JJBS

Volume 13, Number 4, December 2020
ISSN 1995-6673
Pages 475 - 482

Jordan Journal of Biological Sciences

Prevalence of Capsular Polysaccharide Genes and Antibiotic Resistance Pattern of *Klebsiella pneumoniae* in Palestine

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Received: October 17, 2019; Revised: November 29, 2019; Accepted: December 21, 2019

Abstract

Klebsiella pneumoniae (*K. pneumoniae*) is a pathogenic bacteria responsible for a wide spectrum of infections in both hospital and community settings. A total of 66 isolates of *K. pneumoniae* were collected from different clinical sources in Palestine. The aim of this study was to determine the frequency of virulence genes in *K. pneumoniae* isolates using PCR technique, hypermucoviscosity (HMV) phenotype and antibiotic resistance profile. Rate of resistance to antibiotics was as follows: Trimethoprim/sulphamethoxazole (89%), Amoxicillin/clavulanic acid (82%), Aztreonam (77%), Tetracycline (71%), Ceftriaxone (67%), Imipenem (59%), Kanamycin (58%), Ceftazidime (56%), Levofloxacin (44%) and Ciprofloxacin (40%). In addition, isolates recovered from urine samples showed higher resistance ($P < 0.05$) against Imipenem, Ceftriaxone, Ceftazidime and Amoxicillin/clavulanic acid than isolates recovered from throat swabs. The prevalence of multidrug resistant *K. pneumoniae* isolates was 90.9%. Moreover, 5% of isolates were positive for HMV phenotype test. The prevalence of capsular polysaccharide genes among *K. pneumoniae* isolates was as follows: *cps* (100%), *K1* serotype (21.1%), *K2* serotype (11.7%), *p-rmpA2* (15.2%), *c-rmpA* (7.6%), *P-rmpA* (12.1%), and *magA* (0.0%). The results of this study showed that 87% of *rmpA* genes are detected in *non K1/K2* isolates and approximately 25% of tested isolates carried *K1* serotype or *K2* serotype or both serotype genes. Based on distribution of virulence factors, 3 (4.5%) strains were identified as probable hypervirulent *K. pneumoniae*. The presence of *K1* or *K2* or both serotype genes in these isolates together with other genes such as *rmpA* and high level of drug resistance should make bacteria a highly infectious agent, which leads to failure of treatment. Overall, this study demonstrates the significant role of rapid diagnosis and proper treatment of infections caused by this pathogen.

Keywords: *K. pneumoniae*, virulence factor, *rmpA* genes, *K1* serotype gene, *K2* serotype gene, *magA* gene, capsule polysaccharide synthesis (*cps*) gene, Palestine.

1. Introduction

Klebsiella pneumoniae is considered as a widespread human pathogen that is responsible for a broad spectrum of infections in both hospital and community settings. *Klebsiella pneumoniae* is also considered as a common animal pathogen associated with multiple infections, including mastitis in dairy cows (Janda and Abbot, 2009; Pan *et al*., 2015). Pathogenicity of *K. pneumoniae* depends on different virulence factors including lipopolysaccharide antigen (O-antigen), fimbriae, capsular polysaccharides (K antigen) and siderophores (Schembri *et al*., 2005; Vuotto *et al*., 2017). Each of these factors plays a particular function in the pathogenesis depending on the mode of infectivity and the type of infection (Janda and Abbott, 2006).

The incidence of bacterial infections has been increasing in the past few decades. This has led to the continuous and uncontrolled use of antibiotics for prevention and treatment in most parts of the world. Consequently, the emergence of multidrug resistance (MDR) among different strains of pathogens including *K. pneumonia* has increased. One of these mechanisms used for transmitting multi-drug resistance among microbial pathogens is horizontal spread of antibiotic resistance *genes* among bacteria. The efflux pump systems are among the most important causes of MDR (Wasfi *et al*., 2016).

The capsule is considered one of the most essential virulence factors in *K. pneumoniae*, which is associated with biofilm formation and protection of the pathogen from phagocytosis, serum bactericidal activity and antimicrobial peptides (Struve and Krogfelt, 2003; Lin *et al*., 2013; Pan *et al*., 2015). Currently, there are about 79 capsular types recognized in different *Klebsiella* sp. strains (Pan *et al*., 2015). Some of these types are K1 and K2 serotypes, which are considered as the most virulent from non-K1/K2 strains (Lin *et al*., 2004). Another gene, which is known as mucoviscosity associated gene A (*magA*) is restricted to the capsule gene cluster serotype K1 and the chromosomal K2 capsule associated gene A (*k2A*) for the K2 serotype (Yu *et al*., 2006; Doud *et al*., 2009). This gene is more prevalent in strains isolated from human liver abscesses (Fang *et al*., 2004; Lee *et al*., 2006). Existence of this gene in these strains is associated with hypermucoviscosity (HMV) phenotype and eradication resistance by human serum and phagocytosis (Fang *et al*.,

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2004; Lee *et al*., 2006). The *rmp* (regulator of the mucoid phenotype) genes play a pivotal role in hyper-production of mucoid phenotype in *K. pneumoniae* strains. The *rmpA* gene is plasmid-mediated (*p-rmpA*) or chromosomal-mediated (c-*rmpA*), which gives the strains a highly enhanced mucoviscous phenotype, regulates the capsular polysaccharide synthesis and participates in neutrophilic phagocytosis resistance (Yeh *et al*., 2007; Cheng *et al*., 2010; Ko *et al*., 2017). In addition, it was shown that *rmpA* gene is associated with strains related to invasive infections (Yu *et al*., 2006).

Molecular detection of capsule polysaccharide genes and other associated genes has been reported in different countries. A recent study conducted in Brazil showed that the prevalence of K2 serotype and K1 serotype genes among *K. pneumoniae* isolates was 4% and 0%, respectively (Ferreira *et al*., 2019). Several studies carried out in Iran showed that the prevalence of virulence factors in *K. pneumoniae* isolated from different clinical samples was 6.9%-27.82%, 6.96%-32.9%, 13.91%-20.2% and 3.8% for of K1 serotype, K2 serotype, rampA and *magA* genes, respectively (Ranjbar *et al*., 2019; Moghadas *et al*., 2018; Zamani *et al*., 2013; Feizabadi *et al*., 2013). In Iraq, it was found that 100% of clinical isolates of *K. pneumoniae* had capsule polysaccharide synthesis (*cps*) gene ,18.6% had K1 serotype, 32.6% had K2 serotype, 7% had K1\K2 serotypes and 41.9% were non-K1/K2 serotypes. Other genes were also detected such as *magA*, *rmp*A, *rmp*A1 and rmpA2 and the prevalence was 25.6%, 48.8%, 44.2% and 44.2%, respectively (Abdul-Razzaq *et al*., 2014). Also, it was shown that 57.5 % *K. pneumoniae* had K1 capsular serotype, 27.5 % had K2 serotype and 15 % had non-K1/K2 serotype. In addition, the prevalence of *magA*, *k2A* and *rmpA* genes was 57.5 %, 27.5 % and 27.5 %, respectively (Al-Jailawi *et al*., 2014).

A recent study from China, the hypermucoviscosity, as well as *magA*, K1 and K2 serotypes in *K. pneumoniae* isolates accounted to 30.7%, 45.4%, 40.5%, and 19.0%, respectively (Zhang *et al*., 2019). In another study carried out in China, the hypervirulent *K. pneumoniae* was recognized in 31.4% of the infected patients with bacteremia, and in this study 4 serotypes K1, K2, K20, and K57 were identified (Liu *et al*., 2014). In Taiwan, it was found that 98% of *K. pneumoniae* strains recovered from liver abscess were $magA^{+ve}$ (Fang *et al*., 2004). Additionally, it was shown that 38.5% of tested *K. pneumoniae* isolates had a HMV phenotypes. The existence of *rmpA* and/or *rmpA2* gene was confirmed in approximately 91% of these isolates, while these genes found only in about 18% of the isolates did not show HMV phenotype. The K1 and/or K2 serotypes were present in 16.5% of the isolates, the *rmpA* and/or *rmpA2* gene were detected in 46.2% of the isolates, with *rmpA* found in 38.5% and *rmpA2* in 45.1% of the isolates. The *magA* gene was shown to coexist in 8.8% isolates with K1 serotype (Lee *et al*., 2010). Another study conducted in Taiwan showed that the frequency of *K1* and *K2*, *rampA* and HMV phenotype was 0.0% and 7.7%, 0.0% and 0.0% respectively, from *K. pneumoniae* peritoneal dialysis-related peritonitis,while the frequency was 5.6%, 9.3%, 29.6% and 27.8% for *K1*, *K2*, *rampA* and HMV phenotype, respectively, from *K. pneumoniae* isolated from urinary tract infection (Lin *et al*., 2015). In Spain, 53 of invasive and hypermucoviscous phenotypic *K. pneumoniae* isolates, 30.2% of these isolates had a genotype $magA^{+ve}/rmpA^{+ve}$, 22.6% $magA^{-ve}/rmpA^{+ve}$, and the remaining 47.2% $magA^{-ve}/rmpA^{-ve}$. Results of this study showed that all isolates had a genotype $magA^{+ve}/rmpA^{+ve}$ were K1 serotype, while 75% of the isolates that had a genotype $magA^{-ve}/rmpA^{+ve}$ were K2 serotype (Cubero *et al*., 2016).

This study aimed to determine the frequency of virulence factor encoding genes in *K. pneumoniae* isolates, such as *cps*, *K1* serotype and *K2* serotype genes, *magA*, *p-rmpA* ,*c-rmpA*, and *p-rmpA2* using PCR technique, from patients in Northern West Bank-Palestine. Additionally, we wanted to determine the phenotypic characterization including HMV and antibiotic resistant phenotypes for these isolates. This report based on detection of these virulence genes using PCR seems to be the first report from Palestine.

2. Materials and Methods

2.1. Bacterial Strains Collection and Identification

A total of 66 non-duplicate isolates of *K. pneumoniae* were collected from clinical sources as shown in Table 1. These isolates were recovered from in-patients and out-patients from different hospitals in Northern West Bank-Palestine during 2019 (Table 1). Duplicate isolates were excluded. Identification of these isolates was carried out in laboratories of these hospitals by API 20 E system and confirmed using conventional methods in microbiology research laboratory, at An-Najah National University.

Table 1. Source of 66 of *K. pneumoniae* isolates collected from different hospitals.

Hospital	Sample source (n)					Total
	wound	urine	Sputum Trap	swab	Blood	
N	5	5	1	0	1	12
W	0	3	0	0	0	3
T	0	1	0	1	0	2
J	0	6	0	2	0	8
TH	0	2	0	0	0	2
R	0	23	0	10	0	33
S	0	3	2	1	0	6
Total =	5	43	3	14	1	66

N: An-Najah National University Hospital; W: Alwatany Hospital; T: Al-Turk Hospital; J: Jenin Governmental Hospital; TH: Thabet Hospital, R: Rafidia Hospital; S: Nablus Specialist Hospital.

2.2. Antibacterial Resistance

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2017). All *K. pneumoniae* isolates were examined for resistance to Ceftazidime (CAZ, 30µg), Ciprofloxacin (CIP, 5µg), Aztreonam (ATM, 30µg), Imipenem (IPM, 10 µg), Levofloxacin (LEV, 10µg), Ceftriaxone (CFX, 30µg), Trimethoprim/Sulphamethoxazole (SXT, 1.25/23.75µg), Tetracycline (TE, 30µg), Kanamycin (K, 30µg) and Amoxicillin/Clavulanic acid (AMC, 20/10 µg). The plates were incubated at 37°C for 18-24 hrs. The inhibition zones were measured, and isolates were classified as resistant, intermediate or susceptible according to the criteria recommended by CLSI guidelines (CLSI, 2017). The *K. pneumoniae* isolates resistant to three or more classes of antimicrobial agents were considered MDR strains. The

reference strain of *K. pneumoniae* ATCC 13883 was used as a quality control in all of the experiments of antimicrobial susceptibility testing.

2.3. String Test for Hypermucoviscosity

The string test for HMV detection was carried out as described previously (Fang *et al*., 2004). The tested strains were inoculated on 5% sheep blood agar plates and incubated at 37°C overnight. A standard bacteriologic loop was used to stretch a mucoviscous string from the colony. Hypermucoviscosity was defined by the formation of viscous string, which has a length of ≥5 mm, when a loop was used to stretch the colony on blood agar plate (positive string test).

2.4. DNA Isolation and PCR Amplification

2.4.1. DNA isolation

Genome of *K. pneumoniae* was prepared for PCR according to the method described previously (Adwan *et al*., 2013). Briefly, the cells were scraped off an overnight MHA plate, washed with 800 µL of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), centrifuged, and the pellet was resuspended in 400 µL of sterile double distilled H_2O, and boiled for 10-15 minutes. The cells were incubated on ice for ten minutes. The debris were pelleted by centrifugation at 11,500 X *g* for five minutes. The DNA concentration was determined using a nanodrop spectrophotometer (Genova Nano, Jenway). The DNA samples were stored at -20ºC.

2.4.2. PCR Amplification

The presence of 7 virulence genes was investigated using uniplex PCR. Primer sequences, size of amplicons and the annealing temperatures for detection these genes are presented in Table 2. For detection of these genes, each PCR reaction consisted of 12.5 µL of PCR premix with MgCl2 (ReadyMixTM Taq PCR Reaction mix with MgCl2, Sigma), 0.2 µM of each primer, 3 µL (50-100 ng) of DNA template. A negative control without a DNA template and a positive control strain (department collection) possessing a tested genes were used during PCR. The cycling conditions were: initial denaturation for 3 minutes at 94ºC; followed by thirty-five cycles of denaturation at 94ºC for fifty seconds, annealing temperature for each pair of primers is mentioned in Table 2 for fifty seconds, and extension at 72ºC for two minutes, followed by a single final extension step at 72ºC for five minutes. The PCR products were resolved by electrophoresis on 1.5 % agarose gel to determine the size of amplified fragments after staining with a final concentration of 0.5 µg/ml ethidium bromide.

Table 2. Target genes for PCR amplification, primer sequences, size of amplicons and annealing temperatures used.

gene	Primer sequence 5′→3′	Ta*	Amplicon size	
cps	cpsF GCT GGT AGC TGT TAA GCC AGG GGC GGT AGC G cpsR TGT ACA AGA TCC ATT TTC AGC CCC GCT GTC G	59ºC	398	Brisse *et al*., 2004
K1 serotype	K1F GTA GGT ATT GCA AGC CAT GC K1R GCC CAG GTT AAT GAA TCC GT	50ºC	1046	Lin *et al*., 2015
K2 serotype	K2F GGA GCC ATT TGA ATT CGG TG K2R TCC CTA GCA CTG GCT TAA GT	50ºC	1121	Lin *et al*., 2015
p-rmpA2	prmpA2F CTT TAT GTG CAA TAA GGA TGT T prmpA2R CCT CCT GGA GAG TAA GCA TT	50ºC	451	Lee *et al*., 2010
c-rmpA	crmpAF TGG CAG CAG GCA ATA TTG TC crmpAR GAA AGA GTG CTT TCA CCC CCT	53ºC	1006	Fang et al., 2007
p-rmpA	prmpAF TAC TTT ATA TGT AAC AAG GAT GTA AAC ATA G prmpAR CAG TAG GCA TTG CAG CAC TGC	56ºC	441	Fang et al., 2007
magA	magAF TAG GAC CGT TAA TTT GCT TTG T magAR GAA TAT TCC CAC TCC CTC TCC	52ºC	795	Struve *et al*., 2005

*Ta: Annealing temperature

2.5. Statistical Analysis

Generated data was analyzed by Z-test using SPSS software version 20. A $P < 0.05$ values were considered statistically significant**.**

3. Results

3.1. Antibacterial Resistance

In general, the results of this study showed that bacterial isolates had high resistance rate to most antimicrobial agents tested. These isolates showed high resistance rate against Trimethoprim/sulphamethoxazole (89%), Amoxicillin/clavulanic acid (82%), Aztreonam (71%) and Tetracycline (71%), while these isolates showed resistance rate 44% and 40% against Levofloxacin and Ciprofloxacin, respectively. The antimicrobial resistance profile of these isolates is presented in Table 3. Also, results showed that about 91% of the isolates were MDR. In addition, isolates recovered from urine samples showed higher resistance ($P < 0.05$) against Imipenem, Ceftriaxone, Ceftazidime and Amoxicillin/clavulanic acid than isolates recovered from throat swabs. Data are presented in Table 4. However, the prevalence of antibiotic resistance was not significant ($P < 0.05$) between isolates recovered from males and females. Data are presented in Table 5.

Table 3. Antibiotic resistance profile of 66 *K. pneumoniae* isolates recovered from different clinical samples.

Antibiotic	Antibiotic resistance n (%)*		
	S	I	R
Imipenem	20 (30)	7 (11)	39 (59)
Ceftriaxone	17 (26)	5 (8)	44 (67)
Ceftazidime	26 (39)	3 (5)	37 (56)
Aztreonam	13 (20)	2 (3)	51 (77)
Ciprofloxacin	28 (42)	11 (17)	27 (41)
Levofloxacin	36 (54)	1 (2)	29 (44)
Kanamycin	13 (20)	15 (22)	38 (58)
Trimethoprim/sulphamethoxazole	7 (11)	0 (0)	59 (89)
Amoxicillin/clavulanic acid	7 (11)	5 (8)	54 (81)
Tetracycline	19 (29)	0 (0)	47 (71)

*n: number of isolates; S: Susceptible; I: Intermediate; R: Resistant

Table 4. Antibiotic resistance rates according to the source of isolates.

Antibiotic	Sample source (n)**					Total
	Urine n=43	Throat swab n=14	Wound n=5	Sputum trap n=3	Blood n=1	
Imipenem	27 (62.8)*	4 (28.6)*	5 (100)	2 (66.7)	1 (100)	39 (59)
Ceftriaxone	31 (72.1)*	6 (42.9)*	3 (60)	3 (100)	1 (100)	44 (67)
Ceftazidime	26 (60.5)*	4 (28.6)*	3 (60)	3 (100)	1 (100)	37 (56)
Aztreonam	34 (79.1)	10 (71.4)	3 (60)	3 (100)	1 (100)	51 (77)
Ciprofloxacin	16 (37.2)	5 (35.7)	2 (40)	3 (100)	1 (100)	27 (41)
Levofloxacin	20 (46.5)	4 (28.6)	1 (20)	3 (100)	1 (100)	29 (44)
Kanamycin	25 (58.1)	6 (42.9)	3 (60)	3 (100)	1 (100)	38 (58)
Trimethoprim/ Sulphamethoxazole	39 (90.7)	12 (85.7)	5 (100)	2 (66.7)	1 (100)	59 (89)
Amoxicillin/ clavulanic acid	39 (90.7)*	6 (42.9)*	5 (100)	3 (100)	1 (100)	54 (81)
Tetracycline	31 (72.1)	9 (64.2)	4 (80)	2 (66.7)	1 (100)	47 (71)

*significant at $p < 0.05$; **n: number of isolates

Table 5. Antibiotic resistance rates according to the to patients' gender.

Antibiotic	Gender		Total
	Male (38)	Female (28)	
Imipenem	21 (55.2)	18 (64.2)	39 (59)
Ceftriaxone	23 (60.5)	21 (75)	44 (67)
Ceftazidime	18 (47.3)	19 (67.8)	37 (56)
Aztreonam	27 (71)	24 (85.7)	51 (77)
Ciprofloxacin	12 (31.5)	15 (53.5)	27 (41)
Levofloxacin	14 (36.8)	15 (53.5)	29 (44)
Kanamycin	22 (57.8)	16 (57.1)	38 (58)
Trimethoprim/ Sulphamethoxazole	34 (89.3)	25 (89.2)	59 (89)
Amoxicillin/clavulanic acid	31 (51.5)	23 (82.1)	54 (81)
Tetracycline	27 (71)	20 (71.4)	47 (71)

3.2. Hypermucoviscosity Testing

Results of the current study showed that among 66 of *K. pneumoniae* isolates, only 3 (5%) isolates recovered from urine, sputum trap and throat swab displayed hypermucoviscous phenotype. These isolates showed multidrug resistance to different tested antibiotics. In addition to *cps* gene, these isolates carried *K1/K2* (urine sample), *p-rmpA2* (sputum trap sample); however, the third isolate (throat swab) was negative for other tested genes.

3.3. Detection of Virulence Genes

Virulence related genes were studied by uniplex PCR. The prevalence of *cps*, *K1*, *K2*, *p-rmpA2*, *c-rmpA*, *P-rmpA* and *magA* genes among *K. pneumoniae* isolates was 100%, 21.1%, 11.7%, 15.2%, 7.6%, 12.1% and 0.0%, respectively. Data are presented in Figure 1 and Table 6. Results in Table 6 indicate the presence of statistically significant difference ($p < 0.05$) in the prevalence of *p-rmpA2* and *P-rmpA* genes between isolates recovered from urine and throat swab samples in favor of throat swab samples.

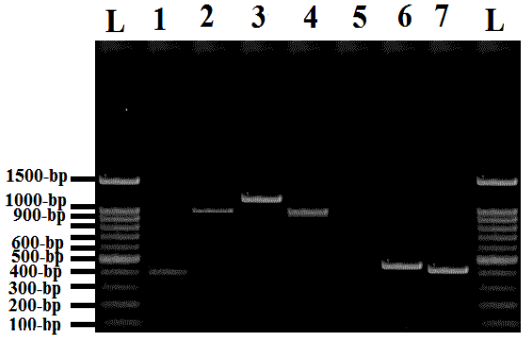

Figure 1. Uniplex PCR profile specific for genes responsible for capsular polysaccharides production. Lanes L represent 100-bp ladder; lane 1 represents *cps* gene (398-bp); lane 2 represents *K1* serotype gene (1046-bp); lane 3 represents *K2* serotype gene (1121-bp); lane 4 represents c-*rmpA* gene (1006-bp); lane 5 repesents *magA* gene (795-bp); lane 6 represents *p-rmpA2* gene (451-bp) and lane 7 represents *p-rmpA* gene (441-bp) .

Table 6. Virulence gene profiles of 66 *K. pneumoniae* recovered from different sample sources.

Virulence gene n (%)**	**Sample source (n)**					Total
	Urine (43)	Throat swab (14)	Wound (5)	Sputum trap (3)	Blood (1)	
cps	43 (100)	14 (100)	5 (100)	3 (100)	1 (100)	66 (100)
K1	10 (23.2)	3 (21.4)	1 (20.0)	0 (0.0)	0 (0.0)	14 (21.2)
K2	9 (20.9)	1 (7.1)	1 (20.0)	0 (0.0)	0 (0.0)	11 (11.7)
p-rmpA2	3 (7)*	5 (35.7)*	1 (20)	1 (33.3)	0 (0.0)	10 (15.2)
c-rmpA	5 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (7.6)
P-rmpA	2 (4.7)*	6 (42.9)*	0 (0.0)	0 (0.0)	0 (0.0)	8 (12.1)
magA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

*significant at $p < 0.05$; **n: number of isolates

The findings of the current study showed that 32 (48.5%) *K. pneumoniae* isolates carried 2 or more virulence genes. The most predominant patterns were *cps*, *K1* serotype, *K2* serotype (10.6%) and *cps*, *p-rmpA2* (7.5%). The data presented in Table 7 indicate the presence of statistically significant difference ($p < 0.05$) in

the prevalence of *cps*, *p-rmpA*, *p-rmpA2* pattern between isolates recovered from urine and throat swab samples in favor of throat swab samples. Also, results showed that 87% of *p-rmpA*, *c-rmpA*, *p-rmpA2* genes are detected in non K1/K2 isolates and approximately 25% of tested *K. pneumoniae* carried *K1* serotype or *K2* serotype or both serotype genes. In addition, distribution of these virulence genes between MDR isolates and non-MDR isolates showed the presence of statistically significant difference ($p < 0.05$) in the distribution of *p-rmpA* gene between isolates recovered from urine and throat swab samples in favor of throat swab samples. Data are presented in Table 8. While distributingon of virulence genes according to patients' gender, results showed the presence of statistically significant difference ($p < 0.05$) in the distribution of *p-rmpA* gene between isolates recovered from males and females in favor of isolates recovered from females. Data are presented in Table 9.

Table 7. Virulence patterns identified among 66 *K. pneumoniae* recovered from different sample sources.

Virulence gene pattern n (%)**	Sample source (n)					Total
	Urine (43)	Throat swab (14)	Wound (5)	Sputum trap (3)	Blood (1)	
cps, K1, K2	5 (11.6)	1 (7.1)	1 (20)	0 (0.0)	0 (0.0)	7 (10.6)
cps, p-rmpA	2 (4.6)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	4 (6.1)
cps, p-rmpA, p-rmpA2	0 (0.0)*	4 (28.6)*	0 (0.0)	0 (0.0)	0 (0.0)	4 (6.1)
cps, K1	2 (4.6)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	4 (6.1)
cps, p-rmpA2	2 (4.6)	1 (7.1)	1 (20)	1 (33.3)	0 (0.0)	5 (7.5)
cps, c-rmpA	3 (7.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (4.5)
cps, K1, K2, c-rmpA	2 (4.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.0)
cps, K2	2 (4.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.0)
cps, K1, p-rmpA2	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)
Cps	24 (55.8)	4 (28.6)	3 (60)	2 (66.7)	1 (100)	34 (51.5)
Total	43 (100)	14 (100)	5 (100)	3 (100)	1 (100)	66 (100)

*significant at $p < 0.05$; **n: number of isolates

Table 8. Distribution of virulence genes in MDR isolates and non-MDR isolates.

Virulence genes	MDR isolates n (%)	Non-MDR isolates n (%)	Total
cps	60 (100)	6 (100)	66 (100)
K1	12 (20)	2 (33.3)	14 (21.1)
K2	10 (16.7)	1 (16.7)	11 (16.7)
p-rmpA2	8 (13.3)	2 (33.3)	10 (15.2)
c-rmpA	5 (8.3)	0 (0.0)	5 (7.6)
p-rmpA	5 (8.3)*	3 (50)*	8 (12.1)

n: number of isolates; *significant at $p < 0.05$

In addition, in this study 3 (4.5%) strains were identified as probable hypervirulent *K. pneumoniae*. These strains carried *cps, K1, K2, c-rmpA* (n=1) and *cps, K1, p-rmpA2* (n=1). All these probable hypervirulent strains were MDR.

Table 9. Virulence gene profiles of 66 *K. pneumoniae* isolates distributed according to patients' gender

Virulence gene n (%)	Gender (n)**		Total
	Male (38)	Female (28)	
CPS	38 (100)	28 (100)	66 (100)
K1	9 (23.6)	5 (17.8)	14 (21.2)
K2	6 (15.7)	5 (17.8)	11 (11.7)
p-rmpA2	2 (5.2)*	8 (28.5)*	10 (15.2)
c-rmpA	4 (10.5)	1 (3.5)	5 (7.6)
p-rmpA	3 (7.8)	5 (17.8)	8 (12.1)
magA	0 (0.0)	0 (0.0)	0 (0.0)

*significant at $p < 0.05$; **n: number of isolates

4. Discussion

Klebsiella pneumoniae is a major pathogen that can cause nosocomial and community acquired infections. This pathogen harbors numerous virulence factors, which help this microorganism to cause infections. The isolates of *K. pneumoniae* had high-level of resistance rate against most antimicrobial agents tested and most of them were MDR isolates. This may be due to misuse of antibiotics at clinical settings. This is also influenced by the deficiency of a clear national antibiotic policy and over-the-counter antibiotic availability in this country (Adwan *et al*., 2014, 2016a; 2016b). Additionally, rates as high as 84% of MDR *K. pneumoniae* isolates were detected in other studies (Ferreira *et al*., 2019). Antibiotic efflux pumps are considered as one of the most important antimicrobial resistance mechanisms used by this pathogen. Existence of the multidrug efflux pump system is significantly correlated with the MDR pattern (Wasfi *et al*., 2016; Ferreira *et al*., 2019).

The results of this study indicate that all tested *K. pneumoniae* isolates carried the *cps* gene, which agrees with previously published research (Abdul-Razzaq *et al*., 2013; 2014). The presence of capsule in *K. pneumoniae* is considered one of the most vital virulence determinants. It helps in biofilm formation and enhances resistance to antibiotics by minimizing the binding of antimicrobial peptides to bacterial surface. In addition, it is an important factor that contributes in protecting the pathogen from phagocytosis process as well as serum bactericidal activity (Struve and krogfelt, 2003; Chung *et al*.,2008; Lin *et al*., 2013; Pan *et al*., 2015; Theophano *et al*., 2017).

In this study, among the 66 *K. pneumoniae* isolates collected from different clinical specimens, 3 (5%) isolates showed HMV phenotype. One of these isolates carried *K1/K2* serotype genes, and the other carried *p-rmpA2* gene. However, the third isolate was negative for all tested genes. Two of these 3 isolates, which showed HMV phenotype, were $magA^{-ve}/rmpA^{-ve}$. This may be due to these strains having mutations in these genes, which might have a potential effect on the primer annealing sites. Our findings agree with Cubero *et al*.,2016, who found that 47.2% of hypermucoviscous isolates were $magA^{-ve}/rmpA^{-ve}$. However, this result is in conflict with previous studies carried out in Taiwan (Yu *et al*.,2006) and Iran (Zamani *et al*., 2013; Shakib *et al*.,2018) which showed that 38.5% of *K. pneumoniae* isolates in Taiwan were positive to HMV phenotype, and 14.3% and 60.95% of *K. pneumoniae* isolates were positive to HMV phenotype in Iran. In a

study carried out by Shakib *et al*.,(2018), only 30% of isolates which had HMV phenotypes were *rmpA*$^{+ve}$ or *magA*$^{+ve}$. Another study in Iran showed that 33.48% of *K. pneumoniae* isolates were positive to HMV phenotype (Nahavandinejad and Asadpour, 2017). In Taiwan, most of the strains which showed positive HMV phenotype (91.4%) were *rmpA*$^{+ve}$ or *rmpA2*$^{+ve}$, while these genes were found only in 17.9% of the isolates without HMV phenotype (Yu *et al*.,2006). In another study in Taiwan, it was found that the frequency of both *rampA* and HMV phenotype was 0.0% from *K. pneumoniae* peritoneal dialysis-related peritonitis. However, the frequency of both *rampA* and HMV phenotype was 29.6% and 27.8%, respectively, from *K. pneumoniae* isolated from urinary tract infection (Lin *et al*,, 2016). In a recent study in China, 45.7% of *K. pneumonia* showed HMV phenotype (Liu and Guo, 2019). Furthermore, *K. pneumoniae* isolates exhibited the hypermucoviscosity phenotype were not limited to *magA* gene (Nahavandinejad and Asadpour, 2017). These conflictions in results could be related to the sample source. In most of those studies, *K. pneumoniae* isolates were invasive and collected from liver abscess and meninges infections.

According to K markers, *K. pneumoniae* can be grouped into 4 serotypes including K1 group, K2 group, K1/K2 group, and non K1/K2 group. Results of this study showed that the prevalence of K1 serotype was higher than *K2* serotype. These results were consistent with a previously published reports (Lee *et al*., 2010; Al-Jailawi *et al*., 2014; Cubero *et al*., 2016; Akbari *et al*., 2017; Thonda and Oluduro, 2018; Ranjbar *et al*., 2019; Zhang *et al*., 2019). However, these were in contrast to other studies (Lin *et al*., 2004; Feizabadi *et al*., 2013; Abdul-Razzaq *et al*., 2014; Lin *et al*., 2015; Moghadas *et al*.,2018; Ferreira *et al*., 2019). In a recent study in Iran carried out by Shakib *et al*., (2018), 70 *K. pneumoniae* isolates collected from different clinical sources, demonstrated that the incidence of *K2* serotype gene among these isolates was 0.0% (Shakib *et al*., 2018). In another study in Iran, the frequency of K1, K2 and non-K1/K2 serotypes was 10.77%, 6.15%, 83.07%, respectively (Akbari *et al*., 2017). In the current research, approximately 25% of the isolates carried the *K1* or *K2* or *K1/K2* serotype genes, which gives an indication that these isolates are more virulent than other isolates.

Outcomes of this research showed that the prevalence of *magA* gene was 0.0% among *K. pneumoniae* isolates recovered from different sources. This result was in contrast to other studies which showed that the prevalence of this gene ranged from 1.4%-98% (Fang *et al*., 2004; Struve *et al*., 2005; Zamani *et al*., 2013; Nahavandinejad and Asadpour, 2017; Shakib *et al*., 2018; Thonda and Oluduro, 2018). The presence of *mag*A gene in clinical isolates of *K. pneumoniae* plays an important role in serious infections such as septicemia, bacteremia, and pneumonia as well as lung and liver abscesses (Chung *et al*., 2007), HMV, protecting the pathogen from phagocyte and serum bactericidal activity (Lee *et al*., 2006). This gene is considered as a diagnostic marker of invasive *K. pneumoniae* strains. Numerous studies have shown that *magA* is more prevalent among *K1* serotype strains (Struve *et al*., 2005; Yu *et al*., 2006; Lin *et al*., 2006; Abdul-Razzaq *et al*., 2014). This may be due to that this gene is located in *cps* gene cluster *K1* of *K. pneumoniae* (Yu *et al*., 2006; Hsueh *et a*l., 2013).

Results of the current study showed that the frequency of *p-rmpA* ,c-*rmpA* ,p-*rmpA2* with the prevalence of (12.1%), (7.6%) and (15.2%) respectively. Most *p-rmpA*, *c-rmpA* and *p-rmpA2* genes are detected in non *K1/K2* isolates. This result is consistent with a previously published study (Abdul-Razzaq *et al*., 2014), which showed that *rmpA* genes were more prevalent in non K1/K2 serotype isolates. Results of our study were in contrast to previously published report (Al-Jailawi *et al*., 2014), which showed that *rmpA* genes were more prevalent in serotype K2. In this study, the frequency of *rmpA* genes among isolates was 28.8%, while the frequency of these genes in literature ranged from 15%-46.2% (Lee *et al*., 2010; Al-Jailawi *et al*., 2014; Nahavandinejad and Asadpour, 2017; Thonda and Oluduro, 2018). The *rmpA* genes are plasmid or chromosomal-mediated (*prmpA or crmpA*), which confers highly enhanced mucoviscous phenotype and participates in neutrophilic phagocytosis resistance (Yeh *et al*., 2007; *K*o *et al*., 2017). The coexistence of *magA* and the *rmpA* or *rmpA2* genes in the *K. pneumoniae* isolates increased the occurrence of expression of the HMV phenotype in these isolates (Lee *et al*., 2010). In addition, it was shown that rmpA^{+ve} extended-spectrum β-lactamases (ESBL-) *K. pneumoniae* strains had greater pathogenic potential than rmpA^{-ve} ESBL-K. pneumoniae and non-ESBL-K. pneumoniae strains (Lin et al., 2016).

It is important to note that genes encoding *rmpA*, K1, or K2 were highly associated with the hypervirulent variant of *K. pneumoniae* (Ferreira *et al*., 2019), which can cause serious community acquired infection. In this study, 3 (4.5%) strains were identified as probable hypervirulent *K. pneumoniae*. These strains carried *cps, K1, K2, c-rmpA* (n=2) and *cps, K1, p-rmpA2* (n=1). All these probable hypervirulent strains were MDR. This result is consistent with a recent report from Egypt, which showed that 6.2% strains were identified as probable hypervirulent *K. pneumoniae* (EL-Mahdy *et al*., 2018). However, this result was in contrast to another study from Brazil, which showed that there was no strains with molecular characteristics of the hypervirulent *K. pneumoniae* (Ferreira *et al*., 2019).

According to the results obtained in this study, approximately 25% of isolates were found to be positive for either *K1* serotype or *K2* serotype or both *K1* and *K2* serotype genes. In addition, the presence of *rmpA* genes in these isolates gave an indication that some of these isolates may be more highly virulent than others. The presence of these virulence factors accompanied by high level of drug resistance should make bacteria a highly infectious agent and lead to failure of treatment. This study explained the significance and the value of rapid diagnosis and proper treatment of infections caused by *K. pneumoniae* in order to achieve prevention of complicated infections.

Acknowledgment

The authors are grateful to Prof. Saleh A. Naser, University of Central Florida, College of Medicine, for language revision.

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