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Low incidence of hypervirulent clinical *klebsiella pneumoniae* producing carbapenemases among Jordanian hospitalized patients

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Abstract

Background: *Klebsiella* species are widely present in the environment and colonize mucosal surfaces of humans. The organism is responsible for various community and hospital-acquired infections. The increased incidence of isolates producing *K. pneumoniae* carbapenemases (KPCs) in infected patients became a significant problem in many countries, especially those new hypervirulent clinical variant (hvKp). This prospective study was intended to detect the incidence, virulence factors and carbapenems resistant gene (blakpc2) in *K. pneumoniae* isolates among Jordanian patients.

Methods: A total of 104 *klebsiella* species isolates were collected randomly from three major hospitals in Amman, Jordan, over the period from September 2013 to October 2014. These isolates were investigated for incidence of *K. pneumoniae*, antimicrobial susceptibility and detection of virulence factors and *kpc* gene using PCR .

Results: A total of 75 (72%) of the collected isolates were confirmed as *K. pneumoniae* using PCR, and 74% of these were MDR to at least 3 antibiotic classes. The percentage of the virulence factors K1, K2, K5, rmpA and aerobactin were 0%, 4%, 0%, 5.3% and 10.7%, respectively. Resistant to cabapenems was detected in 18/75 (24%) of *K . pneumoniae* isolates, and 10 (13.3%) of these have the *kpc* genes .

Conclusion: This study confirms the high incidence rate of MDR *K. pneumoniae* and low incidence of (KPCs) isolates in Jordanian patients. There were few isolates associated with virulence factor hvKp, and no significant correlation demonstrated between the presence of virulence factors and *kpc* gene in these isolates.

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Introduction

K. pneumoniae is the most frequent *Klebsiella* species causing community-acquired and nosocomial infections worldwide [1,2]. During the last few years clinical isolates of *K. pneumoniae* producing carbapenemases (KPCs) became highly multidrug resistant (MDR), including the acquisition of extended-spectrum b-lactamases (ESBLs) and carbapenemases. These b-lactamases are able to hydrolyse the carbapenems and confer resistance to many other antibiotics. In certain countries, KPCs are now considered endemic and many outbreaks were recorded in several countries [2].

To date, nine different variants (KPC-2–KPC-10) of the KPC enzymes have been discovered, with KPC-2 and KPC-3 were the most frequently recovered from clinical specimens [2-3], and re-sequencing of the *blaKPC-1* gene revealed it to be identical to *blaKPC-2* gene [4]. Furthermore, it has been shown that increased incidence of *K. pneumoniae* producing carbapenemases isolates in association with hypervirulent characteristics in infected patients has caused a significant treatment problem [4-5].

The new hypervirulent (hypermucoviscous) clinical variant of *K. pneumoniae* (hvKp) has been increasingly detected over the last 20 years. The first reports about it were from the "Pacific Region", but in recent years more infections caused by hvKp are being reported in other countries [5]. Infection due to hvKp was clinically first observed as community-acquired liver abscess (CA-PLA); affecting patients lacking a history of hepatobiliary disease, and causing metastatic spread to distant body sites in many cases. More recently, hvKp has also been observed to cause a variety of serious extrahepatic abscesses, primary septicemia, meningitis and endophthalmitis in healthy adults. The organism was also associated with a significant mortality rate, ranging from 3–42% [5-6].

This study investigated the general incidence of MDR *K. pneumoniae* isolates and their association with potential virulence factors causing hvKp infection in Jordanian patients.

Material and Methods

Clinical specimens

This prospective study included a total of 104 *klebsiella* isolates from clinical specimens of patients who were admitted to king Hussein Medical Center, Jordan University Hospital (JUH) and Istishari Hospital in Amman, Jordan, over the period September 2013 through October 2014.

Data collection.

A biographical data about each patient was obtained and registered on special form. These include age, gender, name, disease history, and taking of antibiotic at time of sampling and before 2 week of sampling. Ethical approval was obtained from the Institutional Ethical Committees at the three hospitals, and the study was also approved by the Deanship of Scientific Research at the University of Jordan.

Klebsiella identification

All collected *klebsiella* isolates were sub-cultured on MacConkey agar and incubated for 24 hr at 37°C. Five colonies that morphologically represent *Klebsiella* like-growth were selected and sub-cultured on MacConkey again to obtain pure *Klebsiella* isolates and later these were tested by commercial Remel RapID ONE test (Oxoid, England) at the Microbiology Research Laboratories, Faculty of Medicine, The Jordan University. A total of 75 isolates were identified as *Klebsiella pneumoniae.*

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility using a disk diffusion test was performed on 75 *K. pneumoniae* according to the recommendation of the Clinical Laboratory and Standards Institute (CLSI) [7]. The results were interpreted according to the guidelines of CLSI. *Escherichia coli* ATCC 25922 was used as control for susceptibility test.

DNA and plasmid extraction

The bacterial DNA was extracted using the Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol for isolation of bacterial DNA from gram negative bacteria. The DNA quantity of each extract of *Klebsiella pneumoniae* isolate was tested by spectrophotometer (BioRad, USA) to ensure that the DNA yield is significant (>50µg/ml) for PCR test . Extraction of pure plasmid DNA was carried out according to the Zyppy™ Plasmid Miniprep Kit (alkaline lysis method) supplemented by manufactured company (Zyppy™, USA).

Polymerase chain reaction (PCR) and product analysis

Three pairs of primers were used to detect genes(*rpo B, peh X, gyr A*), the first one identified *K. pneumoniae*, the second one for *K. oxytoca*, and the last one for all *klebsiella* species as described by Chander *et al.* [8]. Multiplex PCR was used to identified virulence factors (K1, K2, K5, *rmpA*, and aerobactin) among *K. pneomoniae* isolates as reported by Siu *et al.* [9]. Uniplex PCR was used to identified *KPC* gene in 18 *K. pneumoniae* isolates which were resistant for one or more of ertapenem, imipenem and meropenem as stated by Yigit *et al.* [10]. A strain of *K. pneumoniae* ATCC BAA-1705 was used as a positive control for detection of *KPC* gene, and nuclease free water was used as a negative control.

Sequencing of the *blakpc* PCR products

DNA sequencing was performed by Macrogen Company (Seoul, Korea) to confirm the detected of *blakpc* gene using KPC primers.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS) version 20. Frequency and percentage were calculated for the categorical data, and Pearson's chi-squared test or Fisher's exact test were applied to determine potential factors associated with *k. pnemoniae* and to determine whether there are any statistical differences between virulence factors. The level of significance was set at a p value of ≤0.05 to test the hypothesis of no association. Fisher's exact test replaces chi-squared test when the minimum expected count is less than five.

Results

A total 75 *K. pneumoniae* isolates were examined by use of PCR for detection species specific genes (*rpo B* and *gyr A*.) The distribution of these isolates from patients according to the source of specimens, the gender and age is shown in **Table 1**. The ma-

Table 1. Distribution of 75 *K. pneumonia* from patients according to source of specimens*

Specimens	No.(%)
Urine	52(69.3)
Wound-pus	14(18.7)
Blood	3(4)
Catheter line	3(4)
Bone	1(1.3)
Drain	1(1.3)
Pleural fluid	1(1.3)
Total	75(100)

* Males accounted for 50.7% and females for 49.3% (mean age ± SD = 34 ± 21.9 years)

jority of the isolates (74%) were multidrug resistant to ≥ 3 antibiotic classes **(Table 2)**. Few *K. pneomoniae* isolates were positive for virulence factors genes of *K2* (3; 4%), *rmpA* (4;5.3%) and *aerobactin* (10; 10.7%) **(Table 3)**. A total of 10 /75 (7.5%) *K. pneumoniae* isolates were positive for *KPC* genes. **Table 4** shows the correlation between the type of antibiotics resistance markers and KPC positive isolates.

DNA sequencing of *K. pneomoniae* isolates positive for *blakpc* gene has confirmed the presence of *blakpc* gene using KPC primers (Macrogen Company (Seoul, Korea).

Discussion

This study demonstrates that *K. pneomoniae* is the most common species among *Klebsiella isolates* (72%) from patients in three major hospitals in Amman, Jordan. High rate of antimicrobial resistance was demonstrated and MDR accounted for 74% of the isolates. *K. pneomoniae* positive for *blaKPC* genes among the isolates was low (13.3%), and all these isolates were MDR to 11 tested antimicrobial drugs including resistant to imipenim, meropenem. These results reflect that *K. pneomoniae* carbapenemases (KPCs) is already causing infections in Jordanian patients, since our isolates originating from various clinical samples of three major hospitals in Amman. This result should be considered as alarming sign that other Gram-negative enteric bacteria causing infection in Jordanian patients may also carry *blaKPC* genes.

K. pneomoniae carbapenemases (KPCs) was first reported in USA in the year 2012. Since then, bacterial isolates carrying this resistance mechanism have frequently detected in other countries [11-12]. A recent study from Palestine has describes the first

Table 2. Antimicrobial susceptibility pattern of 75 *K. pneumoniae* isolates

Antibiotic	No. (%) Susceptible	Antibiotic	No. (%) Susceptible
Imipenem	58(77.3)	Amikacin	25(33.3)
Meropenem	54(72)	Azetreonam	24(32)
Cefoxitin	52(69.3)	Ceftazidime	24(32)
Eterapenem	46(61.3)	Cotrimoxazole	24(32)
Gentamicin	30(40)	Ceftriaxone	20(26.7)
Tigecyclin	28(37.7)	Cefotaxime	18(24)
Ciprofloxacin	26(34.7)	Amoxycilin/ Calvulanate	14(18.7)

*A total of 74% were MDR ≥ 3 antibiotic classes

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Table 3. Distribution of virulence factors among 75 *K. pneumoniae* isolates

Virulence Factor	No. (%)	Correlation coefficient Pearson
K1	0	-
K2	3(4)*	0.492
K5	0	-
rmpA	4(5.4)*	0.573
Aerobactin	8(10.7)	0.833

*Associated mostly with hypervirulent *K. pneumoniae*

Table 4. Incidence of *K. pneumoniae* producing carbapenemases (KPCs) isolates and their antimicrobial-resistance markers

Total no. *K. pneumoniae* isolates	No. (%). KPCs-positive isolates	Antibiotics resistance markers
75	10 (13.3)	Amikacin Amoxycilin/ Calvulanate Azetreonam,Ceftazidime Ceftriaxone, Cefotaxime,Eterapenem, Imepenem,Cefoxitin Meropenem Cotrimoxazole

detection of 3 isolates *bla*KPC positive *E. cloacae* and one isolates *K. pneumoniae* in patients, and all 4 isolates were resistant to all β-lactam antibiotics including carbapenems (ertapenem, imipenem and meropenem), co-trimoxazole and gentamicin, while these were susceptible to amikacin and colistin [13] .

Recently, a study from Saudi Arabia reported that multi-drug carbapenem-resistant *K. pneumoniae* infection carrying the OXA-48 gene resulted in an outbreak in a tertiary care hospital in Riyadh [14]. Additionally, there was a report from Lebanon on the first occurrence of oxacillinase-mediated resistance to carbapenems in *Klebsiella pneumoniae* isolates [15]. It is important to note here that carbapenemase-mediated resistance to carbapenems in *K. pneumoniae* may be due to KPC, VIM / IMP and OXA-48 in countries surrounding the Mediterranean [15].

A recent study by Shanmugam *et al*., (2013) in India, reported that *blaKPC* gene was recently detected in clinical isolates of carbapenem resistant *Enterobacteriaceae* in a Tertiary Care Hospital in India [16], whereas a study from china has demonstrated that the majority of carbapenem resistance determinants in *Enterobacteriaceae* (*bla*KPC–2, *bla*VIM–1,and *bla*IMP–4) were transferable by conjugation experiments [17] [*Wang et al. 2015*]. The rapid spread and growing list of pathogens in which the *blaKPC* gene has been isolated is probably due to its carriage on plasmids [18]. In addition, a large multicentre cohort study in Italy had recently showed that infections caused by *K. pneumoniae* strains expressing KPC-2 or KPC-3 are associated with a high mortality rate, and indicated that combination of antimicrobial drugs such as tigecycline, colistin and gentamicin that include meropenem can provide appreciable therapeutic benefits if the meropenem MIC for the KPC-Kp isolate is ≤8 mg/L [19].

The present study showed low distribution of the major virulence factors K1, K2, K5, rmpA and aerobactin among our 75 *K. pneumoniae* clinical isolates, and only few isolates carried the *K2* or *rmp* virulence genes which are frequently present in carbapenem-resistant hypervirulent *K . pneumoniae* (HvKP) strains **(Table 3)**. Additionally, HvKp strains carried *rmpA/rmpA2* genes on large virulence plasmid (180-220 kb) [5].

This finding shows that the prevalence rate of HvKp isolates are still low and not significant in Jordan comparing with other recent studies from china and other countries [19-21]. The occurrence of hypervirulent strains of *K. pneumoniae* (hvKp) associated with abscess formation, commonly hepatic, and metastatic spread have been increasingly detected over the world, and the management of their infections is becoming extremely challenging by presence of carbapenem-resistance [5, 20,21].

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