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Pathogenicity of Carbapenems and Third Generation Cephalosporins Resistant *K. pneumoniae* in Murine Urinary System

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Abstract

One hundred and nine clinical lactose fermenter isolates were collected from different samples (urine, stool, wound swab, blood, and sputum) , in a period from February 2014 till May 2014. All samples gathered from Alyarmok laboratories, Alkadimiya laboratories, and Baghdad teaching laboratories which are situated at Baghdad city. Fifty three (48,62%) isolates were identified as *Klebsiella pneumoniae* depending on microscopic characterization , conventional biochemical tests and then the identification confirmed with API 20E system . The rest of 56(51, 38%) isolates represented other bacteria.Susceptibility test was achieved to all fifty-three *K. pneumoniae* isolates using five antibiotic disks (Ceftazidime, Ceftriaxone, Cefotaxime, Imipenem, and Meropenem). Most of tested isolates were susceptible to Meropenem and Imipenem, 90.5% and 77.3%, respectively, and less susceptible to third generation Cephalosporin. Pathogenicity of Carbapenems and the third generation Cephalosporins resistant *K. pneumoniae* in murine urinary system in this study revealed Cabrapenem resistant isolates (K2 and K3) displayed severe histopathological changes compared to the third generation Cephalosporins resistant isolates (K6 and K46).

Keywords: Carbapenems, *Klebsiella pneumoniae*, Murine urinary system.

امراضية الكليبسيلا الرئوية المقاومة لمضادات الكاربابنيم والجيل الثالث سيفالوسبورين في الجهاز البولي للفئران

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الخلاصة

جمعت 109 عزلة مخمرة لسكر اللاكتوز للمدة من شباط ولغاية شهر ايار 2014 من مختبرات مستشفى اليرموك والكاظمية ومستشفى بغداد التعليمي في مدينة بغداد حيث شملت مختلف العينات السريرية (ادرار و خروج ومسحات جروح وعينات دم وقشع). وكانت نسبة بكتريا *K. pneumoniae* 48,62% تعود لنوع *K. pneumoniae* وباقي انواع البكتريا شكلت نسبة 51,73% . تم تشخيص عزلات *K. pneumoniae* اعتمادا على الصفات المظهرية والمجهرية والفحوصات الكيموحياتية . وقد تم تأكيد التشخيص باستعمال نظام Api 20 E . اظهرت نتائج فحص الحساسية الدوائية لثلاث و خمسين عزلة *K. pneumoniae* باستعمال خمسة انواع من مضادات الحياة (سيفتازديم و سفتراكزون و سيفوتكسايم و اميبينيم و ميروبينيم) ، اظهرت معظم العزلات قيد الدراسة حساسية لمضادي ميروبينيم واميبنيم بنسب مئوية 90.5% و 77.3% على التتابع. واظهرت العزلات حساسية اقل لمضادات الجيل الثالث سيفالوسبورينات. كمابينت دراسة امراضية العزلات المقاومة لمضادات الكاربابنيم ومضادات الجيل الثالث سيفالوسبورين في الجهاز البولي للفئران ، ان

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عزلات الكليبسيلا الرئوية المقاومة للمضادات (K2 و K3) اظهرت تغييرات نسيجية شديدة مقارنة بالعزلات المقاومة للجيل الثالث سيفالوسبورين (K6 و K46).

Introduction

Klebsiella pneumoniae is Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod-shaped bacterium, found as a normal flora of the mouth, skin, and intestine [1]. However, it exists in the respiratory tract and feces of about 5% of normal individuals. It can produce extensive hemorrhagic necrotizing consolidation of the lung and occasionally produces urinary tract infection and bacteremia with focal lesions in debilitated patients [2].

In vitro studies have demonstrated that about 40% of *K. pneumoniae* isolated not only from urine, but also from sputum, blood and wound swabs, were capable to produce biofilm [3]. The development 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 essential to develop new effective therapeutics. During the last two decades, antibiotic resistant mutant strains extended spectrum bet-lactamases creating (ESBLs) as *E. coli* and *K. pneumoniae* have predominated [4].

Beta lactamases genes carrying a *K. pneumoniae* isolate has been revealed to be the most virulent in the murine sepsis model and the stronger biofilm producer, with respect to the non- Beta lactamases genes transport isolates [5]. The 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 [6].

Although *K. pneumoniae* owns only moderate amounts of chromosomal penicillinases, it is a well-known "collector" of multidrug resistance plasmids that commonly encoded resistance to aminoglycosides, till the end of 1980s, while, later, encoding extended-spectrum β-lactamases (ESBLs), mostly Temoniera (TEMs) and Sulfhydryl variable (SHVs) active against last generation Cephalosporins, as well as a diversity of genes conferring resistance to drugs other than β-lactams [7]. The acquisition of these plasmids and the happening of chromosomal mutations that confer resistance to Fluoroquinolones often makes the treatment of *K. pneumoniae* healthcare-associated infections possible only by using Carbapenems as "last-resort of defense" antibiotics [8]. From the early 2000s, multidrug-resistant (MDR) *K. pneumoniae* strains started to produce also "carbapenemases" (KPC) encoded by transmissible plasmids and rapidly disseminated within both acute hospitals and long-term care facilities. Later, other enterobacterial species, including *E. coli*, acquired carbapenemase genes, thus proposing that *K. pneumoniae* may have acted as a pool of β-lactamases [10].Over the past decade, carbapenemases of Classes A, B and D, which are β-lactamases capable to efficiently hydrolyze Penicillins, Cephalosporins, Monobactams, Carbapenems and β-lactamase inhibitors, have progressively spread among *Enterobacteriaceae* [10].

Klebsiella organisms are often resistant to multiple antibiotics. Present evidence implicates plasmids as the primary source of the resistance genes [11]. Antibiotic-resistant bacteria cause a substantial burden on the human population. In addition to morbidity and mortality caused by infections with resistant pathogens. In an environment that has an antibiotic; possession of corresponding resistance genes is clearly beneficial to a bacterium. However, in the lack of antibiotic, resistant genotypes may have lower growth rates than their sensitive counterparts [12].

Aim of this study was to compare the pathogenicity of isolates that resist third generation with those resist Carbapenems through injection the urinary system using mouse as a model.

Materials and Methods

One hundred and nine of lactose fermenter clinical isolates were collected from patients (nineteen to sixty years old) visiting Baghdad hospitals include Alyarmok laboratories, Alkadimiya laboratories and Baghdad teaching laboratories. The clinical specimen comprised urine, stool, sputum, blood, and wound swabs. Identify by biochemical test and confirmed by Api 20E (BioMerieux, France) was employed to confirm the identification results.

Isolation and identification

Identification of isolates was achieved according to Bergey's Manual of Systematic Bacteriology, 2nd edition [13], included morphological characteristics and biochemical tests.

Antibiotic susceptibility test

Five antibiotic disks (BioanalyseTurkey) were used in this study included: Ceftriaxon(CRO), Ceftazidime(CAZ), Cefotaxime(CTX), Imipenem(IPM), and Meropenem(MEM) at concentration 30µg, 30µg , 30µg, 10µg and 10 µg respectively . Antibiotic susceptibility test for *K. pneumoiae* isolates was done according to Baur *et al.* method [14], Afterwards, the inhibition zone was measured and comparison with was accomplished. Results were interpreted according to CLSI [15].

Laboratory animals

Female Swiss white mice (*Mus musculus*) were used at 8 weeks age, weighed 20-25 g, obtained from Al-Nahrian University/Biotechnology center. The animals were classified into six groups (designated 1through 6) and three animals per group. Each animal was put in a single cage; however, all animals were fed *ad libitum* the same food and water.

Pathogenicity of *K. pneumoniae* resists carbapenems and third generation cephalosporins in murine urinary system:

Injection protocol

Depening on Mctaggart *et al*. [16], urinary bladder was discharged from urine and the whole area was disinfected by 70% ethanol. 20 µl of bacterial inoculum (adjusted to 0.5 MacFrland standards) was injected transurethrally via 0.6 mm catheter was placed inside the urethra. Six groups (1-6) of female mice were injected by overnight *K. pneumoniae* resitstant to imipenem, cefotaxime, meropenem, ceftazidime, sensitive to all antibiotics, and bacteria free normal saline (which considered as negative control), respectively. The animals were killed after 3 days and kidney and urinary bladder were stored in 10% formalin.

Tissues sections preparation

According to Humason, [17] method , kidney and urinary bladder were stored in 10% formalin, after fixation the tissues were washed with tap water for few minutes , the tissues then passed in serial concentration of ethanol (50, 60, 70, 80 , 90, and 100% respectively) for 2hr to each concentration, after that the samples were cleared with xylol for 1 hr , then embedded by paraffin wax at 60°C for 3hr, the paraffin blocks were cut as a sliced at size about 5 µm with microtome, then the slices placed on a glass slide and passed on water path. The slices were dehydrated in incubator at 37°C for 2 days, after that stained with haematoxylin (5 min), washed with tap water, stained with eosin (1 min), washed with distilled water, the passed through serial concentration of ethanol (70%, 90%, and 100%, respectively) at 2 min for each one, drops of Canada balsam and cover slides were kept on slide and ready for examination under light microscope [17].

Result and Discussion

Isolation and susceptibility test of *K. pneumoniae*

One hundred and nine lactose fermenter clinical isolates from different clinical specimens, Fifty-three (48.62%) isolates represented *K. pneumoniae*; however, 51.73% represented other bacteria. Susceptibility test was achieved to all fifty-three *K. pneumoniae* isolates using five antibiotic disks (Ceftazidime, Ceftriaxone, Cefotaxime, Imipenem, and Meropenem). Most of tested isolates (90.5% and 77.3%) were susceptible to Meropenem and Imipenem, respectively and less susceptible to third generation Cephalosporin.

Pathogenicity of Carbapenems and third generation Cephalosporins resistant *K. pneumoniae* in murine urinary system

Microorganisms

Fifty three (48.62%) isolates were assigned as *K. pneumoniae* , Four isolates of *K. pneumoniae* vis. K2 (resistant to Imipenem), K6 (resistant to Cefotaxime), K3 (resistant to Meropenem), resistant to Ceftazidime), and K42 (sensitive to all antibiotics), were previously isolated (Rhumaid and Al-Mathkhury, in press)[18].

Depending on Metaggart *et al*. [14], groups 1 to 6 of female mice were injected with *K. pneumoniae* resistant to Imipenem (K2), Cefotaxime (K6), Meropenem (K3), Ceftazidime (K46), sensitive to all antibiotics (K42), and bacteria free normal saline (which considered as negative control), respectively.

Negative control

Kidney sections showed normal glomeruli and renal tubules as mentioned in Figure-1. Similarly, section of urinary bladder appeared normal looking appearance of urinary bladder layers as mentioned in Figure-2.

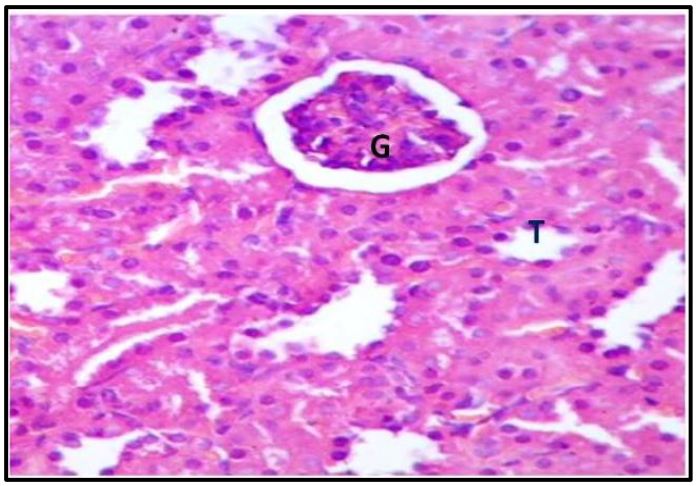

Figure 1- Section of the kidney mouse treated with sterile normal saline, showing normal glomeruli (G) and renal tubules (T). (×400, H&E).

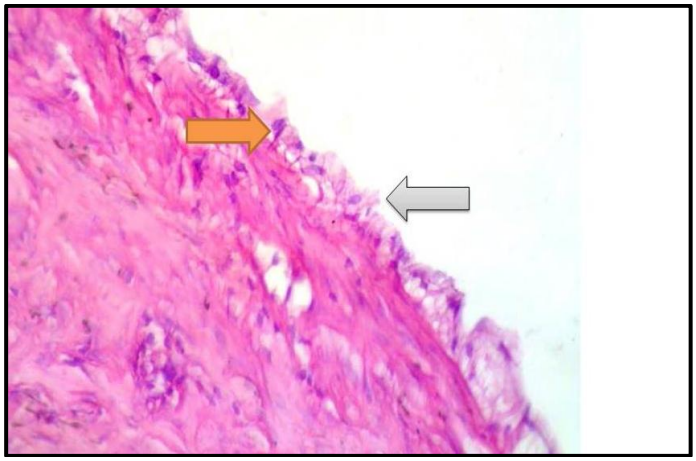

Figure 2- Section of mouse urinary bladder treated with sterile normal saline, showing normal cuticle layer ( ) and normal epithelium appearance ( ) layers (× 200 H&E).

Sensitive isolate

Klebsiella pneumoniae (K42) isolate is sensitive to all tested antibiotics was selected and injected intraurethrally. However, the histological changes in kidney represented by necrosis, prominent chronic inflammatory cells infiltration, oedema, and focal area as shown in Figure-3. Whereas urinary bladder suffered from tissue bladder degeneration, oedema, presence of necrosis, infiltration of inflammatory cells, congestion, and urinary bladder ulceration, as illustrated in Figure-4.

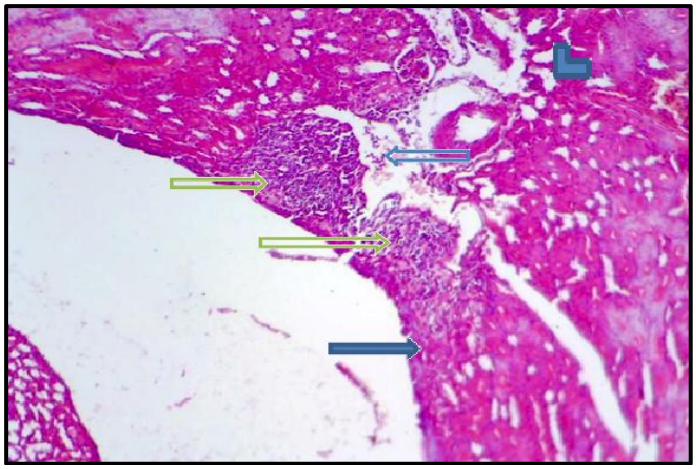

Figure 3- Section of the mouse kidney treated with *K. pneumoniae* (K42) sensitive to all test antibiotics and moderate biofilm producer showing focal area( ) Oedema ( ), Infiltration of inflammatory cells ( ), Necrosis ( ) (X 400 H&E).

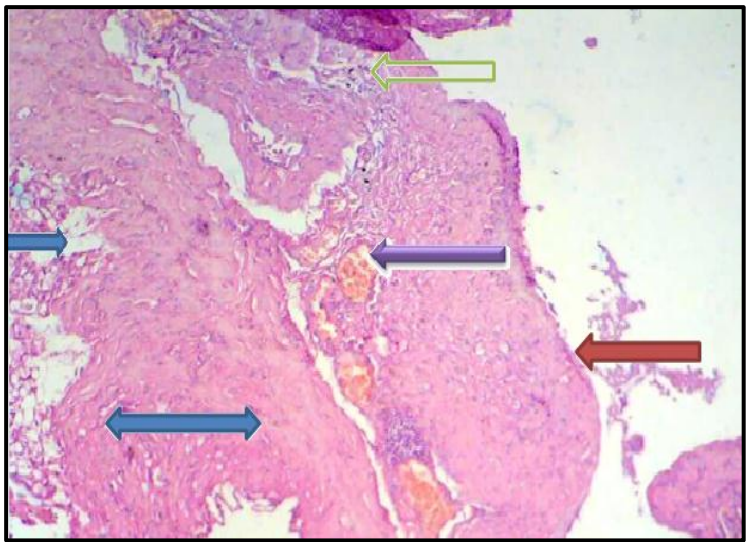

Figure 4- Section of mouse urinary bladder treated with *K. pneumoniae* (K42) sensitive to all antibiotics and moderate produce of biofilm showing: infiltration of inflammatory cells (⇦), congestion (⬅), ulceration (⬅), necrosis (⟷), degeneration (⟷), (X200, H&E).

Imipenem Resistant *K. pneumoniae* (K2) Isolate

Klebsiella pneumoniae (K2) isolate resistant to Imipenem was selected and injected intraurethrally. Several histological changes were noticed in kidney represented by tissue degeneration, necrosis of tubules, inflammatory cell infiltration, and cystic dilation of renal tubules, as depicted in Figure-5.

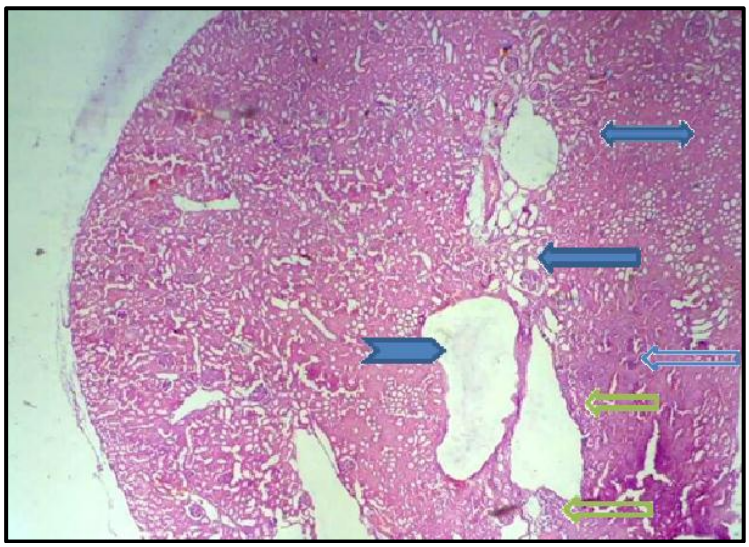

Figure 6- Section of the mouse kidney treated with K2 resistant to Imipenem and moderate biofilm producer showing degeneration (⟷), renal necrosis (⟷), cystic dilation (⟷), Oedema (⟷), Infiltration of inflammatory cells (⇦), (X 100, H&X).

The section of urinary bladder treated with K2 isolate exhibited various histological effects included; mild degeneration, changes of epithelial mucosal lining, necrosis, oedema, and inflammatory cells infiltration Figure-6.

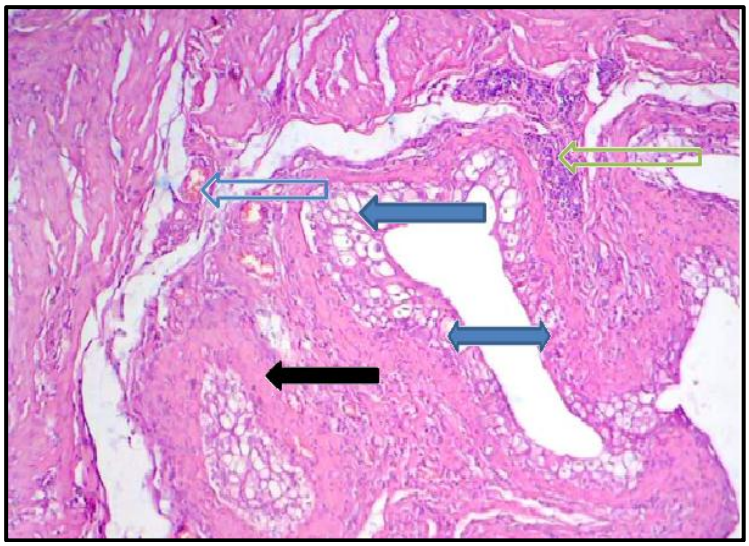

Figure 6- Section of mouse urinary bladder treated with K2 isolate resistant to Imipenem and moderate biofilm producer showing Infiltration of inflammatory cells (⇦), fibrosis (⬅), necrosis (⬅) degeneration (⬌), Oedema (⇦). (X 200, H&X).

Cefotaxime resistant *K. pneumoniae* (K6) isolate

Cefotaxime resistant *K. pneumoniae* (K6) isolate and moderate biofilm producer was selected and injected intraurethrally. Section of mouse kidney revealed mild dilation of renal tubules and mild inflammatory cells infiltration as shown in Figure-7. In regard to urinary bladder normal urinary epithelium, presence of fibrosis, necrosis, and inflammatory cell infiltration were observed Figure-8.

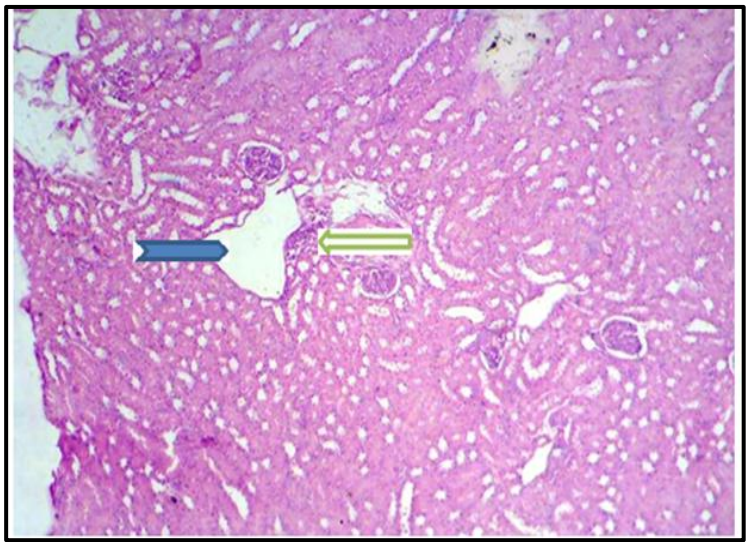

Figure 7- Section of the mouse kidney treated with K6 resistant to Cefotaxime and moderate biofilm producer showing mild cystic dilation of renal tubule(➡), mild inflammatory cells inflitration (⇦), (X100, H&X).

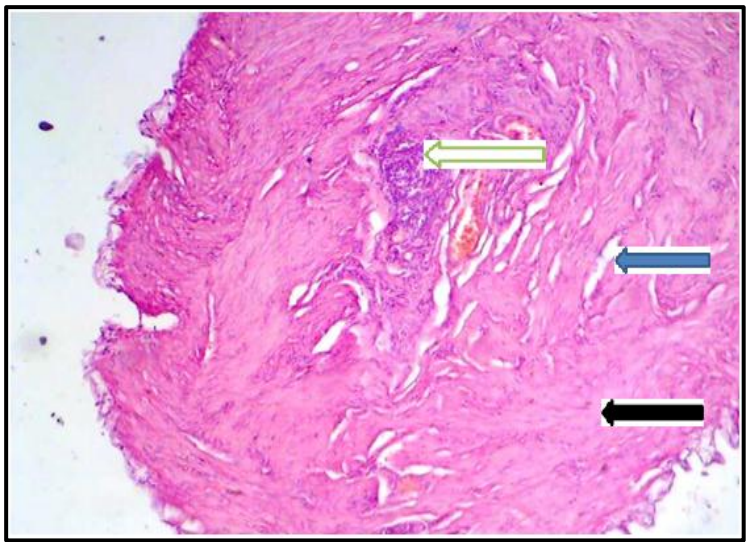

Figure 8- Section of mouse urinary bladder treated with K6 resistant to Cefotaxime and moderate biofilm producer showing Infiltration of inflammatory cells ( ), necrosis ( ), fibrosis ( ), (X 100 H&E).

Meropenem resistant *K. pneumoniae* (K3) isolate

Meropenem resistant *K. pneumoniae* (K3) isolate was selected and injected intraurethrally. Section of kidney in treated mouse demonstrated degenerative changes of renal tubules, cystic dilation, oedema, and congestion, as described in Figure-9.

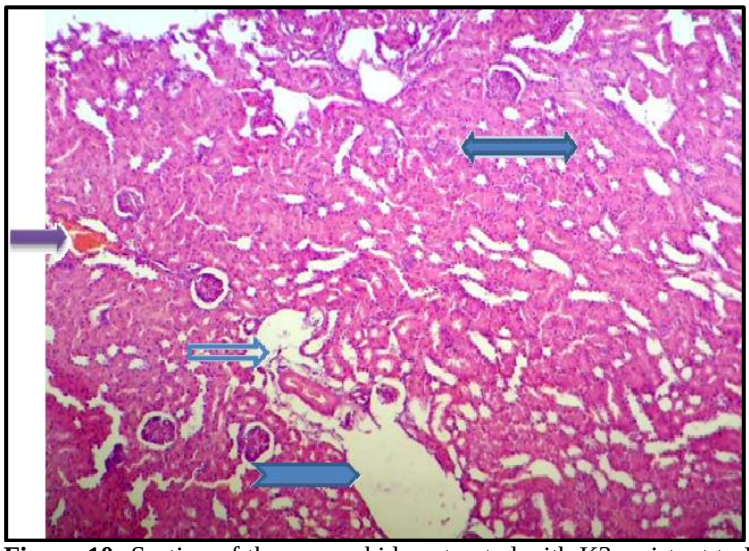

Figure 10- Section of the mouse kidney treated with K3 resistant to Meropenem and moderate biofilm producer showing degeneration ( ) , cystic dilation ( ), Odema ( ), Congesion ( ), (X 100 H&E).

Urinary bladder section of mouse treated with Meropenem K3 isolate showed degeneration, oedema, necrosis of mucosal layer, fibrosis, and abundant of inflammatory cells infiltration, as depicted in Figure-10.

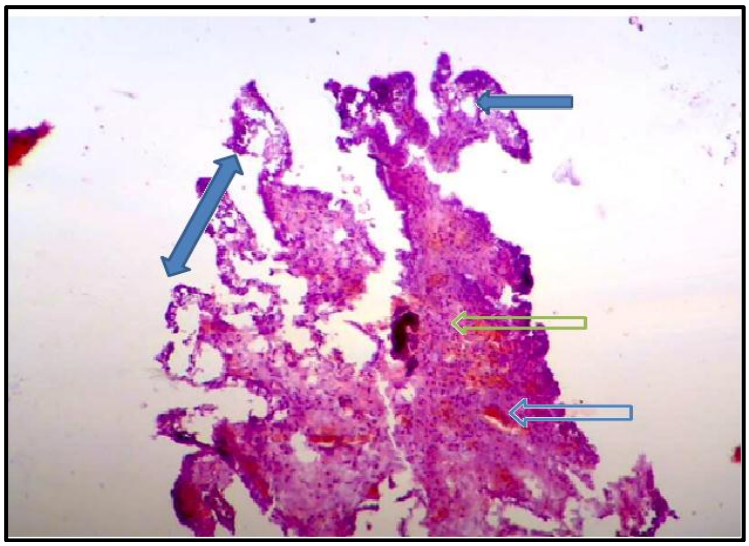

Figure 10- Section of mouse urinary bladder treated with K3 resistant to Meropenem and moderate biofilm producer showing necrosis (⟸), degeneration (⟺) Infiltration of inflammatory cells (⟸), oedema (⟸), (X200H&E).

Ceftazidime resistant *K.pneumoniae* (K46) isolate

Kidney section treated with Ceftazidime resistant K46 isolate revealed histological characterized by scattered dispersed area of degenerative, and necrosis of renal tubules, as clarified in Figure-11. Concerning section of urinary bladder; necrosis of mucosal urinary bladder epithelium, necrosis, and fibrosis were markedly seen as it is presented in Figure-12.

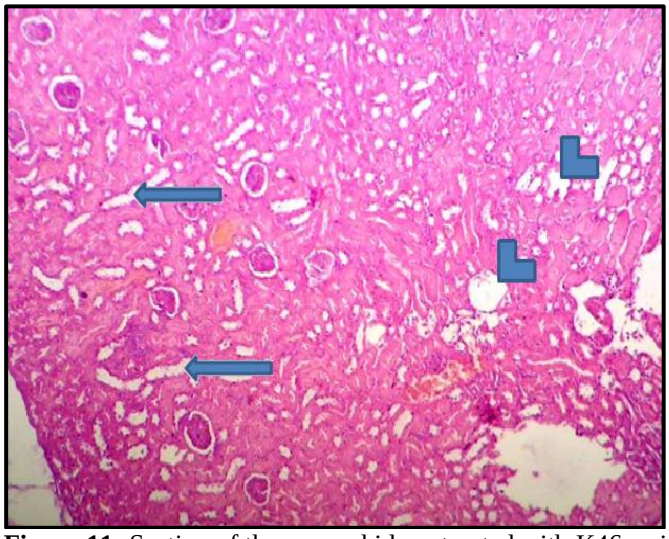

Figure 11- Section of the mouse kidney treated with K46 resistant to Cetazidime and moderate biofilm producer showing Necrosis (⟸), Focal area (⌊) (X 100 H&E).

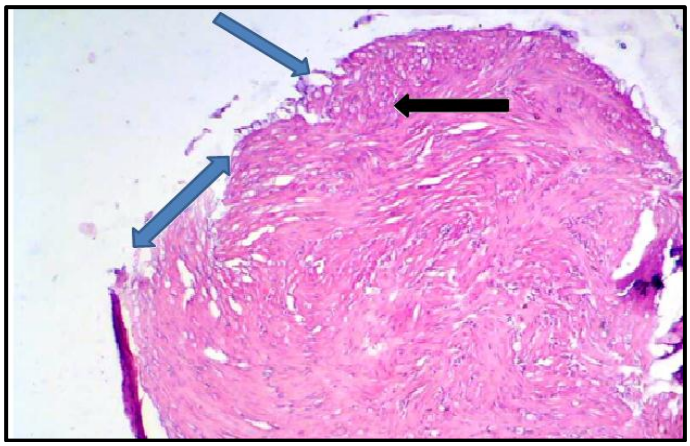

Figure 13- Section of mouse urinary bladder treated with K46 resistant to Ceftazidime and moderate biofilm producer showing necrosis (⟸), degeneration (⟺), fibrosis (⬅), (X 100 H&E).

Imipenem resistant (K2) isolate caused more histological changes in kidney than Meropenem and third generation resistant isolates. Nonetheless, the effect of Imipenem on urinary bladder was similar to that of Meropenem resistant isolate (K3). On contrary, Imipenem resistant K2 isolate afflicted the urinary bladder more than what Cefotaxime (K6) and cetazidime (K46) resistant isolates have done.Kidney of mice was affected by Meropenem resistant isolate (K3) more than K6 and K46 isolates did. However, histopathological changes in urinary bladder were more than it has been seen in case of extended spectrum beta lactam(ESBL) isolates (K6 and K46). Carbapenems and Aztreonam were displayed to be effective in the treatment of peritoneal abscess infection with regard to reduction in bacterial densities and mortality of the animals compared with those of untreated controls. Aztreonam, however, resulted in a more satisfactory outcome overall than that seen with Carbapenems [19].

In a local study achieved by Hammadi [20], mice injected transuretherally with *E. coli* which carried more one β-lactamase gene were compared with a sensitive isolate. The latter caused more damage (hemorrhage, infiltration of inflammatory cells in kidney and infiltration of inflammation cell with absence of cuticle layer of urinary bladder as in present study) than the previous one. Moreover, same author reported that *E. coli*, under the stress of Ceftazidime, established less damage than the sensitive isolates as well as in the case of antibiotic free condition.

Data from studies of Gram-negative bacteria, it is not clear whether the presence of a specific β-lactamase gene weakens the ability of the microorganism to cause damage, in terms of host colonization, invasiveness or fitness costs, and robust conclusions cannot be drawn from the data obtained so far. At least in *E. coli*, the production of a CTX-M-1 enzyme (an ESBL) does not look to affect virulence [21]. Dubois *et al*. [22] reported the isolation, from a patient with neonatal meningitis, of an *E. coli* strain with three different plasmids, one of which produced CTX-M-1 β–lactamase. The plasmid that encoded the β-lactamase did not increase the occurrence of meningitis in a newborn mouse model.

Fernandez *et al.*[23], proved quantitative changes in the peptidoglycan composition in *E. coli* strains expressing OXA-24, OXA-10-like, and SFO-1 (with its upstream regulator AmpR) β-lactamases; the changes were imitated by a decrease in the level of cross-linked muropeptides and an increase in the average length of the peptidoglycan chains. These changes were associated with a statistically significant fitness cost, which was demonstrated both *in vitro* and *in vivo* in a mouse model of systemic infection. The biological cost related with these changes indicates the importance of the interaction between β-lactamases and peptidoglycan metabolism.

Sahly *et al.*[24] demonstrated that Extended-spectrum β-lactamase producing *K. pneumoniae* strains have been recommended to possess higher pathogenic potential than non-producers. They studied the ability of 58 ESBL-producing and 152 non-producing isolates of *K. pneumoniae* to express type 1 and 3 fimbrial adhesins, which are important traits in microbial adherence and invasion of host cells Although the adherence of ESBL- and non-ESBL-producing strains to epithelial cells did not vary significantly, the proportion of strains able to attack ileocecal and bladder epithelial cells was significantly higher among ESBL producers (81% and 27.6%, respectively) than among non- ESBL producers (61% and 10%, respectively). Likewise, the amount of ESBL producers coexpressing both

fimbrial adhesins was significantly higher than that of non-ESBL producers. On acquisition of the ESBL SHV-12-encoding plasmids, two transconjugants started to produce type 3 fimbriae, although expression of type 1 fimbriae was not affected. Overall, the data confirmed that an ESBL plasmid appeared to upregulate the expression of one or more genes, resulting in a higher invasion capacity. It remains unclear whether this effect caused from direct SHV-type expression or expression of another plasmid-borne gene [21].

Koh *et al.* [25] mentioned that the induced Carbapenem mutant strain of *P. aeroginosa* led to failure of spleen invasion. Correspondingly, Skurnik *et al.*[26], showed that the antibiotic resistant strains carried a cost represented by decreased in growth rate and transmission, which mean decreased in bacterial virulence factors.

Marciano *et al.* [27] demonstrated that resistant β-lactam genes in Gram negative bacteria may be responsible for bacterial environment adaptation but not for virulence loose, as in *Salmonella enterica* which lose the virulence when carry the β-lactam resistant genes. *Klebsiella pneumoniae-infected group* (sensitive group), Histological examination of lung tissues in this group exposed edematous pulmonary vasculature, uncovered pseudostratified epithelial cells lining many bronchioles, this is beside multifocal areas of perivascular lymphocytic infiltrates could be seen. Noticeable thickening of alveolar septa and pleura could also be detected. Photomicrograph of lung tissue of *K. pnumoniae* infected-non treated animal, showing interstitial hemorrhagic area, inflammatory cells infiltrate [28].

The possible clarification in this work depends on the theory stated the role of the inflammatory cells infilterates and macrophages in changing elastic fibres profile, where these cells may contribute in discharging proteases and neutrophil elastase which has the potential to preferentially disrupt the elastic network [29]. Likewise, Darwish and Hamouda [28] displayed that in Cefepime treated rat group*:* Mild to moderate degree of improvement comparing to infected non-treated group was detected where peribronchial inflammation and perivascular edema were reduced. However, photomicrograph of lung tissue of *K. pnumoniae* infected – Cefebime treated rat displaying, perivascular edema, pulmonary blood vessel, desquamated cell in the bronchiole lumen, degenerated epithelial cells lining the bronchiole, and emphysema.

In conclusion, Most of tested clinical *K. pneumoniae* isolates (90.5% and 77.3%) were susceptible to Meropenem and Imipenem, respectively and less susceptible to third generation cephalosporin. Carbrapenem resistant isolates (K2 and K3) were revealed severe histopathological changes compared to third generation cephalosporins resistant isolates (K6 and K46).

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