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ORIGINAL ARTICLE

Prevalence assessment of *mag*A gene and antimicrobial susceptibility of *Klebsiella pneumoniae isolated* from clinical specimens in Shahrekord, Iran

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* (*K. pneumoniae*) is an opportunistic microorganism. This study aimed to investigate the presence of *mag*A gene and antimicrobial susceptibility in *K. pneumoniae*.
Materials and Methods: 195 clinical specimens were collected from hospitals of Shahrekord, Iran. Bacterial culture, biochemical diagnostic standard test, determination of antibiotic sensitivity, phenotypic testing hypermucoviscosity (HV) and polymerase chain reaction (PCR) was performed for isolation and characterization of *K. pneumoniae*.
Results: 173 samples were positive for *K. pneumoniae*. The highest and lowest rates of resistance were related to amoxicillin 79.19% and ciprofloxacin 15.60%, respectively. Also 4 samples were positive for *mag*A gene.
Conclusion: Based on our results, *K. pneumoniae* strains were resistant to different antibiotics. Knowing how to identify strains of *K. pneumoniae*, spreading of its virulence and also antimicrobial resistance genes can be useful in treatment of infection caused by this bacterium.

Keywords: prevalence, *mag*A gene, antimicrobial susceptibility, *Klebsiella pneumoniae*

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium that belongs to the Enterobacteriaceae family. This bacterium is an opportunistic microorganism which causes serious diseases such as septicemia, pneumonia, urinary tract infections (UTI), diabetes mellitus, chronic lung disorders and nosocomial infections in immunocompromised patients. Different studies showed the regular use of antibiotics and lack of personal and public hygiene lead to the colonization of this bacterium in patients and its spread in different parts of the hospital. According to the Center for Disease Control (CDC), this bacterium is the main reason for more than 8% of endemic and 3% of epidemic infections in hospitals (1, 2).

Klebsiella bacteriemia and pneumonia arise from community-acquired diseases and clinical infections initiated from surgical wounds. The mortality rate and pneumonia caused by *Klebsiella* has been reported as 20-50% and 50% respectively. *Klebsiella* infection has a significant role in causing septicemia and bacteremia in children and intensive care unit (ICU) admitted patients. This bacterium possesses a number of virulence factors such as adhesion, sid-

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erophore, O antigen and capsule that are involved in its pathogenesis (3). There are 77 types of capsular antigens that K1 and K2 stereotypes are the most important ones. Among these 2 capsular serotypes, K2 is the most common type isolated from patients with pneumonia, bacteriemia and UTI. Regarding to the previous genetic studies, the genomic map of *K. pneumoniae* capsule contains gene clusters as follows: 1. *cps* (capsular polysaccharide synthesis), 2. *rmp*A, *rmp*A1 and *rmp*A2 (regulator of the mucoid phenotype A, A1 and A2, respectively), 3. *wb* (O-specific polysaccharide is directed by the *wb* gene cluster), 4. *mag*A (mucoviscosity associated gene A) (4, 5).

The function of these genes is completely different, as the *cps* gene implements the synthesis of capsular polysaccharide. *rmp*A and *rmp*A2 are responsible for regulating the synthesis of the extracellular polysaccharide capsule. Lipopolysaccharide is synthesized by *wb* gene. These four mentioned genes are conserved in most isolates of *K. pneumoniae*. The *mag*A is a chromosomal gene which plays an important role in serious infection of *Klebsiella* such as septicemia, bacteremia, and pneumonia as well as lung and liver abscesses (6, 7).

*mag*A is 35-Kbp and it has locus with 24 templates translation of mRNA which is homologous with genes involved in biosynthesis, transfer and glycosylation of lipopolysaccaride (8). The chromosomal *mag*A gene has hyperviscous phenotype. It is characterized by forming a muscoviscous string of 5 mm diameter during passing loop through a colony. It also causes increased levels of resistance to phagocytosis. Among 77 characterized capsular serotypes (K), the most isolates separated from hepatic abscesses belong to capsular serotypes K1 and K2. These observations suggest that the genetic locus containing *mag*A is a new pathogencity island responsible for increasing the virulence of *K. pneumoniae* strains (9). Different studies are conducted on different aspect of *K. pneumonia* infection:

Yeh *et al.* (10) showed that in 73 *K. pneumoniae* isolates from liver abscess were collected in Singapore and Taiwan, *magA* was restricted to serotype K1. Lin *et al.* (11) in Taiwan showed that hypermucoviscosity (HV) phenotype and *rmp*A gene was more often found in UTI *K. pneumoniae* isolates, than in those from healthy adults (11). Feizabadi *et al.* (12) in Iran reported that of 89 *K. pneumoniae* samples were isolated from the patients, 10 (11.2%) belonged to K1 and 13 (14.6%) belonged to K2 serotypes, respectively. The aim of this study was to determine the antimicrobial susceptibility and *mag*A gene molecular diagnosis of isolated *K. pneumoniae* from clinical specimens of patients in teaching hospitals of Shahrekord, Iran.

MATERIALS AND METHODS

Samples. One hundred ninety five suspected clinical specimens from patients (98 men and 97 women, ranging 1–80 years) including urine (n=98), blood (n=19)**,** cerebrospinal fluid (CSF) (7 samples)**,** wound (n=25), sputum (n=18), peritoneal fluid (13 samples), ocular fluids (n=5) and catheter (n=10) were collected from hospitals affiliated to Shahrekord University of Medical Sciences during April 2012.

Bacterial culture and identification. All clinical specimens were streaked on the surface of both blood (containing 5% sheep blood) and MacConkey agar (Merck, Germany). The plates were incubated aerobically at 37°C for 24 hours. Those were culture negative after 24 hrs incubation were further incubated for 48hrs. Gram stain and biochemical tests such as indole, methyl red, voges proskauer, citrate (IMViC), oxidase, H_2S production, lysine decarboxylase, lactose fermentation, urea hydrolysis, gas production, catalase and coagulase were used for *K. pneumoniae* detection (13, 14).

Antibiotic susceptibity testing. Kirby-Bauer disc diffusion method was used to determine the susceptibility of isolated organism to amoxicillin (20 µg), tetracycline (30 µg), gentamicin (10 µg), co-trimoxazole (25 µg), imipenem (10 µg), nitrofurantoin (30µg), ciprofloxacin (5 µg), cefazolin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg) and amikacin (30 µg) (Mast, UK) as instructed by Clinical and Laboratory Standards Institute (CLSI) guidelines. *K. pneumoniae* ATCC 1290 was used as control strains (15).

Hypermucoviscosity Test (HV). Each of *K. pneumoniae* isolate was separated and cultivated on blood agar (containing 5% sheep blood) medium (Merck, Germany) and then incubated at 37°C for 24 hrs. After this step, they were investigated for HV using standard bacteriological loops through the bacteri-

Table1- Characteristics of primers used in PCR

Reference	Production size bp	Primer Sequence	Gene
16	1, 282	Forward, 5'- GGTGCTCTTTACATCATTGC- 3' Reverse, 5'- GCAATGGCCATTTGCGTTAG- 3'	magA
17	213	Forward, 5'- ATCTGGTGGACTACTCGC- 3' Reverse, 5'- GCCTCATTCAGTTCCGTT- 3'	bla_{SHV-1a}

al colony. Those colonies which were drawn 5 mm were considered as positive (15).

Polymerase chain reaction. DNA was extracted using kit (Bioneer, Korea) and subjected to PCR using 0.5 mM primers *mag*A gene (16), 0.1 mM of primer bla_{SHV-1a} gene (17), 2.5 µl of buffer 10X, 3 µl $MgCl_2$, 3 ml dNTPs, 2 µl of DNA template and 0.2 enzyme units Taq DNA polymerase in 25 µl reaction mixture with deionized water (DW). The thermocycler program was adjusted as: initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 1 min, 50 °C for 1 min and elongation at 72 °C for 2 min with a final extension at 72 °C for 7 min. The amplicons (25µl) were mixed with 1µl loading buffer and electrophoresed at 280V and 53mA on polyacrylamide gels (6%). After electrophoresis the gel was stained with silver nitrate (0.1%) and DNA bands were photographed. The banding patterns were compared with positive and negative controls. To determine the size of the other PCR products, a molecular marker (Gene Faravaran Company, Iran) was used. In order to enhance the efficiency and reliability of PCR cycles, the samples that were negative in terms of *mag*A gene and have no bands in final electrophoresis, The bla_{SHV-1a} primers were used as internal control PCR as well as gene-specific primers *mag*A (Table 1).

Sequencing of PCR products. One of the PCR products in the desired location (i.e. on the gel was in a position of length 1, 282 bp) was sequenced (ABI Capilary System 3730XL). The sequences were analyzed using the software chromas 233. It was consistent with *mag*A gene sequence. In this study, this sample was used as a positive control in the PCR.

Data analysis. In order to analyze the information, descriptive and inferential methods were utilized. Student's t-test was used in our study to analyze data. All analysis was done by SPSS version16.0 (SPSS, Inc., Chicago, IL, USA) ($p<0.05$).

RESULTS

Bacterial cultures and identification. *K. pneumoniae* were observed as large, blue/gray, mucoid, convex and circular colonies. Strains that were Gram negative, indole-negative, methyl red-negative, voges proskauer-positive, citrate-positive, oxidase-negative, H_2S production-negative, lysine decarboxylase-positive, lactose fermentation-positive, urea hydrolysis- positive, gas production-positive, catalase-positive and coagulase-negative were identified as *K. pneumoniae*. So, out of 195 clinical specimen, 173 (88.71%) were positive for *K. pneumoniae* and these were isolated from; urine (n=93, 94.89%), blood (n=17, 89.47%), CSF (n=5, 71.42%), wound (n=24, 96%), sputum (n=17, 94.44%), peritoneal fluid (n=6, 46.15%), ocular fluids (n=3, 60%) and 8 catheter (n=8, 80%).

Antibiotic susceptibility test results. The rates of resistance to different antibiotics were as 79.19% for amoxicillin, 43.35% for tetracycline, 32.94% for gentamicin, 54.33% for co-trimoxazole, 20.80% for imipenem, 71.09% for nitrofurantoin, 15.60% for ciprofloxacin, 56.64% for cefazolin, 41.61% for ceftriaxone, 49.71% for ceftazidime and 21.96% for amikacin (Table 2).

Hypermucoviscosity (HV) test. Of 173 samples, 73 (42.19%) were positive for HV test and 100 (57.80%) were negative.

PCR assay: The presence of *mag*A gene was investigated among 173 of *K. pneumoniae* isolates. Of 173 isolates of *K. pneumoniae*, 4 (2.31%) were positive and 169 (97.68%) were negative for *mag*A gene (Fig.1).

Data analysis: No significant correlation was found between the presence of *mag*A gene and variables such as age, sex, hospital or community acquired infections, clinical specimen type, the presence of an underlying disease and presence of HV phenotypes (0.05< p).

DISCUSSION

Different diseases such as nosocomial and community acquired infections are caused by *K. pneumoniae*. *K. pneumoniae* infection is often treated with β–lactam antibiotics; also beta-lactam antibiotics are one of the most used resistant antibiotics that created a major crisis in medical clinic in the last two decades (18, 19). Results of antibiotic sensitivity test in our study showed that *K. pneumoniae* strains were resistant to different classes of antibiotics. Beyene and Tsegaye (13) in Ethiopia studied 21 patients with UTI and observed that 50% of them infected with *K. pneumoniae* and the entire isolated organism was found as being resistant to ampicillin and amoxicillin. Also, resistance to nitrofurantion was detected in 2 isolates and the least resistance was observed for ceftriaxone, gentamicin and chloramphenicol**.**

In our study, amoxicillin and nitrofurantoin resistance were 71.19% and 71.09%, respectively. Also 65.89% of *K. pneumoniae* isolates were sensitive to gentamicin. Rate of resistance to these antibiotics was high in both our's and Beyene and Tsegaye's study. Extended spectrum beta-lactamases (ESBLs)

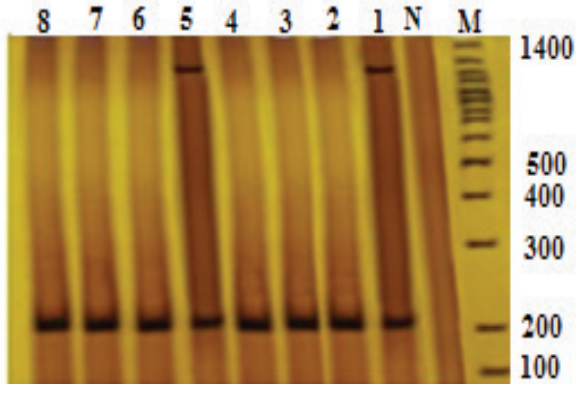

Fig. 1. Result of the PCR assay for identification of *K. pneumoniae mag*A and $bla_{SHV\text{-}1a}$ genes
M, marker 100 bp
N, negative control
Number 1, positive control strains of *K. pneumoniae*
Numbers 1 to 8, isolated *K. pneumoniae* with $bla_{SHV\text{-}1a}$ gene
Numbers 2 to 8, isolated *K. pneumoniae* from patients
Numbers 2, 3, 4, 6, 7 and 8, isolated *K. pneumoniae* without *mag*A gene
Number 5, isolated *K. pneumoniae* with *mag*A gene

hydrolyse β-lactam ring, so it may inactivate cephalosporin and penicillin antibiotics (13). Also some genes are mobile among isolates and they spread in the environment. It is possible that a different mechanism of gene transfer such as horizontal gene transfer between serotypes may cause the spread of resistance genes (20, 21). Kumar *et al.* (22) in the USA showed that *K. pneumoniae* was multidrug-resistant (MDR) to fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxazoles which are similar

Table 2. Antimicrobial susceptibility pattern of *K. pneumoniae* isolates in hospitals of Shahrekord by Kirby-Bauer disc diffusion method. n (%)

Antimicrobial	Resistant	Susceptibility Intermediate	Susceptible
Imipenem	36 (20.80%)	4 (2.31%)	133 (76.87%)
Tetracycline	75 (43.35%)	53 (30.63%)	45 (26.01%)
Cefazolin	98 (56.64%)	8 (4.62%)	67 (38.72%)
Ceftazidime	86 (49.71%)	3 (1.73%)	84 (48.55%)
Amoxicillin	137 (79.19%)	0	36 (20.80%)
Ciprofloxacin	27 (15.60%)	11 (6.35%)	135 (78.03%)
Nitrofurantoin	123 (71.09%)	14 (8.09%)	36 (20.80%)
Co-trimoxazole	94 (54.33%)	3 (1.73%)	76 (43.93%)
Ceftriaxone	72 (41.61%)	4 (2.31%)	97 (56.06%)
Amikacin	38 (21.96%)	4 (2.31%)	131 (75.72%)
Gentamicin	57 (32.94%)	2 (1.15%)	114 (65.89%)

to the result of our study. Different reasons such as large component of the genetic and phenotypic diversity of clinical isolates, additional efflux pumps and multiple mechanisms of fluoroquinolone resistance cause antibiotic resistance in bacteria (22). Antibiotic resistance rates in *K. pneumoniae* were also reported as 19.6% % and 46.6% % by Irajian *et al.* (23) and Mohammadi-mehr and Feizabadi (24) in Iran, respectively. This indicates an increase in resistance to antibiotics by this bacterium.

In comparison with other studies, despite despite increasing resistance over the years, there is still high sensitivity to certain antibiotics. More studies are necessary in different geographical regions to investigate the sensitivity of organisms to antibiotics. Using PCR assay, Yu *et al.* (25) in Taiwan showed that prevalence of HV, *rmp*A, and *mag*A were 38%, 48% and 17%, respectively. Our PCR results proved that out of 173 isolates of *K. pneumoniae*, 2.31 % and 42.19% of samples were positive for *mag*A and HV respectively. Previous had shown that strains carrying *rmp*A were related with the HV phenotype, and had a significant correlation with liver abscess and lung, neck, psoas muscle, or other focal abscess (25). However, in our study study no correlation was observed between the presence of *mag*A and variables such as age, sex, hospital or community acquired infections, clinical specimen type and presence of HV phenotypes.

Using PCR, Zamani *et al.* (15) showed that out of 105 isolated *Klebsiellae* from patients, 96.2% were *K. pneumoniae* and 3.8% were *K. oxytoca* in Iran. They detected *mag*A gene in 4 (3.8%) isolates of *K. pneumoniae*, two of them were positive and two were negative for HV phenotype. Struve *et al.* (9) in Denmark showed that *mag*A is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1. In different studies it was proved that *mag*A gene can specially belong to *K. pneumoniae* (15). Fang *et al.* (18) in Taiwan reported the prevalence of *mag*A gene in invasive and non-invasive *K. pneumoniae* isolates as 98% and 29%, respectively. The lack of concordance among this gene frequency with other studies might be partly due to this reason that *mag*A gene rarely can be seen in other infections caused by *K. pneumoniae* except liver abscesses. In addition, low frequency of *mag*A among our isolates might be due to its low index of iron-uptake system (kfu) which is a proprietary system to absorb acquire iron on the chromosome of this bacterium, because it can be seen mostly on strains of positive *mag*A which induced hepatic abscesses (26).

Presence of *mag*A gene in *K. pneumoniae* in clinical samples is important. In recent decades liver and the meninges curtains infections, bacteremia and septicemia were mainly related with *mag*A gene in *K. pneumoniae*. Therefore *mag*A gene is used as a marker for the diagnosis of invasive *K. pneumoniae* infections. These results mentioned that *mag*A gene can be seen in *K. pneumoniae* capsules with high viscosity. This gene can act as a pathogenicity island and increase the virulence of the bacteria. So presence of this gene in samples without any antibiotic treatment may cause patient's death (9). We used just clinical samples in our study while hepatic abscesses samples were used in some studies and it may a reason for disagreement. Due to the presence of MDR and *mag*A in our samples, further research to combat with MDR and *K. pneumoniae* infection is necessary.

In conclusion, infection with antibiotic resistant *K. pneumoniae* is now a global concern. Based on these results, *mag*A producing *K. pneumoniae* strains were isolated from patients of hospitals, Shahrekord, Iran. So prescribing appropriate antibiotics and detection of *mag*A gene is required and it can be useful in tracking, treating and knowledge of *K. pneumoniae* infection prevalence rate.

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