipenem, cefepime, Azithromycin, Amikacin, levofloxacin, Klebsiella pneumoniae. Inflammation, In vitro study.

Introduction

The evolution of bacteria, which have acquired resistance to the vast majority of antibiotics, is one of the main problems in clinical settings. Challenged by decades of drug exposure, bacteria have evolved through adaptation and natural selection defensive mechanisms that render antimicrobials impotent. Therefore, there is a vital need to develop new effective therapeutics. During the last two decades, antibiotic resistant mutant strains extended spectrum β-lactamases producing (ESBLs) as E. coli and K. pneumoniae have predominated (Iroha et al., 2009); this gave these strains a clinical and therapeutic importance.

On the other hand, in developing countries such as Nigeria, clinical isolates of K. pneumoniae were isolated from urine, stool and blood, where it was found that 36.2% of the isolates were ESBL producers (Iroha et al., 2008). In 2009, another study was done on K. pneumoniae isolated from other two hospitals in Nigeria and reported that the total percentage of ESBL occurrence was 33.5% where it reached in one of both hospitals 61.5% (Iroha et al., 2009).

Nosocomial infections caused by these bacteria are frequently difficult to eradicate using monotherapy antimicrobial agent because of the ability of bacteria to develop resistance (Hosgor-Limoncu et al., 2008) therefore, the need to a combined therapy is a must.

Combination therapy was defined as treatment with more than one antibiotic that are active in vitro against the organism for at least two days. (Korvick et al., 1992). The authors added that the use of synergistic combination in antimicrobial chemotherapy is often used commercially for the treatment of various infections. Evaluation of the therapeutic effectiveness of these drugs against K. pneumoniae in experimental animals is important because such models allow for comparison of antibiotics under similar conditions of intensity and duration of infection (Bakker-Woudenbergen et al., 1982)

The aim of this work was to evaluate the effect of some antibiotics applied as mono and combined therapy on K. pneumoniae in infected rats. The use of animal model in this study will provide better understanding of the potential therapeutic value of these combinations in combating K. pneumonia infections.

Material and Methods

Corresponding Author: Sahar Kamal Amine Darwish, Lecturer in Histology, Histology Department, National Organization of Drug Control and Research (NODCAR).
E-mail: sahardarwish24@yahoo.com
In vitro study:

-Bacterial strains:

Twenty isolates of *K. pneumoniae* were taken from Pathology Laboratory of Kasr El-Ainy Hospital where they were collected from urine specimens in 2009. Suspect colonies, cultured on selective media (McConkey), incubated overnight and were then identified by biochemical tests (Murray *et al.*, 2003). Once identified, the bacterial cultures in TSB (Difco) were mixed with glycerol (20%) (Shinyo Pure Chemicals Co., Ltd. Japan) and stored at -70 °C for further use.

Antibiotic susceptibility:

All *K. pneumoniae* bacterial isolates were tested with antibiotic susceptibility discs by disk diffusion tests (DDT) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2010). Antibiotic discs were purchased from Mast Diagnostics (Mast Group Ltd, Merseyside, U.K) and Oxoid Limited (Oxoid, Hampshire, England). The active antibiotic ingredients were Amikacin (AK), Levofloxacin (Lev), Azithromycin (AZ), Imipenem (IMP) and Cefepime (CPM). They were dispensed onto the surface of the inoculated Mueller-Hinton agar plates. The respective isolate was considered resistant if the diameter of the inhibitory zone was ≤ 14 mm for AK, Lev, AZ, IMP and CPM. Results were interpreted according to recommendations of (CLSI, 2010).

Minimum inhibitory concentration (MIC):

MICs were determined by the micro-agar dilution method for all strains showing complete or decreased inhibition zone diameter in the disk diffusion test (CLSI, 2010). *K. pneumoniae* ATCC 10031 was used for quality control.

Combination by a checkerboard method:

For measuring combination effect of drugs, isolates of *K. pneumoniae* were tested by checkerboard method. Ten serial (0.125-265µg/ml), twofold dilutions of the antibiotics were prepared and added to the wells of each 96-well plate, so that it could contain various concentration combinations of the two antibiotics used.

Mueller-Hinton broth was used to inoculate checkerboard microtiter plates according to the guidelines published by (Eliopoulos and Moellering, 1996).

Fractional Inhibitory Concentration:

The fractional inhibitory concentrations (FICs) for each isolate were calculated by dividing the lowest concentrations of the combination of two agents inhibiting growth by the MIC for each agent alone test. The FIC index was calculated by summing the individual FICs obtained for the two antimicrobial agents. Synergy was defined as an FIC index of < 0.5, additive by an FIC index of > 0.5 to < 4 and antagonism by an FIC index of >4 (Hsieh *et al.*, 1993).

In vivo study:

-Animals:

Female white albino rats (120-150 g) body weight, bred at the Laboratory Animal center of National Organization of Drug Control and Research (NODCAR), were used in the experiments. The animals were kept in metal cages, 6 animals / cage, under controlled temperature and humidity. They were fed commercial pellet diet, and supplied with water *ad libitum*, and kept adapted to the laboratory conditions for two weeks before commencing the experiment.

Experimental pneumonia model in rat:

Mid-logarithmic phase *K. pneumoniae* was suspended in PBS to a final density of 1x10 CFU/ml. The rats were intraperitoneally injected with 50µl of *K. pneumoniae* bacterial suspension. (Pichardo *et al.*, 2005 and Hopkins *et al.*, 1995).
Antimicrobial Treatment:

To evaluate the effectiveness of the different treatment regimens, 48 rats were inoculated with *K. pneumoniae* strains. They were divided into eight groups, each group 6 animals, the first group was kept as frank group, the second group as infected non-treated group, while the other 6 groups received "Amikacin, Azithromycin, Levofloxacin or Cefepime “as mono-therapies and "Cefepime and Amikacin" and "Levofloxacin and Azithromycin " as combined therapies. The antibiotics doses were therapeutic dose calculated for rat with reference to the human as suggested by Paget and Barnes (1964).

The first dose of every antibiotic was administered 24 hrs after inoculation with the following dose schedule:

<table>
<thead>
<tr>
<th>Drug (Manufacturer)</th>
<th>Route</th>
<th>Dose*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin (Bristol-Myers Squibb-Egypt)</td>
<td>intramuscularly</td>
<td>0.27mg/rat/7 successive days</td>
</tr>
<tr>
<td>Cefepime (Bristol-Myers Squibb-Egypt)</td>
<td>intramuscularly</td>
<td>18mg/rat/7 successive days</td>
</tr>
<tr>
<td>Azithromycin (Pfizer-Egypt)</td>
<td>Orally</td>
<td>9mg/rat/7 successive days</td>
</tr>
<tr>
<td>Levofloxacin (Amoun Pharmaceutical Co.)</td>
<td>Orally</td>
<td>9mg/rat/3 successive days</td>
</tr>
</tbody>
</table>

*All doses were calculated for white albino rat with reference to the human therapeutic dose as suggested by Paget and Barnes (1964).

Histological procedures:

The histological features of the pneumonic lesions were studied in infected non-treated and infected-treated groups. Lung tissues were gathered from the animals after anesthesia with diethyl ether, fixed in 10% neutral formalin, proceeded to paraffin sections and stained with haematoxylin and eosin dyes for routine pathological examination (Banchroft, et al., 1996). Orcein stain for elastic fibres (Mcmanus and Mowry, 1964) and PAS technique for goblet cells were also prepared and examined.

In vivo Histochemical Evaluation:

To evaluate the effects of the drugs used in this experiment on the homeostasis of the lung, goblet cells stained with (PAS) was explored according (Culling, 1974).

Histomorphometrical analysis:

The histomorphometrical analysis was done on lung tissues in all experimental groups, to estimate elastic fibres content using orcein stain. The quantification of elastic fibres in alveolar walls was performed using Leica Quin image analyzer computer system. Ten fields from each specimen were chosen; in each field, the elastic fibres were measured using Optical Density measurement tool. The significance differences among groups means were evaluated using oneway Anova-SPSS program (version 11) for windows software.

In vitro results:

Antibiotic susceptibility:

Disk diffusion tests showed a reduced inhibition zone diameter to antimicrobial agents for most of the 20 *K. pneumoniae* isolates.

A relative comparison between MIC results of antimicrobial agents used against *K. pneumoniae* isolates is shown in Table 2.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>% of inhibited bacteria at MIC in μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥256</td>
</tr>
<tr>
<td>Cefepime (CPM)</td>
<td>7/20</td>
</tr>
<tr>
<td>Gentamicin (G)</td>
<td>8/20</td>
</tr>
<tr>
<td>Amikacin (AK)</td>
<td>7/20</td>
</tr>
<tr>
<td>Tobramycin (TOB)</td>
<td>5/20</td>
</tr>
<tr>
<td>Azithromycin (AZ)</td>
<td>2/20</td>
</tr>
<tr>
<td>Levofloxacin (Lev)</td>
<td>8/20</td>
</tr>
</tbody>
</table>

Table 2 shows that the most effective antibiotics were Cefepime and amikacin with lowest effective MIC; while the lowest effective antibiotics was tobramycin, gentamycin and azithromycin with highest MIC.
Table 3: FIC relative values for *K. pneumoniae* isolates (in μg/ml).

<table>
<thead>
<tr>
<th>Antibiotic combinations</th>
<th>Synergetic (if FIC ≤ 0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin + Azithromycin</td>
<td>16/20</td>
</tr>
<tr>
<td>Levofloxacin + Amikacin</td>
<td>12/20</td>
</tr>
<tr>
<td>Levofloxacin + Cefepime</td>
<td>16/20</td>
</tr>
<tr>
<td>Amikacin + Azithromycin</td>
<td>8/20</td>
</tr>
<tr>
<td>Amikacin + Cefepime</td>
<td>16/20</td>
</tr>
<tr>
<td>Cefepime + Azithromycin</td>
<td>8/20</td>
</tr>
</tbody>
</table>

No antagonism was demonstrated between any antibiotic combinations tested. Combinations of levofloxacin with amikacin and azithromycin as well as combination of amikacin with azithromycin demonstrate more synergy against *K. pneumoniae*. Meanwhile, combinations of levofloxacin/cefepime as well as cefepime/amikacin and cefepime/azithromycin demonstrated additive effects against *K. pneumoniae* as shown in table "3".

*In vivo results:*

*K. pneumoniae*-infected group:

Histological examination of lung tissues in this group revealed edematous pulmonary vasculature, denuded pseudostatified epithelial cells lining many bronchioles, this is beside multifocal areas of perivascular lymphocytic infiltrates could be seen. Marked thickening of alveolar septa and pleura could also be detected. (Figs. 1&2).

Monotherapy groups:

*Amikacin treated group:*

*K. pneumoniae*–infected rats, treated with amikacin, revealed moderate curative effect; where the foci of inflammatory cells infiltrate in peribronchial and perivascular areas were reduced and perivascular edema was significantly ameliorated. However, in three out of six animals within the group, fibrotic reaction in peribronchial areas, was noticed together with focal architecture disruption and focal area of emphysema (Figs. 3 &4).

*Cefepime treated group:*

Mild to moderate degree of improvement comparing to infected non-treated group was observed where peribronchial inflammation and perivascular edema were reduced. However, bronchioles with desquamated cells in their lumen, area of thickened alveolar septa with proteinaceous fluid distending the alveolar spaces were seen together with areas of collapsed alveoli. (Fig. 5).

*Azithromycin -treated group:*

In this group, lung tissues display the least effective treatment where moderate to marked thickened edematous wall of blood vessels accompanied with dilated congested peribronchial venules and capillaries; many small foci of macrophages mixed with polymorph and eosinophiles were seen. Marked thickening in the pleura with many macrophages, and marked thickened and congested capillaries in alveolar septa were also observed. (Figs. 6&7).

*Levofloxacin treated group:*

In this group, lung tissues revealed moderate pathological alterations where small discrete inflammatory foci of eosinophiles and macrophages around bronchioles and blood vessels were seen, together with degenerative changes in the epithelial cells lining the bronchioles. This was accompanied with hemorrhagic interstitial areas and pronounced thickening of alveolar septa. Some alveoli spaces filled with eosinophilic proteinaceous fluid were occasionally seen accompanied with disrupted alveolar architecture in many areas. (Fig.8)
Fig 1: Photomicrograph of lung tissue of *K.pnumoniae* infected-non treated animal, showing interstitial hemorrhagic area (arrow), marked thickening of alveolar septa (arrow head). H&E, X: 100.

Fig 2: Photomicrograph of lung tissue of infected non treated animal, demonstrating degenerative changes in bronchiole wall (BL), inflammatory cells infiltrate (Arrow). H&E, X: 200.

Fig 3: Photomicrograph of lung tissue of *K.pnumoniae* infected- Amikacin treated rat showing, perivascular fibrotic area (arrow), alveolar septa with inflammatory cells infiltrate and macrophage (arrowhead). H&E, X: 200
Fig. 4: Photomicrograph of lung tissue of *K. pneumoniae* infected- Amikacin-treated rat showing alveolar septa with congested blood capillary (arrow) macrophage (arrowhead) polymorph in alveolar space (AS). H&E X: 400.

Fig. 5: Photomicrograph of lung tissue of *K. pneumoniae* infected – Cefebim treated rat showing, perivascular edema, pulmonary blood vessel (BV), desquamated cell in the bronchiole lumen (BL), degenerated epithelial cells lining the bronchiole (arrow), and emphysema (E). H&E, X: 200.

Fig. 6: Photomicrograph of lung tissue of *K. pneumoniae* - Azithromax treated rat showing marked thickening of the pleura (arrow), congested blood vessel (arrowhead). H&E, X: 40.

Fig. 7: Photomicrograph of lung tissue of *K. pneumoniae* - Azithromax treated rat showing edematous pulmonary vessel wall (BV), marked thickening of the alveolar septa (arrowhead) desquamated cell in the bronchiole lumen (BL). H&E, X:200

Fig. 8: Photomicrograph of lung tissue of *K. pneumoniae* infected - Levox treated rat showing marked thickening and edema of pulmonary vessels walls (arrow), foci of inflammatory cells aggregates(Arrowhead) thickening of some alveolar septa (thin arrow). H&E, X: 200.

Combined Therapy:

**Levofoxacin + Azithromycin-treated group:**

When this combined treatment was applied to *K. pneumoniae* infected rats, significant curative effects were seen, and wide areas of the examined lung tissues were intact. However, one third of the number of the examined animals within this group, showed the previously mentioned pathological changes (Fig. 9).

**Amikacin + Cefepime treated group:**

The best results were achieved in this group, where in 90% of the examined lung tissues, most of pneumonic areas retained their normal appearance. (Fig. 10).

In vivo Histochemical Studies:

To evaluate the effects of either mono or combined therapy- used in the present experiment on the homeostasis of the lung-goblet cells demonstration using PAS Technique was used. In *K. pneumoniae*-infected non-treated rats, areas of pulmonary bronchi and bronchioles showed significant increase in numbers and PAS-positive intensity of goblet cells (Fig.11). However, in groups treated with levofloxacin, cefepime and azithromycin, moderate PAS-positive cells were clearly seen in pulmonary bronchi and many distal airways. In Amikacin, these cells were significantly reduced in number and stainability (Fig.12). In combined treatment, either levofloxacin with azithromycin or amikacin with cefepime, pulmonary bronchioles still exhibited few functioning goblet cells, meanwhile almost PAS-negative cells were seen in the distal airways.(Fig.13).

Morphometrical results:

Data in table "4" and Figs.14,15,16,17) represent the quantitation of elastic fibres content of the examined lung tissues in all groups.

Significant elevation in elastic fibers in monotherapy groups, compare to *k. pneumoniae* —infected non-treated groups ,Amikacin *k. pneumoniae-*treated group given the most significant outcome, In combined treated groups very highly significant elevation was noticed, However, in levox+Azithromax treated group, this elevation was remarkable compared to Amikacin +Cefepim combined group.

Table 4: Effect of monotherapy and combined therapy on elastic fibres content in lungs of *k. pneumoniae*-infected non treated and infected treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em>-infected non treated</td>
<td>0.648 ± 0.0757</td>
</tr>
<tr>
<td>Amikacin</td>
<td>0.983 ±0.1814</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.899 ±0.09</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.868 ±0.0068</td>
</tr>
<tr>
<td>Levofoxacin</td>
<td>0.681 ±0.00526</td>
</tr>
<tr>
<td>Levox+Azithromax</td>
<td>0.952 ±0.01218</td>
</tr>
<tr>
<td>Amikacin+Cefepim</td>
<td>0.924 ±0.00670</td>
</tr>
</tbody>
</table>
Fig 9: Photomicrograph of lung tissue of *K.pnumonae* infected- (Levofloxacin + Azithromax) treated rat showing congested dilated blood capillary (arrowhead), normal alveolar septa (arrow). H&E, X: 400

Fig 10: Photomicrograph of lung tissue of *K.pnumonae* infected -(Amikacin + Cefibim) treated rat, showing intact pulmonary vessel (BV),mild lymphocytic aggregates (Arrow),intact alveolar septa (Arrow head), bronchiole with shed out cells in the lumen (BL). H&E,X: 200.
**Fig. 11:** Photomicrograph of lung tissue of *K. pneumoniae* infected non treated animal showing significant increase in number and activity of goblet cells (Arrow) bronchiole lumen (BL), pulmonary vessel (BV). PAS method, X:100.

**Fig. 12:** Photomicrograph of lung tissue of *K. pneumoniae* infected treated animal with single antibiotic, demonstrating goblet cells (Arrow) with moderate decrease in number and activity. PAS, X: 200

**Fig. 13:** Photomicrograph of lung tissue of *K. pneumoniae* infected animal treated with combined antibiotics, showing significant reduction of goblet cells numbers. PAS, X: 200

**Fig. 14:** Photomicrograph of lung tissue of *K. pneumoniae* infected non treated animal, showing significant reduction of elastic fibres in alveolar septa. Orcein stain, X:200.

**Fig. 15:** Photomicrograph of lung tissue of *K. pneumoniae* infected rat treated with monotherapy antibiotic demonstrating moderate content of elastic fibres in alveolar septa. Orcein stain, X: 200.

**Fig. 16:** Photomicrograph of lung tissue of *K. pneumoniae* infected rat treated with combined therapy, reflecting elastic fibres content nearly normal. Orcein stain, X: 200.

**Fig. 17:** Elastic fibres content in the infected and infected -treated group.

**Discussion:**

The evolution of bacteria, which have acquired resistance to the vast majority of antibiotics, is one of the main problems in clinical settings. Challenged by decades of drug exposure, bacteria have evolved through adaptation and natural selection defensive mechanisms that render antimicrobials impotent (Iroha et al., 2009). Therefore, there is a vital need to develop new effective therapeutics.

In this study, evaluation of the effect of some antibiotics and combined antibiotics applied to *K. pneumoniae*-infected rats was done in attempt to overcome the resistance of this organism against these therapies. *In-vitro* results of this study confirmed that *K. pneumoniae* isolates had high resistance against most commonly used antibiotics. Disk diffusion tests showed a reduced inhibition zone diameter to antimicrobial agents for most of the twenty *K. pneumoniae* isolates. In addition, antibiotic susceptibility test results showed high resistance percentage. Lower ratios have been reported by many investigators, worldwide. For instance, in January 2011 in Nigeria, analysis of antibiotic susceptibility on *K. pneumoniae* isolates showed resistance percentage from 0%-26% for tobramycin, amikacin, some cephalosporins and gentamycin (Iroha et al., 2011). However, another susceptibility study on 1998 showed much lower percentage (0%-5.3%) when done against some cephalosporins and gentamycin in Slovak Republic.
Hostacka' and Siposova'(1998) stated that similarities and differences in data concerning antimicrobial resistance may be due to the fact that they may have been collected on different periods and different areas.

Most of the isolates showed co-resistance to non-beta-lactam antimicrobials, especially to gentamicin, and levofloxacin. This may agree with Iroha et al.(2011) where K. pneumoniae showed high resistance against gentamicin. However, regarding levofloxacin, most results showed that it is still effective therapy against K. pneumonia (Siegrist et al., 1999).

The considerably high MIC values for Amikacin (AK), Levofloxacin (Lev), Azithromycin (AZ), Tobramycin (TOB) and Cefepime (CPM) as shown in the present study reflect the extent of treatment problem for K. pneumoniae isolates in Egyptian hospitals. However, combination therapies showed hopeful results of synergistic effects (Levofloxacin with Amikacin and Azithromycin) and additive effects (Cefepime with Levofloxacin, Amikacin and Azithromycin) against K. pneumoniae. Generally, in many studies synergistic interaction has been reported between beta-lactams and aminoglycosides (Hosgor-Limocnu et al., 2008 Burgess and Nathisuwan, 2002). For beta-lactam and fluoroquinolone combinations, variable antimicrobial interactions have been reported (Song et al., 2003). Antagonism has occasionally been reported in a small percentage of isolates. It was thought that synergism was obtained only when the organism was susceptible to two antimicrobial agents. However, some studies showed that synergy may occur between two antimicrobial agents although the strains were resistant to the individual antibiotics (Song et al., 2003 and Hosgor-Limocnu et al., 2008).

The variability of the results obtained in this study and those of other investigators may be due to the lack of standardized methods for comparing antimicrobial agents alone and in combination using the checkerboard assay (Hsieh et al., 1993). However, a study conducted by Mackay et al. (2000), comparing the checkerboard method with time-kill methodology after 24 h reported that the calculation of the FIC index was a good indicator of synergistic bactericidal activity. The term pneumonia refers to an inflammatory reaction of the lower respiratory tract, i.e., within the terminal bronchioles, alveolar spaces and/or the alveolar walls (septa) of the lung. As with any inflammatory process, the pathophysiology of pneumonia is characterized by vascular changes and movement of fluid and white blood cells out of the microcirculation. In the present work, the in vivo experiment data demonstrate that pneumonia in the rat, induced by K.pneumonia achieved most of the histological features of pneumonic lesions were reported previously by many investigators (Bakker-Woudenberg et al., 1982 Bernedet et al., 1976; Grims, 2007). Also, Pichardo et al. (2005) reported acute pneumonia was resulted in mice groups inoculated with strains of K. pneumonia and showed acute inflammation characterized by diffuse and/or focal affectation of all lobes, with a mild to severe inflammatory infiltration of polymorphonuclear cells, and with mild to moderate infiltration of alveolar macrophages, and alveolar hemorrhage.

In antibiotics monotherapy phase, the in vivo data demonstrated the efficacy of Amikacin in resolving the pneumonia infection, and this efficacy was higher than Cefepim, Levofloxacin, or Azithromax.. These results were confirmed by in vitro results showed in table“2”, and agree with Pattharachayakul et al.(2003), who stated that, Amikacin demonstrated the fastest killing rate. In other way, K.pneumonia strain was uniformly susceptible to all cephalosporins and aminoglycosides (Wang et al., 1998; Chang et al., 2000). Levofloxacin has demonstrated a wide range of activity against gram –positive, gram negative organism both in vitro and in vivo (Davis and Bryson, 1994; Fu et al., 1992). One could attribute the moderate efficacy of the tested antibiotics treatment as monotherapy, either to the resistance of the bacteria which is evident by the in vitro study, or to the vascular necrosis and edema in the lung prevented access of antibiotics both from the airways and the circulation (Bernedet, 1976).

The remarkable improvement in lung tissues of rats in combined therapy groups, which again strongly supported the in vitro data, where the additive and synergistic activity between bet –lactams (Cefepim) and aminoglycosides (Amikacin), was in agreement with many workers (Burgess et al., 2003; Burgess et al., 2002; Bosso, et al., 1990).

To elucidate the role of the different antibiotics used in the present study, in abrogate the destructive effects of the bacteria and helping to restore lung functions, the change in goblet cells profile (as mucous secretory cells) was demonstrated. Goblet cells are found among the epithelial lining in the respiratory tracts, they synthesize, store and secret mucin that influence mucociliary clearance and innate defence of the lung (Ishikawa et al., 1994; Salathe et al., 2002). In rats, goblet cells are said to be very infrequent in peripheral airways (Jones et al., 1973). It is well established previously that numbers and activity of goblet cells are induced by a variety of acute and chronic inflammatory stimuli (Chen et al, 2009). In the present work, goblet cells reacted positively with PAS reaction, in lung tissues in either infected, non-treated and infected treated groups, was used as a tool to demonstrate the effect of both mono and combined therapy phases on goblet cells number and activity. Hence one can estimate that the significant increase in goblet cells stainability and number in K.pnemoniae infected group, met with significant reduction in mono therapy, and in combined therapy almost normal condition was observed. This change in goblet cells profile suggests the role of either antibiotics monotherapy, to some extent and combined therapy in restoring lung tissues homeostasis.
In rodents, goblet cells were few in number and reduced size in bronchi, bronchioles, the increase in number and PAS-reactivity came along with the inflammatory status of the animals (Murata et al., 1996; Finkelman et al., 2005), hence the changes in number, size and reactivity reflect the response of the lung tissues of the experimental animals to the received either monotherapy or combined therapy. The present data indicate significant reduction in goblet cells number and size in monotherapy groups, while in combined therapy, this reduction was very significant.

In normal lung, elastic and collagen fibres are responsible for compliance and lung distensibility (Lang et al., 1993). The collagen and elastic systems, the major fibrous components of the extracellular matrix, have been addressed in previous reports. In an attempt to establish a correlation between alterations in their content and possible deleterious consequences for pulmonary function (Rozen et al., 2005). In several studies by Parra et al. (2009) demonstrated that lung collagen and elastic content are increased in both acute and chronic respiratory bronchiolitis interstitial lung disease. In the present work, the elastic fibres content in the k.pneumoniae-infected non-treated group was significantly lower than in the treated groups either mono or combined therapy groups. These results disagree with many investigators, who reported increase in elastic fibres content in many lung diseases (Saldiva et al., 1989; Rocco et al., 2001). The possible explanation in this work depends on the theory stated the role of the inflammatory cells infiltrates and macrophages in changing elastic fibres profile, where these cells may contribute in releasing proteases and neutrophil elastase which has the potential to preferentially disrupt the elastic network (Dona et al., 2003, Cotran et al., 1994). Valenca et al. (2004) showed that mice exposed to cigarette smoking exhibited an increased number of alveolar macrophages as evident in the present data, where macrophages were predominant. The macrophages produce and release cathepsin B, D, L and S, matrillisin, gelatinase A and B, and MMP-12 (Shapiro, 1994; Ohnisi et al., 1998). All these proteases exert a highly proteolytic role in the lung, and some are capable of inhibiting or inactivating or even degrading al-protease inhibitor and other anti-proteases, largely contributing to the elastolytic process (Valenca et al., 2004).

Alveolar macrophages metalloelastase, not neutrophil elastase, degrade elastin (Niewoehner, 1988; Evans and Pryor, 1994; Shapiro, 1994). This enzyme, the primary elastase enzyme of the macrophage, can hydrolyze a broad spectrum of extracellular matrix components and has been seen as necessary for macrophage-mediated proteolysis of the extracellular matrix during invasion (Shipley et al., 1996). It is probable that other enzymes have participation in the continuity of the degradation of septal extracellular matrix components (Valenca et al., 2004).

One can speculate that the ability of the antibiotics used in this work to kill bacteria and hence render the inflammatory responses is the key role in overcome goblet cells hypersecretion and activity and in restoring elastic fibres contents.

In conclusion, the present work, showed that infection with these highly resistant isolates are increasing and spreading in hospitals and community. This may be due to antibiotic misuse and low hospital finances for controlling the spread of these isolates. Thus we recommend further researches for controlling resistance and for sparing other combination therapies of least side effects for emergencies and stubborn cases.

Acknowledgment

The authors thanks dr.Dalia M.Marzouk for providing the authors with bacterial isolates.

References


CLSI, 2010. Performance standards for antimicrobial susceptibility testing. *Clinical and Laboratory Standards Institute*, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 1898 USA.


