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RESEARCH ARTICLE

Molecular Detection and PCR-RFLP Analysis of Mucoviscosity-Associated Gene A (*magA*) in Clinical Isolates of Multidrug-Resistant *Klebsiella pneumoniae* in Bangladesh

Md. Hazrat Ali[1,*], Saeed Anwar[1], Nusrat Jahan Toma[1,¶], Ikram Rafid[1,¶], Md. Kamrul Hasan[2] and Md. Javed Foysal[1,3]

[1]*Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh*
[2]*Department of Biochemistry and Molecular Biology, Tejgaon College, National University, Gazipur 1704, Bangladesh*
[3]*School of Molecular and Life Sciences, Curtin University, Kent Street, Bentley, Perth, WA6102, Australia*

Abstract:

Background and Objective:

The mucoviscosity associated gene A (*magA*) in the hypermucoviscous variants of *K. pneumoniae* is reported to be associated with invasive infections and considered a virulence factor. We sought to analyze the *magA* genes in *K. pneumoniae* isolates in the clinical specimen collected from Bangladesh.

Methods:

We established a multicenter cohort of patients with *Klebsiella* infection hospitalized at 05 different hospitals between September 2016 and April 2017. We collected 313 *K. pneumoniae* isolates from patients who consented to participate in the study. The isolates were evaluated for harboring the *magA* genes using a single-tube multiplexed polymerase chain reaction. The *magA* genes were analyzed by PCR-RFLP technique using two enzymes, namely PciI and SmaI. Antibiogram assay using 12 commercially available antibiotic discs was performed on all the isolates.

Results:

The presence of *K. pneumoniae* specific gene (*ureD*) was confirmed in all the isolates. The percentage of isolates harboring the *magA* gene was 7.34%(23 isolates), the majority of which was collected from the patients admitted in intensive care units (16 isolates, 69.6%), and infectious diseases wards (5 isolates, 21.7%). PCR-RFLP analysis revealed that for 7 out of 23 isolates, where Sma1 could not cleave the *magA* gene. All the isolates showed resistance to ampicillin, carbenicillin cefradine, chloramphenicol, erythromycin, kanamycin, and sulphamethoxazole, though the extent was varying. However, imipenem showed 100% sensitivity to all the tested isolates.

Conclusion:

This study demonstrates the presence of the *magA* gene in multidrug-resistant clinical isolates of *K. pneumoniae* collected from Bangladesh.

Keywords: *Klebsiella pneumoniae*, Polysaccharide capsule, *ureD*, *magA*, Multidrug-resistance, PCR-RFLP, Imipenem, Antibiogram.

1. INTRODUCTION

Klebsiella pneumoniae is an encapsulated gram-negative bacterium of the Enterobacteriaceae family [1]. As a component of the human gut microbiota, this bacterium is usually harmless to the body. It turns into a threat only if

transmitted into other parts of the body. Nevertheless, infections are infrequent as healthy immune systems of men can resist the bacterium effectively. However, in immunocompromised patients, it causes severe diseases, *e.g.*, septicemia, pneumonia, urinary tract infections (UTI), liver abscess, chronic lung disorders, and nosocomial infections [1, 2].

K. pneumoniae are intrinsically resistant to ampicillin. Besides, they ubiquitously possess an inducible chromosomally encoded ß-lactamase (cephalosporinase), making them resistant

¶ These authors contributed equally to this work.
* Address correspondence to this author at Department of Genetic Engineering and Biotechnology, 211 - Academic Building E, Shahjalal University Avenue, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh; Tel: +88 01716 299 245; E-mail:hazratsust05@gmail.com

to first and second-generation cephalosporins [3, 4]. In addition, clinical reports indicate that they are increasingly becoming resistant toward tigecycline, colistins, and polymyxin antibiotics, turning out to be a pan drug-resistant bacteria, or in other words, a 'superbug' [5 - 8]. Consequently, once the modest bacterium, has now turned into a serious threat to public health [9, 10].

Among the Gram-negative bacteria, *K. pneumoniae* are the second-most frequent hospital-acquired and nosocomial pathogens [11, 12]. According to a recent report, these bacteria are the primary reason for 3 – 15% of all hospital-acquired infections, and the carrier rate can be as high as 77% among hospitalized patients [13, 14]. The constant unregulated use of antibiotics and improper hygiene managements are among the foremost reasons behind this spread of multi-drug resistant strains [13]. Proper diagnosis and transmission management have been the key to control the spread of this pan drug-resistant bacterium [15 - 18]. In clinical settings, *K. pneumoniae* infections are usually diagnosed by examining samples of the infected tissue, *e.g.*, sputum, urine, or blood by culture-based methods [15, 19]. Depending on the site of infection, ultrasounds, X-rays, and computerized tomography (CT) can also be useful [20]. However, these diagnosis techniques have several limitations, including turnaround time, narrow application, low specificity, and sensitivity [15, 19]. Cutting-edge technologies, *e.g.*, Sanger and next-generation sequencing, have gained a foothold in health care for diagnosis of infection at the individual level, outbreak detection, transmission mapping, and source tracing [21, 22]. However, high-throughput screening through targeted sequencing is expensive, labor extensive, and requires expertise. Simple molecular techniques, *e.g.*, PCR, PCR-RFLP assays are, in contrast, rapid and easy to perform, but have considerable sensitivity, specificity, and wide-range of application in infection detection [15, 23].

Several virulence factors, *e.g.*, adhesion, siderophore, O antigen, and capsule attribute to its pathogenesis [24]. Among the 77 forms of capsular antigens, K1 and K2 are mostly studied. Between these two, K2 is the most common type isolated from patients with pneumonia, bacteremia, and UTI. Previous genetic studies identified that the genomic map of *K. pneumoniae* capsule contains a gene cluster subsuming the *cps* (capsular polysaccharide synthesis), *rmpA*, *rmpA1* and *rmpA2* (regulator of the mucoid phenotype A, A1, and A2, respectively), *wb* (the wb gene cluster codes for O-specific polysaccharide), and *magA* (mucoviscosity associated gene A) [25, 26]. Functional aspects of these genes include the synthesis of capsular polysaccharide, the regulation of the extracellular polysaccharide capsule's synthesis, and the production of lipopolysaccharide, respectively. In most of the

isolates of *K. pneumoniae*, these four genes remain conserved. Among the genes mentioned above, *magA* is a novel virulent gene that causes acute infection of *Klebsiella, e.g.*, septicemia, bacteremia, pneumonia, and lung and liver abscesses [27, 28]. It spans about 1 kb region and involves in biosynthesis, transfer, and glycosylation of lipopolysaccharide [27]. The presence of chromosomal *magA* gene confers a hyper-viscous phenotype, which can be characterized by a positive string test (formation of a mucoviscous string of 5 mm diameter during passing loop through a colony) and resistance to phagocytosis. Among 77 characterized capsular serotypes (K), a major portion belongs to K1 and K2 serotypes. Based on these observations, the genetic locus containing *magA* can be targeted as a new pathogenicity island responsible for increasing the virulence of *K. pneumoniae* strains [29].

In the present study, we sought to investigate the presence of *magA* gene in isolates of *K. pneumoniae* obtained from patients from different wards of Bangladeshi hospitals.

2. MATERIALS AND METHODS

2.1. Samples

From September 2016 to April 2017, we collected 313 clinical specimens from patients, including urine, blood, trachea, and wounds. Authorized personnel collected the specimen at the bedside from the patients (one isolate per patient) admitted in 05 hospitals of 05 different regions, namely Sylhet, Dhaka, Mymensingh, Rajshahi, and Khulna districts of Bangladesh. All isolates were subjected to standard confirmatory tests, which included microscopic evaluation, Gram staining, oxidase test, catalase test, and growth on SIM (sulfide, indole, motility), lysine iron agar, phenylalanine agar, urea agar, malonate, blood agar, and MacConkey agar. Bacterial isolates were stored in tryptic soy broth (TSB) supplemented with 20% glycerol at -20°C.

2.2. Design of Primers

Primer3Plus tool was utilized to design the specific primers for PCR-based identification of *magA* and *ureD* genes in *K. pneumoniae* isolates [30]. The *ureD* (urease D, an accessory gene in the urease gene cluster) was coamplified as an internal control, as it is a specific gene for *K. pneumoniae* [31]. Since we took advantage of a single tube multiplexed PCR for this study, careful optimization of T_a of the primers was critically important. We used Primer3Plus, OligoAnalyzer 3.1, and Multiple Primer Analyzer tools to optimize the annealing temperature (*Ta*) of the primers (Table **1**) [30, 32 - 34]. The designed primers were commercially purchased from Integrated DNA Technologies.

Table 1. List of primers used for the detection of *ureD* and *magA* genes in *K. pneumoniae* isolates.

Target	Primer	Tm °(C)	Ta °(C)	Product size (bp)
ureD	Forward: ATCGTCACTGAGGAAACGC	54.4	49	590
	Reverse: ACCCGGAAGAAGAGACCT	54.3		
magA	Forward: TGGCTTAGACCGATGGTTG	53.9		1057
	Reverse: ACATTGCCGCTACTACAGG	53.6		

2.3. Genomic DNA Extraction

We extracted genomic DNA from the obtained isolates using a commercially available bacterial DNA isolation kit pursuing the manufacturer's instructions (GenElute™ Bacterial Genomic DNA Kit, Sigma-Aldrich, Cat.: NA2120). The DNA quality was examined by electrophoresis on 0.7% agarose gel (Ultra-pure Agarose, Invitrogen, Cat. No.: 6500-25).

2.4. Single Tube Multiplex PCR Amplification of *ureD* and *magA* Genes

The single-tube multiplexed PCR was carried out with 3.0 µL of template DNA, 1 µL of each primer (10 µM), 12.5 µL of master mix (Promega Hot Start, Cat No.: M7122) and 5.5 µL of nuclease-free Water (Promega, USA) in a total volume of 25 µL. The *magA* and *ureD* genes were amplified simultaneously using the ProFlex™ 2 x 96-well PCR System (Applied Biosystems, Cat No.: 4484076). The PCRs were initially heated to 96°C for 7 minutes, followed by 35 cycles of denaturation (96°C, 1 minute), annealing (49°C, 1 minute), and extension (72°C, 1 minute). A final extension was carried at 72°C for 7 minutes. PCR products were evaluated by electrophoresis on 1.2% agarose gels ((Ultra-pure Agarose, Invitrogen, Cat. No.: 6500-25). 1-kb DNA ladder (Promega, Cat. No.: G5711) was used to compare DNA band sizes.

Using commercially available DNA purification kits, we purified 15 randomly selected PCR products of both genes from the TBE buffered agarose gels (GeneJET Gel Extraction Kit, Cat.: K0692). Purified PCR products were sent to Macrogen Corp. (Geumcheon-gu, Seoul, Rep. of Korea) for Sanger sequencing. The sequence outcomes were aligned and compared with the reference sequences of the genes using the BLAST program at the web interface of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [35]. The confirmed samples were used as the control for the rest of the analysis.

2.5. Restriction Digestion

10 µL of the PCR products, which produced positive outcomes for both the genes, were transferred to a separate 0.5 µL-Eppendorf vial and added with 17 µL of nuclease-free water. Then 1.5 µL of each of PciI (Thermo Fisher Scientific, Cat: ER1871) and SmaI (Thermo Fisher Scientific, Cat: ER0661) restriction enzymes were added and mixed carefully

to the solution and incubated at 32°C for an overnight period (Table **2**). The incubation temperature was set at the mean temperature of the optimum incubation temperatures of the enzymes mentioned above. Using UV illumination, the restriction fragments were compared against a 1-kb plus DNA ladder (Thermo Scientific, Cat. No.: FERSM1331) on 1.5% agarose gels. This step was repeated multiple times to confirm that the restriction digestion was not incidental.

2.6. Antibiogram Assay of the Isolates

A disc diffusion method was adopted to investigate antibiotic profiling of *K. pneuomoniae* isolates using 11 commercially available discs [36, 37]. High-quality absorbent paper discs impregnated with the following antibiotics were used: ampicillin (10 µg/disc), carbenicillin (10 µg/disc), cefradine (25 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (30 µg/disc), erythromycin (15 µg/disc), gentamycin (10 µg/disc), imipenem (10 µg/disc), kanamycin (30 µg/disc), levofloxacin (5 µg/disc), streptomycin (10 µg/disc) and sulphamethoxazole (25 µg/disc). A fresh bacterial culture inoculated on Tryptocasein Soy Agar plates (Sigma-Aldrich, Cat.: 22091) by spreading. An antibiotic disc and a blank disc (as negative control) were placed carefully onto each culture plates and incubated overnight at 37°. After the incubation period, the zone of inhibition due to the activity of antibiotic-containing discs was measured following the protocol described by Sharmeen et al. [38].

2.7. Statistical Analysis

All data were analyzed using IBM-SPSS version 17.0. We evaluated statistical significance using both one-tailed and two-tailed experiments by Student t-test and χ^2 test at 99% ($p \leq 0.01$) and 95% ($p \leq 0.05$) confidence intervals.

3. RESULTS

3.1. Patient Characteristics and the Isolates Obtained

In the current study, 313 isolates of *K. pneuomoniae* were obtained from the intensive care units (ICU), internal medicine and infectious diseases wards of the five hospitals (Table **3**). Out of these 313 samples, 192 isolates (61.3%) were collected from females and the rest (n = 121, 38.7%) were from males. The age of the patients ranged from 13 to 84 years, with a mean of 49.50 ± 18.24 years.

Table 2. Restriction enzymes used for restriction digestion.

Restriction enzyme	Target	Cutting site	Cutting positions	Fragment sizes (bp)
SmaI	PCR amplified *ureD* gene of *K. pneumoniae*	CCC↓GGG	453/454	453, 372
PciI	PCR amplified *magA* gene of *K. pneumoniae*	A↓CATGT	958/962	958, 269

Table 3. General characteristics of the patients and the specimen collected.

Features	Values
Total no. of isolates (n)	313
Age (years) of the patients (mean ± SD, range)	49.50 ± 18.24, 13 - 84
Female: male	1.0: 0.63 (192: 121)

(Table 3) cont.....

Features	Values
Regions from where specimen were collected (n, %)	-
Sylhet	94, 30.03
Dhaka	69, 22.04
Mymensingh	57, 18.21
Rajshahi	52, 16.61
Khulna	41, 13.1
Hospital units from where specimen was collected (n, %)	-
Intensive care unit (ICU)	144, 46
Internal medicine unit	94, 30
Infectious diseases wards	75, 24
Type of specimen from where isolates were collected (n, %)	-
Urine	159, 50.7
Trachea aspirates	64, 20.4
Blood	49, 15.6
Wound	41, 13.1

SD = standard deviation.

3.2. Multiplex PCR Outcomes

All the isolates carried the *ureD* gene (served as an internal control), and 23 isolates harbored the *magA* gene (Fig. **1**). Out of these 23 isolates, 07 were collected from Dhaka (10.14%, n = 69). Among the rest 16 isolates, 05 were from Mymensingh (8.77%, n = 57), 08 were from Sylhet (8.51%, n = 94) and 03 were from Rajshahi (5.77%, n = 52). None of the isolates collected from the Khulna region harbored the *magA* gene.

Hence, the overall prevalence of the *magA* gene in our studied subjects was 7.34% (n = 313). The isolates harboring *magA* were mostly isolated from the patients hospitalized in the intensive care unit (ICU) (Table **4**). Twenty-three out of the 313 isolates harbored the *magA* gene, and most of these isolates were collected from the patients admitted in intensive care units (16 isolates, 69.6%) and infectious diseases wards (05 isolates, 21.7%).
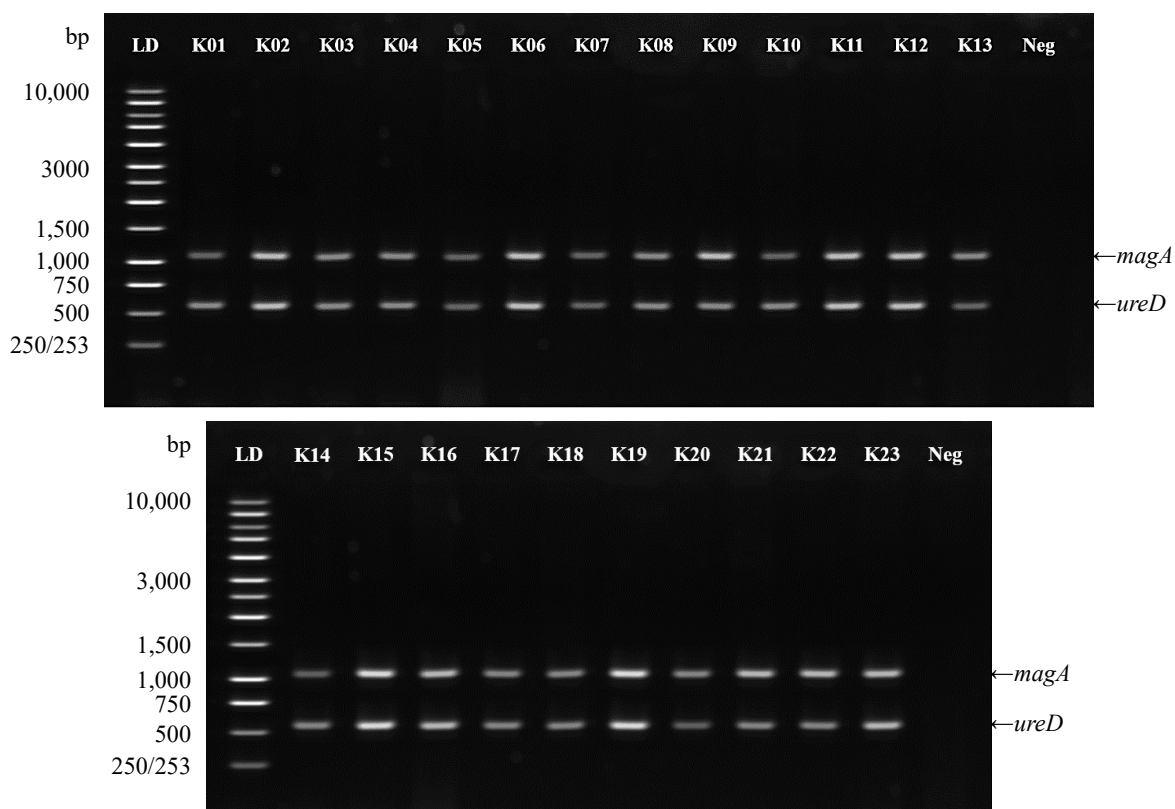

Fig. (1). Identification of *ureD* and *magA* genes by single-tube multiplex PCR amplification. K1 - K13 and K14 – K23 denotes different isolates that contained *magA* gene. Neg denotes negative control (water), which served as a loading control.

Table 4. The frequency of *magA* genes in *K. pneumoniae* isolates based on the clinical samples and hospital wards.

Features	No (%)
Total	23 (7.34)
Hospital units from where specimen was collected (n, %)	-
Intensive care unit (ICU)	16 (69.6)
Infectious diseases wards	5 (21.7)
Internal medicine unit	2 (8.7)
Type of specimen from where isolates were collected (n, %)	-
Urine	8 (34.8)
Trachea aspirates	6 (26.1)
Blood	5 (21.7)
Wound	4 (17.4)
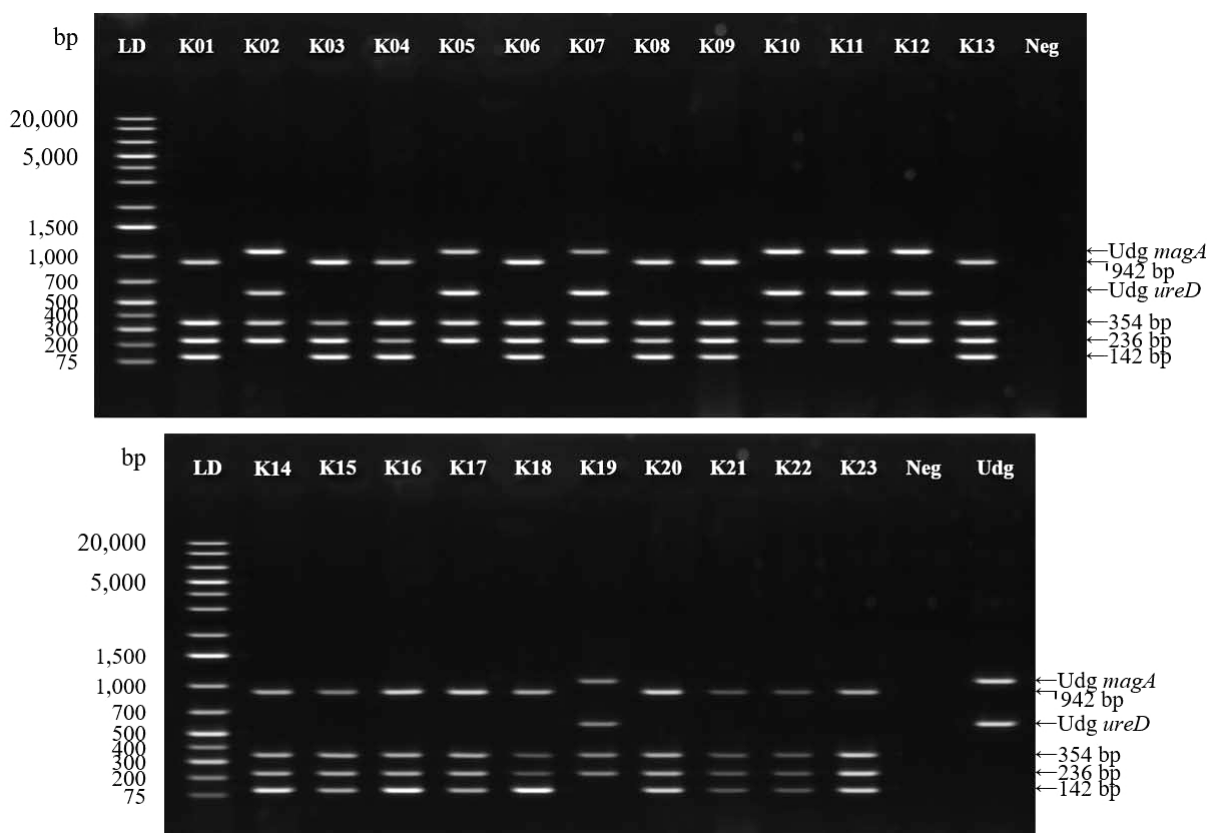
Fig. (2). PCR-RFLP analysis of *ureD* and *magA* genes. K1 - K13 and K14 – K23 denotes different isolates that were double digested with SmaI and PciI restriction enzymes. Neg denotes negative control (water), which served as a loading control. Udg refers to DNA solution that contained undigested *ureD* and *magA* genes.

3.3. PCR-RFLP Analysis

After overnight incubation, PciI could digest the *magA* gene into two fragments (958 and 269 bp) in 16 out of the 23 isolates. On the contrary, SmaI digested the *ureD* gene into the desired fragments (453 and 372 bp) in all 23 isolates (Fig. **2**).

3.4. Antibiotic Sensitivity of *K. pneumoniae* Isolates

All the isolates exhibited resistance to ampicillin, carbenicillin, cefradine, chloramphenicol, erythromycin, kanamycin, and sulphamethoxazole. Ciprofloxacin and levofloxacin showed ~75% sensitivity, whereas gentamycin and streptomycin exhibited ~12% sensitivity. Two isolates, both collected from Mymensingh district, showed a 10% sensitivity for streptomycin. Imipenem was the only antibiotic used, which showed 100% sensitivity to all the tested isolates (Fig. **3**). Among the 23 isolates that contained the *magA* gene, imipenem, ciprofloxacin, levofloxacin showed 100%, 69.6%, and 65.28% sensitivity, respectively. The sensitivity of both gentamycin and streptomycin was 13.04% (Fig. **3**).
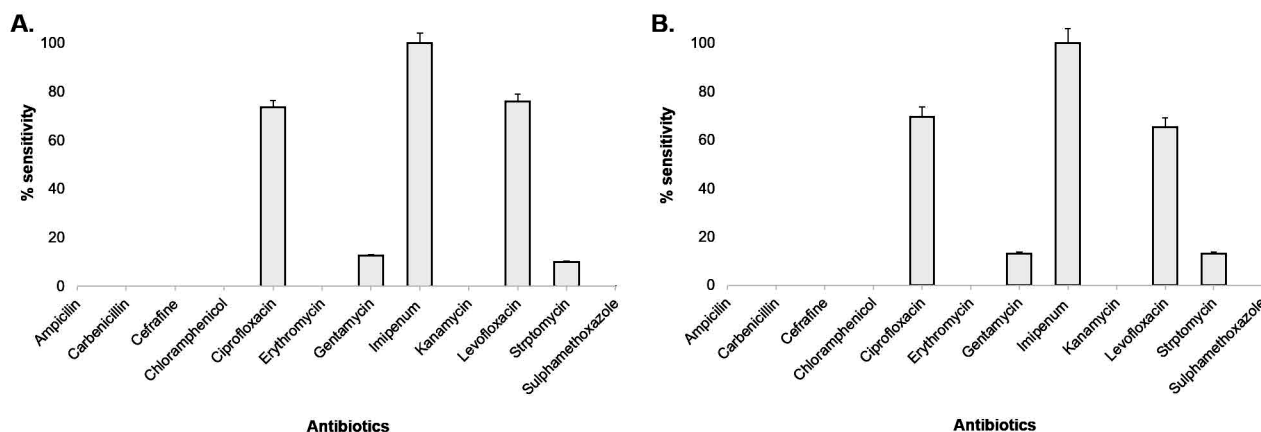
Fig. (3). Antibiotic susceptibility analysis of the *K. pneumoniae* isolates. **A.** Overall antibiotic susceptibility analysis of the *K. pneumoniae* isolates (n = 313). This includes both *magA* containing and *magA* not-containing isolates. **B.** Antibiotic susceptibility of the *magA* containing isolates (n = 23).

4. DISCUSSION

K. pneumoniae are responsible for several nosocomial infections among the debilitated elderly and children [39]. They cause significant morbidity following urinary tract infections, septicemia, and pneumonia due to high resistance to extensively administered antibiotics [9, 40].

The *magA* gene, which plays a critical role in polysaccharide capsule production of the bacterium, is one of the critical factors in *K. pneumoniae* associated virulence [41]. Previous studies reported that the secondary acquired infections, *e.g.*, eye infection, bacteremia, sepsis, and meningitis, are *magA* related diseases [42, 43]. In the present study, over 7% isolates were positive for the presence of *magA* gene (Table **3**). Amrai *et al.*, studied 173 *K. pneumoniae* isolates collected from different clinical samples taken from Iran and reported 2.3% prevalence of the *magA* gene [44]. The prevalence of the gene in Bangladeshi subjects was significantly higher ($p = 0.0203$; $\chi^2 = 5.386$). Previous studies on clinical samples of *K. pneumoniae*, invasive and non-invasive metastatic infections reported that the prevalence of the *magA* gene was 08 – 100% [45 - 50]. Another remarkable observation was found studying isolates collected from the liver abscess that none of the samples were positive for the presence of the *magA* gene.

Previously, the *magA* gene was considered one of the primary reasons for the prevalence of these bacteria in East and South Asian countries [51]. Several reports have suggested the *magA* gene as a root among geographical location, genetic predisposition, and susceptibility to the infections [51 - 54]. Struve *et al.*, sorted out that only *K1* serotype *of Klebsiella* spp. isolated from patients with liver abscesses carries the *magA* gene [29]. In the current study, most of the species containing *magA* gene were isolated from patients in ICUs, and trachea and urine samples. It was noticeable that long-time hospitalization and the use of invasive medical tools enhanced the susceptibility of patients towards a *Klebsiella* infection. Our study suggests that the evaluation of *magA* could be considered a suitable index for rapid molecular identification and diagnosis of these bacteria. Besides, it can be used for the timely treatment of infections caused by *K. pneumoniae.*

This study describes a PCR-RFLP based technique for precise detection and analysis of *magA* gene in *K. pneumoniae* isolates collected from clinical specimens. Our analysis revealed that all the isolates were uniformly cleaved by Pci1. Nearly 1 in every 3 isolates (30.43%) produced negative results for Sma1. This variation by restriction endonuclease digestion of PCR products exerted significant diversity in the *magA* gene. What it translates into is, the *magA* gene in *K. pneumoniae* is significantly evolving in clinical isolates of Bangladesh.

Previous studies affirmed that *K. pneumoniae* are resistant to most of the antibiotics and thus, they are termed as multidrug-resistant. Our study revealed that *K. pneumoniae* isolates collected from clinical samples are 100% resistant to several antibiotics, including ampicillin, cefradine, chloramphenicol, erythromycin, kanamycin, and sulphamethoxazole. Only imipenem was found fully effective against all the isolates. These outcomes support previous reports from Dhaka and Sylhet regions of Bangladesh [23, 55, 56]. Our study suggests that multi-drug resistant strains of *K. pneumoniae* are prevalent across the country.

The antibiotic-resistant pattern has continuously been increasing at an alarming rate, primarily due to the overdose and unregulated use of antibiotics. Nowadays, powerful antibiotics are prescribed in the treatment of most of the diseases, contributing to the spread of multidrug-resistant bacteria [36]. For effective treatment, it is critically important to evaluate the antibiotic-sensitivity status of the causative agents [36, 37].

CONCLUSION

Infection with multi-drug resistant *K. pneumoniae* is now a global public health concern. Our study suggests that *magA* producing *K. pneumoniae* strains are prevalent in the clinical samples of Bangladesh. It also points out that this gene continues to evolve in *K. pneumoniae* strains in this country. We suggest that the *magA* gene, coupled with the *ureD* gene (as an internal control) in the single-tube multiplexed PCR, can be used as a reliable genetic marker for the rapid diagnosis of multi-drug *K. pneumoniae* infections. Considering the presence

of the *magA* gene among clinical isolates of *K. pneumoniae*, especially in the isolates collected from ICUs, it is necessary to adopt suitable infection controlling tools and appropriate therapeutic approaches to prevent further spread of this virulent organism in our hospital settings.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethical and Animal Care Committee of the Shahjalal University of Science and Technology.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Written informed consent was obtained from all the participants.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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