MOLECULAR CHARACTERIZATION OF CAPSULAR POLYSACCHARIDE & PUTATIVE SERIENE PROTEASE GENES OF KLEBSIELLA PNEUMONIAE ISOLATED FROM PUBLIC HOSPITALS IN BAGHADAD - IRAQ

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ABSTRACT
Gram negative pathogens are an important cause of hospital acquired infections throughout the world. Klebsiella pneumoniae is an important medicals pathogens responsible for causing nosocomial infections. About 158 bacterial samples & specimens have been collected [urine and sputum]. A total of 50 bacterial isolated were identified as Klebsiella pneumoniae in bacteriology department of Baghdad teaching hospital. The specimens were collected depending on the standard methods from hospitalized patients suffered from varaities of nosocomial infections. The study was conducted at Baghdad teaching hospital (medical city) from the period of January 2015 until June 2015 in order to investigate the role of capsular polysaccharide and putative serine protease genes as virulence factors of Klebsiella pneumoniae in nosocomial infections. The bacterial isolates were identified depending on the golden characterization of morphology and biophysiology. Results of biochemical tests were confirmed by using the API 20E and VITEK2 system which has been done in Central Public Health Laboratory (CPHL). Klebsiella pneumoniae clinical isolates were tested for antimicrobial sensitivity. The multiplex Polymerase chain reaction (Multiplex PCR) and gel-agarose electrophoresis has been done in Uruk Lab For Molecular & Serological tests in order to investigate the prescence and detection of [K1,K2,K57 capsular polysaccharide genes] and [htrAgene (putative seriene protease)] using PCR specific primers. It was found that only five isolates (10%) were positive to K2 cluster where as [K1,K57] not detected by PCR. K2 capsular type was commonly found among submitted isolates. The htrA gene was detected in 48 (96%) isolates from hospitalized patients with nosocomial pneumonia, ventilator-associated pneumonia (VAP), and catheters...
of urinary tract infections UTIs patients in the Intensive Care Units ICUs. It has found that this gene played an important and vital role in the *Klebsiella pneumoniae* virulence. The multiplex PCR method provide a rapid mean of characterization and typing of this important agent of community-acquired and nosocomial infections.

**KEYWORDS:** *Klebsiella pneumoniae*, Baghdad, varaities of nosocomial, biophysiology.

**INTRODUCTION**

*Klebsiella pneumoniae* is an important common gram-negative medical pathogen that responsible for causing both community-acquired and nosocomial infection.[1] It can cause a wide spectrum of infection like pneumonia; septicemia; urinary tract infections (UTIs); meningitis; and purelent abscess at a various sites of the body. [2] The invasive nature of *Klebsiella pneumoniae* strains that appear in a relation with an extreme “stickness” of these colonies that can be seen on an agar plates. This phenomenon known as hypermucoviscosity phenotype.[3] *Klebsiella pneumoniae* that produces on a primary isolation mucoid colonies ; improve that there is a prescence of a large capsule which that surrounding the individual cells.[4] The degree of mucoidy has been shown a relation with the establishment of an infections. [5] *Klebsiella pneumoniae* produce virulence factors that play an important role in the pathogenicity. Two virulence factors are essential for the microorganism and have the ability to spread through the blood and causes sepsis, these factors are 1) capsular polysaccharide (CPS); 2) lipopoly saccharide(LPS).

The capsular polysaccharide that are produced from the clinical and environmental isolates; have been regarded and consider the main determinant of the pathogenicity of *Klebsiella*. [6] It mainly involved in resistance to phagocytosis its role act as a physical barrier; and may participate in the resistance of complement system, while lipopoly saccharide (LPS) is required in order to resist complement mediated killing. *Klebsiella pneumoniae* has focused on the capsule serotype; serotype *K1* and *K2* considered the most virulent to human.[7] In addition there is a prescence of some capsular types like *K54* and *K57*. The *htrA* gene play a vital role in the virulence of *Klebsiella pneumoniae*.[8] *htrA* is a member of serine proteases which have an important role in several biological and pathological process.

The Multiplex PCR assays using a set of primers that responsible for encoding different genes of aminoglycoside modifying enzymes which revealed the prescence of *(K2)*,[9] and *(htrA)*. PCR products confirmed that *Klebsiella pneumoniae* carried the targets *(K2* capsular
polysaccharide gene and \((htrA);\) putative serine protease], that has been shown after extraction of DNA. There for Multiplex PCR was confirmed to be successfully approach to identify the \(K2\) and \(htrA\) gene in addition to sensitivity test.

The aim of the present work is to evaluate the role of some virulence factors associated with \(Klebsiella pneumoniae\) in an Iraqi hospital acquired infections through investigation of the capsular polysaccharide and putative serine protease genes.

**MATERIAL AND METHODS**

**Isolation and Identification of \(Klebsiella pneumoniae\) isolates:** \(Klebsiella pneumoniae\) were isolated from patients with nosocomial infections admitted to the main two hospitals in Baghdad city – Iraq, from the period between January to June 2015. They were cultivated on conventional laboratory media in bacteriology department of Baghdad Teaching Hospital. The identification of each bacterial isolate has been done through characterization and biochemical reaction, API-20E (Brooks et al., 2007).[10]

**Antimicrobial susceptibility testing:** Susceptibility testing was done for several types of antibiotics were performed according to Kirby-Bauer (Disk diffusion) using muller-Hinton agar according to the guidelines of the clinical and Laboratory Standards Institute 2011.[11] The antibiotics used in this study: (Amikacin 30\(\mu\)g, Amoxicillin 20\(\mu\)g, Nitrofurantion 300\(\mu\)g, Chloramphenico 130\(\mu\)g, Gentamicin 30\(\mu\)g, Imipenem 10\(\mu\)g, Trimethoprim + Sulfamethazaxole 75\(\mu\)g, Tetracycline 30\(\mu\)g, Azetreonam 30\(\mu\)g, Ampicillin 10\(\mu\)g) (Bioanalyses / Turkey).

**DNA Extraction:** DNA was extracted using a promega DNA purification kit (Promega, Germany) and accordance with manufactures protocols.

The primers that used in this study are listed in Table 1. were prepared depending on the manufacturing company (Promega, Germany).

**Polymerase chain reaction(PCR):** was performed in a total volume of 20 \(\mu\)l containing 1.0 \(\mu\)l of both forward and reverse of the primers, 12.5 \(\mu\)l master mix, 3.5 \(\mu\)l free water nuclease and 2.0 \(\mu\)l of the extracted DNA (as DNA template), then the DNA amplification was carried out with thermal cycler. Oligonucleotide primers were prepared depending on the manufacturing company (Promega, Germany).

**Table (1): Primers used in this study.**
Programs of Multiplex PCR thermocycling conditions: PCR is performed for 35 cycles which has been done in Uruk Lab For Molecular & Serological tests. Table 2 & Table 3 shows the Programs of Multiplex PCR thermocycling conditions.

Table (2): PCR amplification program for K1,K2,K57 capsular polysaccharide gene detection in K.pneumoniae.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>Initial Denaturation</td>
<td>95°C</td>
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<td>1</td>
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<tr>
<td>Second</td>
<td>I Denaturation</td>
<td>94°C</td>
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<td>35</td>
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<td>II Annealing</td>
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<td></td>
<td>III Extension</td>
<td>72°C</td>
<td>1:30 min</td>
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<tr>
<td>Third</td>
<td>Final Extension</td>
<td>72°C</td>
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Table (3): PCR Amplification Program for htrA gene detection in K.pneumoniae.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>First</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>5 min</td>
<td>1</td>
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<tr>
<td>Second</td>
<td>I Denaturation</td>
<td>94°C</td>
<td>30 s</td>
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<td></td>
<td>II Annealing</td>
<td>56°C</td>
<td>1:30 min</td>
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<td></td>
<td>III Extension</td>
<td>72°C</td>
<td>1:30 min</td>
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<tr>
<td>Third</td>
<td>Final Extension</td>
<td>72°C</td>
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Agarose Gel preparation and DNA loading
This method was carried out according to (Sambrook J et al., 2001)[16]

Statistical analysis
Data were revised, coded, and analyzed using the computer program. “SPSS version 18.0”, the 0.05 level was used as the cut-off value for statistical significance.

RESULTS AND DISCUSSION
From different clinical specimens have been collected; a total of 50 bacterial isolated were identified as Klebsiella pneumoniae collected from hospitalized Iraqi’s patients that suffered from different nosocomial infections within different age group and gender and according to
the standard properties of cultural and biochemical features of *K.pneumoniae* isolates were identified.

**Gene’s detection by multiplex PCR as the following**

(1) **capsular polysaccharide genes**

The multiplex polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in the molecular biology, it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source of DNA material.[17]

In our study the capsular polysaccharide gene (cps) was investigated through specific primer for (50 isolates), it was found that five isolates (10.0%) have K2 cps gene. In fact, the presence of cps genes are correlated with the presence of capsule in *Klebsiella pneumoniae* that played a vital role in the resistance of action of phagocytosis (Ghorashet *et al.*, 2011).[18] More ever, the presence of K2 capsular poly saccharide genes in isolated bacteria means that all these isolates may contain the genes of cps biosynthesis as recorded in (Lin *et al.*, 2011).[19] The prescence of K2 cps gene reduces the binding of antimicrobial peptides to the bacterial surface and this will stimulate the resistance of the bacteria to antibiotics (Kyong and Jae, 2008)[20], as well as five isolates (10.0%) were given positive PCR product for K2 capsular poly saccharide gene, and the other cps genes (K1,K57) had given negative results ( See Figure-1 & 2 below).

So K2 capsular polysaccharide gene indicate the isolates were highly virulent than the other, these result are similar to the results that mentioned by (chen and his co-workers 2008).[21] *Klebsiella pneumoniae* isolates revealed mucoid phenotype and this suggested that the cps genes is very important tool for identifying of the bacteria and this will indicated that the genotype is strongly associated with highly invasive disease.

(2) **htrA gene (putative serien protease)**

*htrA* represents the first well-studied protein that acts in an ATP-independent manner, furthermore, it has previously suggested that *htrA*-like sequences have been entered the metazoan lineages through horizontal acquisition from prokaryotes.[22] Klebsiella pneumoniae *htrA* was diagnosed by using specific primer that mentioned in table 1. In this study, a specific primer set from sequence of the heat shock protein gene *htrA*, a gene coding for high temperature-requirement A (*htrA*) protein and used it for multiplex PCR inorder for detecting
of *K. pneumoniae* isolates. Our results as shown below in figure (3,4 and 5) respectively; confirms the presence of *htrA* gene in local cases that had been isolated, fifty strains that isolated from nosocomial infection cases revealed that *htrA* gene is expressed and generated positive result in 48 cases (96.0%) while about two cases revealed negative results as shown in figure (4 and 5), the present study revealed that *htrA* gene play an important role in the *K. pneumoniae* virulence among the different clinical and environmental samples that has been isolated from a varieties of nosocomial infections and has been contributed to the pathogenicity.

Our results improved that the high temperature requirement is a major virulence factor for *K. pneumoniae*. The bacterial *htrA* gene product is one of the most well-characterized proteins of the HtrA family and its presence is necessary for bacterial thermotolerance.[23] It has recently been shown that bacterial *htrA* has dual roles, acting as a chaperone at normal temperature; we identified that *htrA* gene as new secreted vitulence and invading factor from human pathogen, especially in patients that infected with nosocomial pneumonia and nosocomial UTIs, these data suggested that the appearance of *htrA* gene in *K. pneumoniae* patients is an important growth factor and; has specific functions during an infections and has an impact in the folding of virulence.

![Figure (1): Gel electrophoresis of PCR product of (K1,K2,K57cps)genes (1.5% agarose, 120 Volt/1hr) line M:100bp ladder, visualized under UV. Lines (11,12,14,19) represent positive results for K2 genes with 641bp amplicon by using (oligonucleotide primers), while line (15,16,17,18,20) show negative results for K1,K2,K57.](image-url)
Figure (2): Gel electrophoresis of PCR product of \((K1,K2, K57 \text{ cps})\) genes (1.5\% agarose, 120 Volt/1hr) line M:100bp ladder, visualized under UV. Line(44) represent positive result with \(K2\) gene 641bp amplicon by using (oligonucleotide primers) while line (41, 42,43,45, 46,47,48, 49,50) show negative results for \(K1,K2,K57\).

Figure (3): Gel electrophoresis of PCR product of \((\text{htrA putative seriene protease})\) gene (1.5\% agarose, 120 Volt/1hr) line M:100bp ladder, visualized under UV. Lines (1,2,3,4,5,6,7,8,9,10) represent positive results \(\text{htrA gene}\) with (221bp). by using (specific primers).

Figure (4): Gel electrophoresis of PCR product of \((\text{htrA putative seriene protease})\) gene (1.5\% agarose, 120 Volt/1hr) line M:100bp ladder, visualized under UV. Lines (11,12,13, 14,15,16,17,18,19,20, 22,23,24,25, 26,27,28,29,30) represent positive results \(\text{htrA gene}\) with (221bp). While line(21) represents negative results by using (specific primers).
CONCLUSIONS

The molecular diagnosis of Klebsiella pneumoniae revealed the incidence of K2 capsular polysaccharide genes were found in five bacterial isolated; K1 and K57 cps have not been detected, while the htrA gene positive strains were detected in (48) various specimens that have been isolated from patients with nosocomial infections in Iraqis' hospitals. The incidence of htrA gene in Klebsiella pneumoniae isolates play an important role in K.pneumoniae virulence. The present study suggested that htrA gene as a new secreted virulence and an important growth factor and has a specific functions during an infections; especially among community acquired nosocomially infection patients.

RECOMMENDATIONS

Use of animal model as a critical element in the study of Klebsiella pathogenicity by providing vital information that cannot be providing by in vitro studies, further studies are require to investigate other Klebsiella virulence factors such as type 1 and type 3 pilli, serum resistance lipopolysaccharide and siderophore.

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