Original Research Article

Molecular characterization of capsular polysaccharide genes of *Klebsiella pneumoniae* in Iraq

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ABSTRACT

*Klebsiella pneumoniae* is important medical pathogen responsible for some health problems especially nosocomial infections; pneumonia, septicemia and urinary tract infection. Study of genetic and phenotypic properties that related to virulence factors associated with pathogenicity of *K. pneumoniae* isolated from some clinical cases. The different clinical specimens were collected by standard methods from 158 patients. The bacterial isolates were identified according to golden characterization of morphology and biophysiology for this microorganism. Molecular detection and genotyping were done by polymerase chain reaction and gel-agarose electrophoresis. The molecular detection of capsular polysaccharide gene (cps) was investigated using PCR specific primers. It was found that only 43 isolates were positive for this marker. Genotyping of these isolates were also carried out by using K1, K2 specific PCR primers. PCR technique showed that 8 isolates were positive to K1 serotype, 14 isolates to K2 serotype, 3 isolates were positive for both K1 and K2, so they were designated as K1/K2 and 18 isolates were negative for both markers. The magA gene was investigated by gel-agarose electrophoresis, and it was found that 11 isolates were positive for magA marker. Also, *rmpA*, *rmpA1* and *rmpA2* markers were investigated. The *rmpA* is found in 21 isolates, whereas *rmpA1* and *rmpA2* were positive in 19 isolates. The *cps* gene is common in *K. pneumoniae* which reflect the presence of capsular polysaccharide. We have developed a simple and useful capsular genotyping method for *K. pneumoniae* based on K markers. *MagA* genes and *RmpA* genes are detected in most local isolates. Local Klebsiella isolates are divided according to their serotypes into K1, K2 and K1/K2 groups.

Keywords

*Klebsiella pneumoniae*, capsular polysaccharides genes, genotyping.

Introduction

*Klebsiella pneumoniae*, an important nosocomial pathogen, causes a wide range of infections, including pneumonia, bacteremia, urinary tract infection, and life-threatening septic shock [Alves *et al.*, 2006, Hentschke *et al.*, 2010]. The clinical isolates of *K. pneumoniae* usually produce a large amount of capsular polysaccharide (cps), which confers not only a mucoid phenotype but also resistance to engulfment.
by professional phagocytes or to serum bactericidal factors [Bach et al., 2000].

The capsular polysaccharides have been regarded as the main determinant of Klebsiella pathogenicity, and about 77 different capsular serotypes have been reported by Chen and co-workers [Chen et al., 2009]. K. pneumoniae strains belonging to serotypes K1 and K2 are the most virulent to mice [Fang et al., 2009]. Most strains of K. pneumoniae have K markers; K1 and K2 were found to be significantly more resistant to phagocytosis than non-K1/K2 isolates [Lin et al., 2004]. The capsular polysaccharides (cps) genotyping K1/K2 K. pneumoniae strains are important responsible for nosocomial infections. The strains of K. pneumoniae isolated from patients produce large amounts of capsular polysaccharides (cps) as reflected by the formation of glistening mucoid colonies with viscid consistency. The degree of mucoidy has been shown to positively correlate with the establishment of infection [Friridich and Whitfield, 2004].

The importance of K. pneumoniae capsular serotype in virulence and phagocytosis resistance has been reported before showing that serotype K1 or K2 played a more determinant role in virulence than those of magA (mucoviscosity-associated gene A) and rmpA (regulation of capsular polysaccharide synthesis) in pathogenic strains. Sequence analysis of a pathogenicity island carried has revealed a locus named rmpA, which has been reported to enhance the colony mucoidy of various serotypes of K. pneumoniae [Ghorashi et al., 2011]. These findings indicated that rmpA2 functions as a trans-acting activator for the cps biosynthesis. Due to the essential role of cps in K. pneumoniae pathogenesis, it would be important to understand how rmpA2 exerts its activation to the K2 cps biosynthesis. Virulence gene; magA was first identified in pathogenic strains isolated from Taiwan causing liver abscess [Rahan et al., 2004; Evrard et al., 2010].

The aim of the present work is to study the phenotypic and genotypic characterization of K. pneumoniae isolated from patients in Hilla city, Iraq. The aims was achieved by detecting of genes responsible for capsular polysaccharides such as Cps, K1, K2, K1/K2, non K1/K2, mag A, rmpA, rmpA1, and rmpA2 using molecular methods (PCR).

Materials and Methods

Patients

A total of 158 patients were admitted to the main two hospitals in Hilla city: Teaching Hilla hospital and teaching Babylon hospital -IRAQ.

Collection of specimens

The different specimens were generally collected from patients suffering from urinary tract infection, burns, wound skin infection and respiratory tract infection. The specimens were collected by standard methods as mentioned in [Forbes et al., 2007].

Identification

They were cultivated on conventional laboratory media. Identification each bacterial isolate by phenotypic characterization and biochemical reactions, and by commercial kits, API-20E [Collee et al., 1996; McFaddin, 2000].

Detection of capsule

The capsule was detection in local isolates according to procedure that described in [Murray et al., 2003].
DNA extraction

DNA was extracted using a Promega DNA purification kit (Promega, USA) and in accordance with the manufacturer's protocols.

Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl containing 2.5 µl of both the forward and the reverse of the primers, 12.5 µl master mix, 2.5 µl free water nuclease and 5 µl of the extracted DNA (as DNA template), then DNA amplification was carried out with the thermal cycler. The lyophilized oligonucleotide upstream and downstream primers (see table -1 below) were prepared according to the manufacturing company (Alpha, USA). PCR is performed for 30 cycles. After the electrophoresis is complete, the molecules in the gel can be visible by UV-transilluminator [Lodish et al., 2005; Chuang et al., 2006].

Ethical approval and consent:

The ethical approval was obtained from the Babylon health Office. Moreover, all patients involved in this work were informed and the agreement was obtained from each one prior the collection of samples.

Results and Discussion

A total of 158 clinical samples were collected from different infections. Only 46 (29.1%) isolates of K. pneumoniae were identified according to morphological characterization and biochemical tests. According to standard phenotypic properties of the cultural and biochemical features of K. pneumoniae isolates were identified as mentioned by Collee et al., 1996 and Macfaddin, 2000.

Detection of capsular polysaccharide gene

In our study the capsular polysaccharide (cps) gene was investigated through specific primer for 46 isolates that identified by biochemical tests. It was found that forty three isolates of K. pneumoniae have cps gene whereas three isolates have not (Fig.-1). This will confirm that these three isolates may not belong to K. pneumoniae for that they are excluded from other experiments. In fact, the presence of cps genes are correlated with the presence of capsule in K. pneumoniae, and the overproduction of mucoid phenotypes may correlate with the presence of rcs genes which are chromosomally located and rmpA genes which are mostly plasmid encoded cps gene may have a large role in resistance of action of phagocytosis and serum resistance (Ghorash et al., 2011). This result is in agreement with the result of Debroy and co-workers [2005] who stated that cps genes are available in most K. pneumoniae isolates.

The presence of cps genes in most isolated bacteria means that all these isolates may contain the genes of cps biosynthesis as that mentioned in [Lin et al., 2011]. However, the presence of cps reduces the binding of antimicrobial peptides to bacterial surface and this will promote the bacterial resistance to antibiotics [Kyong and Jae, 2008]. The genomic organization of chromosomal cps region is responsible for cps biosynthesis in K. pneumoniae [Pan et al., 2008]. Chen and co-workers [2009] indicate that the majority of clinical isolates of K. pneumoniae express pronounced capsule polysaccharide that is essential to virulence.

Genotyping of K. pneumoniae clinical isolates

By molecular detection of K markers, the
eight isolates give positive PCR products for K1, fourteen isolates give positive with K2, three isolates with K1/K2, and the rest 18 isolates are (non K1/K2). However, 25 isolates give positive for K1 and K2 antigen (as in table-2). The molecular technique for detection of K1 gene (Fig.2) and K2 gene (see Fig.3) in isolates of K. pneumoniae was confirmed by gel-agarose electrophoreses which ensure that isolates are highly virulent than others. These results are similar to results mentioned by Chen and co-workers[2008]. So, Klebsiella isolates are distributed according to their serotypes into four genogroups ; K1 group, K2 group, K1/K2 group, and non K1/K2 group. It was seen that the prevalence of K2 isolates are higher than K1 isolates. This result is in agreement with [Lin et al.,2004; Maldin et al., 2009] show that K1 and K2 isolates more virulent than other serotypes. However, this result is in disagreement with [Turton et al., 2006, Hsueh et al., 2013] who finds that K1 serotype is striking that general prevalence of the K1 serotype is significantly higher. The our result is unlike Pan and co-workers[2008] who found that K1 most common serotype found in community acquired and nosocomial K. pneumoniae infections. On the other study, 21 isolates where classed as non K1/K2. These isolates may belong to other serotypes such as K3, K14, K20 [Whitehouse et al.,2010].

However, all K. pneumoniae isolates reveal mucoid phenotype regardless of genotype the polysaccharide (cps) is a very important tool identifying and in genotyping of these bacteria indicates that genotype is strongly associated with high invasive disease[Lin et al., 2011]. The genotypes particularly K1 and K2 are common in our province. However non K1/K2 could not be classified into other K antigen groups rather them K1 or K2. The results are agreed with Fang and co-workers[2009]. According to the results obtained by this study, only 22 isolates are found to be positive for K1 or K2 or K1/K2 that give an indicator that these isolates are highly virulent than the other. Regarding to this study, K2 is found to be more prevalent than K1. This is in agreement with Whithouse and co-workers[2010] who have indicated that K2 is more predominant in human infections but is very rarely identified in the natural environment. In addition to that, three isolates are found to have K1, and K2 and these isolates are considered as high virulent strains because of their possession of K1 and K2 antigens as observed in reference[Lin et al., 2006].

Furthermore, no specific pattern for differentiation of K. pneumoniae K1 or K2 isolates from urinary tract infection or from respirator aspirates could be detected pyogenic UTI or RTI caused by K. pneumoniae with distant metastasis to urinary tract and respiratory tract that may be due to virulent capsule or host factors which could not be found in this study. Further analysis of the cps gene and host factors may be provide due to understanding the pathogenesis of K. pneumoniae in UTI and RTI [Evrard et al., 2010].

In study [Rozalski ,2007] shows the K. pneumoniae isolates belong to serotypes K1 and K2 are the most virulent. However, the predominance of serotypes of K1 and K2 in pyogenic cases suggest that those serotypes have more invasive capsular antigen. To differentiate between UTI, RTI, burns, wounds capsular of K. pneumoniae serotypes K1, K2 and non K1/K2 to compare phagocytic rate between them. There is no significant difference of phagocytic of non K1/K2 was absorbed in all sample but in K1, K2 there are significant phagocytic in all sample isolated for K. pneumoniae [Granier et al., 2002; Lin et al., 2006].

This result is similar to study [Rozalski,
2007] who showed that has been demonstrated that strain with capsule such as (K₁, K₂) are virulent to human, whereas serotypes without capsule are less virulent or without virulent.

**Detection of magA gene in clinical isolates:**

The molecular detection for magA gene by PCR was also investigated through gel-agarose electrophoresis (as in Fig.4). A total of 43 isolates of *K. pneumoniae*. It was found that 11 isolates were positive for magA gene, of which 6 isolates were found among K₁ serotype, 4 isolates among non K₁/K₂ serotype and only one in K₂ serotypes as shown in table-3. Although this gene is highly important for *K. pneumoniae* which confirm the bacteria mucoid viscosity, but it is prevalence and among *K.pneumoniae* local isolates is not high. This means that there are other means play a role in formation the mucoid viscosity.

Many studies have indicated that magA is more prevalent among K₁ serotype. MagA gene has been recently investigated by Rozalski[2007] who find that the prevalent of this gene is high among K₁ isolate and the presence of mag A is connected with K₁ serotype of *K. pneumoniae*. Also, Lin and co-workers[2006] state that it is observed that magA gene is not prevalent among K₂ serotype isolates and only one isolate is positive to that, This may be attributed in the fact that magA is highly present in K₁ serotype but not in K₂ serotype mentioned. Also, other study find that magA gene is not prevalent in K₂ serotype but high prevalent in K₁ that may be attributed that magA is located in cps gene cluster K₁ of *K.pneumoniae* and it is restricted to serotype K₁ isolates[ Granier et al., 2002, Hsueh et al.,2013].

**Molecular detection of rmpA markers in Klebsiella isolates**

The genes; rmpA₁, rmpA₂ and rmpA₂ were detected by using PCR markers. The molecular detection for rmpA in local isolates was shown in figure-5. It is found that rmpA give the present in 21 isolates and absent in 22 isolates, also rmpA₁ is found to be available in 19 isolates and absent in 24 isolates, and the results are the same for rmpA₂ where 19 isolates are positive, for this gene and 24 are negative for it. The rmpA gene encodes for the regulation of mucoid phenotype which may be located on bacteria chromosome or on plasmid and so the of absence a such genes may be related where these genes are located in Klebsiella genome[Lin et al., 2011]. According to serotypes, it is found that rmpA₁ and rmpA₂ is prevalent more in K₂ than and also in non K₁/K₂ isolates this will enhance the severity of *K.pneumoniae* isolates are shown in table-4.

The Rmp genes play a role in over production of mucoid phenotype in this bacteria .However the deletion of rmpA₁ reduce capsular polysaccharide (cps) biosynthesis and result in decreases colony mucoid and virulence[Pan et al;2008]. Also, the multi-copy plasmid carrying rmpA restored cps production in the rmpA₁ or rmpA₂ mutant. Transformation of rmpA detection mutant with an rcsB carrying plasmid also fails to enhance cps production suggesting that a cooperation of rmpA with an rcs gene is required for regulatory activity. This is further cooperated by the demonstration of interaction between rmpA and rcsB using two hybrid analysis [Whitehouse et al., 2010, Timofte et al.,2014].
Although, the previous study on *rmpA* are restricted to serotype K<sub>2</sub> strain. This study shows that *rmpA* also exists in serotype, other than K<sub>2</sub>. Besides, among *rmpA* positive isolates, the K<sub>1</sub> or K<sub>2</sub> group has more significant and high lethality than the non K<sub>1</sub>/K<sub>2</sub> group. This difference may be related to a higher prevalence of virulent serotypes in certain regions [Synder et al., 2004, Heutschke et al., 2010] Rapid detection of the virulent K<sub>1</sub> serotype will be most helpful in diagnosis and treatment to decrease the risk of severe metastatic infections, as well as in epidemiological studies. Traditional sera-typing is cumbersome and requires access to high quality antisera. Our results show that *magA* is restricted to isolates of the K<sub>1</sub> serotype. We therefore suggest detection of *magA* by PCR analysis as an easy, fast and highly specific diagnostic method for identification of the *K. pneumoniae* K<sub>1</sub> capsule serotype.

Table.1 The oligonucleotides primers sequences that used in the present study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cps</em></td>
<td>5-GTCGTTAGCTGTAAAGCCAGGGCCGGTAGGG-3</td>
<td>5-TATTCATCAGAAGCAGCAGCTGGGAGAGGCC-3</td>
<td>418</td>
</tr>
<tr>
<td><em>magA</em></td>
<td>5-TAGGAGCCGTATTTTGGTTTGT-3</td>
<td>5-GAATATTCCTCACTCCCTCTCC-3</td>
<td>1282</td>
</tr>
<tr>
<td>K&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5-GTAGTTATTGCAACGCATGC-3</td>
<td>5-GGCAGGTTAATGAAATCCGT-3</td>
<td>1283</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5-GGAGCCATTATATTGGTGTG-3</td>
<td>5-TCCCTAGCAGTGGCTTAAGT-3</td>
<td>646</td>
</tr>
<tr>
<td><em>rmpA</em></td>
<td>5-GCATTTAATGCCTGGAGTACCTCGT-3</td>
<td>5-GTTTACATCGGCTACATTCTTTTCTTAAG-3</td>
<td>553</td>
</tr>
<tr>
<td><em>rmpA&lt;sub&gt;1&lt;/sub&gt;</em></td>
<td>5-CTGTGTCATCATTGGTG-3</td>
<td>5-GATAGTTCACCTCCTCCTCC-3</td>
<td>448</td>
</tr>
<tr>
<td><em>rmpA&lt;sub&gt;2&lt;/sub&gt;</em></td>
<td>5-TGGCCAGCAGGCAATACTGTC-3</td>
<td>5-GAAGAGTGCTTTACACCCT-3</td>
<td>496</td>
</tr>
</tbody>
</table>

**Figure.1** Agarose gel electrophoresis of PCR products for detection of Capsular polysaccharide (*cps*) gene amplicon product in *K. pneumoniae*. Lanes 1-12 refer to isolates’ number. L refers to allelic ladder.
Table 2 Distribution of local isolates (43) of *K. pneumoniae* into genotypic groups

<table>
<thead>
<tr>
<th>Local isolates(No.)</th>
<th>Genogroups</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> (43)</td>
<td>K1</td>
<td>8</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (43)</td>
<td>K2</td>
<td>14</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (43)</td>
<td>K1/K2</td>
<td>3</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (43)</td>
<td>Non-K1/K2</td>
<td>18</td>
</tr>
</tbody>
</table>

Figure 2 Agarose gel electrophoresis of PCR products for detection of K1 gene amplicon product of *K. pneumoniae*. Lanes 1-7 refers to isolates’ number. L refers to allelic ladder.

Figure 3 Agarose gel electrophoresis of PCR products for detection of K2 gene amplicon product
**Figure 4** A agarose gel electrophoresis of PCR products for detection of *MagA* gene amplicon product.

**Table 3**: Distribution of local isolates of *K. pneumoniae* according *magA* genotypic marker.

<table>
<thead>
<tr>
<th>Local isolates</th>
<th>Genetic markers</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>K1</td>
<td>6</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>K2</td>
<td>1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>K1/K2</td>
<td>0</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Non-K1/K2</td>
<td>4</td>
</tr>
</tbody>
</table>

**Figure 5** Agarose gel electrophoresis of PCR products for detection of *rmpA* gene amplicon product in *Klebsiella pneumoniae*, lane 1-7 refers to isolates' number. L-refers to allelic ladder.
Table 4: Distribution of local isolates of *K. pneumoniae* according *rmpA* genotypic markers

<table>
<thead>
<tr>
<th>Local isolates</th>
<th>Genotype</th>
<th><em>rmpA</em></th>
<th><em>rmpA1</em></th>
<th><em>rmpA2</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> K1</td>
<td>K1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> K2</td>
<td>K2</td>
<td>16</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> K1/K2</td>
<td>K1/K2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> Non-K1/K2</td>
<td>Non-K1/K2</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total no.</td>
<td></td>
<td>21</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

The *Cps* gene is common in *K. pneumoniae* which reflects the presence of capsular polysaccharide. We have developed a simple and useful capsular genotyping method for *K. pneumoniae* base on *K* markers. *Mag*A genes and *Rmp*A genes are detected in most local isolates. Local *Klebsiella* isolates are divided according to their serotypes into K1, K2 and K1/K2 groups.

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