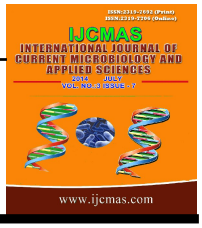
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 7 (2014) pp. 224-234**
http://www.ijcmas.com



Original Research Article

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

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K e y w o r d s

Klebsiella pneumoniae, capsular polysaccharides genes, genotyping.

A B S T R A C T

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 *mag* A gene was investigated by gel-agarose electrophoreses, and it was found that 11 isolates were positive for *mag* A marker. Also ,*rmp*A, *rmp*A1 and *rmp*A2 markers were investigated. The *rmp*A is found in 21 isolates, whereas *rmp*$A_1$and *rmp*A2 were positive in 19 isolates. *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* base 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.

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* pathogencity, and about 77 different capsular serotypes have been reported by Chen and co-workers [Chen *et al*., 2009]. *K. pneumoniae* strains belonging to serotypes K_1 and K_2 are the most virulent to mice[Fang *et al*., 2009]. Most strains of *K.pneumoniae* have K markers; K_1 and K_2 were found to be significantly more resistant to phagocytosis than non-K_1/K_2 isolates [Lin *et al*., 2004]. The capsular polysaccharides (cps) genotyping K_1/K_2 *K. pneumoniae* strains are important responsible for nosocomial infections. The strains of *K. pneumoniae* isolated from patients are 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 K_1 or K_2 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 *rmp*A, which has been reported to enhance the colony mucoidy of various serotypes of *K. pneumoniae* [Ghorashi *et al.*, 2011]. These findings indicated that *rmp*A_2 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 *rmp*A_2 exerts its activation to the K_2 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, K_1, K_2, K_1/K_2, non K_1/K_2, *mag* A ,*rmp*A,*rmp*A_1, and *rmp*A_2 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 phynotypic 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 *rmp*A 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 K_1, fourteen isolates give positive with K_2, three isolates with K_1/K_2, and the rest 18 isolates are (non K_1/K_2). However, 25 isolates give positive for K_1 and K_2 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 his co-workers[2008].So, Klebsiella isolates are distributed according to their serotypes into four genogroups ; K_1 group, K_2 group , K_1/K_2 group, and non K_1/K_2group. It was seen that the prevalence of K_2 isolates are higher than K1 isolates. This result is in agreement with [Lin *et al*.,2004; Maldin *et al.*, 2009] show that K_1 and K_2 isolates more virulent than other serotypes. However, this result is in disagreement with [Turton *et al*., 2006, Hsueh *et al*., 2013] who finds that K_1 serotype is striking that general prevalence of the K_1 serotype is significantly higher. The our result is unlike Pan and co-workers[2008] who found that K_1 most common serotype found in community acquired and nosocomial *K. pneumoniae* infections. On the other study, 21 isolates where classed as non K_1/K_2 .These isolates may belong to other serotypes such as K_5, $K1_4$, K_{20} [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 K_2 are common in our province. However non K_1 /K_2 could not be classified into other K antigen groups rather them K_1 or K_2. 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 K_1 or K_2 or K_1/K_2 that give an indicator that these isolates are highly virulent than the other . Regarding to this study, K_2 is found to be more prevalent than K_1 .This is in agreement with Whithouse and co-workers[2010] who have indicated that K_2 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 K_1, and K_2 and these isolates are considered as high virulent strains because of their possession of K_1 and K_2 antigens as observed in reference[Lin *et al*., 2006].

Furthermore, no specific pattern for differentiation of *K. pneumoniae* K_1 or K_2 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 provided 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 K_1 and K_2 are the most virulent. However, the predominance of serotypes of K_1 and K_2 in pyogenic cases suggests that those serotypes have more invasive capsular antigen. To differentiate between UTI, RTI ,burns ,wounds capsular of *K. pneumoniae* serotypes K_1, K_2 and non K_1/K_2 to compare phagocytic rate between them .There is no significant difference of phagocytic of non K_1/K_2 was absorbed in all sample but in K_1 , K_2 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 (K_1, K_2) are virulent to human, whereas serotypes without capsule are less virulent or without virulent.

Detection of *mag*A gene in clinical isolates:

The molecular detection for *mag*A 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 *mag*A gene, of which 6 isolates were found among K_1 serotype , 4 isolates among non K_1/K_2 serotype and only one in K_2 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.pnemouniae* 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 *mag*A is more prevalent among K_1 serotype. *Mag*A gene has been recently investigated by Rozalski[2007]who find that the prevalent of this gene is high among K_1 isolate and the presence of *mag* A is connected with K_1 serotype of *K. pneumoniae*. Also, Lin and co-workers[2006] state that it is observed that *mag*A gene is not prevalent among K_2 serotype isolates and only one isolate is positive to that, This may be attributed in the fact that *mag* A is highly present in K_1 serotype but not in K_2 serotype mentioned. Also, other study find that *mag*A gene is not prevalent in K_2 serotype but high prevalent in K_1 that may be attributed that *mag* A is located in *cps* gene cluster K_1 of *K.pneumoniae* and it is restricted to serotype K_1 isolates[Granier *et al*., 2002, Hsueh *et a*l.,2013] .

Molecular detection of *rmp*A markers in *Klebsiella* isolates

The genes; *rmp*A, *rmp*$A_1$and *rmp*A_2 were detected by using PCR markers. The molecular detection for *rmp*A in local isolates was shown in figure-5. It is found that *rmp*A give the present in 21 isolates and absent in 22 isolates, also *rmp*A_1 is found to be available in 19 isolates and absent in 24 isolates, and the results are the same for rmpA_2 where 19 isolates are positive, for this gene and 24 are negative for it. The *rmp*A 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 wherethese genes are located in Klebsiella genome[Lin *et al*., 2011]. According to serotypes , it is found that *rmp*A_1 and *rmp*A_2 is prevalent more in K_2 than and also in non K_1/K_2 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 *rmp*A_1 reduce capsular polysaccharide (cps) biosynthesis and result in decreases colony mucoid and virulence[Pan *et al*;2008]. Also, the multi-copy plasmid carrying *rmp*A restored cps production in the *rmp*A1 or *rmp*A_2 mutant. Transformation of *rmp*A detection mutant with an *rcs*B carrying plasmid also fails to enhance cps production suggesting that a cooperation of *rmp*A with an *rcs* gene is required for regulatory activity. This is further cooperated by the demonstration of interaction between *rmp*A and *rcs*B using two hybrid analysis [Whitehouse *et al*., 2010, Timofte *et al*.,2014].

Although, the previous study on *rmp*A are restricted to serotype K_2 strain. This study shows that *rmp*A also exists in serotype, other than K_2. Besides, among *rmp*A positive isolates, the K_1 or K_2 group has more significant and high lethality than the non K_1/K_2 group. This difference may be related to a higher prevalence of virulent serotypes in certain regions[Synder *et a*l., 2004, Heutschke *et al*.,2010] Rapid detection of the virulent K_1 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_1 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_1 capsule serotype.

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

Genes	Forward primer	Reverse primer	Amplicon size
cps	5-GTCGGTAGCTGTTAAGCCAGGGGCGGTAGCG-3	5-TATTCATCAGAAGCACGCAGCTGGGAGAAGCC-3	418
*mag*A	5-TAGGACCGTTAATTTGCTTTGT-3	5-GAATATTCCCACTCCCTCTCC-3	1282
K_1	5-GTAGGTATTGCAAGCCATGC-3	5-GCCCAGGTTAATGAATCCGT-3	1283
K_2	5-GGAGCCATTTGAATTCGGTG-3	5-TCCCTAGCACTGGCTTAAGT-3	646
*rmp*A	5-GCAGTTAACTGGACTACCTCTG-3	5.GTTTACAATTCGGCTAACATTTTTCTTTAAG-3	553
rmpA_1	5-CTGTGTCCACATTGGTGGG-3	5-GATAGTTCACCTCCTCCTCC-3	448
rmpA_2	5-TGGCAGCAGGCAATATTGTC-3	5-GAAAGAGTGCTTTCACCCCCT-3	496

Figure.1 Agarose gel electrophoresis of PCR products for detection of Capsular polysaccharide (cps) gene amplicon product in *K.pneumonia*e. Lanes 1-12 refer to isolates' number. L refers to allelic ladder

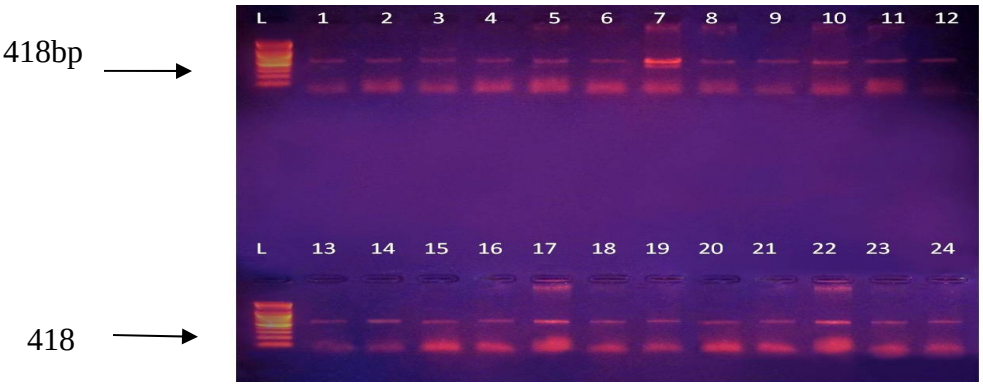

Table.2 Distribution of local isolates (43) of *K.pneumoniae* into genotypic groups

Local isolates(No.)	Genogroups	No. of isolates
K.pneumoniae (43)	K1	8
K.pneumoniae (43)	K2	14
K.pneumoniae(43)	K1/K2	3
K.pneumoniae(43)	Non-K1/K2	18

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

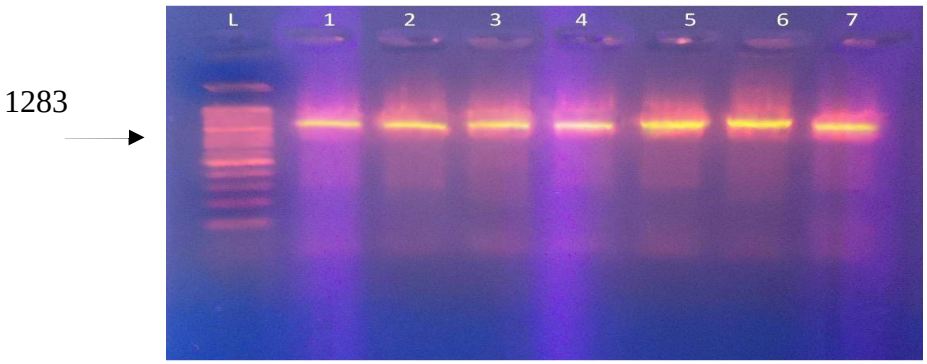

Figure.3 Agarose gel electrophoresis of PCR products for detection of K2 gene amplicon product

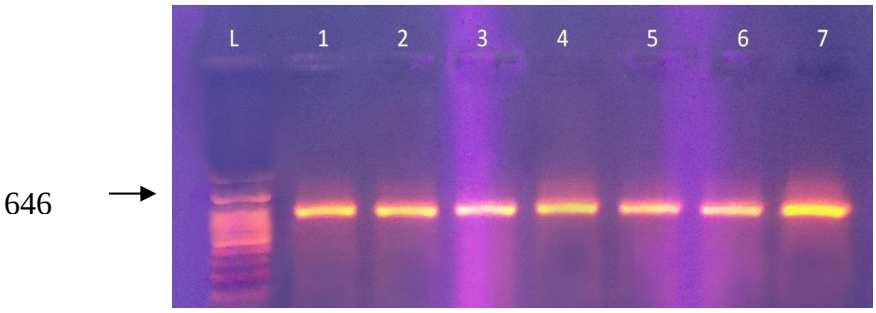

Figure.4 A garose gel electrophoresis of PCR products for detection of *Mag*A gene amplicon product.

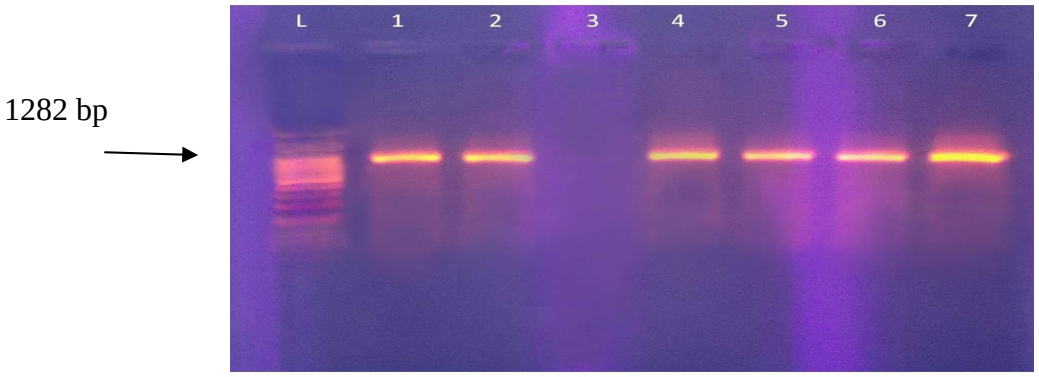

Table.3: Distribution of local isolates of *K.pneumoniae* according *mag*A genotypic marker.

Local isolates	Genetic markers	No. of isolates
K.pneumoniae	K1	6
K.pneumoniae	K2	1
K.pneumoniae	K1/K2	0
K.pneumoniae	Non-K1/K2	4

Figure.5 Agarose gel electrophoresis of PCR products for detection of *rmp* A gene amplicon product in *Klebsiella pneumonia*e, lane 1-7 refers to isolates' number. L-refers to allelic ladder

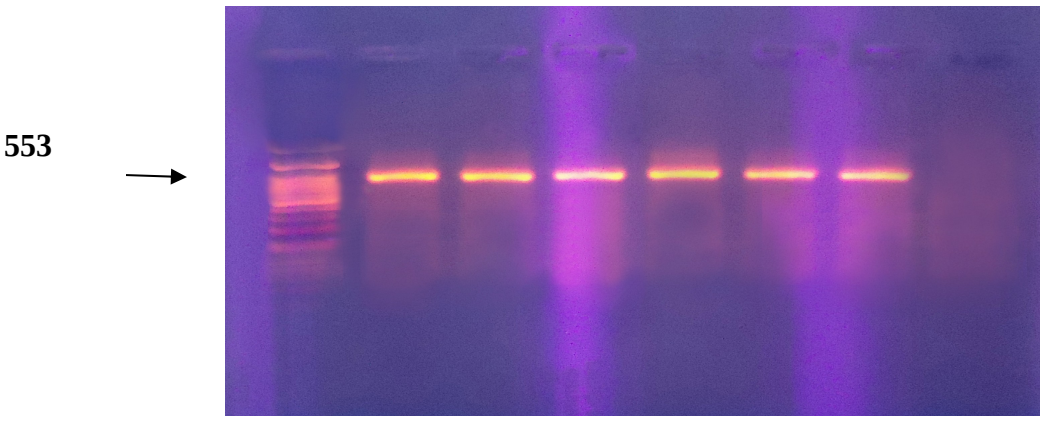

Table.4 Distribution of local isolates of *K.pneumoniae* according *rmp*A genotypic markers

Local isolates	Genotype	*rmp*A	*rmp*A1	*rmp*A2
K.pneumoniae	K1	1	2	1
K.pneumoniae	K2	16	12	13
K.pneumoniae	K1/K2	0	0	0
K.pneumoniae	Non-K1/K2	4	5	5
Total no.		21	19	19

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.

Acknowledgments

We would like to thanks the microbiology department in college of Medicine, Babylon University for support and providing the entire needed essential for the completion of this work. We also deeply indebted to staff and patients of Hilla hospitals for their kind assistance.

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