HEAVY METAL RESISTANCE AND PLASMID PATTERNS OF ANTIMICROBIAL RESISTANCE AMONG KELBSIELLA ISOLATED FROM URINARY TRACT INFECTION

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Background: Klebsiella species particularly Klebsiella pneumoniae are important opportunistic nosocomial pathogens causing a variety of infections including urinary tract infections, pneumonia, septicemia, wound infections and infections in the intensive care units. The increasing incidence of resistance to a wide range of antibiotic agents by a variety of organisms is a major concern facing modern medicine. Microorganisms that are resistant to both antibiotics and metals have been isolated from urinary tract infection. Most of the hospital infections including Klebsiella pneumoniae that is resistant to heavy metals harbor the plasmids with different molecular sizes. The aim of this study is to Evolution of Heavy Metal Resistance and plasmid pattern of Antimicrobial resistance among Kelbsiella isolated from urinary tract infection.

Materials and methods: 144 of K. pneumonia strains were isolated from UTI samples were identified after collecting from the hospitals and clinical laboratories. Primary screening of β-Lactam resistant strains performed by using of disk diffusion, Oxoid combining Disk, double disk (DD) synergy methods. Plasmids were extracted from resistant strains and PCR was performed for TEM and SHV genes. Then MIC values for heavy metals including Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} were determined. The resistant strains to both antibiotics and heavy metals were selected for plasmid extraction.

Results: The results showed that all the Klebsiella strains showed multiple-drug resistance to least 7 antibiotics (Ampicillin, Gentamicin, tetracycline, cephalixin, amikacin, sulfamethoxazole, Ciprofloxacin). Either 61/81% (89strain)
of the isolates were resistant to antibiotics it was indicated that 42.69% (38 strain) of these strains were β-lactamase-producers (Possetive ESBL). After performing PCR, from 38 strains of Possetive ESBL, 28.94% of Klebsiella strains harbored SHV gene, 34.21% of them harbored TEM gene.

**Conclusion:** The isolates with plasmids for heavy metal resistance showed resistance to the mentioned antibiotics too. Probably, the genes responsible for resistance to both heavy metals and antibiotics are harbored by plasmids in some of the bacterial agents of (UTIs). So, the importance of investigations on the genes responsible for resistance to extended-spectrum β-lactam antibiotics and heavy metals is revealed.

**Key words:** Heavy metal, TEM, SHV, Antimicrobial resistance.

**Introduction**

Plasmids encoding SHV-1 and TEM-1 beta-lactamases, which can be seen more in *E. coli* and *Klebsiella pneumoniae*, are leading to rapid antibiotic resistance by rapid spread through bacteria populations causing hospital infections, urinary tract infections and bacteremia, and so sometimes result in more than 90% mortality (1-3). In this regard, Nakahara et al., found that multiple-antibiotic resistance can be observed along with resistance to heavy metals. Zeroual et al. also reported that resistance to different concentrations of heavy metals such as mercury, lead, copper and cadmium in some of the bacteria causing nosocomial infection occurs simultaneously with the development of antibiotic resistance, indicating co-spread of metal and antibiotic resistance determinant genes through a joint plasmid (4-6). The aim of this study was molecular detection of simultaneous occurrence of antibiotic resistance and heavy metals in *K. pneumoniae* isolated from urinary tract infection.

**Materials and Methods:** This descriptive and analytical study was conducted on 845 patients referred to the wards of urology, obstetrics and gynecology at Imam Ali hospital and Clinics in Gerash County, Iran. Urine samples were collected from all patients and delivered aseptically to laboratory.

**Biochemical identification of *K. pneumoniae* strains**

Urine samples were cultured on E.M.B, Blood agar and TSI media to isolate the bacteria. Cultures were confirmed using supplementary media such Simon citrate, MRVP, SIM, urea broth, lysine decarboxylase and also single fermentation of sucrose, lactose and glucose. *K. pneumoniae* strains were identified based on differential tests using standard diagnostic and biochemical methods (7).
Assessment of antibiotic resistance in isolated *K. pneumonia*: To detect antibiotic-resistant bacteria, some pure colonies were inoculated into tubes containing Muller Hinton broth medium, and incubated for 24 hours at a temperature of 36-37°C. Then, suspension was spread on Mueller Hinton agar medium (Merck) by means of swab and disc diffusion test was carried out according to C.L.S.I. standard technique using antibiotics discs, including ampicillin (10µg), chloramphenicol (30µg), trimethoprim (5µg), sulfamethoxazole (5µg), amikacin (30mg), nalidixic acid (30mg), kanamycin (30mg), tetracycline (30µg), ciprofloxacin (5µg), gentamicin (10mg), ceftazidime (30mg), imipenem (110mg), cephalothin (30µg).

ESBL-producing *K. pneumoniae* isolates were assessed by ampicillin, gentamicin, cefixime, sulfamethoxazole and imipenem antibiotics using synergistic double discs method and the oxoid combining disc method (8). Ceftazidime (30mg), cefotaxime (30mg) and co-amoxiclav (10µg) antibiotic discs were used to synergistic double discs method with a distance of 20 mm from each other. Also Ceftazidime (30mg) and ceftazidime along with clavulanic acid (10mg) at a distance of 20 mm were used for combined disc method.

Finally, after 18 hours incubation and measuring the diameter of inhibition zone, ESBL-producing *K. pneumoniae* strains were determined according to NCCLS standards (9).

**Extraction of plasmid DNA and detection of SHV-1 and TEM-1 genes**

DNA extraction kit (Biospin plasmid extraction, Bioflux, Japan) was used to prepare plasmid. Some colonies of the bacteria identified by synergistic and combined disc methods were inoculated in 10 ml Tryptcase soy broth (Merck) for 24 hours at 36-37°C; and then bacterial suspension transferred to 2 ml microtubes and centrifuged at 10000 rpm for 30 seconds to separate the supernatant. All subsequent stages of extraction were carried out according to the kit instructions.

In order to do PCR, 3 ml of plasmid DNA samples along with 0.5µl from each pair of primers specific for two genes listed in Table 1 were used for each strain of bacteria under temperature conditions (Table 1).

Materials required to PCR were prepared from Cinna Gen Company; the reaction mixture in a 25µl volume included 2.5µl PCR buffer, 0.75µl Mgcl₂, 0.5µl dNTP, 0.5µl of each pair of primers, 0.25µl Taq polymerase enzyme and 3 µl of plasmid DNA. *K. pneumonia ATCC700603* and *E. coli ATCC35218* strains were respectively used as positive and negative controls at all stages. Finally, PCR products were electrophoresis on 1.5% agarose gel; 1016bp and 1074bp bands, which respectively show the existence of SHV-1 and TEM-1 genes, were observed using Gel Doc device (Gel document, Bio...
Under UV light (10-12). Primers used for detection of SHV-1 and TEM-1 genes have been respectively given below:

**Table 1: Primers used for detection of SHV-1 and TEM-1 genes.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>F: 5’-GAAGACGAAAGGCCTCGTG-3 R: 5’-GGTCTGACAGTTACCAATGC-3</td>
<td>1074</td>
</tr>
<tr>
<td>SHV-1</td>
<td>F: 5’-CGCCGGGTATTCTTATTGTTCGC-3 R: 5’-TCTTTCCGATGCCGCCCCAGTCA-3</td>
<td>1016</td>
</tr>
</tbody>
</table>

**Figure 1:** Line 1 is positive sample for the gene SHV-1, line 2 is positive control (related to standard K. pneumoniae), line M is 100bp marker sizes, line 3 is negative control and line 4 is positive sample for gene TEM-1.

**Determination of MIC for heavy metals in antibiotic-resistant isolates**

Antibiotic-resistant strains positive for beta-lactamase were selected for evaluation and determination of resistance to heavy metals and inoculated with the maximum concentration of heavy metals to LB broth for 24 hours, and then transferred to the LB broth with lower concentrations of the same heavy metal to be assessed at different levels of resistance and tolerance to heavy metals. In this study, heavy metal salts including mercury (HgCl₂), Lead (Pb(NO₃)₂), Cadmium (CdO₄) and copper (CuSO₄) were used in tubes containing different concentrations from the highest to the lowest levels to evaluate MIC for heavy metals. Various concentrations involved 20,30,35,40,45,55,65 and 80 mg/l for mercury; 40,50,100, 200, 250, 300 and 400 mg/l for cadmium; 400, 450, 500, 600, 800, 950, 300 and 350 mg/l for lead and
Results

144 (34.75%) of 328 bacterial isolates in this study were identified as *K. pneumoniae* strains and the most antibiotic resistance pattern in these bacteria through the antibiogram test was: 53.50% amikacin, 57.89% trimethoprim, 36.84% ciprofloxacin, 45.61% sulfamethoxazole, 47.36% tetracycline, 51.75% cephalexin and 57.01% gentamicin, so that antibiotic resistance was found in 89 strains (61.81%). Also, the lowest antibiotic resistance was related to imipenem (11.55%) and nalidixic acid (27.19%); susceptibility of strains was 88.45% to imipenem. 38 strains (42.69%) resistant to antibiotics mentioned was found positive for ESBLs using synergistic and combined disc methods. Beta-lactamase genes (TEM-1 and SHV-1) were detected in 24 (63.16%) of 38 ESBL-positive strains by PCR technique, which 14 (58.34%) had plasmid genes and 10 (41.66%) had chromosome genes, 25% SHV-1 genes and 16.66% TEM-1 genes were chromosomal; there was statistically significant relationship (P=0.003) between strains resistant to antibiotics and beta-lactamase genes using statistical ANOVA analysis. Isolation of antibiotic-resistant *K. pneumoniae* strains with plasmid is confirmed by the presence of 1016bp and 1074bp gene bands, respectively for SHV-1 and TEM-1 genes. As can be seen in Figure 1, TEM-1 and SHV-1 genes in ESBLs-positive strains were respectively isolated based on specific TEM-1 and SHV-1 primers. In this study, TEM-1 and SHV-1 genes were detected positive for ESBL among *K. pneumoniae* isolates. Line 1 is positive sample for the gene SHV-1, line 2 is positive control (related to standard *K. pneumoniae*), line M is 100bp marker sizes, line 3 is negative control and line 4 is positive sample for gene TEM-1; PCR results indicated that 1016bp in line 1 and 1074bp in line 2 were related to SHV-1 and TEM-1 genes, respectively (figure1). Heavy metals tolerance was carried out on 89 antibiotic-resistant strains, which the tolerance findings were as follows: 48 cases to 35µg/ml mercury, 39 cases to 200µg/ml cadmium, 45 to 350µg/ml lead and 38 to 650µg/ml copper. In concentrations above, of 38 ESBLs-positive strains, the growth rate respectively was 28 cases in mercury, 21 isolates in cadmium, 25 cases in copper and 15 bacteria in lead. The beta-lactamasers-positive strains (ESBLs) showed metal tolerance to more concentration. Significant relationship (p=0.012) was statistically observed between strains resistant to antibiotics and heavy metal resistance using ANOVA analysis. It was also observed that antibiotic-resistant *K. pneumoniae* strains compared to non-resistant strains did not show the same tolerance to heavy metal concentrations.
The strains resistant to antibiotics (N=89) showed tolerance to heavy metal concentrations, whereas ESBLs-positive bacteria (N=38) were able to grow in these concentrations and presented tolerance to higher concentrations in. Also, the strains containing TEM-1 and SHV-1 genes tolerated high concentrations, suggesting antibiotic-resistant bacteria can tolerate more metal concentrations. Fisher's exact test showed significant association between antibiotic resistance and metal tolerance (p=0.015, r=0.28) and so that more metal tolerance was observed among strains with beta-lactamase gene (Table 2,3).

**Discussion**

Gram-negative bacteria resistance to antibiotics has led to an increase in mortality throughout the world, suffering heavily annual cost of treating this infections. *K. pneumoniae* as a gram-negative bacterium plays clinically important role in the incidence of nosocomial infection and urinary tract infection (15,16). Unfortunately, the indiscriminate use of antibiotics in recent decades has increased the emergence of multi-drug resistance in gram-negative enterobacteriaceae such as *K. pneumoniae* (17). So that the bacteria producing ESBL enzymes (ESBLs) against antibiotics such as cephalosporins, penicillin, ciprofloxacin and cefotaxime show that the presence of lactamases-encoding gene and its transmission through gram-negative enteric bacteria is considered as a major threat to consumers of wide range cephalosporins. The highest resistance among strains of *K. pneumoniae* in this study was observed in amikacin (53.50%), trimethoprim (57.89%), ciprofloxacin (36.84%), sulfamethoxazole (45.61%), tetracycline (47.36%), cephalexin (51.75%) and gentamicin (57.01%), in fact totally in 89 strains (61.81%). Antibiotic susceptibility was also obtained in imipenem (88.45%) and nalidixic acid (27.19%). 42.69% of *K. pneumoniae* strains resistant to antibiotics were detected positive for beta-lactamase enzymes applying synergistic double discs and combined discs methods. Aladag et al. in 2009 in Turkey identified ESBLs in 44% of 87 *K. pneumoniae*. Numerous and extensive studies have been reported about the production of beta-lactamase enzymes among gram-negative bacteria including *E. coli, K. pneumoniae* and *P. aeruginosa* causing infections (18). Ullah et al., in 2009 reported that 54 strains (58.7%) of 92 *K. pneumoniae* had ESBLs (19). Al-charrakh et al. in 2011 showed that of 88 strains of *K. pneumoniae*, 65 ESBLs-producing cases (73.8%) were resistant to beta-lactam antibiotics (20). Manikandan et al. in 2011 reported that enteric gram-negative bacteria causing urinary tract infections exhibit resistance to antibiotics such as nalidixic acid, trimethoprim, amoxicillin and imipenem (21). Mirsaleh et al. in 2006 stated that ESBL production among strains of *K. pneumoniae* has been reached to 76% in Iran (22). Also Hujer et al.
in 2006 isolated TEM-1 gene from 40% of the bacteria isolated from a hospital in America (23). While, Hui et al. in 2009 observed TEM-1 gene in 81.5% of hospital strains of the bacteria (24). Graham et al. in 2010 based on a study believe that the global prevalence of antibiotic resistance among bacteria is due to the rapidly increasing and uncontrolled use of agricultural chemicals and fertilizers together with metal compounds entering the water resources and rivers promote drug and metal resistance in microorganisms (25). Today, most of studies indicate that the production of beta-lactamases enzymes in K. pneumoniae strains is on the rise, for example 63% in Turkey, 13.5% in Taiwan and 83.4% in America (26, 27). By comparing the findings of this study and others, available statistics indicate that ESBL-producing strains of bacteria can be increased as a result of overdose and prolonged broad-spectrum cephalosporins. Ram and Galas also stated that multi-drug resistant pathogenic bacteria have plasmids containing several resistance genes, causing development of resistance by gene transfer (28, 29 and 30). In accordance with the results of PCR, beta-lactamase genes (TEM-1 and SHV-1) were observed in 24 (63.16%) of 38 strains of ESBLs positive- K. pneumoniae, which 14 (58.34%) plasmidal genes and 10 (41.66%) chromosomal genes. Espano et al. in 2002 conducted a study which reported that 58% of gram-negative bacteria had TEM-1 gene, but 75% in K. pneumoniae (31). Song et al. in 2007 reported TEM-1 gene in 80 samples and SHV-1 gene in 40 samples in ESBL-positive K. pneumoniae strains and Patzer et al., found SHV-1 gene in 56% of K. pneumoniae strains (32, 33). Tasli et al. in 2005, in Turkey found TEM-1 and SHV-1 genes in 52.7% and 32.4% of K. pneumoniae strains, respectively (34). As can be seen in Table 3, 54.16% TEM-1 gene and 45.8% SHV-1 gene were identified in K. pneumoniae strains positive for ESBL. Raihan et al. in 1992 showed that antibiotic-resistant bacteria may tolerate concentrations of metals, such as Pseudomonas aeruginosa CMG 58 can grow in the presence of 500µg/ml mercury and 300µg/ml lead as well as 400µg/ml cobalt (35). Zeroual et al. reported in 2001 that K. pneumoniae strains having reductase enzymes are able to tolerate and resist concentration of 2400 micromoles of mercury (36). Spangler in 1973, Filali in 2000 and Zeroual et al. in 2003 presented reports based on the resistance of metals such as nickel, cobalt, lead, copper and cadmium in ESBL-positive K. pneumoniae strains (37-39). Karbasizaed et al. in 2003 investigated resistance and tolerance of K. pneumoniae strains to concentrations of mercury, copper, lead and cadmium (15). The results of our study, which was conducted to assess sustainability and resistance to heavy metals in K. pneumoniae strains for beta-lactam antibiotics, showed that more resistant strains with antibiotic resistance genes can tolerate heavy metals such as mercury, copper, lead and cadmium in concentrations of 400, 700, 45 and 250 mg/l,
respectively, and the growth was seen at less concentrations. Also, all *K. pneumoniae* strains with antibiotic resistance genes could culture at concentrations of metals, whereas those without the resistance genes were unable to grow at a presence of heavy metals (Table 2 and 3).

**Table-2: The frequency of ESBL s-positive bacteria and tolerance to heavy metal concentrations.**

<table>
<thead>
<tr>
<th>Heavy Metals</th>
<th>Heavy metals tolerance concentrations (mg/l)</th>
<th>ESBL-positive <em>K. pneumoniae</em> strains (N=38)</th>
<th><em>K. pneumoniae</em> strains with TEM,SHV-1 (N=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium (CdO₄)</td>
<td>250</td>
<td>(42.02)16</td>
<td>(42.01)11</td>
</tr>
<tr>
<td>Mercury (HgCl₂)</td>
<td>45</td>
<td>(39.47)15</td>
<td>(39.47)10</td>
</tr>
<tr>
<td>Lead (Pb(NO₃)₂),</td>
<td>400</td>
<td>(31.55)12</td>
<td>(21.05)8</td>
</tr>
<tr>
<td>Copper (CuSO₄)</td>
<td>700</td>
<td>(42.02)16</td>
<td>(31.57)7</td>
</tr>
</tbody>
</table>

**Table3: The frequency of SHV-1/ TEM-1 Genes in ESBLs-positive bacteria.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>SHV-1 Gene (%) n</th>
<th>TEM-1 Gene (%) n</th>
<th>Total Genes (%) N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal Genes</td>
<td>(25) 6</td>
<td>(16.66)4</td>
<td>(41.66)10</td>
</tr>
<tr>
<td>Plasmidal Genes</td>
<td>(20.80) 5</td>
<td>(37.5) 9</td>
<td>(58.33)14</td>
</tr>
<tr>
<td>Total</td>
<td>(45.83)11</td>
<td>(54.16)13</td>
<td>(100)24</td>
</tr>
</tbody>
</table>

**Conclusion**

According to the results of our study and other reports can be argued that antibiotic resistance genes are transmitted faster among bacterial nosocomial pathogens with the indiscriminate use of antibiotics. However, the use of metal compounds and chemical fertilizers in the environment as well as simultaneous transmission of antibiotic resistance and heavy metal genes (38-39) can be a big threat for patients with nosocomial and urinary tract infections, which beta-lactam family are third-generation cephalosporins antibiotics are used to treat. Therefore, some suitable therapeutic solution against
pathogens must be provided by better strategies to reduce metal contaminations in the environment and identify resistant strains.

References


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