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GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF SOME VIRULENCE GENES OF *KLEBSIELLA PNEUMONIAE* IN HILLA CITY

Massar A. M. AL-A`ammary*, Jawad K. Al-Khafaji and Ifad K. Al-Shibly

Department of Microbiology, College of Medicine, University of Babylon, Iraq.

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***Corresponding Author**
Massar A. M. AL-A`ammary
Department of Microbiology, College of Medicine, University of Babylon, Iraq.

ABSTRACT

Klebsiella pneumoniae is a gram-negative, non-motile, lactose fermenting, rod-shape organism. Although found in the microbiota, *K. pneumoniae* is frequently associated with hospital-acquired infections and can progress into severe illness. This study aims to detect some genes of virulence of *Klebsiella pneumoniae* isolated from different clinical samples in Hilla city, Iraq. A total (200) clinical samples were collected from different sources of patients from both sexes. Primary bacterial isolation was done, followed by phenotypic detection of some virulence factors, then molecular detection of virulence genes was done by genomic DNA extraction and Polymerase Chain Reaction (PCR). From a total of (200) clinical samples, only twenty isolates (10%) of *Klebsiella pneumoniae* were identified according to the cultural characteristics and biochemical tests. Most isolates were in male gender, mean age of isolation was 14.9 years. Phenotypically all *Klebsiella pneumoniae* isolates were positive for urease, siderophore, colonization factors antigens and capsule production. Similarly, all isolates had strong ability for stone formation after incubation in normal sterile urine. While only 40% of isolates had high capacity for biofilm formation. Genotypic study revealed that among total of 20 local isolates of *Klebsiella pneumoniae*, all of the twenty isolates (100%) were negative for *mag* A gene, *Kfu* gene was detected in 5 isolates of *Klebsiella pneumoniae* (25%) and absent in 15 (75%) isolates. While *Uge* gene was found in two isolates (10%) only.

KEYWORDS: *Klebsiella pneumoniae*, *mag* A gene, *Kfu* gene, *Uge* gene.

INTRODUCTION

Klebsiella pneumoniae is encountered as pathogen for humans and other mammals, colonizing different clinical sites including the gastrointestinal tract, skin, and nasopharynx; it is also found in various environmental niches (soil, water, etc.).[1] The pathogenicity of *K. pneumoniae* is multifactorial including LPS, capsule, urease, adhesins, outer-membrane proteins and biofilms.[2] Mucoviscosity-associated gene A (*mag*A), has been identified in *Klebsiella pneumoniae*.[3] The m*ag*A is detected in a vast majority of *K. pneumoniae* liver abscess isolates and is associated with hypermucoviscosity (HV) and resistance to killing by human serum and phagocytosis.[4] The *kfu* gene encodes an iron-uptake system that is associated with a hypermucoviscosity phenotype and increased virulence.[5] While *K. pneumoniae* typically expresses both smooth lipopolysaccharide (O antigen) and capsule polysaccharide (K antigen) on its surface and both LPS and capsule contribute to the pathogenesis of this species. A single mutation in a gene that codes for a UDP-galacturonate 4-epimerase *(uge)* renders a strain with the O−K− phenotype (lack of capsule and LPS without O antigen molecules and outer core oligosaccharide).[6] This study aims to detect some genes of virulence of *Klebsiella pneumoniae* isolated from different clinical samples in Hilla city, Iraq.

MATERIALS AND METHODS

A total (200) clinical samples were collected from different sources of patients from both sexes who attended or admitted to the main three hospitals in Hilla city /Babylon province (General Teaching hospital, Medical Teaching hospital of Merjan and Babylon hospital for Maternity and Children) for a period from September 2016 to December 2016. The different clinical specimens (sputum, sinus, tonsils, urine and vagina) were taken by a sterile containers, then stored in a cool place until transport to the laboratory. Primary bacterial isolation was done by streaking a loopful of culture on MacConkey agar for primary selection of pathogenic *Klebsiella pneumoniae.* The plates were incubated overnight at 37°C. The developed colonies which showed characteristic growth, colors, mucoid phenotype were transferred to a new plate for further tests.

Phenotypic detection of virulence factors was done individually for each virulence factor including capsule production by Indian ink test, Urease production by inoculating urea medium, Colonization factor antigen by the slide agglutination technique, Siderophore / Hemolysin production by inoculating a blood agar medium, Biofilm Production by tissue

culture plate method (TCP) assay and In Vitro Stone Formation by inoculating a fresh urine sample.

Molecular detection of virulence genes was done by Genomic DNA extraction followed by Polymerase Chain Reaction (PCR). The primers, which were used in PCR assay in this study with their sequences, and the DNA amplicon size the PCR conditions was carried out through the thermal cycling profile listed in table (1).

Table 1: the primers used in the present study

Markers	Oligonucleotide sequence 5`-3`	Size of amplicon	Thermal cycler conditions	Reference
MagA-F *MagA* -R	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1280 pb	94C° for 1 min. 94C° for 30 sec. 59C° for 45 sec (30 cycle) 74C° for 2 min. 72C° for 6 min.	Fang *et al*. (2004)
Kfu –F *Kfu* - R	GAAGTGACGCTGTTTCTGGC TTTCGTGTGGCCAGTGACTC	797 pb	94C° for 5min. 94C° for 60 sec. 54C° for 45 sec.(35 cycle) 72C° for 60 sec. 72C° for 7min.	Ma *et al*., (2005)
Uge – F *Uge* - R	TCTTCACGCCTTCCTTCACT GATCATCCGGTCTCCCTGTA	534 pb	94C° for 5 min. 94C° for 60 sec. 54C° for 45 sec.(35 cycle) 72C° for 60 sec. k72C° for 7 min.	Regue *et al*., (2004)

RESULTS

From a total of (200) clinical samples, only twenty isolates (10%) of *Klebsiella pneumoniae* were identified according to the cultural characteristics and biochemical tests as presented in Figure (1).

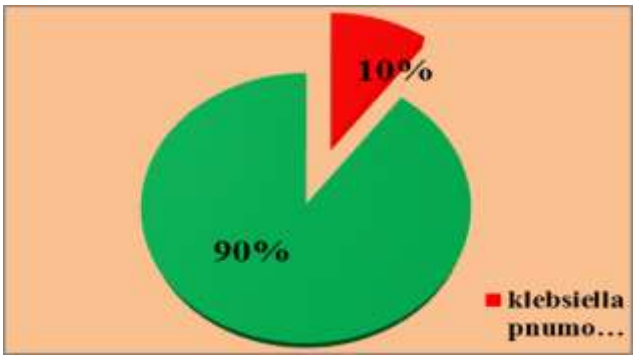

Figure (1): Distribution of *Klebsiella pneumoniae* isolates out of total clinical samples (N=200).

In the present study the *Klebsiella pneumoniae* isolates were distributed regarding different site of collection as the following: 8(40%) isolates from sputum, 5(25%) isolates from urine, 3(15%) isolates from tonsil, 2(10%) isolates from sinus, and also 2(10%) isolates from vagina as showed in Table (2).

Table (2): Distribution of *Klebsiella pneumoniae* isolates according to the site of infection (N=20).

Site of infection	No. of specimens	No. of *K.pneumoniae*	Percentage (%)
Sputum	70	8	40
Tonsil	55	3	15
Sinus	25	2	10
Vagina	20	2	10
Urine	30	5	25
TOTAL	**200**	**20**	**100**

In the present study the gender distribution of the *Klebsiella pneumoniae* isolation was studied and it was found that, *Klebsiella pneumoniae* in males formed 12 isolates(60%) and 8 isolates(40%) in the females as presented in Figure (2).

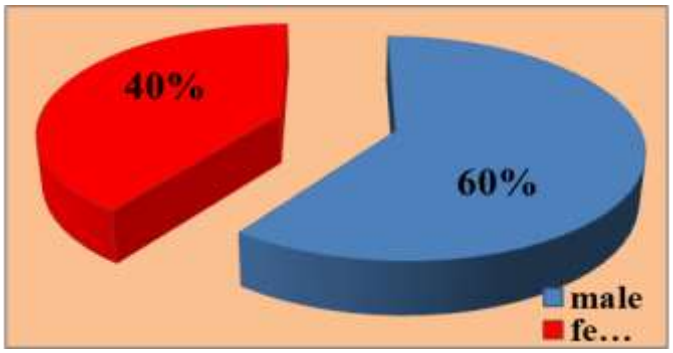

Figure (2): Distribution of *Klebsiella pneumoniae* isolates out of total clinical samples according to gender of patients

In the existent study the relationship between *Klebsiella pneumoniae* isolation rate and age of patients was investigated as presented in Table (3). The age-groups of patients who involved in present study was ranged from one year to thirty years old. Our results showed that the mean age of isolation was 14.9 years with standard deviation of 10.96.

Table (3): Mean and standard deviation of the age of *Klebsiella pneumoniae* patients (N=20).

Variable	Means ±SD	Range
Age (years)	14.9±10.96	(1-30)

Concerning Urease production, The results in the present study showed that all *Klebsiella pneumoniae* isolates (100%) were positive for urease production.

In the present study, capsular phenotype was investigated among *Klebsiella pneumoniae* isolates, the results showed that all *Klebsiella pneumoniae* isolates (100%) were positive for capsule production.

The results of this study about siderophore production revealed that (100%) of Klebsiella pneumoniae isolates were able to produce siderophore (in presence of dipyridil) and none was able to produce hemolysin enzyme as shown in Table (4).

Table (4): The siderophore and hemolysin production by *K.pneumoniae*.

No. of *Klebsiella pneumoniae* isolates	Hemolysin production no.(%)	Siderophore production no.(%)
20	0(0%)	20(100%)

The colonization factors antigens study results through this work revealed that all isolates (100%) of Klebsiella pneumoniae that have been isolated from urine, sputum, vagina, sinus, and tonsil samples have the ability to express the colonization factor antigens (CFA/I, and CFA/III) as shown in Figure (3).

Figure (3): Colonization Factor Antigens of *Klebsiella pneumoniae*

Biofilm formation ability of *Klebsiella pneumoniae* was investigated in this study and the results showed that 8 isolates (40%) had high capacity of biofilm formation due to strong adherence, 7 isolates (35%) had moderate capacity of biofilm formation, while only 5 isolates (25%) were weak producers of biofilm, the results presented in table (5).

Table: (5) Biofilm formation of *Klebsiella pneumoniae.*

Degree of Biofilm formation	No. of isolates	%
Strong	8	40
Moderate	7	35
Weak	5	25

In the present study, the stone formation ability has been investigated among urinary tract infection isolates of *Klebsiella pneumoniae* results showed that all isolates had strong ability for stone formation after incubation in normal sterile urine samples as shown in Figure (4).

Figure (4): The stone forming ability of *K. pneumoniae* isolated from urine samples.

Three selected genes were investigated molecularly in this study to explore their role in the pathogenesis of *Klebsiella pneumoniae* associated diseases. These genes were *mag* A gene (mucoviscosity associated gene A), *Kfu* gene (iron uptake system), and *Uge* gene (smooth LPS and O-antigen and K-antigen) (UDP-galacturonate 4-epimerase gene) by PCR technique. Among total of 20 local isolates of *Klebsiella pneumoniae*, the molecular detection for *mag* A gene by PCR revealed that all of the twenty isolates (100%) were negative for this gene. Regarding *Kfu* gene it was detected in 5 isolates of Klebsiella pneumoniae (25%) and absent in 15 (75%) isolates. While *Uge* gene results showed that only two isolates (10%) were positive for this gene out of the total twenty as presented in Figure (5), Figure (6), and Figure (7).

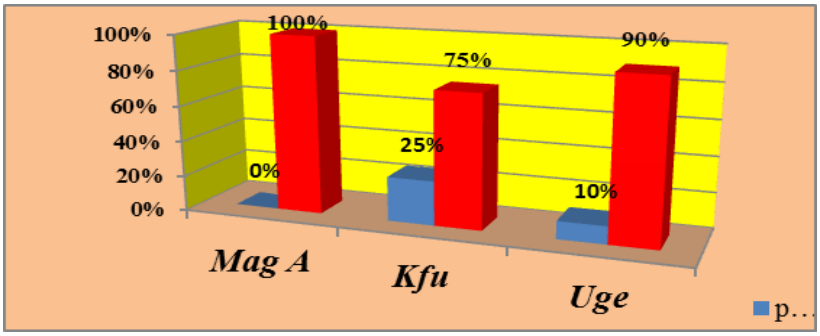

Figure (5): Distribution of the virulence gene (*Mag A*, *Kfu* and *Uge*) among the *Klebsiella pneumoniae* isolates (N=20).

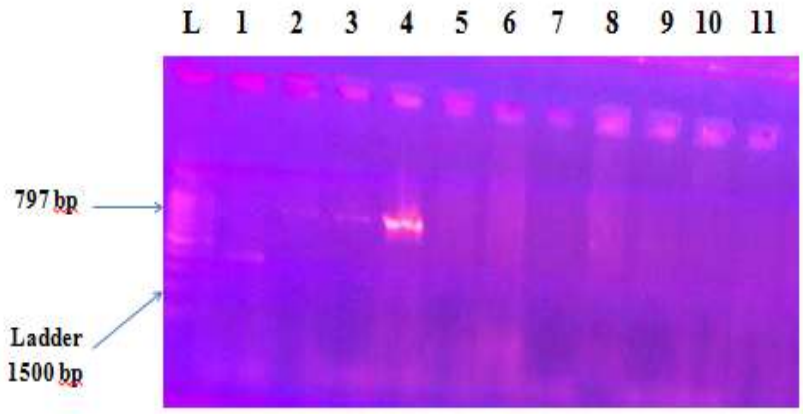

Figure (6): Agarose gel electrophoresis of PCR product of (*Kfu*) gene amplicon product in *Klebsiella pneumoniae*. Lane 1-20 refer to isolate`s number.

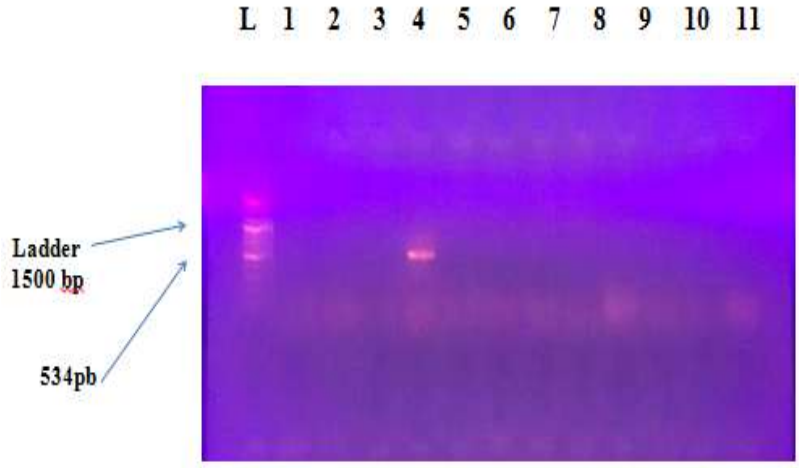

Figure (7) Agarose gel electrophoresis of PCR product of *Uge* gene amplicon product in *Klebsiella pneumoniae* lane 1-12 refer to isolate`s number.

The distribution of virulence genes (*kfu, uge*) according to the site of collection among *Klebsiella pneumoniae* isolates had been studied, results presented in Table (6) and Table (7).

Table (6): Distribution of *Kfu* gene by source of infection (N=20).

Source of collection	*Kfu* gene		Total Number (%)
	Positive Number (%)	Negative Number (%)	
Sputum	3(15%)	5(25%)	8(40%)
Vagina	0(0.0%)	2(10%)	2(10%)
Sinus	1(5%)	1(5%)	2(10%)
Tonsil	1(5%)	2(10%)	3(15%)
Urine	0(0.0%)	5(25%)	5(25%)
TOTAL	5(25%)	15(75%)	20(100.0%)

Table (7): Distribution of *Uge* gene by source of infection (N=20).

Site of collection	*Uge* gene		Total Number (%)
	Positive Number (%)	Negative Number (%)	
Sputum	0(0.0%)	8(40%)	8(40%)
Vagina	0(0.0%)	2(10%)	2(10%)
Sinus	0(0.0%)	2(10%)	2(10%)
Tonsil	0(0.0%)	3(15%)	3(15%)
Urine	2(10.0%)	3(15%)	5(25%)
TOTAL	2(10.0%)	18(90.0%)	20(100.0%)

DISCUSSION

This study results about the percentage of *Klebsiella pneumoniae* isolation rates were confirmed by the findings obtained by[7] and[8] who illustrated that immunocompromised patients were more frequently colonized by Klebsiella spp., and agreed with the findings obtained by[9] who instituted that Klebsiella spp. were responsible for many nosocomial infections in adults.

Results about the demographic data distribution were agreed with many previous studies such as that established by[10],[11], who found that K. *pneumoniae* was the commonest member of Klebsiella spp. that can cause severe infections. Among all patients diagnosed with K. *pneumoniae* pneumonia,[12] established that there was a male predominance thus more strengthening the results obtained in this current work.

A gender distribution difference was observed in this study, results came in agreement with[13] who reported that males are more susceptible to infection with Klebsiella spp. than females. Otherwise, these results did not agreed with other study which found that the percentage of *Klebsiella pneumoniae* in female was higher than male[14]

Results about the age distribution in the present study coming inside with research results of[15] who found a dominance of infection in age group range 16-30 years. Although other study found the percentage of *Klebsiella pneumoniae* is high in ages with 45-60 years.[16] Also, results about urease production agreed with[17], who found that urease production is considered as an important virulence factor in bacterial pathogenicity because urease material elevated the pH due to the ammonia generated by this enzyme has important ramifications for medicine. Urease is a virulence factor in pathogenic bacteria that cause gastric ulceration, urinary stone formation, pyelonephritis, and other human health–related problems.[18]

The results about capsule production in the present study agreed with[17], who found that capsule production is considered as an important virulence factors in bacterial pathogenicity because capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers.

Likewise, this study results about siderophore and heamolysin production are similar to those reported by[19] and[20], who found that Klebsiella spp. have the ability to uptake iron through production siderophore but not hemolysin production.

All *Klebsiella pneumoniae* isolates (100%) produced CFA/III by hemagglutination of red blood cell group A in presence of tannic acid. This result agrees with finding obtained by[21] who finds that Klebsiella mediates mannose-resistant hemagglutination tannin treated erythrocyte.

The results of the present study about biofilm production came in agreement with[22] who found that a high value of biofilm formation is strong in urine sample, one of the most commonly studied properties of biofilms is their increased resistance to the effect of antibiotics.[23] And also this results agreed with[24] who found the biofilm formation should be considered in antimicrobial therapy.

Urinary stones may develop as a result of urinary tract infections. The latter plays an important role in urolithiasis. Whereas all non-infectious urinary stones originate because of metabolic disturbances or through changes so far unknown (for example in kidney tissue), coming inside with this study finding about the ability of *Klebsiella pneumoniae* for stone formation other study found that the commonest organisms isolated from urinary stone were Klebsiella in 12%.[25]

The results obtained in the current study about mag A gene among *Klebsiella pneumoniae* means that there are other means play a role in formation the mucoid viscosity. Although mag A gene is highly important for *Klebsiella pneumoniae* which confirm the bacteria mucoid viscosity, but it is prevalence and among *Klebsiella pneumoniae* local isolates is not high.[26] Many studies have indicated that mag A is not prevalent among K. *pneumoniae* isolates.[27],[18]

The *Kfu* gene which codes for an iron uptake system is a putative pathogenic gene, significantly associated with the virulent hypermucoviscosity phenotype and purulent tissue

infections.[28] Results about the prevalence of *Kfu* gene among *Klebsiella pneumoniae* in the existing study agreed with[29] who found the percentage of *kfu* gene is 25%. And these results disagreement with[30] the percentage is slightly higher than our study 66%.

Among the total of 20 isolates of K. *pneumoniae*, the molecular technique for *Uge* gene as done by PCR and detected in only two isolates (10%). Although this results disagree with[31] who found the percentage of uge gene higher than our study.

CONCLUSION AND RECOMMENDATION

The conclusions that can be extrapolated by this study are that, among this study the local isolates of *Klebsiella pneumoniae* are a common among males than females especially in age group 14.9. Stone formation capacity of Klebsiella pneumoniae isolates may acts as contributing factor in their virulence. The *mag* A gene is absent among Klebsiella pneumoniae, other alternative virulence factor may play more important role in our local isolates. While the prevalence of *Kfu* gene and *uge* gene is relatively low among local Klebsiella pneumoniae isolates.

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