Molecular Analysis of *Klebsiella Pneumoniae* Isolated from UTI Patients in Erbil City

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1. INTRODUCTION

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**ABSTRACT**

Fifty isolates of *Klebsiella pneumoniae* were identified using cultural, morphological and biochemical characteristics. Three-hundred and fifty urine samples were collected from patients with urinary tract infection admitted to Hawler Teaching, Rizgary, Raparin and West Emergency hospitals in Erbil city during the period of 2nd of June to 5th of October 2012.

Antibiotic sensitivity testing performed for all isolates using amikacin (AK₃₀), ampicillin (AMP₁₀), aztreonam (ATM₃₀), Cefixime (CFM₃), Ceftriaxone (CI₃₀), Ciprofloxacin (CIP₃₀), Cefoxitin (CN₃₀), Trimethoprim/sulfamethoxazole (COT₂₅), Cefotaxime (CTX₅), Gentamicin (GEN₁₀), Imipenem (IPM₁₀), Nalidixic acid (NA₃₀), Nitrofurantoin (NIT₃₀₀), Norfloxacin (NOR₁₀) and Trimethoprim (TMP₅). The resistance percentage of the isolates were (8, 100, 64, 52, 20, 86, 50, 60, 34, 0, 24, 58, 24 and 60) %, respectively. Plasmid profiling was done for all isolates and it was revealed that 48% contained one plasmid, 44% possessed two plasmids, 6% had three plasmids and 2% of the isolates contained four plasmids. The process of transformation was done for the laboratory strain *Escherichia coli* DH5α by using purified plasmid of the most resistant isolate (K50) to determine the location of the genes which are responsible for resistance to antibiotics in that isolate. The transformed colonies appear to be resistant to the following antibiotics (AMP₁₀, CFM₅ and CTX₅) which indicated that the genes encoding resistance to these antibiotics were located on transferrable plasmid.

Plasmids are extra-chromosomal DNA molecules that replicate independently of the chromosome. They are typically supercoiled, circular and double stranded DNA molecules (Mascaretti, 2003). Bacterial plasmids can range in size from 1 kb to more than 1000 kb (Finan *et al.*, 2001). Resistance plasmids contain genes encoding resistance to one or more antibiotics. Genes encoding resistance to several families of antibiotics, particularly the β-lactams, are commonly disseminated by the incorporation of transposable elements and integron gene cassettes into plasmids (Bennett, 2008).

*Klebsiella pneumoniae* (*K. pneumoniae*), is a Gram-negative bacterium belongs to the Enterobacteriaceae family. It is the second Gram negative causative agent of UTI.
(Schembri et al., 2005). There is a growing concern regarding antimicrobial resistance worldwide, particularly in \textit{K. pneumoniae} and other causative agents of UTIs (Saderi et al., 2006).

The predominant mechanism for resistance to \(\beta\)-lactam antibiotics in gram-negative bacteria is the production of \(\beta\)-lactamase. In addition, production of extended-spectrum \(\beta\)-lactamases (ESBLs) is another important mechanism which is responsible for resistance to the third-generation cephalosporins (Paterson et al., 2003). \textit{Klebsiella} species contain many plasmids that differ in numbers and molecular weight, carrying different types of genes (Essack et al., 2004).

It has been reported that a multi-resistant \textit{K. pneumoniae} strain isolated from neonates harboured a plasmid of 48 kb with genetic determinants for resistance to amikacin, ampicillin, kanamycin, streptomycin and tobramycin (Tolmasky et al., 1988). Moreover, an isolate of \textit{K. pneumoniae} carried two plasmids of 17 and 90 kb encoded resistance for \(\beta\)-lactam antibiotics, aminoglycosides, trimethoprim and chloramphenicol was studied by (Hanson et al., 1999).

Transformation is the process whereby a cell takes-up and expresses exogenous DNA (Summers, 1996). It was the first mechanism of bacterial gene exchange to be described and played an important role in the initial identification of DNA as the genetic material (Chakraborty, 2003).

Crucial stages in the process of transformation are the acquisition of competence, binding of DNA to the cell surface, uptake and chromosomal integration or re-circularization of the transforming molecule (Summers, 1996).

Mawlad, (2006) observed that after transformation process of \textit{K. pneumoniae} isolate K32, the resistance for the following antibiotics were located on plasmid, amikacin, ampicillin, cefotaxime, gentamicin, trimethoprim, tetracycline, amoxicillin, cephalothin, kanamycin, nalidixic acid, streptomycin, keflex and pan-cloxacillin.

It was investigated that a multiple antibiotic resistance strain of \textit{K. pneumoniae} isolated from urine was resistant to 36 antibiotics and sensitive to cefotaxime and imipenem. The resistance to ampicillin, nitrofurantoin, rifampicin, streptomycin, tetracycline, trimethoprim, chloramphenicol, bacitracin, cefprozil, cloxacillin, cephalexin, fusidic acid, methicillin, co-trimoxazole and colistin were plasmid encoded (Barman et al., 2008).

It was determined that the genes responsible for amikacin, ampicillin, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, gentamicin and trimethoprim/sulfamethoxazole resistance in \textit{K. pneumoniae} were located on plasmid and successfully transferred to the laboratory strain \textit{E. coli} DH5\(\alpha\) through transformation (Khoshnaw, 2010). In the present study, antibiotic sensitivity testing of the isolates of \textit{Klebsiella pneumoniae} was done to determine their resistance pattern and plasmid profiling of all of the \textit{Klebsiella pneumoniae} isolates was done to determine the site of antibiotic resistance genes by transformation.

2. MATERIALS AND METHODS

Three hundred and fifty urine samples were collected from patients admitted to Hawler Teaching, Rizgary, Raparin and West Emergency hospitals in Erbil city during the period of 2nd of June to 5th of October 2012. Fifty isolates were identified as \textit{Klebsiella pneumoniae} depending on cultural, morphological and biochemical characteristics. Furthermore, the identity of the isolates confirmed by using Vitek 2 systems.

2.1 Antibiotic Sensitivity Testing
The effect of different antibiotics on previously identified isolates of *K. pneumoniae* was studied by using disc diffusion method using (AK, AMP, ATM, CFM, CTX, CN, CI, CIP, GEN, IPM, NA, NIT, NOR, TMP and COT). After incubation, the inhibition zone diameter around each antibiotic disc was measured in millimeter (mm) and compared with standard inhibition zone diameter (CLSI, 2011).

### 2.2 Plasmid DNA extraction

Plasmid DNA was extracted from 5 millilitre (ml) of an overnight culture of the isolates of *K. pneumoniae* grown in LB broth medium containing an appropriate antibiotic (Ampicillin 0.1gm/ml) by using plasmid DNA purification kit (DNA-spin Plasmid DNA Purification Kit/ iNtRON/ Korea). The extracted plasmid DNA was stored at -20°C until using.

The method described by Sambrook and Russell (2001) was used for gel electrophoresis. The extracted plasmids were electrophoresed in 0.7% agarose gel with Tris-Borate Ethylene-Diamine Tetra-Acetic acid (TBE), stained with ethidium bromide and visualized under UV-light.

### 2.3 Transformation

The process of transformation was done for the most resistant isolate of *K. pneumoniae* (K50) to determine the location of antibiotic resistance genes. The purified plasmid from isolate (K50) transferred to the sensitive strain (*E. coli* DH5α). Competence in *E. coli* DH5α was induced by chemical treatment with ice-cold CaCl2 followed by a brief heat shock (Casali and Preston, 2003).

### 3. RESULTS AND DISCUSSION

Among 350 urine samples collected from patients admitted to Hawler Teaching, Rizgary, Raparin and West Emergency hospitals in Erbil city during the period of 2nd of June to 5th of October 2012, fifty isolates were identified as *K. pneumoniae*.

Antibiotic sensitivity testing was done for all fifty, previously identified, isolates of *K. pneumoniae* using fifteen different antibiotics which were AK$_{30}$, AMP$_{10}$, ATM$_{30}$, CFM$_{5}$, CI$_{30}$, CIP$_{30}$, CN$_{30}$, CTX$_{5}$, COT$_{25}$, GEN$_{10}$, IPM$_{10}$, NA$_{30}$, NIT$_{300}$, NOR$_{10}$ and TMP$_{5}$.

Figure (1) shows the percentage of resistance of all *K. pneumoniae* isolates toward different antibiotics used. The resistance percentages were 100% for AMP, 60% for CTX and CI, 64% for ATM, 50% for COT, 52% for CFM, 86% for CN, 8% for AK, 34% for GEN, 20% for CIP, 58% for NIT, 24% for NA, 60% for TMP, 24% for NOR and 0% for IPM (which was the most effective one).

IPM10 was the most effective antibiotic against *K. pneumoniae* isolates of the present study and 100% of the isolates were susceptible to it. This result is in agreement with Khoshnaw, (2010), Harini and Ananthan, (2012), and Ahmed et al., (2013). All of them found that 100% of *K. pneumoniae* isolates included in their studies were susceptible to IPM. The high level of sensitivity to IPM among isolates of the current study may be due to the fact that carbapenems (especially IPM) are not so widely used in our area that make these bacteria develop resistance against them. Thus, IPM can be used as a drug of choice for treatment of *K. pneumoniae* UTIs.

The isolates included in the current study showed 100% resistance to AMP. This is agreeing with the studies performed by Al-Agamy, (2012), Akpaka and Swanston, (2013), and Elsharkawy et al., (2013). All these studies
reported that their isolates appeared as resistance to AMP. However, this high level of resistance of \textit{K. pneumoniae} to AMP is not surprising due to constitutive expression of a chromosomally encoded \(\beta\)-lactamase (\textit{bla}\textsubscript{SHV-1}) which confers resistance to ampicillin, amoxicillin, carbenicillin and ticarcillin (Haeggman et al., 2004).

As it was illustrated in the figure (1), there is considerably high level of resistance against third generation cephalosporin (3GCs) CTX5, CI\textsubscript{30} and CFM\textsubscript{30}. The results were recorded as 60\% for both CTX and CI and 52\% for CFM. These results are in a good agreement with that of Ahmed \textit{et al.}, (2013). It is also concordant with 68\% of resistance for CTX reported by Khoshnaw, (2010).

Increasing resistance to broad-spectrum cephalosporins in \textit{Klebsiella} species predominantly is due to the production of ESBLs. This is considered as a growing therapeutic problem in most developed and developing countries (Paterson \textit{et al.}, 2003; Mekki \textit{et al.}, 2010). Resistance of \textit{K. pneumoniae} isolates of the current study to quinolones, NA\textsubscript{30}, NOR\textsubscript{10} and CIP\textsubscript{30} were 24\% for both NA\textsubscript{30} and NOR\textsubscript{10} and 20\% for CIP\textsubscript{30}. These results agree with that of Behroozi \textit{et al.}, (2010) in which the resistance percentage of \textit{K. pneumoniae} urinary isolates for NA was 21\%.

\textit{K. pneumoniae} is highly resistant to clinically used antibiotics and causing a wide spectrum of infections (Echeverri-Toro \textit{et al.}, 2012). Efflux pumps are one of the major mechanisms of antibiotic resistance in \textit{K. pneumoniae}. Among these mechanisms, increasing efflux by efflux pumps is considered to be one of the most important contributors to bacterial antibiotic resistance (Li and Nikaido, 2009) and may be responsible for resistance to either one specific class of antibiotics or a large number of unrelated antimicrobial agents (Lynch, 2006). The main causes of \textit{K. pneumoniae} resistance to CIP are mutations occurring in the target enzymes of quinolones, namely DNA gyrase and topoisomerase IV as well as efflux pumps (Aathithan and French, 2011).

The resistance to the monobactam, ATM\textsubscript{30} among isolates of the current study was 64\%. It
is completely agree with the results reported by Ahmad et al., (2009) who demonstrated that 63.5% of their *K. pneumoniae* isolates were resistance for ATM, respectively.

It was revealed that the resistance percentage of the isolates included in the present study for CN30 was 86%. This result is in concordance with those of Al-Jebouri and Mdish, (2013) who reported resistance to CN as 92%, 89.3% and 80% among *K. pneumoniae* isolates, respectively. The present result disagrees with that of Elsharkawy et al., (2013). In their study, resistance of *K. pneumoniae* to CN reported as 60%.

The isolates included in the current study showed 58% resistance to NIT300. This finding is consistent with Maina et al., (2013), who reported that 50% of their *K. pneumoniae* isolates were resistant to NIT. On the other hand, this result shows dissimilarity with that of Ahmed et al., (2013) and Al-Jebouri and Mdish, (2013). In their studies, the resistance percentages of *K. pneumoniae* to NIT were 27.5% and 12%, respectively. This high level of resistance can be attributed to abuse as well as increasingly use of this antibiotic in the treatment of UTIs.

The resistance percentage to TMP5 among *K. pneumoniae* isolates of the present study was 60%. This result is in contrast to the studies of Al-Agamy, (2012) who showed that 79% of the isolates include in their studies were resistant for TMP.

Finally, 50% of the isolates were resistance to COT25. This is agreeing with the results of Ahmed et al., (2013). Their result was 48.2% respectively.

The antimicrobial susceptibility patterns of urinary pathogens have been changed over years. There is a growing global concern regarding antimicrobial resistance among the members of Enterobacteriaceae, particularly *K. pneumoniae* and other causative agents of UTI. *K. pneumoniae* are known for high resistance to various antibiotics. They harbour a series of antibiotic resistance genes which can be transferred horizontally to other bacteria (Behroozi et al., 2010).

*K. pneumoniae* is one of the main multidrug-resistant pathogens that cause nosocomial infections. The emergence of multidrug resistant strains has become a major clinical problem (Van der Donk et al., 2011). Moreover, patients with prior antibiotic exposure exhibit more resistance and are associated with increased mortality Johnson et al., (2011).

### 3.1 Plasmid profiling

Plasmid profiling of all of the isolates under study revealed that out of fifty isolates of *K. pneumoniae*, 24 (48%) contained one plasmid, 22 (44%) had two plasmids, 3 (6%) contained three plasmids and one isolate had four plasmids (figure 2). The current result partly disagrees with Mohamed (2011) who determined plasmid profile of *Klebsiella*. It was revealed from her study that 35.7% of the isolates had the same profile, containing one plasmid of 23.13 kb while, 35.7% had three plasmids of 23.13, 3.5 and 2.3 kb which is in concordance with the current result, 14.2% harboured four plasmids and 14.2% possess five plasmids. Furthermore, Akingbade et al., (2013) found that only 37.03% of the multidrug resistance isolates of *Klebsiella* possess plasmid. The molecular size of these plasmids ranged from 700-892 bp.

The present result is higher than that reported by Aladag and Durak, (2009) who studied the frequency and distribution of plasmids among 74 *K. pneumoniae* strains. They revealed that most of the strains (76.7%)
carried from one to five plasmids. The group of strains most frequently encountered was that with only one plasmid (37%). In addition, Aladag et al., (2009) showed that among ESBL producing urinary isolates of K. pneumoniae, 76% contain plasmid which range in size from 1.6 to 30.1 kb.

Figure (2): Agarose gel electrophoresis (0.7% agarose for 90 minutes) of plasmid profile of K. pneumoniae isolates

Lane L: 1500 bp DNA ladder

Lane no. 1-34: Plasmid content of the K. pneumoniae isolates

Figure (3): Agarose gel electrophoresis (0.7 agarose for 60 minutes) of plasmid DNA of K. pneumoniae isolate (K50) and DH5α (before and after transformation).

Lane L: 1500 bp DNA ladder

Lane 1: Plasmid content of K. pneumoniae isolate K50

Plasmid-mediated transfer of resistance genes can be considered as one of the most important mechanisms for the spread of antibiotic resistance. In K. pneumoniae, plasmid-mediated resistance has been associated with the spread of genes conferring resistance to several classes of antibiotics including the fluoroquinolones and aminoglycosides, particularly the β-lactams via the dissemination of genes encoding β-lactamases. The acquisition of resistance plasmids provides a direct mechanism by which a bacterium can become resistant to multiple classes of antibiotics (Kiratisin et al., 2008).

3.2 Transformation process

Transformation experiment was performed to determine the site of the genes conferring antibiotic resistance in K. pneumoniae isolate (K50) which was the most resistant isolate (resistant for thirteen antibiotics).

After success of transformation and growth on ampicillin containing plate, several colonies of transformed DH5α were chosen to be purified on LB agar. This step is essential to ensure the stability of resistance phenotype to antibiotics in transformed colonies and regular maintenance of the plasmid after purification and then these colonies were subjected to antibiotic sensitivity testing by using disc diffusion method. The genes which are responsible for resistance to these antibiotics (AMP₁₀, CTX₃₀ and CF₅₃) were located on plasmid. The current result is in concordance with that of Khoshnaw, (2010). In his study, AMP, CTX and NA, AMP and AMP and CTX resistance genes were located on plasmid and successfully transferred to the laboratory strain E. coli DH5α through transformation, respectively. The plasmid of transformed colonies and isolate (K50) were run in agarose gel (figure 3).
Lane 2: Plasmid content of E. coli DH5α laboratory strain before transformation

Lane 3: Plasmid content of E. coli DH5α after transformation process

4. CONCLUSIONS

It could be concluded that most of the isolates of *Klebsiella pneumoniae* were resistant to more than one antibiotic and all of them were sensitive to Imipenem. Most of the studied isolates contained one or two plasmids. It was also concluded that there is not a clear relationship between number of plasmids and antibiotic resistance pattern of the *Klebsiella pneumoniae* isolates. Finally, the genes responsible for (AMP$_{10}$, CFM$_{3}$ and CTX$_{3}$) resistance in *Klebsiella pneumoniae* were located on plasmid and successfully transferred to laboratory strain *E. coli* DH5α through transformation process.

Conflict of Interest

There is no conflict of interest.

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