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Detection of Quinolone Resistance Genes of *Klebsiella pneumoniae* Isolated from Patients with Urinary Tract Infection attending Some Selected Hospitals in Irbid, Jordan

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Abstract
The incidence of resistant clinical bacteria associated with urinary tract infection (UTI) has been on the increase worldwide. Urinary tract is the most common site of infection for Klebsiella spp. Clinical isolates of fluoroquinolone-resistant Enterobacteriacea are emerging worldwide. The present study was designed to detect the presence of quinolone resistance genes: Chromosome and Plasmid mediated genes (PMQR) in Klebsiella pneumoniae isolated in patients attending some selected hospitals in Irbid, Jordan. One hundred and twelve isolates (112) of K. pneumoniae were collected from patients diagnosed with UTI from cultures available at the Microbiology laboratory at King Abdullah University Hospital (KAUH), Princess Basma Hospital, Prince Rashed Hospital and King Hussein Medical center between January 2014-June 2014. The bacterial isolates were identified as K. Pneumoniae morphologically and biochemically. Kirby-Bauer disk diffusion method was used to test the antimicrobial susceptibility to the fluoroquinolone antimicrobial agents: Ciprofloxacin, Ofloxacin, Norfloxacin, Lomefloxacin, Levofloxacin, Enoxacin, Moxifloxacin and Gatifloxacin. Conventional polymerase chain reaction (PCR) with specific primers to the fluoroquinolone genes was carried out to detect the presence of the genes: gyrA, parC, aac (6')-Ib- cr and qepA. Sequencing of the positive strains for the quinolone resistance genes was carried out to determine the nucleotide sequence and compare it with the nucleotide sequence of the reference strains. Plasmid profile was conducted to relate the number and size of plasmids with antimicrobial resistance pattern of the isolates. Antimicrobial resistance ranged from 23.2% for Levofloxacin and Enoxacin to 28.6% for Moxifloxacin. Out of the 38 resistant isolates 19(50 %) expressed the PMQR gene aac (6')-Ib- cr, none of the isolates expressed the efflux pump PMQR gene qepA, 5(13 %) expressed gyrA and 6(16 %) expressed parC chromosome genes. Plasmid profile of PMQR positive and negative strains showed plasmids with the same size (23,130bp). Nucleotide sequence of genes of positive strains in our study showed high percentage of identity with sequence of the reference strains. The most effective fluoroquinolones against urinary tract K. pneumoniae the isolate is Enoxacin, Levofloxacin and Ofloxacin. Our study found high prevalence of the PMQR gene aac (6')-Ib-cr in the urinary K. pneumoniae (50%), this is alarming as the genes are located on plasmid which could be easily transferred by conjugation.
Key words: *Quinolone Resistance genes, Klebsiella pneumoniae, UTI.*

INTRODUCTION

One of the major hazards facing patient requiring long time hospitalization in intensive care unit (ICU) is the spread of multidrug resistant (MDR) gram negative pathogens (Rice, 2009). Widespread occurrence of infections due to multidrug resistant organisms is alarming and a cause for great concern among health care professionals (Filippa *et al.,* 2013). The most medically important specie of the genus Klebsiella, *K. pneumoniae,* is responsible mainly for nosocomial Klebsiella infections. Urinary tract is the most common site of infection for Klebsiella *spp*. The pathogen accounts for 6 to 17% of all nosocomial UTIs with higher incidence shown in specific groups of patients at risk, e.g. patients with diabetes mellitus or with neuropathic bladders (Bennett *et al.,* 1995). The aim of this study was to examine the presence of PMQR *(aac (6')-Ib-cr, qepA)* and chromosome quinolone resistance genes *(gyrA and ParC)*.

Fluoroquinolones are used against most bacterial infections because of their broad-spectrum activity (Hopkins *et al.,* 2005). In enterobacteria, the major mechanisms of resistance to quinolones involve mutations of chromosomal genes encoding DNA gyrase and/or topoisomerase IV, mutations of genes regulating the expression of efflux pumps and a decrease in the permeability of the bacterial cell wall (Nikaido, 2003), all of which are chromosomally mediated. In addition, Plasmid-mediated quinolone resistance (PMQR), especially among the various species of the Enterobacteriaceae, has been increasingly reported in many regions of the world (Robicsek *et al.*, 2006). The *qepA* gene encodes an efflux pump that confers reduced susceptibility to fluoroquinolones such as norfloxacin and ciprofloxacin; and the *aac(6')-Ib-cr* gene that encodes modified aminoglycoside acetylating enzymes, which can inactivate aminoglycosides and fluoroquinolones. Although these PMQR genes have been associated with low-level quinolone resistance, they may cause high-level quinolone resistance by facilitating the selection of chromosomal mutations with low-level quinolone resistance, they may cause high-level quinolone resistance by facilitating the selection of chromosomal mutations (Nazik, 2011).

MATERIALS AND METHODS

Study Area and Samples Collection

One hundred and twelve *K. pneumoniae* isolates from urine of UTI patients were collected from cultures available at the Microbilogy laboratory of KAUH, Princess Basma Hospital, Prince Rashed Hospital and King Hussein Medical center Irbid, Jordan. The isolates were sub-cultured on MacConkey agar and incubated overnight at 37°C. The isolates were identified morphologically as lactose fermenting colonies (pink), with mucoid/viscous appearance and yeasty odor. These were later confirmed as *K. pneumoniae* biochemically with Microgen™ STREP ID system (catalogue# MID-62, Microgen, UK).

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed using commercial antibiotic disks (HiMedia, India). Kirby-Bauer disk diffusion antibiotic test was conducted in accordance with the clinical and laboratory standard institute (CLSI) guidelines (CLSI 2013). Isolates susceptibility to the following antibiotics; ciprofloxacin, Ofloxacin, Norfloxacin, Lomefloxacin, Enoxacin, Levofloxacin, Gatifloxacin and Moxifloxacin were tested. *E.coli* ATCC25922 and *K.pneumoniae* ATCC 700603 were used as quality control strains.

Plasmid and Genomic DNA Extraction and Quantification

Bacterial cells were cultured in Luria-Bertani broth and were subjected to total plasmid DNA extraction with commercially available kit of (Promega pure yield™ Plasmid Miniprep system (catalogue#A1223,USA), and chromosomal DNA extraction with Wizard®Genomic DNA Purification Kit (catalogue#A1120)) according to manufacturer's instructions.

The Plasmid and genomic DNA extracted were quantified indirectly by measuring the absorbance at 260nm using Thermo Scientific NANODROP 1000 Spectrophotometer USA.

Polymerase Chain Reaction (PCR)

Conventional PCR was performed to screen for PMQR and chromosomal genes using iNtRON 2x PCR Master Mix solution (*i*-MAX II catalogue# 2566, containing *i*-MAX II DNA polymerase, dNTPs, PCR reaction buffer and gel loading buffer).

Conditions for the PCR of the genes are: *aac (6')-Ib- cr*; 94°C for10 minutes,94°C for 45 second and 72°C for 10 minutes. For *qepA;* 94°C for 10 minutes 94°C for 1 min 60°C for 1min and 72°C for 10minutes . For *gyr* gene *:* 94° C for 10minutes, 94°C for 1min 55°C for 45sec 72°C for 1min 72°C for 10minutes. Condition for amplification of *parC* gene is 95°C for 10minutes 95°C for 1min 54°C for 45sec 72°C for 30sec 72°C for 10min

The PCR products were separated by electrophoresis in 2% Agarose (containing 1x Tris-borate EDTA buffer (TBE)) and visualised under UV light (Woravit *et al.*, 2013).

Plasmid Profiling

Plasmid profiling was conducted to relate the number and size of plasmids with antimicrobial resistance pattern of the isolates. Plasmid profile is important in epidemiological surveillance of disease outbreak and investigation of antimicrobial resistance transmission (Podschun and Ullmann, 1998). An appropriate volume of 12 micro litre plasmid DNA with four micro litre Kapa loading dye was loaded in 0.8% Agarose gel for 2 hours at 100V.

DNA Sequencing of Genes

DNA sequencing of some of the positive strains for the quinolone resistance genes was carried out to determine their nucleotide sequence and compare it with the reference strain using the BLASTN algorithm available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

RESULTS

Two hundred (200) isolates were initially obtained and 112 were confirmed to be *K. pneumoniae*. The remaining 88 were discarded based on the test procedures adopted.

Out of the 112 isolates of *K. pneumoniae* (66.1%), isolates were sensitive to the fluoroquinolone antimicrobial agents tested in this study and 33.9% isolates were resistant. Thirty-one (27.7%) isolates were resistant to Ciprofloxacin, 26 (23.2%) isolates were resistant to Enoxacin, 28 (25 %) isolates were resistant to Gatifloxacin, 32(28.6 %) isolates were resistant to Lomefloxacin, 26 (23.2 %) isolates were resistant to Levofloxacin, and 32 (28.6%) isolates were resistant to Moxifloxacin. Figure 1 below shows the susceptibility and resistance percentages.

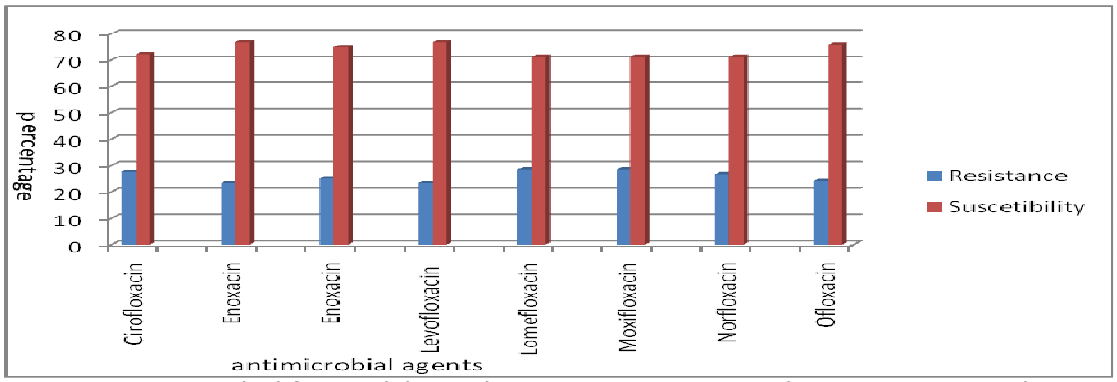

Figure 1: Antimicrobial Susceptibility and Resistance percentages of *K. pneumoniae* to the fluoroquinolones antimicrobial agent.

The electrophoresis of PCR product showed that 50% among quinolone resistant isolates were positive for *aac (6')-Ib- cr* and none of the isolate was positive for *qepA*. Among the quinolone resistant isolates, 13% were positive for *gyrA* and 16% were positive for *ParC*. Some of the genes amplified are shown in figure 2 and 3 below.

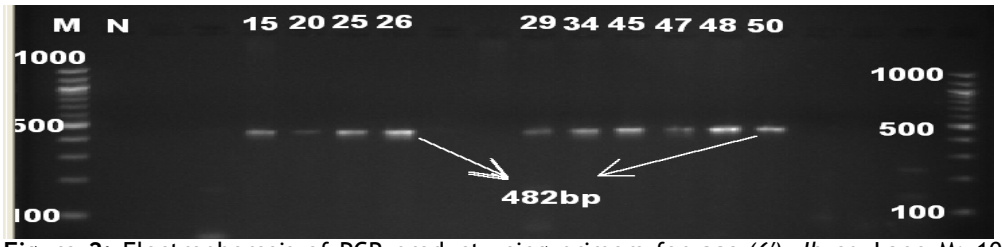

Figure 2: Electrophoresis of PCR product using primers for *aac (6')- Ib-cr*. Lane M: 100bp ladder. Lane N: Negative control. Sample: 15, 20, 25,26,29,34,45,47,48 and 50 are positive for the gene, with size of 482bp.

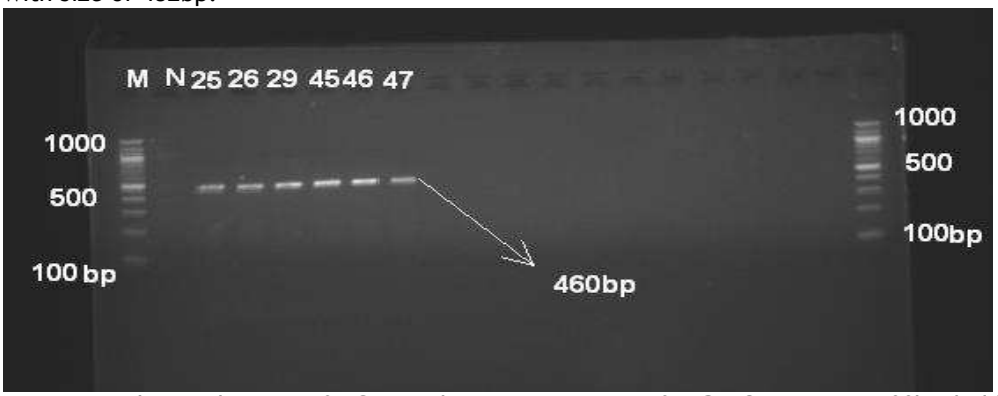

Figure 3: Electrophoresis of PCR product using primers for *ParC*. Lane M: 100bp ladder. Lane N: Negative control. Sample: 25, 26,29,45,46 and 47 are positive for the gene.

Plasmid profile result showed plasmids with size of 23,130bp as shown in figure 4.

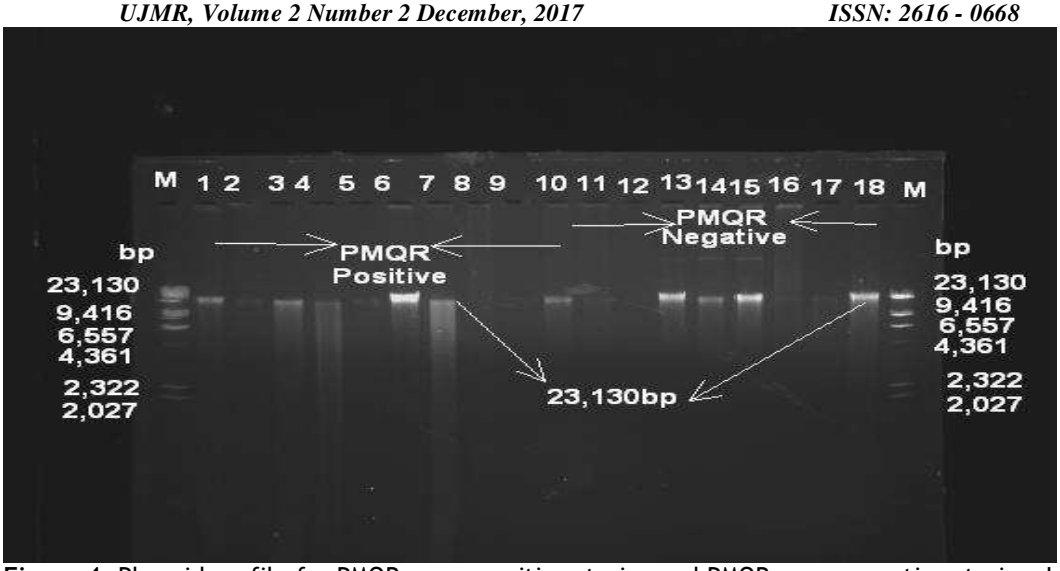

Figure 4: Plasmid profile for PMQR genes positive strains and PMQR genes negative strains. Lane M: Lambda-Hind III marker. Lane 1-10: PMQR positive. Lane 11-18: PMQR negative strains.

Figure 5 below shows the result of DNA sequencing for the positive strains with high percentage of identity to the reference strains.

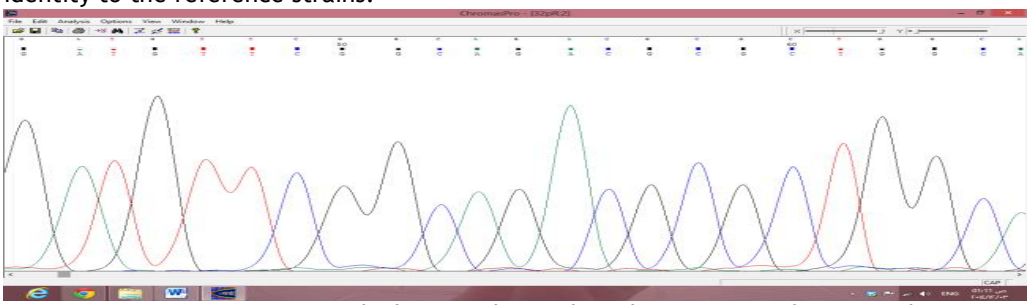

Figure 5: DNA sequencing graph showing the nucleotide sequence of *aac (6')-ib-cr gene* compared to reference strain.

DISCUSSION

The antimicrobial susceptibility test result revealed that resistance to Ciprofloxacin, Lomefloxacin and Moxifloxacin were high among the urinary isolates of *K. pneumoniae* with percentage 27.7% and 28.6%, while the most active fluoroquinolones antimicrobial agent against the isolates were Enoxacin, Levofloxacin and Ofloxacin respectively. The resistance rates in *E. coli* and *K. Pneumoniae* increased from <2% in 1996 to ≥20% in 2009 ; the resistance rates of fluoroquinolones for *P. mirabilis* remained almost constant throughout the years at ≤2%. Enterococci demonstrated frequently resistance against fluoroquinolones although resistance rates decreased between 2002 and 2009 (Dalhoff, 2012).

In a previous study in Slovenia, ciprofloxacin resistance increased from 50% to 88% between 2000 and 2005 and percentage of those with immediate resistance phenotype decreased to 12% from 50% (Avguštin *et al.*, 2007). Resistance to ciprofloxacin in the present study (27.7%) is far lower than another study in Brazil by Minarini (2012), which showed high resistance to ciprofloxacin 72.3% associated with presence of the chromosomal quinolone resistance genes; *gyrA* and *par*C.

The occurrence and rapid increase of drug-resistant *K. pneumoniae* isolates is a threat to antimicrobial management worldwide. The quinolone resistance genes detected in this study, include chromosomal genes and PMQR genes; *gyrA, parC, aac (6')- Ib-cr* and *qepA*, 50% of the resistant isolates expressed the PMQR gene *aac (6')- Ib-cr*, none of the resistant isolates expressed the plasmid mediated quinolone resistance efflux pump gene *qepA*, 13% expressed the chromosomal genes *gyrA* and 16% expressed *parC*. Out of the 19 strains positive for the *aac (6')- Ib-cr*,95% were resistant to Ciprofloxacin and Norfloxacin, this is due to N-acetylation of the piperazinyl ring of the two fluoroquinolones by the gene (Carattoli,2008). Among the PMQR genes positive strains in our study, 47% were resistant to all fluoroquinolones and 73% of the strains were found resistant to at least four fluoroquinolones.

This shows strong relationship between the plasmid genes and quinolone resistance. Also, 6/19(31.6%) PMQR gene positive strains have the chromosome genes *gyrA* and *parC*.

The result for *aac* (6')-*Ib-cr* gene is consistent to the result of Yang *et al*. (2014) who detected 77.5% of the gene,but differ slightly in qepA gene with 3.9%. In 2013, a study in Thailand also reported high percentage of the PMQR genes (*aac (6)-Ib-cr* and *qnr*) 49/75(65.3%) from urinary *Klebsiella pneumoniae* isolates (Woravit *et al.,* 2013). The occurrence of *aac (6)-Ib-cr* was lower (9.3%) in *Enterobacter cloacae* (member of Enterobacteriacea) isolated from china compared to other species tested (Yang *et al.,* 2008), and also compared to our result,this may be due to the difference in the genetic make-up of *E. Cloacae and K. pneumoniae* . In a study carried out in Egypt, the percentage of PMQR genes *aac (6)-Ib-cr* was 23.3% and *qepA* gene was 6.6% (Hassan and Domany, 2012).

Another study conducted in Turkey on urinary tract isolated *E.coli* which is also a member of Enterobacteriacea family had slightly lower percentage of *aac (6)-Ib-cr* (45.9%) compared to our study, but different percentage of *qepA* (5.7%) (Nazik, 2011). The PMQR gene *aac (6)-Ib-cr* from food poisoning patients in china, was found to co-existed with B-lactamase gene and chromosome located quinolone resistance genes *gyrA, parC* and *parE* (Hao *et al.,*2012).

Absence of *qepA* in our study is contrary to a study conducted in china which shows high prevalence of the PMQR gene between *E.coli* isolated from food producing animals, absence of this gene is not surprising, since the determinant is rarely found worldwide (Liu *et al.*,2008) .

DNA sequencing of some positive strains for the genes in our study showed high percentage of identity with the reference strains.

Plasmid profile is important in epidemiological surveillance of infection outbreaks and in tracing of antimicrobial resistance transmission (Guo *et al.,* 2012) . The result of plasmid profile in our study, showed a single plasmids with the same size (23,130bp) in both PMQR gene positive and negative strains, three strains among the PMQR negative strains showed additional plasmid with larger size above the size marker used in this study. The PMQR negative strains could get their plasmids transferred from PMQR positive strains by conjugation which occur frequently between Enterobacteriacea. Also, it is possible to have the same size plasmids with different genetic make-up, different function or different antimicrobial resistance pattern (Guo *et al.,* 2012). The landscape of fluoroquinolones resistance is getting diverse, from chromosomal mutations causing high level fluoroquinolone resistance to PMQR responsible for low level quinolone resistance. The most effective fluoroquinolones against urinary tract *K. pneumoniae* isolate were Enoxacin, Levofloxacin and Ofloxacin. Our study found high prevalence of the PMQR gene *aac (6')-Ib-cr* in the urinary *K. pneumoniae* (50%), this was alarming as the genes are located on plasmid which could be easily transferred by conjugation.

This shows that hospital isolates are important source for spreading antimicrobial resistance determinants among gram negative pathogens in particular between enterobacteriacea. Plasmid profile in our study showed no relationship between plasmid size and number with antimicrobial resistance pattern of the strains. DNA sequencing of the genes showed high percentage of identity with the genes in the reference strains.

Therefore, it is necessary to monitor for the spread of PMQR genes of clinical isolates and to ensure careful antibiotic use in a hospital setting.

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