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Molecular Detection of Capsular Genes in Multidrug Resistance *Klebsiella pneumoniae* Isolated from Different Clinical Sources

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Abstract: *Klebsiella pneumoniae* is an opportunistic pathogen. This bacterium has resistance to many classes of antibiotics, which leads to severe infections associated with the community and hospitals. It is found naturally in the human digestive system as a normal flora. At the same time, they cause many opportunistic infections, such as nosocomial infections, which include Pneumonia, Urinary tract infection, bacteremia, and Burns and Wounds. For this reason, it is possible to isolate *K. pneumoniae* bacteria from different clinical sources. 190 samples were collected from different health institutions in Diyala Governorate, including Baquba Teaching Hospital, Al-Batoul Teaching Hospital, the Advisory Clinic, Burn and Wound wards, and Educational laboratories for the period from September 2022 to January 2023. The samples included Urine, Sputum, Swab from Wounds, Burns and Blood. All isolates were diagnosed using biochemical tests and confirmed by the VITEK2 compact system. It was found (26.3% n=50) that *K. pneumoniae* (34% n=17) isolates were Multidrug Resistance (MDR). In this study MDR *K. pneumoniae* showed high resistance against different types of antibiotics. It was as follows : AMP 100%, AMC 68%, ATM 46%, CAZ 70%, CTX 60%, IMP 40%, MEM 48%, AK 38%, LEV 30%, CIP 38%, STX 60% and AZM 40% . All 17 isolates of resistant *K. pneumoniae* bacteria underwent a capsular detection test and this process was done by Microscopic test using Indian Ink. The appearance of bacterial cells in the form of bacilli and surrounded by a white halo indicates the positivity. The molecular Capsular Polysaccharide genes found (94.11% ; n=16) and isolates were positive for *wzm* gene, 100% (n=17) isolates were negative for *magA* gene, 35.29 % (n=6) isolates were positive for *k2wzy* gene.

Keywords: *Klebsiella pneumoniae*, Multidrug Resistance, Virulence Factors Genes

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Introduction

Klebsiella pneumoniae is a gram-negative bacterium and belongs to the family Enterobacteriaceae, containing capsule, non-

motile (Jose *et al.*, 2019), lactose fermented, facultative anaerobe (Ryan and Ray, 2014). *Klebsiella pneumoniae* is one of the most important

types of the genus *Klebsiella* that are found naturally in the human digestive system and can also cause many opportunistic infections, as it has been associated with patients with Pneumonia and. Urinary tract, Bacteremia, Purulent Liver abscess, Wound infection, Burns, and Diarrhea, so they can be isolated from different clinical sources (Falih *et al.*, 2016). Most of the Newborns, the Elderly, and immune-compromised individuals are susceptible to infection with this bacterium. However, the bacterium is also responsible for an increasing number of community-acquired infections (Bengoechea and Sa Pessoa, 2019). The mucous surfaces of the digestive system and pharynx are usually colonized in humans by these bacteria, and it can show high degrees of virulence and antibiotic resistance (Josepha *et al.*, 2021), where it is multi-resistant, meaning that it is resistant to more than one type or more than one group of antibiotics, and it is called Multidrug Resistance (MDR) (Wang *et al.*, 2022). *K. pneumoniae* bacteria possess many virulence factors that make them able to invade the body and then cause infection. Capsule is one of the important virulence factors in addition to Lipopolysaccharides (LPS), Siderophores, Biofilm and Fimbriae, which are important virulence factors that help bacteria on adhesion, colonization, and invasion of the body (Al-Timimi, 2021). The capsule is one of the most important virulence factors possessed by *K. pneumoniae* bacteria, which works to inhibit phagocytosis as well as prevent complement attachment with the bacterial cell membrane and is responsible for the bright mucoid texture on the surface of the agar (Cortes *et al.*, 2002). Lipopolysaccharides Capsular polysaccharide (CPS) helps bacterial colonization and biofilm formation (Chiu *et al.*, 2013). There are many genes responsible for capsular formation, including *K1*, *K2*, *magA*, and *cps*, which are considered virulence factors involved in bacterial pathogenesis (Lin *et al.*, 2012) and helps in transfer of genetic material between bacterial cells and thus increases the resistance of bacteria to antibiotics (Trubenova *et al.*, 2022).

The aim of present study was to investigate the phenotypic and genotypic characterization of *K. pneumoniae* isolated from different clinical sources and study the genetic diversity of some genes of the capsule such as *wzm*, *magA*, *K₂wzy* using molecular methods.

Materials and Methods

Sample Collection and Identification:

190 samples were collected from different health institutions in Diyala Governorate, including Baquba Teaching Hospital, Al-Batoul Teaching Hospital, the Consulting Clinic, Burn and Wound wards, and Educational Laboratories for the period from September 2022 to January 2023. These samples were distributed between 80 urine samples for people suffering from urinary tract infections and 45 Sputum samples for people with Respiratory infections. 28 Wound samples were collected from the Wound wards, in addition to 22 Burn samples. Also, 15 Blood samples were collected from inpatients in Baquba General Hospital. These samples were cultured on MacConky Agar medium and Blood Agar medium, and incubated at 37 °C for a period of 24 h. The bacteria were diagnosed visually. Noting the mucosal lactose fermenting bacteria on MacConky medium and then transferred on EMB medium in order to differentiate them from other bacterial species belonging to the same family. A number of biochemical tests were performed on the collected samples, and the tests included the oxidase test, catalase test, urease test, indole test and methyl red test, For final confirmation Biochemical tests embedded in VITEK 2 compact system (Bio merieux / USA) were performed.

Antimicrobial Susceptibility Test:

A susceptibility test was carried out on 50 *Klebsiella pneumoniae* isolates to determine their resistance to antibiotics. The following antigens were used: Ampicillin (AMP), Amoxicillin/Clavulanic acid (AMC), Azetroname (ATM), Ceftazidime (CAZ), Cephalosporine (CTX), Imipenem (IPM), Meropenem (MEM), Amikacin (AK), Levofloxacin (LEV), Ciprofloxacin (CIP),

Table 1: The primers sequencing of capsular genes

Primer Name	Sequence 5'-3'	Annealing Temp (°C)	Product size (bp)	References
<i>wzm</i> -F	TGCCAGTTCGGCCACTAAC	62	168	Vuotto <i>et al.</i> (2017)
<i>wzm</i> -R	GACAACAATAACCGGGATGG			
<i>magA</i> -F	GGTGCTCTTTACATCATTGC	60	1283	Compain <i>et al.</i> (2014)
<i>magA</i> -R	GCAATGGCCATTTGCGTTAG			
<i>k₂wzy</i> -F	GACCCGATATTCATACTTGACAGAG	50	642	Turton <i>et al.</i> (2010)
<i>k₂wzy</i> -R	CCTGAAGTAAATCGTAAATAGATGGC			

Azithromycin (AZM), Trimethoprim – sulfa-methoxazole (STX).

Phenotypic detection of capsule:

The appearance of the presence of the Capsule was detected, where a small amount of bacterial growth was taken from a 24-hour-old bacterial colony by a wooden stick and then placed on a glass slide where it was mixed with Indian ink dye and then left to dry slightly on the air and tested under a microscope. The appearance of a white halo (unpigmented) surrounding the bacterial cell indicates that the bacteria are surrounded by Capsule.

Molecular study of Klebsilla pneumonia:

DNA Extraction:

DNA was extracted from bacterial cells growing on brain and heart infusion agar liquid, where these cells were grown on this medium for 24 h at 37 °C, and the extraction process was carried out according to the ABIOPure Extraction protocol. The concentration of the DNA extract and purity was determined by measuring absorbance by Quants Fluorometer.

PCR detection of virulence associated genes:

The Polymerase Chain Reaction (PCR) was used to detect the presence of capsule genes in 17 multidrug-resistant isolates out of 50 *K. pneumoniae* isolates using primers assigned to each gene. All these primers were supplied by the

Macrogen company in a lyophilized form. Lyophilized primers were dissolved in a nuclease-free water to give a final concentration of 100 pmol/μl as a stock solution. A working solution of these primers prepared by adding 10 μl of primer stock solution (stored at 20 °C) to 90 μl of nuclease-free water to obtain a working primer of 10 pmol/μl. Table 1 shows the sequence of primers for the genes used in the study.

Results and Discussion

190 clinical samples were collected from inpatients, 120 patients, representing 63.15%, and non-inpatients, 70 patients, 36.84%, from health institutions affiliated to Diyala Governorate. The ages of patients ranged between 1-80 years, and these samples were implanted on the medium of MacConkey Agar, where the results of transplantation were 121 samples with a rate of 63.7% which showed Clear bacterial growth on the culture medium, and 69 samples with a rate of 36.31% did not give any bacterial growth. The results of the bacterial culture on the MacConkey Agar medium according to the source of the sample were Urine 57 out of a total of 80 samples that appeared positive for culture with a percentage of 71.3%, Sputum out of a total of 45 samples appeared 20 positive for culture with a percentage 44.4%, and Wounds 19 out of a total of 28 samples appeared positive for implantation, at a rate of 67.9%, and Burns out of a total of 22 samples appeared 15 positive for implantation, at

Table 2: Resistant of *K. pneumoniae* isolate to different antibiotics

Antibiotic groups	Antibiotics	Code	Percentage
β-Lactam	Ampicillin	AMP	100%
	Amoxicillin+Clavulanic acid	AMG	68%
	Azeteroname	ATM	46%
	Ceftazidime	CAZ	70%
	Cephalosporine	CTX	60%
	Imipenem	IPM	40%
	Meropenem	MEM	48%
Aminoglycosides	Amikacin	AK	38%
Quinolones	Levofloxacin	LEV	30%
	Ciprofloxacin	CIP	38%
Sulfonamide	Trimethoprim-sulfamethoxazole	STX	60%
	Azithromycin	AZM	40%

a rate of 68.2%. Blood samples from a total of 15 samples appeared 10 positive for culture, with a rate of 66.6%. Among the results of the current study the percentage of infection that appeared as a bacterial infection was higher in Urine samples among all samples collected from different clinical sources, and this is consistent with Al-Ezzi (2019) who showed that among 152 samples of Urine 115 samples gave a positive result for implantation at a rate of 75.65%. The results of Al-Saady (2023) also showed that among 108 samples, Urine samples were the most, with a rate of 37%. The spread of *K. pneumoniae* and pathogenic importance comes through the events of urinary and respiratory tract infection which may be because these bacteria are from the normal flora that lives in the intestinal wall.

Antibiotic Susceptibility Tests:

The sensitivity of *K. pneumoniae* isolates under study, which numbered 50 was studied, as they showed high resistance towards a number of antibiotics that are used in health institutions and are widely used to treat different types of infections. By measuring the diameter of the inhibition zone surrounding the antibiotic discs, the results were compared with the standard tables contained in CLSI-2021. The results of the current study showed the sensitivity of *K. pneumoniae* isolates towards the group of β-

lactam antibiotics, where the resistance to Ampicillin (AMP) was 100%, Amoxicillin + Clavulanic acid (AMG) 68%, Azetrename (ATM) 46%, Imipenem (IPM) 40%, Ceftazidime (CAZ) 70%, Meropenem (MEM) 48% and Cephalosporin (CTX) 60%. As for the percentage of isolates' resistance to Amikacin (AK), which belongs to the group of Aminoglycoside antibiotics, it was 38%, and the results of the resistance of *K. pneumoniae* to Quinolone antibiotics, it was 30% for Levofloxacin (LEV) and 38% for Ciprofloxacin (CIP). As for the Sulfonamide group, Trimethoprim-sulfamethoxazole (STX) 60%, Azithromycin (AZM) 40%. This is illustrated in Table 2. These results are consistent with the findings of Al-Zubaidi (2020), as he found that the percentage of resistance of *K. pneumoniae* bacteria to Beta-lactam antibiotics was Ampicillin 100%, Amoxicillin + Clavulanic acid 73.36%, Azeteroname 72.46%, Imipenem 27.53%, and Ceftazidime 62.32%. The results of this study converge with the findings of El-Badawy *et al.* (2017), where the resistance rate to Levofloxacin 38.59% and to Ciprofloxacin 44.73%.

The relative difference in *K.pneumoniae* resistance patterns towards antibiotics is due to several reasons, such as the size of the sample studied, the source of the sample, geographical differences, and the site of infection, in addition to

that, the patient's predisposition to disease (Abdelraheem, 2020).

Phenotypic detection of capsular:

The results of the current study showed that all 17 resistant isolates of *K. pneumoniae* bacteria are 100% surrounded by the capsule, which is the most important factor of virulence possessed by bacteria, as it inhibits phagocytosis and inhibits complementary binding with the bacterial cell membrane and colonization of mucous membranes (Dorman et al., 2018). The results of the current study are consistent with the results of Al-Zubaidi (2020), who showed that all 18 isolates resistant to *K. pneumoniae* bacteria are surrounded by the capsule. The study is also in conformity with findings of AL-Zinca (2013). This study is inconsistent with the results of Al-aajem (2021), which showed that out of 30 resistant isolations, only 5 isolates were surrounded by the capsule, at a rate of 16.3%, and 25 isolates were without capsules (83.7%).

Molecular detection of Capsular genes of K.pneumoniae:

Capsular genes were detected in 17 *K.pneumoniae* multidrug-resistant MDR isolates using Polymerase Chain Reaction (PCR) technique, and the detected capsular genes include *magA*, *k₂wzy*, *wzm*.

Detection of wzm gene:

The PCR results of the DNA sequence of the *wzm* gene, using the primer designated for it, showed that 94.11% (n=16) isolates from *K. pneumoniae* out of 17 carriers of the *wzm* gene (168bp) (Fig. 1).

The *wzm* gene is one of the genes of the synthesis of lipopolysaccharide (LPS), which is one of the important and main components of the outer membrane of the bacteria *K. pneumoniae* and consists of two main regions: one is a lipid A region and a polysaccharide, which in turn consists of the core polysaccharide or antigen O and the specific polysaccharide side chain (Jawtetz et al., 2010). Seven groups of antigen O serotypes

(O1, O2, O3, O4, O5, O8, O12), three of which (O1, O2, O3) are identified as the most common and cause bacterial infections depending on the K-antigen (Fu et al., 2018). O-antigen enzymes are encoded on the *rfb* site, which consists of a set of genes (including *wzm*, *wzt*) and these sites encode the membrane components and ATP binding components of the transporter ABC-2 (Bronner et al., 1994).

Detection magA gene:

The second capsular gene detected in *K.pneumoniae* isolates is the *magA* gene, which belongs to the capsular genes of *K.pneumoniae* belonging to the K1 serotype (Lin et al., 2012). Genetic detection of the *magA* gene in *K.pneumoniae* multidrug resistant (MDR) bacteria was carried out using the primers designated for it. The results of the study showed that all isolates lacked this gene (Fig. 2).

The results of the current study agree with the study of Al-Saady (2023), as it has been reported that the *magA* gene was not detected in any isolate of the total number of 53 bacterial isolates of *K. pneumoniae* used in the study. The incidence of the *magA* gene was 0.2%, as its presence was detected in only one isolate out of 370 isolates. Al-aajem (2021) showed that the *magA* gene was not found in any of the isolates used in the study, which numbered 30 isolates. The results of the current study do not agree with the study of Al-Zubaidi (2020) as it showed that the presence of the *magA* gene was detected in 8 isolates out of a total of 18 isolates of *K. pneumoniae* multidrug resistance (MDR) with a percentage of 44.44%.

Detection k₂wzy gene:

The third *K.pneumoniae* capsular gene that was detected is *k₂wzy*, which is a capsular polysaccharide (CPS) gene that aids bacterial colonization and biofilm formation at the site of infection (Chiu et al., 2013). All 17 MDR-resistant *K.pneumoniae* isolates were subjected to detection of the K2wzy gene using the primers assigned to it. The results of the current study showed that 6 isolates, with a percentage 35.29 % of *K.*

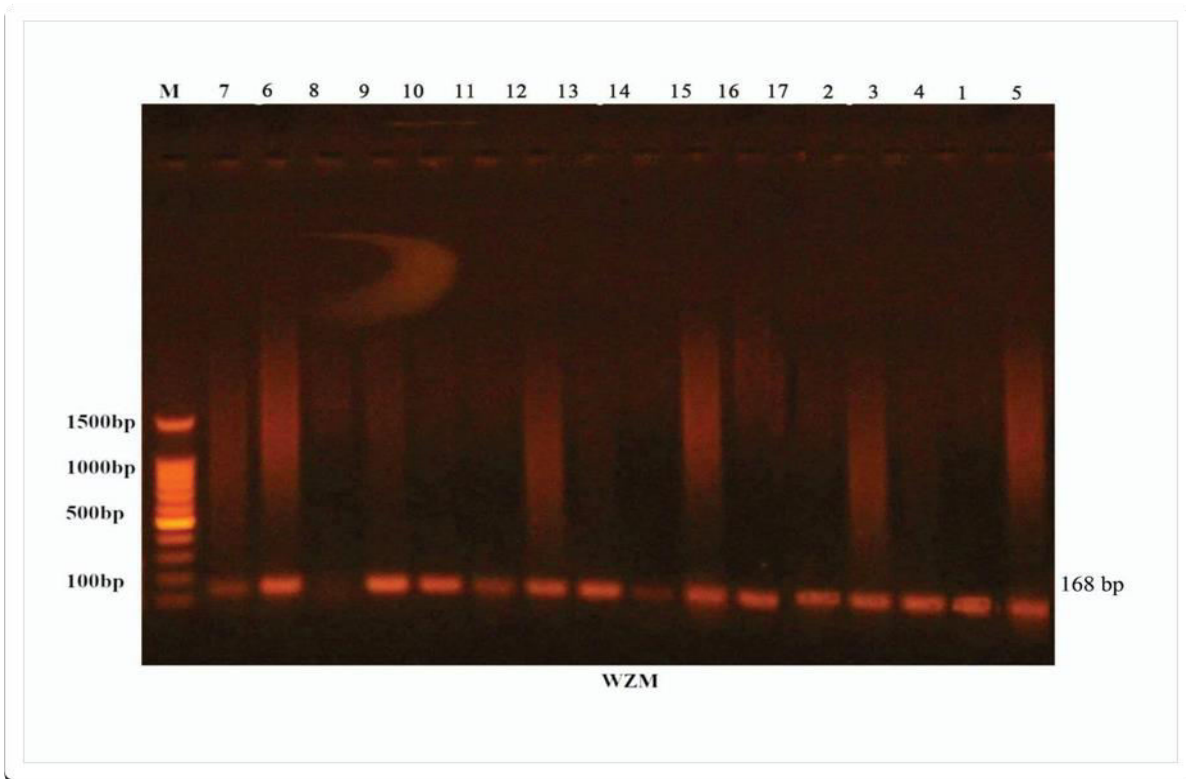


Fig. 1: Amplification of *wzm* gene of *Klebsiella pneumoniae* samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker.

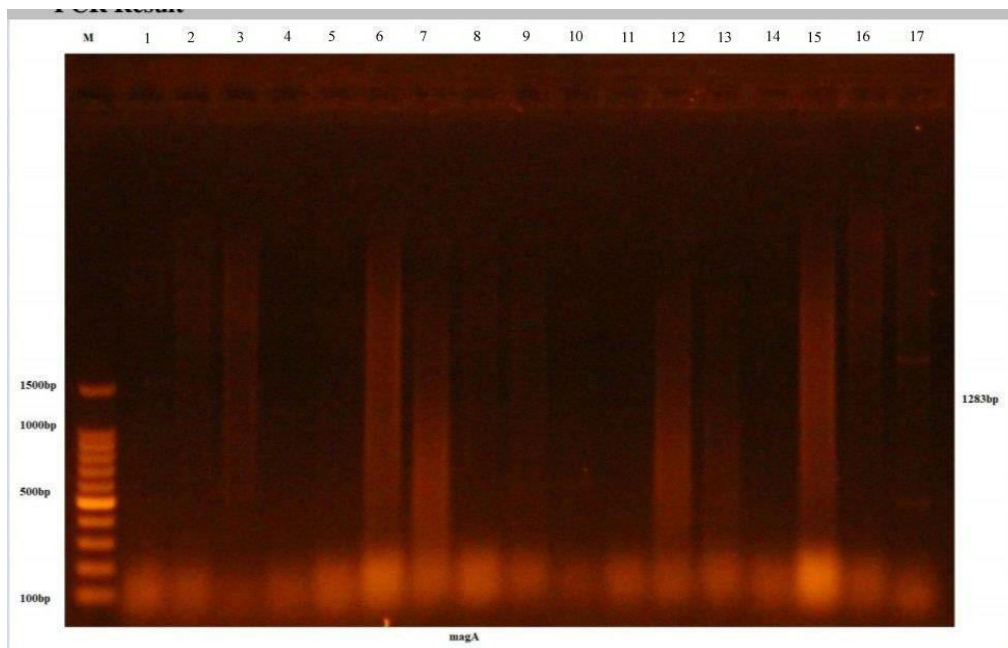


Fig. 2: Amplification of *magA* gene of *Klebsiella pneumoniae* samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder.

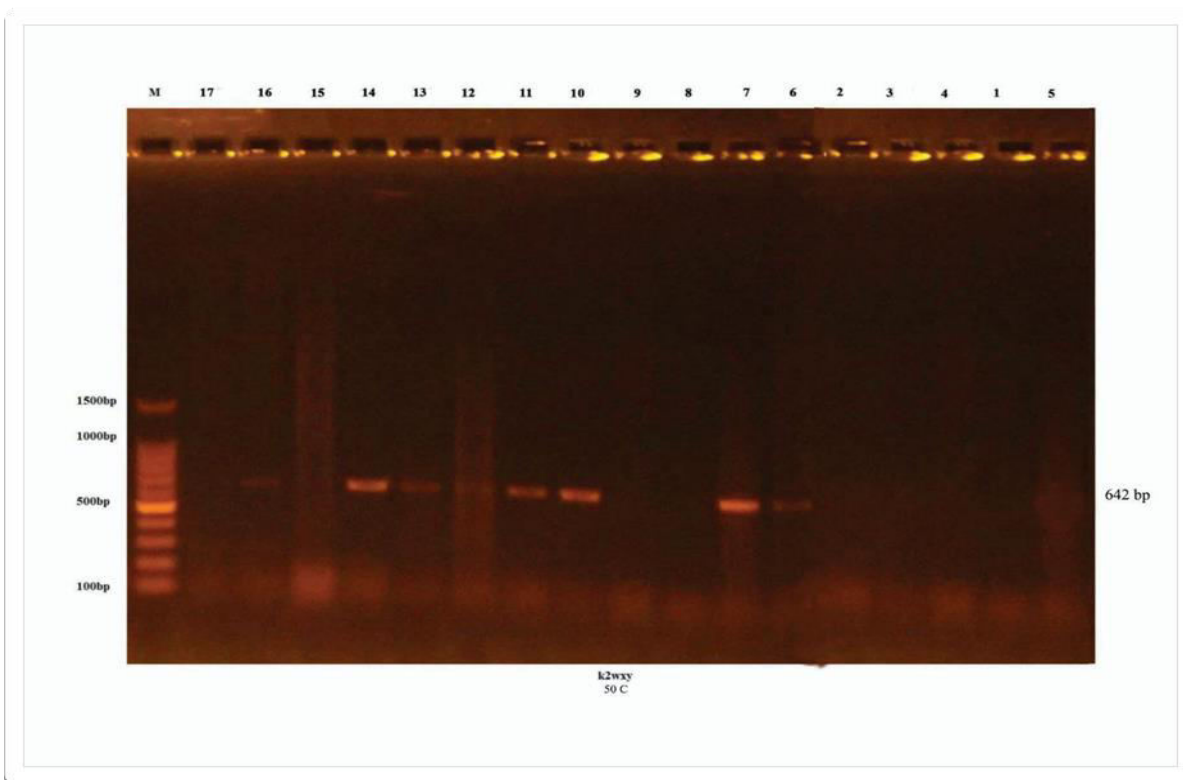


Fig. 3: Amplification of *k2wxy* gene of *Klebsiella pneumoniae* samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder.

pneumoniae isolates, contained this gene out of a total of 17 isolates (Fig. 3).

The results of the current study are consistent with the study of Rastegar *et al.* (2021), where the study showed that 27.3% of the isolates of the *K.pneumoniae* bacteria contain the *K1/K2* serotypes, while the *K20* serotype was the most common serotype in the capsule of the Bacterium *K.pneumoniae*. The results of the current study are close to the study of Al-Musawi (2018) who showed that the most common capsular genotype is *K2* non *K1* by 61.42%, while the *K2* serotype came in second place with a presence rate of 38.57%.

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