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Molecular characterization of capsular polysaccharide genes of *Klebsiella pneumoniae* in Palestine/West Bank

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Dedication

To the laurel crown that I wear ....who taught me to establish life stairs

wisely and patiently…. My dearest father

To the Spring that never stops giving, to my mother who weaves my

happiness with strings from her merciful heart.... My mother

To the flower that spread her perfume in my life…. My sister

To whose love flows in my veins, and my heart always remembers them….

My brothers

Acknowledgements

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الإقرار

أنا الموقعة أدناه، مقدمة الرسالة التي تحمل العنوان:

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Molecular characterization of capsular polysaccharide genes of
Klebsiella pneumoniae in Palestine/ West Bank

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Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: اسم الطالبة:

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List of Abbreviations

ATM	Aztreonam
AMC	Amoxicillin/clavulanic acid
CAZ	Ceftazidime
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CRO	Ceftriaxone
ESBL	Extended-spectrum beta-lactamase
HV	Hypermucoviscosity
IPM	Imipenem
K	Kanamycin
k2A	K2 capsule associated gene A
LEV	Levofloxacin
MagA	Mucoviscosity associated gene A
MgCl2	Magnesium chloride
MHA	Mueller Hinton agar
MR-VP	Methyl red Voges Proskauer
NA	Nutrient agar
NOR	Norfloxacin
rcs	Regulator of capsule synthesis
Rmp	Regulator of the mucoid phenotype
SIM	Sulphide indole motility medium
SXT	Trimethoprim/sulphamethoxazole
TE	Tetracycline
TSB	Tryptone soy broth
VP	Voges-Proskauer
WcaG	GDP-L-fucose synthase

Molecular characterization of capsular polysaccharide genes of *Klebsiella pneumoniae* in Palestine/

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Abstract

Klebsiella pneumoniae (*K. pneumoniae*) is a significant pathogen responsible for a wide spectrum of infections in both hospital and community settings. Investigation of genetic properties that associate with the virulence factors related to the pathogenicity of *K. pneumoniae* isolated from some clinical cases was conducted. A total of 66 non-duplicate isolates of *K. pneumoniae* were collected from different sources and from different hospitals in Northern West Bank-Palestine. This study was carried out to detect certain genes responsible for capsular polysaccharides such as *cps*, *K1*, *K2*, *magA*, *p-rmpA*, *c-rmpA*, and *p-rmpA2* using PCR technique. In addition, to detect phenotypic characterization such as hypermucoviscosity phenotype and antibiotic resistance profile for these isolates. Results of this study showed that these isolates had high-level of resistance to all antimicrobial agents tested and (60/66, 90.9%) of the isolates were multi-drug resistant (MDR). Results also showed that 5% of isolates were positive for hypermucoviscosity phenotype test. According to virulence genes among *K. pneumoniae* isolates, the prevalence of *CPS* was 100%, approximately 25% of tested isolates carried *K1* or *K2* or both genes, and 35% of isolates carried *rmpA* genes, while the prevalence of *magA* was 0.0%.

This study has characterized *K. pneumoniae* isolated from different clinical sources in the West Bank-Palestine, the presence of these *K1* or *K2* serotypes or both are probably important in clinical specimens. Presence of these virulence factors accompanied by high level of drug resistance should make bacteria highly infectious agent and lead to failure of treatment.. This study explains the significance and the value of rapid diagnosis and proper treatment of infections caused by *K. pneumoniae* in prevention of complicated infections.

Chapter One
Introduction

1.1. General background

1.1.1 *Klebsiella pneumoniae*

Species of the genus *Klebsiella* are excessively distributed in nature, belong to the family *Enterobacteriaceae*. The different species of this genus can be found in environment as a free-living state for prolonged period. In addition, they are found as normal flora in the digestive tract of different living organisms such as vertebrates and mammals (Janda and Abbot, 2009). The genus *Klebsiella* includes human pathogens such as *K. pneumoniae* subspecies *pneumoniae, rhinoscleromatis, ozaenae, K. granulomatis*; *K. oxytoca*; *K. singaporensis* and *K. variicola. Klebsiella. terrigena, K. planticola* and *K. orinthinolytica* have been relocated to the another genus called *Raoultella* (Janda and Abbott, 2006; Abbott, 2007).

Species of *Klebsiella* genus are rod-shaped Gram-negative bacteria, lactose fermenter, most are nonmotile, usually have a polysaccharide capsule, Voges-Proskauer (VP) test positive and indole test negative (except for *K. oxytoca*). These bacteria produce lysine decarboxylase enzyme but not ornithine decarboxylase. Other traits that are traditionally associated with many/most *Klebsiella* spp. include fermentation of *m*-inositol and urea hydrolysis, a carbohydrate-like compound that is not commonly utilized by many other enterobacteriaceae groups. These species of bacteria are generally facultative anaerobic, have a size from 0.3 to 1.0 μm in width and

0.6 to 6.0 μm in length, and often occur as mucoid colonies (Janda and Abbott, 2006; Abbott, 2007; Janda and Abbot, 2009).

Klebsiella pneumoniae, is considered a widespread human pathogen that is responsible for a broad spectrum of infections in both hospital and community settings. This pathogen is associated with nosocomial infections, such as septicemia, urinary tract infections and pneumonia. In addition, it can cause community-acquired infections, including urinary tract infections, pneumonia and pyogenic liver abscess complicated with meningitis and endophthalmitis. *Klebsiella pneumoniae* is also considered an important animal pathogen and associated with different infections, including mastitis in dairy cows (Janda and Abbot, 2009; Pan *et al*., 2015).

1.1.2 Capsular Polysaccharide Genes

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 virulence factors plays a particular function in the pathogenesis depending on the mode of infectivity and the type of infection (Janda and Abbott, 2006).

The capsule is considered one of the most significant virulence factors in *K. pneumoniae*, which associates with biofilm formation and protects the pathogen from phagocyte activity, serum bactericidal activity and antimicrobial peptides (Struve and krogfelt, 2003; Cortes *et al*., 2002; Lin *et al*., 2013; Pan *et al*., 2015). Now, about 79 capsular types have been

recognized in different *Klebsiella* sp. strains (Pan *et al*., 2015). Of these types, *K1* serotype is considered the most virulent from that of non-*K1* strains, in which *K. pneumoniae* genotype *K1* pathogen is capable of causing disastrous septic ocular or central nervous system complications from pyogenic liver abscess independent of underlying diseases in the host (Fang *et al*., 2007). It was found that most strains of *K. pneumoniae* that carry *K1* and *K2* determinants were more resistant to the phagocytosis process than those strains which have not *K1* and *K2* (non-K1/K2 strains) (Lin *et al*., 2004). Other gene called 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). The expression of extracapsular polysaccharide (*cps*) gene clusters in *K. pneumoniae* is controlled and modulated by a complex group of several regulators such as the *Rcs* system, *RmpA*, *RmpA2* and others (Lin *et al*., 2013).

The *magA* gene is detected in the majority of invasive strains of *K. pneumoniae* recovered from liver abscess. The existence of this gene in invasive *K. pneumoniae* strains is associated with hypermucoviscosity and resistance of this pathogen to killing by human serum and phagocytosis (Fang *et al*., 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 a plasmid- 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, 2017). In addition, it was shown that *rmpA* gene is associated with strains related to invasive infections (Yu *et al*., 2006). Transformation of the *rmpA* deletion mutant with an regulator of capsule synthesis *B* (*rcsB*) carrying plasmid showed that these cells failed to increase capsule production. A cooperation between *rmpA* gene and *rcsB* may be required for regulatory activity (Cheng *et al*., 2010). Remove or deletion of the *rmpA* gene can decrease virulence in mouse lethality tests by 1000-fold (Nassif *et al.,* 1989). The gene *rmpA2* is considered as a *trans*-acting activator for the capsule biosynthesis. According to serotypes , it is found that *rmpA1* and *rmpA2* are more predominant in *K2* and in non *K1/K2* strains this will increase and enhance the severity of *K. pneumoniae* strains (Abdul-Razzaq *et al*., 2014). It was found that *K. pneumoniae* CG43 strain highly reduces its mucoidy and pathogenicity if this strain lost the plasmid which carries the *rmpA2* gene (Lai *et al*., 2003). In addition, It was shown that pathogenic potential is greater in *rmpA*$^{+ve}$ ESBL-*K. pneumoniae* strains than in *rmpA*$^{-ve}$ ESBL-*K. pneumoniae* and non-ESBL-*K. pneumoniae* strains (Lin *et al*., 2016)

1.2. Literature review

Molecular detection of capsule polysaccharide genes and other associated genes has been reported. In a recent study carried out in Iran, the frequency of virulence factors in clinical urine isolates of *K. pneumoniae* was 32.9%, 20.2%, 6.9% and 16.2% for capsular type *K2*, *rmpA*, capsular type *K1*, and GDP-L-fucose synthase (*WcaG*), respectively (Moghadas *et al*., 2018). In

other study carried out in the same country showed that the prevalence of *magA* gene in clinical isolates of *K. pneumoniae* recovered from different sources was 3.8% (Zamani *et al*., 2013). In other study conducted in Iran, it was found that the frequency of *K1* and *K2* serotypes from clinical *K. pneumoniae* isolates recovered from urine samples was 11.25 and 14.6%, respectively (Feizabadi *et al*., 2013). In Iraq, it was found that all of clinical *K. pneumoniae* isolates have *cps* gene, 18.6% had *K1*, 32.6% had *K2*, 7% had *K1/K2* and 41.9% were non-*K1/K2*. Other genes were also detected such as *magA*, *rmpA*, *rmp*A1 and *rmpA2* and the prevalence was 25.6%, 48.8%, 44.2% and 44.2%, respectively (Abdul-Razzaq *et al*., 2014). In other study from the same country conducted in 2014, 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).

In China, the hypervirulent *K. pneumoniae* was recognized in 31.4% of the infected patients with *K. pneumoniae* bacteremia. Four serotypes *K1, K2, K20, and K57* were identified in this study (Liu *et al*., 2014). In Taiwan, it was found that 38.5% of tested *K. pneumoniae* isolates showed a hypermucoviscosity phenotypes. The existence of *rmpA* or *rmpA2* gene was found in approximately 91% of the *K. pneumoniae* isolates with the hypermucoviscosity phenotype, while these genes found only in about 18% of the isolates which did not have hypermucoviscosity phenotype. The *cps K1/K2* was present in 16.5%, with *K1* in 8.8% and *K2* in 7.7% of the total

isolates. The *rmpA* or *rmpA2* gene was 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 isolates (8.8%) with the *cps K1* serotype (Lee *et al*., 2010). In other study from the previous country, it was found that 98% of *K. pneumoniae* strains recovered from liver abscess were *magA*$^{+ve}$ (Fang *et al*., 2004). In other study from Taiwan, Lin *et al*., (2015) found that the frequency of *K1* and *K2*, *rmpA* and hypermucoviscosity phenotype was 0.0% and 7.7%, 0.0% and 00.0% respectively, from *K. pneumoniae* peritoneal dialysis-related peritonitis. While, the frequency of *K1* and *K2*, *rmpA* and hypermucoviscosity phenotype were 5.6%, 9.3%, 29.6% and 27.8%, respectively, from *K. pneumoniae* isolated from urinary tract infection.

In Spain, 53 of invasive and hypermucoviscous phenotypic *K. pneumoniae* isolates, 30.2% of these isolates had a genotype *magA*$^{+}$/*rmpA*$^{+}$, 22.6% *magA*$^{-}$/*rmpA*$^{+}$, and the remaining 47.2% *magA*$^{-}$/*rmpA*$^{-}$. Results of this study showed that all isolates had a genotype *magA*$^{+}$/*rmpA*$^{+}$ were serotype *K1*, while 75% of the isolates had a genotype *magA*$^{-}$/*rmpA*$^{+}$ were serotype *K2* (Cubero *et al*., 2016).

1.3. Aims of the Study

The aim of this work study the genotypic and phenotypic characterization of *K. pneumoniae* isolated from patients in Northern West Bank-Palestine. This study was carried out to detect the genes responsible for capsular polysaccharides such as *cps, K1, K2, magA, p-rmpA ,c-rmpA, and p-rmpA2*

using PCR technique. In addition, to detect phenotypic characterization such as hypermucoviscosity phenotype and antibiotic resistance profile for these isolates. These genes were not studied previously in Palestine.

Chapter Two
Materials and Methods

2.1. Bacterial Strains collection and Identification

A total of 66 non-duplicate isolates of *K. pneumoniae* were collected from different sources such as urine (n=43),wound (n=5), throat swab (n=14), blood (n=1), and sputum trap (n=3). These isolates were recovered from in-patients and out-patients from different hospitals in Northern West Bank-Palestine. The hospitals were An-Najah National University Hospital-Nablus (n=12), Al-Turki Hospital-Tubas (n=2), Rafidia Hospital-Nablus (n=33), Al-Watani (The National) Hospital-Nablus(n=3), Nablus Specialist Hospital-Nablus (n=6), Jenin Governmental Hospital-Jenin (n=8) and Thabet hospital-Tulkarm (n=2) (Table 2.1). These isolates were collected from these hospitals during 2019. Identification of these isolates were carried out in laboratories of these hospitals by API 20 E system and confirmed using different biochemical tests (growth on MacConkey at 5°C and 44.5°C, motility test, Gram stain, Indole test, Voges-Proskauer test) in microbiology research laboratory, at An-Najah National University.

Table 2.1. A sample source of 66 of *K. pneumoniae* isolates collected from different hospitals

Hospital	Sample source (n)					Toatal
	wound	urine	Sputum Trap	throat 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. Media Preparation

2.2.1. MacConkey agar

MacConkey agar (HIMEDIA, India) was prepared according to the manufacturer's instructions. A 1 L bottle containing 500 ml deionized water and 25 g MacConkey agar was heated and stirred until the agar dissolved. The solution was allowed to boil for 1min, and then autoclaved at 121°C for 15 min. After that it will be allowed to cool to about 50°C, and the agar was poured into sterile Petri dishes to have 25 ml that covered and left overnight. The following morning the Petri dishes were turned upside down and stored in the refrigerator at 4°C.

2.2.2. Sulphide indole motility (SIM) medium

SIM medium (Acumedia, India) was prepared according to the manufacturer's instructions. A 0.5 L bottle containing 250 ml deionized water and 7.5 g of SIM agar was heated and stirred until dissolved. Medium was dispensed into tubes to a give depth of about 4-5 cm each and plugged them with a piece of cotton. Then the medium in tubes was autoclaved at 121°C for 15 min, allowed to cool and stored the refrigerator at 4°C.

2.2.3. Mueller Hinton agar (MHA)

Mueller Hinton agar (BD, Baltimore) was prepared according to manufacturer's instructions. A 1 L bottle containing 500 ml deionized water and 19 g of MHA was heated and stirred until agar completely dissolved. The solution was allowed to boil for 1 minute and then autoclaved at 121°C for 15 minutes. After that, it was allowed to cool to about 50-60°C and poured in sterile Petri dishes to have approximately 30-25 ml each, then covered and left overnight at room temperature. The following day, the Petri dishes were turned upside down and stored in the refrigerator at 4°C.

2.2.4. Nutrient agar (NA)

Nutrient agar (ACUMEDIA, India) was prepared according to manufacturer's instructions. In a 1 L bottle, 500 ml deionized water was heated and mixed with 11.5 g NA until the agar dissolved. The solution was allowed to boil for 1 minute, and then autoclaved at 121°C for 15

minutes. After that it was allowed to cool to about 50-60°C. The agar was poured into sterile Petri dishes to have 20-25 ml, then covered and left overnight at room temperature. The following morning the Petri dishes were turned upside down and stored in the refrigerator at 4°C.

2.2.5.Tryptone soy broth(TSB)

Tryptone soy broth (OXOID, England) was prepared according to manufacturer`s instructions labeled on the bottle. In a 0.5 L bottle, 250 ml deionized water was mixed and heated with 7.5 g of TSB medium until completely dissolved. The broth was then distributed into tubes to have approximately 10 ml each and plugged them with a piece of cotton. The tubes were autoclaved at 121°C for 15 min, then allowed to cool and stored in the refrigerator at 4°C.

2.2.6. Methyl red Voges Proskauer(MR-VP)

MR-VP (HIMEDIA, India) was prepared according to manufacturer's instructions. A 0.5 L bottle containing 250 ml of deionized water were heated with 4.3g of MR-VP medium until completely dissolved. The broth was then dispensed into tubes to have approximately 5-8 ml each and plugged with a piece of cotton. The tubes were autoclaved at 121°C for 15 min, allowed to cool and then stored in the refrigerator at 4°C.

2.2.7. Blood agar

Blood agar was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 20 g blood agar base (Oxoid,

USA) and 455 ml deionized water were heated and stirred until the agar dissolved. Then, the mixture was autoclaved at 121°C for 15 min and allowed to cool to about 50°C-60°C. After that, 25 ml of sterile defibrinated sheep blood was added aseptically and mixed thoroughly. The agar was poured into Petri dishes to have 20-25 ml each, then covered and left overnight at room temperature. In the next day, the Petri dishes were stored in the refrigerator at 4°C.

2.3. Bacterial Sample Identification

2.3.1. Gram staining

Gram staining was performed to distinguish Gram-positive bacteria from Gram-negative bacteria. A thin smear of bacteria was made on a clean glass slide by picking the isolates from marked colonies after 24 hours incubation on Mueller-Hinton agar plate and mixed with a drop of distilled water. The smear was heat fixed by passing through a flame 2 or 3 times. After cooling, the slide was flooded with primary stain (crystal violet) and left for one minute then washed with tap water. Gram's iodine solution was then added for one minute and again washed off with a tap water, decolorized with acetone alcohol from 10 to 20 seconds and then again washed with tap water. Finally, the slide was flooded with a counter stain (safranin) for 1 minute then washed with tap water and let to dry. The smear was observed under 100x objective lens of the light microscope (Cappuccino and Sherman, 1996).

2.3.2. Motility test

A motile organism, caused turbidity (diffused growth) in the SIM agar deep tube inoculated by means of stab method and the stab line is obscured. But in case a non motile organism, the growth was restricted to the stab line and the medium was very clear (Johnson and case, 1998). After pure organism inoculated into the SIM agar deep tube by a stabbing method, the tubes were incubated at 37°C/24 h. The presence of turbidity (diffused growth) in the SIM agar deep tube, the organism was considered a motile.

2.3.3. Indole test

Indole test was carried out by adding 0.5 ml (10 drops) of Kovac's reagent into SIM agar deep tube inoculated by mean of stab method and incubated 37°C/24 h (Cappuccino and Sherman, 1996). A cherry-red ring (pink to red color) appeared on the top of medium after adding the indicator was considered as a positive result, while negative result showed no color change after the addition of the indicator.

2.3.4. VP test

MR/VP broth was inoculated with a pure culture of the *K. pneumoniae* and incubated for 24 hours at 37°C. Test was carried out by adding 0.5 ml (10 drops) of Barritt's reagent A, the culture was shaken, then immediately 0.5 ml (10 drops) of Barritt's reagent B was added, then culture was shaken and re-shaken after every 3-4 min. Formation a cherry red color after 15 min indicates a positive result for VP test while a yellow-brown or no

change in color indicates a negative result (Cappuccino and Sherman 1996).

2.3.5. Growth at 5°C and 44.5°C

Each *K. pneumoniae* isolate was subcultured on 2 MacConkey agar plates, one was incubated at 5°C, while the other at 44.5°C for 24 h.

2.4. String Test for Hypermucoviscosity

The string test for Hypermucoviscosity 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 $37^{o}C$ overnight. A standard bacteriologic loop was used to stretch a mucoviscous string from the colony. Hypermucoviscosity (HV) was defined by the formation of viscous string have a length ≥5 mm when a loop was used to stretch the colony on blood agar plate (positive string test).

2.5. 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 zone of inhibition was measured in millimetres using a calliper. Isolates were classified as resistant 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 multi-drug resistant (MDR).

2.6. DNA Isolation and PCR Amplification

2.6.1. DNA isolation

Genome of *K. pneumoniae* was prepared for PCR according to the method described previously (Adwan *et al*., 2013). Briefly, cells were scraped off an overnight MHA plate with a sterile loop, washed with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was resuspended in 0.5 ml of sterile distilled H_2O, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. DNA concentration was determined using a nanodrop spectrophotometer (Genova Nano, Jenway), then the samples were stored at -20ºC until use for PCR analysis.

2.6.2. PCR Amplification:

The presence of 7 virulence genes (Abdul-Razzaq *et al*., 2014) were investigated using uniplex PCR. The amplicon size, primer sequence and annealing temperature for these genes are presented in Table 2.

For detection of these genes, the PCR reaction was performed with a final volume of 25 μl containing 12.5 μl of PCR premix with MgCl2 (ReadyMixTM Taq PCR Reaction mix with MgCl2, Sigma), 0.2 μM of each primer, 3 μL (100-200 ng) of DNA template. The DNA amplification was performed with a thermal cycler (Mastercycler Personal, Eppendorf)

using the following conditions: initial denaturation for 3 minutes at 94ºC; followed by 35 cycles, each cycle consisted of denaturation at 94ºC for 50 seconds, annealing temperature for each pair of primers is included in Table 2.1 for 50 seconds and extension at 72ºC for 2 minutes, was followed by a final extension step at 72ºC for 5 minutes. The PCR products were resolved by electrophoresis through 1.5 % agarose gel to determine the size of amplified fragment after ethidium bromide (0.5 μg/ml) staining.

Table 2.2. Target genes for PCR amplification, primer sequence, amplicon size and annealing temperature that were used in this study.

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*., 2005 This study
K1	K1F GTA GGT ATT GCA AGC CAT GC K1R GCC CAG GTT AAT GAA TCC GT	50ºC	1046	Lin *et al*., 2015
K2	K2F GGA GCC ATT TGA ATT CGG TG K2R TCC CTA GCA CTG GCT AAG 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	crmpA F TGG CAG CAG GCA ATA TTG TC crmpA R GAA AGA GTG CTT TCA CCC CCT	53ºC	1006	Fang et al., 2007
p-rmpA	prmpA F TAC TTT ATA TGT AAC AAG GAT GTA AAC ATA G prmpA R 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

Chapter Three
Results

3.1. Identification of *Klebsiells pneumoniae* isolates

A total of 66 isolates of *Klebsiells pneumoniae* were isolated and identified in laboratories of hospitals in Northern West Bank-Palestine. Identification of these isolates in laboratories of these hospitals were carried out by API 20 E system. These isolates were confirmed using conventional methods in Microbiology laboratories at An-Najah National University-Nablus, Palestine. On MacConkey agar, all isolates showed typical pink mucoid colonies (lactose fermentation positive). All these isolates on MacConkey agar have ability to grow at 44.5°C but not at 5°C. In addition, bacterial cells of these isolates showed Gram-negative reaction and rod shape. Also all isolates showed indole test negative, H2S production negative, VP test positive and typically were non motile microorganisms.

3.2. Antibacterial Resistance

All tested antibiotics were classified into 8 different classes according to their chemical structure: Carbapenems (Imipenem), Cephalosporins (Ceftriaxone and Ceftazidime), Monobactams (Aztreonam), Fluoroquinolones (Ciprofloxacin and Levofloxacin), Aminoglycosides (Kanamycin), Sulfonamides (Trimethoprim/sulphamethoxazole), Penicilline-type antibiotic (Amoxicillin/clavulanic acid) and Tetracycline (Tetracycline). In general, results of this study showed that these isolates had high-level of resistance to all antimicrobial agents tested. In the

current study, *K. pneumoniae* isolates showed high resistance to Trimethoprim/sulphamethoxazole (89%), Amoxicillin/clavulanic acid (82%), Aztreonam (77%) and Tetracycline (71%), while these isolates showed more susceptible to Levofloxacin (55%). The resistance pattern of these isolates against ten chosen antimicrobial agents is presented in Table 3.1. In addition, results showed that (60/66) 90.9% of the isolates were MDR. The most predominant MDR patterns were IPM,CIP,CRO,CAZ,K, LEV,SXT,ATM,AMC,TE   (11/60,   18.3%)   and CIP,CRO,CAZ,K,LEV,SXT, ATM,AMC,TE (9/60, 15%). The MDR patterns are presented in Table 3.2.

Table 3.1. Antibiotic resistance profile of 66 *K. pneumoniae* isolates recovered from different clinical samples.

Group	Antibiotic	Antibiotic resistance n (%)		
		S	I	R
Carbapenems	Imipenem	20(30 %)	7 (11 %)	39 (59 %)
Cephalosporins	Ceftriaxone	17 (26 %)	5 (8 %)	44 (67 %)
	Ceftazidime	26 (39 %)	3 (5 %)	37 (56 %)
Monobactams	Aztreonam	13 (20%)	2 (3%)	51 (77%)
Fluoroquinolones	Ciprofloxacin	28 (42 %)	11 (17 %)	27 (40 %)
	Levofloxacin	36 (55%)	1(2%)	29(44%)
Aminoglycosides	Kanamycin	13 (20%)	15 (23%)	38 (58%)
Sulfonamides	Trimethoprim/sulphamethoxazole	7 (11%)	0 (0%)	59 (89%)
penicillines	Amoxicillin/clavulanic acid	7 (11 %)	5 (8%)	54 (82%)
Tetracycline	Tetracycline	19 (29%)	0(0%)	47 (71%)

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

Table 3.2. Multidrug resistance patterns of 60 *K. pneumoniae* isolates recovered from different clinical samples.

	Resistance pattern	No. of isolates
1	IPM,CIP,CRO,CAZ,K,LEV,SXT,ATM,AMC,TE[*]	11
2	CIP,CRO,CAZ,K,LEV,SXT,ATM,AMC,TE	9
3	CRO,CAZ,K,LEV,SXT,ATM,AMC,TE	1
4	CRO,ATM,SXT,TE	2
5	TE,ATM,SXT	1
6	TE,ATM,SXT,K,CRO	1
7	TE,AMC,ATM,SXT,CAZ,CRO	1
8	TE,AMC,ATM,SXT,K,CRO	1
9	TE,AMC,ATM,SXT,K	1
10	IPM,CIP,CRO,CAZ,K,LEV,SXT,ATM,AMC	2
11	IPM,CIP,CRO,CAZ,K,LEV,ATM,AMC	1
12	TE,AMC,ATM	1
13	CRO, K, SXT,ATM,AMC	1
14	IPM, CRO, K, SXT,ATM,AMC,CAZ	2
15	CRO,SXT,ATM,AMC,TE	1
16	IPM, CRO,SXT,ATM,AMC,TE,CAZ	2
17	IPM,CIP,CRO,CAZ, LEV,SXT,ATM,AMC,TE	1
18	IPM,AMC,TE	1
19	IPM,CAZ,SXT,ATM,AMC,TE	1
20	IPM, CRO,CAZ,K,SXT,ATM,AMC,TE	2
21	IPM,CIP,CRO,CAZ,K, SXT,ATM,AMC,TE	1
22	IPM,CRO,CAZ,K,SXT,ATM,AMC,TE	2
23	IPM,SXT,AMC,TE	3
24	SXT,AMC,TE	1
25	IPM, CRO,CAZ,K,LEV,SXT,ATM,AMC,TE	1
26	IPM,CRO,K,SXT,ATM,AMC,TE	1
27	IPM,CRO,SXT,ATM,AMC	1
28	IPM,CRO,K,SXT,AMC,TE	1
29	IPM,K,SXT,AMC,TE	1
30	IPM,K,SXT,AMC	2
31	IPM,CIP, LEV,SXT,AMC,TE	1
32	IPM,SXT,ATM,AMC	1
33	IPM,CRO,CAZ,AMC	1
	Total	60

[*]Imipenem, IPM; Ceftriaxone, CRO; Ceftazidime, CAZ; Aztreonam, ATM; Ciprofloxacin, CIP; Levofloxacin, LEV; Kanamycin, K; Trimethoprim/sulphamethoxazole, SXT; Amoxicillin/clavulanic acid, AMC; Tetracycline,TE.

3.3 Hypermucoviscosity

Results of this study showed that among 66 of *K. pneumoniae* isolates, only 3/66 (5%) isolates were positive for hypermucoviscosity test. The sources of these isolates were one urine sample, one sputum trap and one throat swab.

3.4. Detection of Virulence gene

Virulence related genes were studied by uniplex PCR. The capsular polysaccharide gene (*CPS*) was detected in all studied isolates of *K. pneumoniae*. In this study, 24.2% of tested *K. pneumoniae* carried *K1* or *K2* or both genes. The prevalence of isolates that carried *K1*, *K2*, *K1/K2* and non-*K1/K2* genes was 7.6%, 3%, 13.6% and 75.8%, respectively. Results are presented in Figure 3.1 and Table 3.3. In addition, isolates were carrying *p-rmpA*, *c-rmpA*, *p-rmpA2* with the prevalence of (12%), (8%) and (15%) respectively. Most *p-rmpA* ,*c-rmpA*, *p-rmpA2* genes are detected in *non K1/K2* isolates. Results are presented in Table.3. This study showed that none of *K. pneumoniae* isolates carried *magA* (0%) gene.
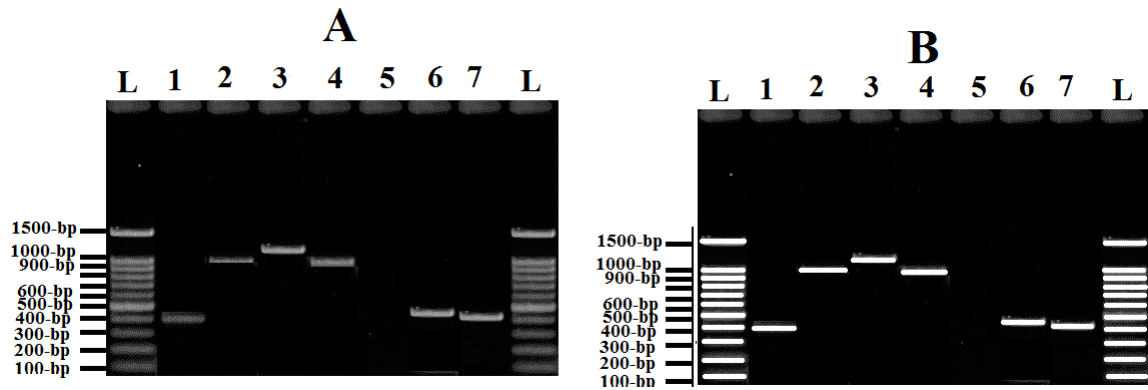
Figure 3.1. Uniplex PCR profile specific for genes responsible for capsular polysaccharides production. Lanes L represent 100 bp ladder; lane 1 represents *cps* gene (398-bp); lane 2 represents *K1* gene (1046-bp); lane 3 represents *K2* gene (1121-bp); lane 4 represents c*rmpA* gene (1006-bp); lane 5 repesents *magA* gene (795-bp); lane 6 represents *prmpA2* gene (451-bp) and lane 7 represents *rmpA* gene (441-bp) . Figure 3.1 A and B are the same but bands are demarcated for clarity.

Table 3.3. Distribution of 66 *K. pneumoniae* isolates into genotypic groups (according to the presence of *K* genes).

K. pneumoniae isolates (66)	
Genogroups	No. of isolates (%)
K1	5 (7.6%)
K2	2 (3%)
K1/K2	9 (13.6%)
Non-K1/K2	50 (75.8%)
Total	66 (100%)

Table 3.4. Distribution of *K. pneumoniae* isolates according *rmp*A genotypic markers.

Genotype	*K. pneumoniae* isolates (66)		
	p-rmpA	*c-rmpA*	*p-rmpA2*
K1	0	0	1
K2	0	0	0
K1/K2	0	2	0
Non-K1/K2	8	3	9
Total	8	5	10

Chapter Four
Discussion

Klebsiella pneumoniae is a major pathogen that can cause nosocomial and community acquired infections. This pathogen harbors numerous virulence factors which help this microorganisms to cause infections. Results of this study showed that the isolates of *K. pneumoniae* had high-level of resistance to all antimicrobial agents tested and most of them were MDR isolates. This may be due to selective pressure of antibiotic resulting from uncontrolled, extensive incorrect and misuse of antimicrobial agent in hospitals as well as in the country as a whole. This is promoted by the lack of national antibiotic policy and over-the-counter antibiotic availability in this country (Adwan *et al*., 2014, 2016a; 2016b)

Result of the current research showed that all *K. pneumoniae* isolates carried the *CPS* gene. This result is in agreement with previously reported results (Abdul-Razzaq *et al*., 2013; 2014). The presence of capsule in *K. pneumoniae* is considered one of the most vital virulence determinant. It helps in biofilm formation, enhances resistance to antibiotics by minimizing the binding of antimicrobial peptides to bacterial surface, important factor that contribute in protection of the pathogen from phagocyte and serum bactericidal activity (Chung *et al*.,2008; Theophano *et al*.,2017; Struve and krogfelt, 2003; Cortes *et al*., 2002; Lin *et al*., 2013; Pan *et al*., 2015).

In this study, among the 66 *K. pneumoniae* isolates collected from different clinical specimens, 3 (5%) isolates showed hypermucoviscosity. The sources of these isolates were urine sample, sputum trap and throat swab. These isolates were *magA^{-ve}/rmpA^{-ve}*. Result of this research is in agreement 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 hypermucoviscosity phenotypes, and 14.3% and 60.95% of *K. pneumoniae* isolates showed a hypermucoviscosity phenotypes in Iran. The study was carried out by Shakib *et al.,*(2018), only 30% of isolates which had hypermucoviscosity phenotypes were *rmpA* or *magA* positive. Other study in Iran showed that 33.48% of *K. pneumoniae* isolates strains showed an hypermucoviscosity-positive phenotype (Nahavandinejad and Asadpour, 2017). In Taiwan, most of the strains which showed hypermucoviscosity phenotypes (91.4%) were essentially associated with *rmpA* or *rmpA2* gene, while these genes found only in 17.9% of the isolates without hypermucoviscosity phenotype (Yu *et al.*,2006). In other study in Taiwan, it was found that the frequency of both *rmpA* and hypermucoviscosity phenotype was 0.0% from *K. pneumoniae* peritoneal dialysis-related peritonitis. However, the frequency of both *rmpA* and hypermucoviscosity phenotype was 29.6% and 27.8%, respectively, from *K. pneumoniae* isolated from urinary tract infection (Lin *et al,*, 2016). Furthermore, *K. pneumoniae* isolates exhibited the

hypermucoviscosity phenotype were not limited to *magA* gene (Nahavandinejad and Asadpour,2017). These contradictions 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 ;*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 (Turton *et al.,* 2006; Lee *et al.,* 2010; Al-Jailawi *et al.*, 2014; Cubero *et al.*, 2016; Akbari *et al.*, 2017; Thonda and Oluduro; 2018). However, these were in contrast to other studies (Moghadas *etal.*,2018; Abdul-Razzaq *et al.*, 2014; Feizabadi *et al.*, 2013; Lin *et al.*, 2004). 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 prevalence of *K2* gene among these isolates was 0.0% (Shakib et al.,2018). In other recent 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 current research, approximately 25% of the isolates carried the genes *K1* or *K2* or *K1/K2* and this gives indication that these isolates are more virulent than other isolates. Nevertheless that, the isolates that carried both genes *K1* and *K2* (*K1/K2*) are considered more harmful strains than those that carried only one gene, because they have both *K1* and *K2* antigens (Lin *et al.*, 2004). In Taiwan, Lin *et al.*, (2015) found that the frequency of *K1* and *K2*

was 0.0% and 7.7%, respectively, from *K. pneumoniae* peritoneal dialysis-related peritonitis, while the frequency of *K1* and *K2* was 5.6%, 9.3%, respectively, from *K. pneumoniae* isolated from urinary tract infection (Lin *et al*., 2015).

Results of the current study 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). 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). The presence of this gene plays a vital role for *K. pneumoniae* strains in hypermucoviscosity, protection the pathogen from phagocyte and serum bactericidal activity (Lee *et al*., 2006).

Results of the current study showed that the frequency of *p-rmpA* ,*c-rmpA* ,*p-rmpA2* with the prevalence of (12%), (8%) and (15%) respectively. Most *p-rmpA* ,*c-rmpA* ,*p-rmpA2* genes are detected in non *K1/K2* isolates. This results 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 this study was in contrast to other study (Al-Jailawi *et al.*, 2014), which showed that *rmpA* genes were more prevalent in serotype *K2*. In this study, the frequency of *rmpA* genes was 35%. The frequency of *rmpA* genes ranged from 15%-46.2% (Lee *et al.*, 2010; Al-Jailawi *et al.*, 2014; Nahavandinejad and Asadpour, 2017; Thonda and Oluduro, 2018). The *rmpA* gene is a plasmid or chromosomal-mediated (*p-rmpA or c-rmpA*), which confer highly enhanced mucoviscous phenotype and participates in neutrophilic phagocytosis resistance (Yeh *et al.*, 2007;*Ko 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 hypermucoviscosity phenotype in these isolates (Lee *et al.*, 2010). Corporate of *rmpA* with *RcsB* required for regulate capsular polysaccharide synthesis (Cheng *et al.*,2010). In addition, It was shown that pathogenic potential is greater in *rmpA*[+ve] ESBL-*K. pneumoniae* strains than in *rmpA*[-ve] ESBL-*K. pneumoniae* and non-ESBL-*K. pneumoniae* strains (Lin et al., 2016).

According to the results obtained in this study, approximately 25% isolates were found to be positive for either *K1* or *K2* or both. These results gave an indication that these isolates of *K. pneumoniae* are highly virulent than the other.

This study characterized *K. pneumoniae* isolated from different clinical sources in the West Bank-Palestine, the presence of these *K1* serotype or *K2* serotype or both serotypes are probably important in clinical specimens. 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 explains the significance and the value of rapid diagnosis and proper treatment of infections caused by *K. pneumoniae* in prevention of complicated infections.

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أ. د. غالب عدوان

الملخص

تم الحصول على 66 عزلة من بكتيريا الكلبسيلة الرئوية من مستشفيات مختلفة من منطقة نابلس، جنين، طوباس، طولكرم /فلسطين خلال الفترة ما بين كانون أول 2018 إلى شباط 2019. تهدف هذه الدراسة إلى الكشف عن جينات عديدة السكاريات مثل CPS، K1، magA،K2، P- rmpA، c- rmpA 2،P - rmpA باستخدام تقنية سلسلة تفاعل البلمرة .

إلى جانب الكشف عن ظاهرة فرط المخاطية، ومقاومة المضادات الحيوية لهذه العزلات. أظهرت النتائج أن جين CPS كان سائدا بنسبة 100% وأن 25% من العزلات كانت تحمل K1، أو K2، أو الإثنين معا. و35% من العزلات تحمل جينات rmpA. ولكن magA لم يوجد في أي عزلة.

كما وأظهرت النتائج أن 90,9% من العزلات كانت مقاومة لثلاثة أو أكثر من المضادات الحيوية، و5% من العزلات كانت لها خاصية فرط المخاطية. إن وجود العوامل العدائية إلى جانب مقاومة المضادات الحيوية تجعل البكتيريا شديدة العدوى وغير قابلة للسيطرة.

هذه الدراسة تؤكد أهمية التشخيص السريع واستخدام المضادات الحيوية الفعالة عند الإصابة بالبكتيريا الكلبسيلة الرئوية.