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Characterization of multi-drug resistant *Klebsiella pneumoniae* isolates from urinary tract infected-women in Sylhet city, Bangladesh

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ABSTRACT

Aim: *Klebsiella pneumoniae* is considered to be one of the most frequent bacterial species associated with urinary tract infections (UTIs) and recurrent UTIs (RUTIs) worldwide. The present study aimed to comprehensively characterize *K. pneumoniae* isolates from women suffering from UTI and RUTIs.
Methodology and results: A total of 15 clinical isolates, collected from different hospitals in Bangladesh, were tested for biochemical features, and amplified by PCR. Antibiogram assay was performed by disk-diffusion assay. Phylogenetic and functional features were analyzed using bioinformatics platform. XLSTAT was used for principal component analysis (PCA). PCR amplification using *Klebsiella* hemolysin gene (*khe*) confirmed the presence of *K. pneumoniae* in agarose gel with expected product size of 486 kb. Antibiogram assay revealed all *K. pneumoniae* isolates to be completely resistant to six out of ten relevant drugs namely ampicillin, cephradine, chloramphenicol, erythromycin, kanamycin and sulfamethoxazole used for treating UTIs in Bangladesh. Sequencing of 16S rRNA gene of clinically significant *K. pneumoniae* isolates showed a high level of sequence divergence among the isolates from UTI and RUTIs as well as functional features such as SNP variants and restriction sites.
Conclusion, significance and impact of study: We surmise that the results could be used as a pipeline for further research in the identification of *K. pneumoniae* associated with UTI and RUTIs, and treatment of infection.

Keywords: Klebsiella pneumoniae, *khe* gene, PCR, 16S rRNA sequencing, phylogenetic analysis

INTRODUCTION

Urinary tract infection (UTI) is a microbial infection, which is also the most commonly encountered hospital-acquired infection. It affects different parts of the urinary tract, with an occurrence both in males and females. However, women are more vulnerable to the infections owing to their reproductive physiology, i.e., shorter urethra in females allows a rapid transportation of bacteria from the anus to reach the bladder as compared to the male counterpart (Kolawole *et al.*, 2009; Vasudevan, 2014). The literature reveals more than 60% of women to have a high risk of developing UTI in their lifetime; however, some of the infections remain asymptomatic for a prolonged period (Minardi *et al*., 2011). The term "RUTIs" was coined from the concept of re-isolating the bacteria responsible for causing infection during young age (Badr and Shaikh, 2013). In cases where bacteria exhibit tolerance to antibiotic treatment leading to their prolonged survival in the body, RUTIs can be lethal (Badr and Shaikh, 2013). A study revealed 27% of re-infection in women for the second time after 6 months of follow-up, whereas 2.7% of them reported having a third recurrent infection by the same bacteria (Hooton, 2001). A large group of bacteria is associated with UTI including strains from *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus, Pseudomonas* sp., *Proteus* sp., and *Enterobacter* sp. Among these, *K. pneumoniae* is reported to associate with 10 to 14% of global UTIs, where the pathogenicity and persistence were reported higher than the bacterial counterpart *E. coli* (Kazemnia *et al*., 2014; Mahmudunnabi *et al*., 2018).

The most common problem that hinders the treatment of UTIs is drug resistance in clinical isolates which poses a constant challenge to treat UTIs with commercially available antibiotics. A multi-drug resistance (MDR)

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isolate displays complete resistance to three or more commercially available drugs that were reported to have increased sensitivity earlier (Nikaido, 2010). Insensitivity or resistance to antimicrobials has emerged as a common problem in the densely populated, low-income countries with poor hygiene and sanitation. The situation is aggravated owing to improper drug regime and frequent use of antibiotics to treat common infections (Mahmudunnabi *et al*., 2018). The ability of multidrug-resistant bacteria to combat attack by antimicrobial drugs leads to ineffective treatment, consequently resulting in the persistence and spreading of infections (Tanwar *et al*., 2014; Oliver *et al*., 2015).

Recent advancements in molecular methods have enabled rapid and comprehensive detection of various pathogens from clinical samples. Among numerous techniques, polymerase chain reaction (PCR) is most frequently used to identify bacteria (Barghouthi, 2011). The identification of targeted virulence factors and amplification of 16S rRNA are the two most common practices in characterizing pathogens (López-banda *et al*., 2014; Srinivasan *et al*., 2015). The multi-functional and multi-factorial behavior of virulent genes makes them a key component in determining the pathogenicity of any isolate (Zhan and Zhu, 2017). The 16S rRNA gene of bacteria is associated with the advantage of providing large-scale sequence informatics on bacterial phylogeny and taxonomy. These act as low-resolution profiles in the traditional biochemical characterization and other plate-based methods (Janda and Abbott, 2007). Moreover, computer-aided bioinformatics tools and statistical packages make the data analysis and interpretation easier (Allali *et al*., 2017).

In Bangladesh, the detection of *K. pneumoniae*-associated UTIs is still based on traditional phenotypic tests. These culture-based methods are time-consuming, require significant technical skills to interpret the data, often resulting in false-positive results (Yasmeen *et al*., 2015; Setu *et al*., 2016). The widespread use of molecular tools is extremely limited due to the lack of knowledge and technical skills. Since the selection of an appropriate treatment approach largely depends on finding the causative agent, therefore a comprehensive set of guidelines is necessary for precise identification of *K. pneumoniae* in Bangladesh.

MATERIALS AND METHODS

Collection of samples

A total of 15 clinical samples were collected from three different hospitals from Sylhet, Bangladesh, namely Sylhet MAG Osmani Medical College and Hospitals, Popular Hospitals and Diagnostic Centre, and Jalalabad Ragib Rabeya Medical College and Hospital, over a period of six months, from January to June 2017. The information of patients positive for UTI and *K. pneumoniae* were retrieved from the patient consent forms (Table 1). Then the samples were transported to the laboratory by maintaining cold condition (4 °C) for further analysis. The samples were initially cultured in chromogenic agar medium (Sigma-Aldrich, Germany), followed by and sub-cultured onto nutrient agar (NA) medium (Sigma-Aldrich, Germany) and incubated at 37 °C for 24 h. The overnight bacterial cultures were examined for biochemical characterization and genomic DNA extraction.

Table 1: List of *K. pneumoniae* isolates with isolation history

Id no.	Isolates	Age	Weight	Income†	Physical status of the patient	Infection type	Hospitals□
2667	K11	36	58	300	Secondary bacterial infection	First time	1
619	K12	30	59	516	Healthy	First time	1
702	K13	35	62	480	Secondary bacterial infection	First time	2
1775	K14	28	55	600	Fever, stomachache	First time	1
1078	K15	41	60	500	Secondary bacterial infection	First time	3
937	K16	35	58	500	Fever, stomachache	First time	3
235	K17	38	58	360	Malnutrition	First time	2
1140	K18	65	65	480	Secondary bacterial infection	First time	3
1235	K19	30	61	300	Fever, stomachache	First time	3
785	K20	32	65	360	Pregnancy complicacies, Secondary bacterial infections	Re-current	3
396	K21	45	62	240	Stomachache, flatulence	First time	2
896	K22	37	57	270	Secondary bacterial infection	First time	2
905	K23	68	59	600	Malnutrition	First time	3
1526	K24	65	62	580	Secondary bacterial infection	First time	2
759	K25	40	58	240	Stomach pain, flatulence	First time	3

†Income values are presented as thousand BDT. □Hospitals = [1]JRRMC = Jalalabad Ragib Rabeya Medical College and Hospitals, [2]SMAGOMC = Sylhet MAG Osmani Medical College, [3]PHDC = Popular Hospital and Diagnostic Centre, Sylhet.

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Biochemical characterization of *K. pneumoniae* isolates

All isolates were subjected to several microbiological and biochemical tests according to the standard methods for identification of *K. pneumoniae* from clinical samples (Bergey and Holt, 1994; Hansen *et al*., 2004). The isolates were assayed for Gram's test, catalase, oxidase, methyl-red, Voges-Proskauer, citrate, urease, H_2S, and indole tests, motility, fermentation with glucose, sucrose, and maltose. Following the biochemical tests, positive isolates of *K. pneumoniae* were preserved and cultured.

Antibiogram profiling of *K. pneumoniae* isolates

Drug sensitivity assay with 10 commercially available antibiotics was conducted following disk-diffusion assay (Balouiri *et al*., 2016). The overnight bacterial culture (30 µL) was poured onto NA plates and spread uniformly with an L-shaped glass rod. Then antibiotic discs were added aseptically onto the culture plate inside biosafety cabinet using sterile forceps and incubated at 37 °C overnight. Antibiotic disks (Oxoid, Thermo Scientific, USA) used in this study included ampicillin (AMP, 10 µg/disk), cephradine (CEP, 30 µg/disk), chloramphenicol (C, 30 µg/disk), ciprofloxacin (CP, 10 µg/disk), erythromycin (ERY, 15 µg/disk), kanamycin (K, 5 µg/disk), levofloxacin (LEV, 5 µg/disk), streptomycin (S, 10 µg/disk), sulfamethoxazole (SXT, 25 µg/disk), and tetracycline (T, 10 µg/disk). The zone of inhibition was measured according to previously described standard method following CLSI method (Rota *et al*., 2008).

Genomic DNA extraction and quantification

Genomic DNA was extracted using the ATP Genomic DNA Mini Kit (ATP Biotech. Inc., Taiwan) following manufacturer's instructions. Proteinase K and RNase A were added to samples and incubated at 37 °C for 30 min to remove the impurities and enhance the quality of the extracted DNA. Extracted DNA was quantified using NanoDrop (ThermoFisher Scientific, 2000c) by measuring the DNA–protein absorbance. The extracted DNA was then diluted accordingly to reach the final concentration of 30 ng/µL. Both stock and diluted DNA were then stored at –20 °C until use for PCR.

Amplification of *khe* and 16S rRNA gene

For *khe* PCR, the final master mixture volume was adjusted to 30 µL that contained 15 µL of 2 × master mixture (Fermentas, USA), 1.5 µL of each *khe* forward and reverse primers (5'-TGATTGCATTCGCCACTGG–3' and 5'-GGTCAACCCAACGATCCTG–3'), 2 µL of template DNA and 10 µL of nuclease-free water (Jian-li *et al*., 2017). Finally, a total of 30 cycles of reaction was programmed in MultiGene Gradient Thermal Cycler (Labnet International Inc., USA) with an initial denaturation temperature of 94 °C for 4 min; denaturation step at 95 °C for 1.5 min, annealing at 58 °C for 1.5 min, an extension at 72 °C for 1.5 min, followed by a final extension step at 72 °C for 5 min and final storage at 4 °C. *K. pneumoniae* ATCC®33495 and nuclease-free water were used as positive and negative control for the validation of PCR amplification. For 16S rRNA, the universal 27F and 1492R universal primers (5'-AGAGTTTGATCCTGGCTCAG–3' and 5'-AGGAGGTGATCCAACCGCA–3') were used with the same volume of master mixture and concentration of reagents described above while the PCR conditions were slightly modified (Srinivasan *et al*., 2015). The 32 cycles of reactions were programmed with the annealing at 52 °C for 30 s. All PCR products were separated by electrophoresis (Bio-Rad Laboratories Inc.) on 1.0% agarose gel containing 6% gel red. The amplified bands were visualized using the gel documentation system (FujiFilm LAS–4000 Image Analyzer, Boston Inc.).

Sequencing of 16S rRNA gene of *K. pneumoniae*

The amplified full-length PCR products of 16S rRNA of 1,465 bp were visualized on an agarose gel and purified using the PureLink PCR purification kit (Thermo Scientific, USA) according to the manufacturer's instructions. The purified 16S rRNA PCR products were then sequenced from "1st BASE" sequencing center, Malaysia. Raw sequences were analyzed using various software and web-based sequence analysis tools.

Analysis of sequence data and phylogenetic relationship

After sequencing, raw sequences were extracted, primarily edited, and assembled using BioEdit (v7.0.4) (Carlsbad, California, USA), Chromas (v2.01) (Technelysium Pty Ltd, Helensvale, QLD, Australia), and SeqMan Pro (v15.0) (Madison, Wisconsin, USA) tools (Momtaz *et al*., 2018). DECIPHER (v9.20) (Sanger Institute, Hinxton, UK) was used to remove chimeras from the raw sequences. The edited DNA sequences in FASTA format were then run for homology in the NCBI nucleotide database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast). Multiple alignment of the assembled sequences was performed in MEGA 7.0 (Pennsylvania State University, USA) using ClustalW and extracting the raw file in MEGA format. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA 7.0. The GenBank accession numbers of *E. coli* species indicate their parenthesis to most similar sequences. Evolutionary divergence among sequences was calculated as *p*-distances after avoiding gaps and taking both transitions and trans-versions. SNP variants and restriction sites (REs) in the three *K. pneumoniae* sequences were analyzed in Geneious (v11.07) (Biomatters Ltd, Auckland, New Zealand) using default parameters.

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Statistical analysis

To analyze the correlations among the set of observations (variables), principal component analysis (PCA) test was done using XLSTAT (Addinsoft, New York, USA) considering the number of antibiotic resistant as the primary component. The *K. pneumoniae* isolates were set as observation level while other variables (age, weight, hospitals and number of antibiotic resistance) were run for Pearson (n) correlation rank test in PCA. Correlations mono-plot created after avoiding of missing data in default parameters in separate sheet.

RESULTS

Biochemical characterization of *K. pneumoniae* isolates

All isolates were initially supplied as *K. pneumoniae* in the ESBL chromogenic medium. Morphological and biochemical characterization confirmed them to belong to *K. pneumoniae* species. All of them were negative for Gram's test, indole, methyl-red, citrate, urease, and H_2S tests and motility; they tested positive for catalase, oxidase, and Voges-Proskauer tests. They could also successfully ferment glucose, sucrose, and maltose.

Drug resistance in *K. pneumoniae* isolates

In the present study, all *K. pneumoniae* isolates characterized from UTI exhibited a high percentage of drug resistance. These were completely resistant to ampicillin, cephradine, chloramphenicol, erythromycin, kanamycin, and sulfamethoxazole. Isolates K13, K19, and K20 were completely resistant to all of the tested antibiotics in the present study. The *in vitro* disk diffusion assay revealed levofloxacin and ciprofloxacin to be two most effective drugs with 87% and 80% sensitivity, respectively. However, no drug was found to potentially inhibit all *K. pneumoniae* isolates (Figure 1).

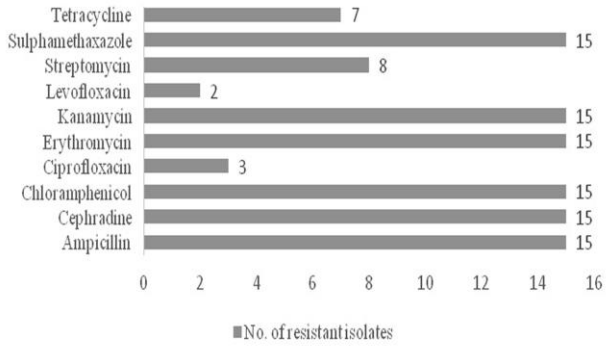

Figure 1: Antibiotic resistance in *K. pneumoniae* isolates. The numerical values in right side indicate the number of resistant isolates.

Amplification of *khe* and 16S rRNA gene by PCR

All isolates (K11–K25) that were positive for *K. pneumoniae* in biochemical tests were also found to be positive for *khe* gene by PCR amplification showed the expected 486 bp band (Figure 2). Three isolates, namely K13, K19, and K20 that showed complete resistance to all antibiotics and were associated with other complications were selected for 16S rRNA gene amplification using 27F and 1492R universal sequencing primers. All isolates upon amplification displayed expected product size of 1,465 bp on an agarose gel.

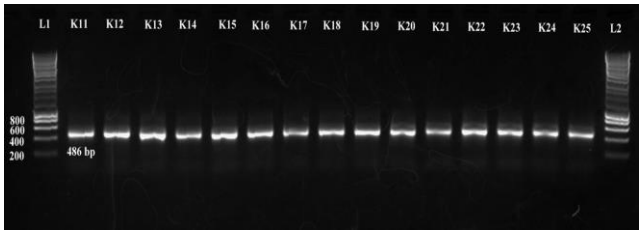

Figure 2: Amplification of *khe* gene of *K. pneumoniae* by PCR. Lane 1 (L1) and lane 2 (L2) are 1kb DNA ladder (HyperLadder™, Bioline).

Sequence and data analysis

The present study found 99% homology of the sequenced isolates to clinical *K. pneumoniae* species in BLASTn at NCBI. Also, the study of phylogenetic tree revealed a close clustering of study isolates with strains of *K. pneumoniae* associated with UTIs (Figure 3). Evolutionary divergence data revealed fair distances within and among the study sequences (0.002-0.004), where the isolate *K. pneumoniae* K13 demonstrated a negligible detachment from *K. pneumoniae* subsp. *ozaenae* ATCC 11296 (0.001), a bacterium associated with UTI. Significant evolutionary divergence (0.237-0.285) was observed among other *K. pneumoniae* strains and two bacteria, namely *Actinobaculum massiliense* and *Staphylococcus saprophyticus*.

The SNP variant study in the 16S rRNA sequence of K20 strain found 28 SNPs, abundantly present all over the sequences (Figure 4). Restriction sites analysis found significant dissimilarities in the number and positions of the enzyme in strain K20. Analysis from position 1,078 to 1,096 revealed six digestion sites (*Ava*II, *Hpa*II, two *Msp*I, and two *Hae*II) in K20 compared to three in K13 and K19 (*Hpa*II, *Msp*I, and *Hae*II). The study sequence is available in the GenBank databank of NCBI under the accession number of MG890348.1 (K13), MG890349 (K19), and MG890350 (K20). The PCA among variables found a correlations among antibiotic resistance, weight, and the sources of sample collection where the relationship was strong between drug resistance and weight compared to drug resistance and hospital sources. Whereas the number of drug resistance were independent of age and personal income. A distinct pattern of clustering for *K. pneumoniae* isolates also observed in the plot where

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characteristically similar isolates (K13, K19, K20, and K21) were clustered together. In the mono-plot, the axis F1 and F2 described 70.42% of variables, fair enough to make conclusive comments (Figure 5).

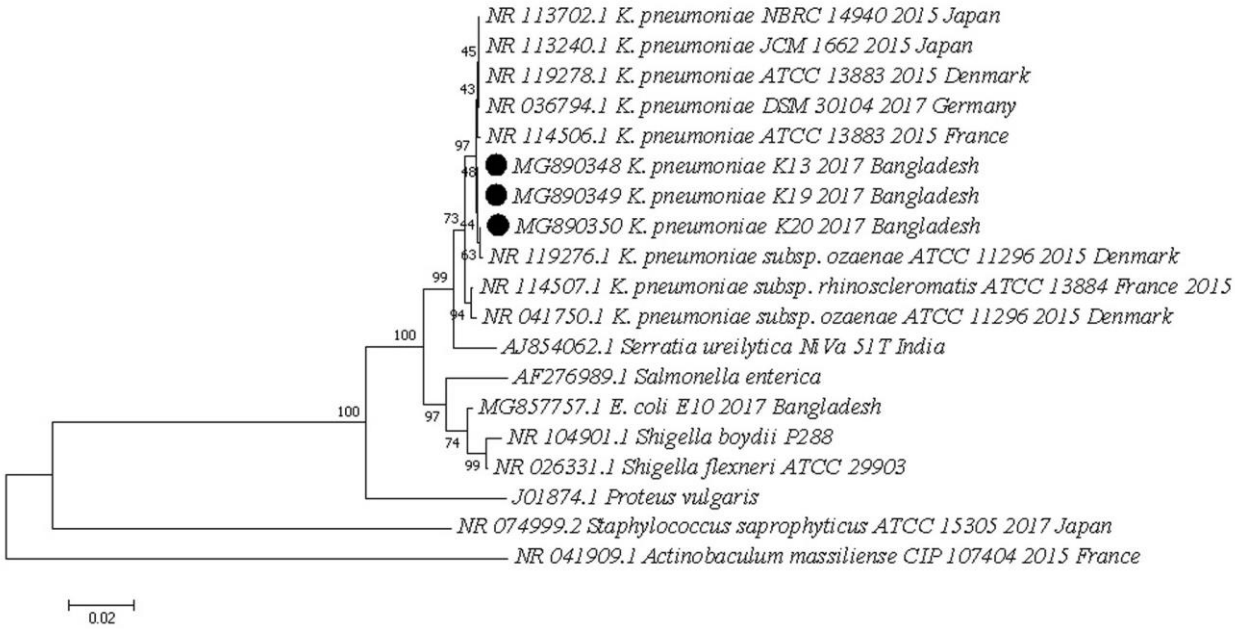

Figure 3: Evolutionary relationships of *K. pneumoniae* isolates associated with UTI. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Figure 4: SNPs heat-map (first image) of the study *K. pneumoniae* isolates with other UTI associated *K. pneumoniae* strains. Out of 28, only two SNP variants (green box) were common in three study isolates. Predicted restriction (RE) sites (second image) found six sites in RUTIs associated K20 isolate (MG890350) compared three in two UTI causing isolates.

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DISCUSSION

Humans are prone to a wide variety of infections caused by *K. pneumoniae*, including UTIs. The pathogenicity of *K. pneumoniae* associated with UTI is influenced by diverse factors, including population density, living conditions, proper hygiene practice, sanitation, diagnosis and treatment strategies, and physical and immune status of the patient (Zaffanello *et al*., 2010; Gupta and Trautner, 2013).

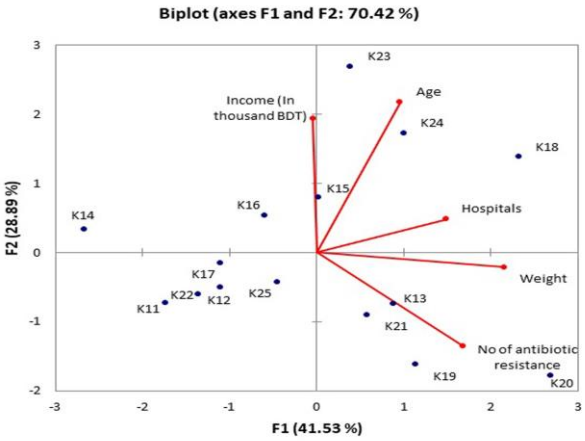

Figure 5: Principal component analysis (PCA) mono-plot showing the relationship between variables.

These factors are also responsible for causing RUTIs and secondary bacterial infections by *Chlamydia* and mycoplasmas (Matuszkiewicz-rowińska *et al*., 2015). The observed uncharacterized secondary bacterial infections in half of (47%) the UTI patients in the present study signify the importance of these factors and the diagnostic limitations in Bangladesh. The spread of multi-drug resistant bacteria is attributed to misdiagnosis or late diagnosis of UTIs (Jian-li *et al*., 2017; Hawkey *et al*., 2018). The present study identified circulating *K. pneumoniae* from UTI and RUTIs in women using virulence factor-based PCR and 16S rRNA sequence-analysis. The gene *khe* is reported to be a highly conserved virulent gene for *K. pneumoniae* that can be detected even at a very low concentration of genomic DNA (<100 copies) precisely and accurately (Clifford *et al*., 2012). All *K. pneumoniae* isolates tested positive for *khe* indicating this gene to be a better identification marker for the pathogenic features of bacteria. Here, we selected three of the most drug-resistant isolates that were also associated with complications for evolutionary informatics and found substantial sequence divergence in the 16S rRNA data, especially in the isolate associated with RUTIs. These distances possibly arise from the differences in SNP variants and restriction sites. SNP variants, responsible for genetic differences in human, and have been found to be associated with an individual's drug response and susceptibility to a particular disease (Gamazon *et al*., 2010; Roden *et al*., 2012). The increasing number of SNP variants in a DNA sequence raises the probability of mutations within or near the regulatory genes directly involved in disease progression and reinfections through alteration of gene function (Li *et al*., 2014; Ma *et al*., 2015). The presence of two more SNP variants in RUTIs causing *K. pneumoniae*, therefore, could be responsible for other phylogenetic and functional differences as compared to other two isolates. Moreover, an analysis of restriction sites is significant in analyzing the multi-drug resistant strain as well as the recurring strains in clinical infections (Tahmasebi *et al*., 2012). Drug-specific receptors are usually more susceptible to point mutations, thereby leading to antibiotic resistance (Gorgani *et al*., 2010). Hence, we hypothesize that the modifications in RE sites from positions 1,078 to 1,096 possibly initiated from DNA sequence variants in *K. pneumoniae* associated with RUTIs and drug resistance.

The present research could find no antibiotic with a potential to inhibit or kill experimental *K. pneumoniae* isolates. In addition, sensitivity to the drug was found to be specific to isolates and depended on patient's response and concomitant factors including type of infections, habitat, age, etc. Alarming drug resistance in *K. pneumoniae* associated with UTI become a major health concern in Bangladesh in recent years (Mahmudunnabi *et al*., 2018). The results indicate that analyzing the health status of the patient and the causative agent are equally important before selecting the appropriate treatment strategy for UTI and RUTIs. Finally, the correlations among antibiotic resistance with other variables in this study signifying that a better health status, life style and living conditions could help to minimize the risk of UTI infection in women.

CONCLUSION

The current study describes a set of rapid, reliable, and accurate techniques for early and easier detection of *K. pneumoniae* in UTI and RUTIs. Besides, we also found a positive correlations among antimicrobial resistance, SNP variants, and RE sites of clinical *K. pneumoniae* isolates associated with UTI and RUTIs in women. However, the sample sizes was the major limitation of the study to make any conclusive comments based on research findings. Besides, this was a complete regional study where samples were collected from the Eastern part of Bangladesh. Therefore, it may not reflect the global scenario. Further long-term studies are required to analyze the drug resistance mechanism of UTI and RUTIs causing *K. pneumoniae* in Bangladesh.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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