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. pneumoniae* 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 that can aid the bacteria in overcoming the immune system and causing a variety of diseases (Moghadas et al., 2018). *K. pneumoniae* 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 absence 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.
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. pneumoniae

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>TSI agar</td>
<td>A/A</td>
</tr>
<tr>
<td>Indole test</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td>-</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>-</td>
</tr>
<tr>
<td>Lactose Fermentation</td>
<td>+</td>
</tr>
</tbody>
</table>

(+): 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°C on a heat block for 15 min and then immediately cooled on the ice cooled 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°C 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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequence (5’ to 3’)</th>
<th>Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>F-ATTTTGAAGAGGTTGCAAAACGAT</td>
<td>130</td>
<td>(Albasha et al., 2020)</td>
</tr>
<tr>
<td></td>
<td>R-GCAAATGCGCATTTGGCTTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MagA for K1</td>
<td>F-GGTGCCTTTACATATTGC</td>
<td>1283</td>
<td>(Zedan and Al-Jalawli, 2019)</td>
</tr>
<tr>
<td></td>
<td>R-GCAAATGCGCATTTGGCTTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K2A for K2</td>
<td>F-CAACCATGGGTGTCCTTATTAG</td>
<td>531</td>
<td>(Alyassari et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>R-TGGTAGCCATATCCCTTTGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Temperature (°C)/Time</th>
<th>Initial denaturation</th>
<th>Cycling conditions</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>94°C/5 min</td>
<td>94°C/50 s</td>
<td>57°C/45 s</td>
<td>72°C/90 s/10 min</td>
</tr>
<tr>
<td>magA for K1</td>
<td>95°C/5 min</td>
<td>94°C/45 s</td>
<td>55°C/60 s</td>
<td>72°C/1 min/10 min</td>
</tr>
<tr>
<td>K2A for K2A</td>
<td>94°C/5 min</td>
<td>94°C/60 s</td>
<td>58°C/1 min</td>
<td>72°C/10 min/10 min</td>
</tr>
</tbody>
</table>

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 Figure 1.

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

<table>
<thead>
<tr>
<th>Capsular typing</th>
<th>Positive No. (%)</th>
<th>Negative No. (%)</th>
<th>Total No</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>6 (12)</td>
<td>44 (88)</td>
<td>50</td>
</tr>
<tr>
<td>K2</td>
<td>4 (8)</td>
<td>46 (92)</td>
<td>50</td>
</tr>
<tr>
<td>Non K1/K2</td>
<td>40(80)</td>
<td>10 (20)</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 2. 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: (1,18) 130 bp K. pneumoniae.

Figure 3. PCR amplification of the K2A (531 bp) primer for K2 serotype, the PCR product visualized on 2% agarose gel electrophoresis with an 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 bacteria 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 (Figure 1) 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 hypermucoviscosity 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 hypermucoviscosity 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.

REFERENCES


