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Microbiological and Molecular Study of K1, K2 Genes among *Klebsiella pneumoniae* isolated from Urine Specimens in Duhok city, Iraq.

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Abstract

***Klebsiella pneumoniae* is a common cause of infections, particularly urinary tract infections (UTIs). Urine samples were taken from 800 patients with clinical indications of UTI who visited the inpatient and outpatient clinics from governorate hospitals and private clinics, namely, Azadi Teaching hospital, Duhok Emergency hospital, Heevi Pediatrics Teaching hospital and Vajeen Private hospital in Duhok city. Standard bacteriological methods were used to identify isolated colonies from urine samples cultured on bacterial media. A species-specific PCR assay was used to identify *K. pneumoneae* isolates at molecular level PCR assays and capsular type counting of the *K1* and *K2* genes were detected among 50 *K. pneumoniae* isolates. The furthermost predominant genes amongst all isolates were *K1* gene (12%) followed by *K2* gene (8%). This study emphasized on *K. pneumoniae* as sources of UTIs that displayed to harboring *K1* and *K2* genes. The goal of this work was to investigate the mucoviscosity of *K. pneumoniae* isolated from a urinary tract infection and to screen for certain virulence factor genes such as *K1* and *K2* genes.**

Keywords: *Klebsiella pneumoniae, Urinary tract infection, capsular typing, PCR*

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I. INTRODUCTION

Klebsiella pneumoniae is one of the most common opportunistic bacteria in humans, causing a vast range of infections in hospitals and the community settings (Pan *et al*., 2015).

It can cause a range of infections, The most common etiological agents of UTIs are *Escherichia coli* and *K. pneumoniae*, both belonging to the Enterobacteriaceae family (Ali and Al-dahmoshi, 2021). The hypermucoviscosity phenotype of *K. pneumoniae* colonies on agar plates has been linked to the invasiveness of these strains (Dubey *et al*., 2013). The pathogenicity of *K. pneumoniae* is due to the synthesis of several virulence factors can aid the bacteria in overcoming the immune system and causing a variety of diseases (Moghadas *et al*., 2018). *K. pneu*moniae produces different virulence factors including lipopolysaccharides, fimbriae for adhesins, antiphagocytic capsule (K antigen), and siderophores for iron acquisitions from the host (Vuotto *et al*., 2017).

The capsule is a critical virulence factor for *Klebsiella* since it shields evading bacteria from host killing by phagocytosis and also stops the immune response of the host (Patro and Rathinavelan, 2019). There are at least 77 capsular serotypes identified, and infection severity varies according to the type of serotype. *K1* and *K2* are further pathogenic to humans than non *K1-K2* serotypes due to their polysaccharide capsule, which helps them avoid being phagocytosed by macrophages (Yu *et al*., 2007; Paczosa and Mecsas, 2016). The absences of mannose repeats on its capsule, avoiding it from being detected by macrophages. mucoviscosity associated gene A (*magA*) is only found in the *K1* capsule gene cluster, but the chromosomal *K2* capsule associated gene A (*K2A*) is found in the *K2* serotype (Yu *et al*., 2006; Doud *et al*., 2009).

Fang *et al*. (2010) described the *magA* gene for the first time in 2004 and revealed that the invasive *K. pneumoniae* strains had higher levels of hypermucoviscosity and *magA*, while mutant strains lacking *magA* lost their exo-polysaccharide. Similar to the *magA* capsule gene cluster of capsular serotype *K1* isolate, the *K2A* gene of capsule gene cluster *K. pneumoniae* might be utilized as a very specialized technique to recognize the capsule *K2* serotype (Chuang *et al*., 2006).

The goal of this study was to investigate phenotypic colony mucoviscosity characterization and PCR detection of *K1* and *K2* genes of *K. pneumoniae* in UTI patients that might have a part in the bacteria's pathogenicity.

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II. Materials And Methods

A. Samples Collection

From September 2021 to January 2022, 800 urine samples were collected from symptomatic out/in patients with urinary tract infections from Duhok city involved hospitals and private clinics, namely, Azadi Teaching hospital, Duhok Emergency hospital, Heevi Pediatrics hospital and Vajeen Private hospital. Each sample was cultured on distinct labelled culture media and then distinguished from other bacteria grown on the same medium using phenotyping criteria and biochemical assays (Shoaib *et al*., 2020).

B. Ethical approval

The study proposal was accepted by the ethic committee, and the Duhok Polytechnic University and Duhok General Health Directorate gave their informed consent.

C. Isolation and Identification of K. pneumoniae

All urine samples that grown on blood and selective agar media, then phenotypically identified to the species level using standard bacteriological and biochemical techniques with expected reference results as shown in Table (1) (Mahon *et al*., 2019).

Table 1. Conventional biochemical tests for identification of *K. Pneumonia*e

Test	Results
Urease	+
TSI agar	A/A
Indole test	-
Voges Proskauer	+
Motility	-
Citrate Utilization	+
Catalase test	+
Oxidase test	-
lactose Fermentation	+

(+) a positive result, (-) negative result. * A/A= ferment lactose/sucrose

D. String test

To detect the hyper-mucoviscosity of *K. pneumoniae*, the string test was done on MacConkey agar as described by (Shon *et al*.,2013).

E. Genomic DNA extraction

Genomic DNA was extracted by using boiling method. About 2 to 3 colonies of bacteria grown on MacConkey agar plate were suspended in 300 µl of double-distilled water in an Eppendorf tube, then vortex and heated to 100℃ on a heat block for 15 min and then immediately cooled on the ice at -20°C for 10 min. Followed by centrifugation at 14000 rams for 15 min, and supernatants were transferred into another sterile Eppendorf tube. Finally stored at -20℃ and used as a DNA template for PCR amplification (Compain *et al*., 2014; Al-Kraetay *et al*., 2020).

F. Determination the concentration and purity of DNA

The concentration of DNA was determined using a Nanodrop spectrophotometer at 260nm, DNA purity was determined using the ratio of sample optical density at 260 and 280 nm. A ratio of ~1.8 is considered pure DNA (Green and Sambrook, 2012).

G. PCR amplification

Molecular detection of *K. pneumoniae* isolates in this study was performed by PCR amplification in the Dohuk Central laboratory. specific primers (Bioneer/Korea) were used to amplify segments from the 16S ribosomalRNA 130 bp as a target of *K. pneumoniae*-specific gene), *magA* primer for detection of *magA* gene (*K1* serotype-specific gene), and *K2A* primer as a target of *K2A* among *K2* serotype). as shown in Table 1. The reaction was performed in a 20 µl volume, containing 10 µl of master mix (2x), 2 µl of both the forward and the reverse of the primers (10 pmol), 2 µl DNA template, and 6 µl of free water nuclease to complete the volume. Then DNA amplification was carried out with the thermocycler programs installed as in (Table 3). The PCR application products were separated in 2% agarose gel containing Green safe dye (0.6 µg/ml) at 8 to 5 V/Cm for 75 min. DNA bands were visualized by UV transilluminator.

Table 2. primers size and sequence of *K. pneumoniae* used in this study.

Primers	sequence (5' to 3')	Size (bp)	References
16S rRNA	F-ATTTGAAGAGGTTGCAAACGAT	130	(Albasha *et al*., 2020)
	R-GCAATGGCCATTTGCGTTTGC GTTAG		
MagA for *K1*	F-GGTGCTCTTTACATCATTGC	1283	(Zedan and Al-jailawi, 2019)
	R-GCAATGGCCATTTGCGTTTGC GTTAG		
K2A for *K2*	F-CAACCATGGTGGTCGATTAG	531	(Alyassari *et al*., 2019)
	R-TGGTAGCCATATCCCTTTGG		

Table 3. PCR conditions for *K. pneumoniae* species-specific primer and Capsular typing.

Gene name	Temperature (℃)/Time				
	Initial denaturation	Cycling conditions			Final Extension
		denaturation	annealing	Extension	72 ℃/10 min
16S rRNA	94 ℃/5 min	94 ℃/30 s	57 ℃/45s	72℃/90 s	
	35 Cycle				
magA for *K1*	95℃/5 min	94 ℃/ 45 s	55℃/ 60s	72 ℃/ 1min	72℃/ 10 min
	35 Cycle				
K2A for *K2A*	94℃/5 min	94℃/60s	58 ℃/1min	72 ℃/1min	72℃/10 min
	35 Cycle				

III. Results

A. Morphologically characterization and biochemical tests

Out of 800 urine samples 12.5% (100/800) has been identified as *K. pneumoniae* collected from different hospitals in Duhok city. All isolates identified and characterized as *K. pneumoniae* on the basis morphological, physiological and biochemical characteristics shown as in Table 1. (Mahon *et al*., 2019). Out of 50 *K. pneumoniae* were 28% (14) found as

hypermucoviscous and 72% (36) as non-hypermucoviscous by string test.

B. Molecular identification of K. pneumoniae

Fifty isolates of *K. pneumoniae* were selected for molecular study were subjected to PCR amplification using species-specific primers (16S rRNA) for *K. pneumoniae*; Results revealed that entirely 50 isolates yielded positive results (130 bp bands) as shown in Figure1.

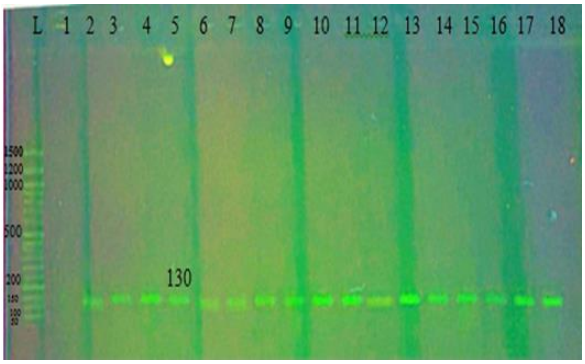

Figure1. PCR amplification of the 16SRNA (130 bp) specific primer for 50 *K. pneumoniae* identification. the PCR product visualized on 2% agarose gel electrophoresis with a 8 to 5 V/Cm. Lane L: is a (50-1500bp) ladder, lane: (1-18) 130 bp *K. pneumoniae*.

C. Molecular capsular serotyping of K. Pneumoniae

K. pneumoniae serotypes were confirmed at molecular level by PCR analysis using specific primer of *magA* gene specific for *K1*serotype with molecular weight 1283 bp, and specific primer for detection of *K2* gene (531 bp) as shown in (Table 4). The results showed that out of a total of 50 isolates, 6 isolates (12%) give positive PCR products for K1, 4 isolates (8%) give positive with *K2*, while 40 isolates (80%) were observed as non *K1/K2* as shown in (Figure 2,3).

Table 4. Comparison of different serotyping of *K1*, *K2*, and Non- *K1/K2*

Capsular typing	Positive No. (%)	Negative No. (%)	Total No
K1	6 (12)	44 (38	50
K2	4 (8)	46 (42	50
Non *K1/K2*	40(80)	10 (20)	50

Figure2. PCR amplification of the *magA* (1283 bp) primer for *K1*serotype PCR product visualized on 2% agarose gel electrophoresis with a 8 to 5 V/Cm for 75 minutes. Lane L is a (50-1500 bp) ladder, line:1 negative control, lane: (4,6, and 9) is positive *magA* (*K1*) serotype, lane: (2,3,5,7,8,10,11,12,13,14,15,16,17,18, and 19) are negative for *magA* (*K1*) serotype of *K. pneumoniae*.

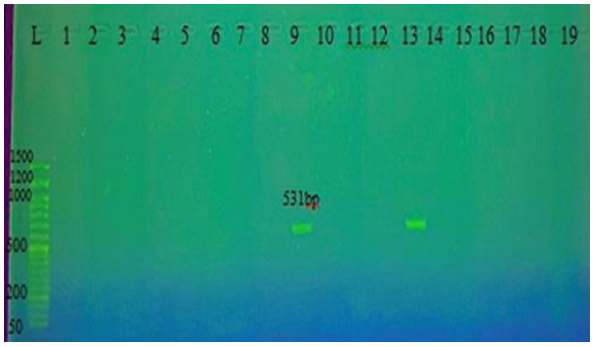

Figure3. PCR amplification of the *K2A* (531 bp) primer for *K2* serotype. the PCR product visualized on 2% agarose gel electrophoresis with a 85 volt for 75 minutes. Lane L: (50-1500 bp) ladder, lane: 1 negative control, lane: (9, and 13) is positive *K2A* (*K2*) serotype, lane (2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 14, 15, 16, 17, 18, and 19) are negative for *K2A* (*K2*) serotype of *K. pneumoniae*.

IV. Discussion

Urinary tract infections (UTIs) are relatively frequent infectious disorders that affect a large percentage of the population and are a major source of worry in the healthcare system (Moustafa *et al*., 2018).

Based on morphological and biochemical characteristics, 12.5 % *K. pneumoniae* were discovered from clinical urine samples in our investigation and those people had a clinical indication of UTI.

Our findings close to study conducted by Hasan *et al*. (2021) in Baghdad found 13.2% of 336 urine samples had UTI caused by *K. pneumoniae.* Another study in Duhok by (Al-Naqshbandi *et al*., 2019) found that 14.3% of 530 urine samples tested positive for *K. pneumoniae* UTI. While Al-Gasha'a *et al*. (2020) study reported 22.45% of UTIs caused by *K. pneumoniae*. The presence of these bacterium in UTI could be attributed to the fact that these germs are frequently found in the resident flora and possess a variety of virulence components that lead to their pathogenicity (Ali *et al*., 2017). However, the difference in prevalence with other studies could be due to the number of samples taken and the study's time period. Molecular biology approaches, according to (Osman *et al*., 2020), could be used as critical diagnostic tools in microbiology laboratories.

The findings of current study revealed that all fifty isolates subjected to molecular technique for identification of *K. pneumoniae* ssp. was gave successful amplification of specific 16S rRNA region with 130 bp (Figure1) as well as capsular *K1* and *K2* serotypes have been identified using particular primers for it. The 16S ribosomal RNA gene is a highly proper and adaptable technique for identifying microbes to the species level, even when the species in question is normally hard to detect by biochemical procedures, to prevent being misidentified (Osman *et al*., 2020).

magA gene is positioned within an operon specific to serotype *K1* capsule cluster gene independent of the origin (Fang *et al*. 2010). According to our findings, the *magA* gene was found

to be present in six isolates (12 %) successfully gave amplicon with 1283 bp in size. All six *K1*serotype isolates were found to be hypermucoviscusity strains by string test. Remya *et al*. (2018) analyzed 146 *K. pneumoniae* and discovered that *magA* is exclusive to the *K. pneumoniae* capsule serotype *K1* gene cluster, with all non *K1* strains being *magA* negative. As a result, PCR investigation for *magA* provides a fast, precise method for diagnosing *K. pneumoniae* serotyping *K1* isolates.

As part of this study, the presence of *K2A* gene among selected isolates has been studied. The obtained results of molecular study showed that the *K2A* gene was detected in 4 (8 %) of *K. pneumoniae* isolates. The result of molecular study showed that *K2A* gene was detected in 4 (8 %) of *K. pneumoniae* isolates in this study. These findings indicated that isolates have a *K2* serotype. The *K. pneumoniae* serotype *K2* open reading frame (ORF)–9 area *K2A*, which matches to *mag A* region in *K1* isolates capsule gene cluster, could be utilized as a extremely specific genetic analytic technique that identify *K. pneumoniae* capsule *K2* serotyping (Cheng *et al*., 2013). These four *K2* serotypes (8%) are also hypermucoviscusity strains.

In this investigation, all Non *K1* / *K2* (40) isolates were *magA* and *K2A* negative. In serotyping, non-*K1*/*K2* cross-reacts with *K1* and *K2* by serotyping method, but does not produce *magA* or *K2A* specific amplicons. The lack of cross-reactions in molecular serotyping may be a benefit over classical serotyping (Cheng *et al*., 2013).

In comparable to another study conducted in Iraq found that the prevalence of serotype *K1* (23.2%) was greater than serotype *K2* (20.9%) (Adwan *et al*., 2020). These findings in this study matched to previous publication study in Iran, which reported the same results for *K1* and *K2* serotypes 12 (23.52) and 4 (7.84), respectively, in 51 clinical urine samples (Ranjbar *et al*., 2019). However, findings in current study were in direct conflict with previously published data by Zedan and Al-jailawi (2019), who found that serotypes *K1*, *K2*, and Non *K1* / *K2* reported for 23/40 (57.5%), 11/40 (27.5%), and 15/40 (6%) (15/49) of all *K. pneumoniae* isolates, respectively. In our study, the prevalence of hypermucoviscous 28% (14/50) as hypermucoviscous and 72% (36/50) as non- hypermucoviscous. The differences in the frequency of capsular serotypes could be attributed to differences in the types of samples collected in another study (Hasani *et al*., 2020).

V. Conclusions

The most common *Klebsiella* species found in UTI cases is *Klebsiella pneumoniae*. In all the investigated isolates, the use of a 16SrRNA primer resulted in accurate identification. It's worth noting that the virulence factors *K1* and *K2* aid in the spread of the organisms in urinary infections.

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