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Original Article

Molecular detection and PCR-RFLP analysis using *Pst*1 and *Alu*1 of multidrug resistant *Klebsiella pneumoniae* causing urinary tract infection in women in the eastern part of Bangladesh

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ABSTRACT

Klebsiella pneumoniae is the second leading causative agent of UTI. In this study, a rapid combined polymerase chain reaction and restriction fragment length polymorphism analysis was developed to identify *K. pneumoniae* in women, infected with urinary tract infection in the Sylhet city of Bangladesh. Analysis of 11 isolates from women at the age range of 20–55 from three different hospitals were done firstly by amplification with *K. pneumoniae* specific ITS primers. All of the 11 collected isolates were amplified in PCR and showed the expected 136 bp products. Then, restriction fragment length polymorphism analysis of 11 isolates were conducted after PCR amplification by 16s rRNA universal primers, followed by subsequent digestion and incubation with two restriction enzymes, *Pst1* and *Alu1*. Seven out of 11 isolates were digested by *Pst1* restriction enzymes, six isolates digested by *Alu1*, and while others were negative for both enzymes. Data results reveal that, women at age between 25 and 50 were digested by both enzymes. A woman aged over than 50 was negative while bellow 20 was digested by only *Pst1*. The results could pave the tactic for further research in the detection of *K. pneumoniae* from UTI infected women.

1. Introduction

Klebsiella pneumoniae is the second most potential agent of urinary tract infection after *Escherichia coli*, however, the pathogenicity is higher than its counterpart [1]. Approximately 12% of UTI infection caused by *K. pneumoniae* and the number is increasing at an alarming rate all over the world, particularly in Asia, due to spread of antibiotic resistant and extended beta-lactamase strains [2]. Women are eight times more vulnerable to UTI infection due to their position of reproductive organs and many of the infections remain asymptomatic for prolonged period [3]. The incidence rate increases with age, recurrent infections (very common for women), and during pregnancy period [4,5]. In Bangladesh, due to geographical position, weather, food habit, early age pregnancy, and lack of awareness about UTI: the numbers of patients infected by *K. pneumoniae* have been proliferated in the last couple of years [6,7]. Several researches have been conducted on *Escherichia coli* associated UTI, but molecular based approach for the detection and analysis of *K. pneumoniae* causing UTI in women has yet to be developed.

PCR alone or sometimes in combination with RFLP has been extensively used for precise detection and analysis of pathogens for many years [8]. Traditional culture based technologies are time consuming, labor intensive, and sometimes frequent use of antibiotics may affect culture positive isolates thus difficult to interpret data correctly [9]. However, PCR based molecular approaches are independent of antibiotics, more rapid, reliable, and sensitive, thus routinely used as molecular tools for pathogen identification [10]. 16s-23s internal transcribed spacer (ITS) unit of *K. pneumoniae* facilitating precise identification of this organism by polymerase chain reaction (PCR) [11]. Restriction endonuclease digestion of

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PCR products enables species determination and analysis of genome variability [12]. The sequence specific RFLP pattern of bacteria amplified from 16s rDNA primers varied widely from species to species, and the conserved sequence likely to be differentiated by PCR-RFLP method [13]. Restriction endonuclease digestion of bacterial DNA by *Pst1*, *Alu1*, and *Mob1* have been used to confirm etiological agents in some earlier studies [14–16].

Multidrug resistant *K. pneumoniae* cause an emerging health threat worldwide, especially in least developed, and densely populated countries [17]. Current treatment practice commonly prescribe powerful antibiotics resulting spread of multidrug resistant bacteria and thereby reducing therapeutic efficacy [18]. In order to implement a successful treatment strategy for UTI, it is of great importance to know the current antibiotic resistant profile of the causative agents [17,18]. Early detection of *K. pneumoniae* from UTI could minimize the widespread use of antibiotics in prevention and control programs as well as reduce the medical cost. The objective of this study was to evaluate 16s-23s ITS primer and PCR-RFLP method as a tools for the identification of multi-drug resistant (MDR) *K. pneumoniae* causing UTI in women.

2. Materials and methods

2.1. Collection and culture of bacterial isolates

A total of 11 bacterial isolates were collected from three different hospitals of Sylhet city of Bangladesh: Sylhet MAG Osmani Medical College and Hospitals, Popular Hospitals and Diagnostic Centre, and Jalalabad Ragib Rabeya Medical College and Hospitals. Immediately after collection, isolates were transported to USDA project laboratory of the Department of Genetic Engineering and Biotechnology of Shahjalal University of Science and Technology by maintaining cool chain. Isolates were cultured in ESBL medium and incubated overnight at 37 °C. Isolates were then numbered numerically from K1 to K11 for further studies. UTI patient's data (Table 1) were collected from doctor's consent form and recorded for future analysis.

2.2. Genomic DNA extraction

All of the bacterial isolates were streaked in trypticase soy (TCS) agar medium for colony formation and incubated at 37 °C for overnight. A single colony was picked and grown over night at 37 °C on TCS broth in a shaker incubator for genomic DNA extraction. DNA of 11 bacteria were extracted by following the instructions of commercial genomic DNA extraction kit (Bio Basic Inc., 160 Torbay Road, Markham Ontario, Canada). Additionally, proteinase K and RNase A added after incubation step for purified DNA according to the guidelines of extraction kit. Extracted DNA were quantified by gel electrophoresis with lambda (λ-DNA marker as well as in a spectrophotometer as a ratio of DNA-protein absorbance. DNA was then stored at −20 °C for further use.

2.3. Identification of Klebsiella pneumoniae by PCR

For identification of *K. pneumoniae*, 16s-23s ITS primer was used to amplify DNA sequence in this study [11]. PCR master mixture was prepared in 50 μl volume containing 25 μl of 2X master mixtures (Fermentus, Gene Ruller™, USA), 2.5 μl of each forward and reverse primer (Table 2), 5 μl of template DNA (100 ng) and 15 μl of nuclease free water. PCR conditions consisted of an initial denaturation temperature of 94 °C for 4 min; denaturation step of 94 °C for 1 min, annealing for 1 min at 55 °C, and an extension at 72 °C for 1.5 min, a final extension step of 72 °C for 10 min and 4 °C for final storage. A total of 35 serial cycles of amplification reaction was performed in a MultiGene Gradient Thermal Cycler (Labnet International Inc., USA). PCR products were separated on 1.5% agarose gel followed by subsequent staining in ethidium bromide solution and visualized in a gel documentation system.

2.4. Amplification of bacterial 16s rDNA by universal PCR

PCR master mixture was adjusted at 30 μl final volume contained 15 μl of 2X master mixtures (Fermentus, Gene Ruller™, USA), 1.5 μl of each universal 27F forward and 1540R reverse primers (Table 2), 2 μl of template DNA and 10 μl of nuclease free water. Here 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 of 95 °C for 1.5 min, annealing for 1.5 min at 58 °C for, an extension at 72 °C for 1.5 min, a final extension step of 72 °C for 5 min, and 4 °C for final storage.

2.5. Restriction digestion

After 16s rDNA PCR, 10 μl of PCR product was transferred to a separate eppendorf and 18 μl of nuclease free water added. Then, 2 μl of *Pst1* and *Alu1* restriction enzymes (Table 2) premixed with BSA were added carefully to the solution. Restriction enzyme added samples were then spin gently for few seconds and incubated at 37 °C for 2 h in a water bath [12]. Fragments then analyzed in 2% agarose on 10% TBE under UV illumination. A molecular weight marker (1kb DNA ladder, Fermentus, GeneRuller™, USA) was added for each of the gel run.

2.6. Antibiogram assay of the isolates

Antibiotic profiling of the *K. pneumoniae* isolates to 10 commercial antibiotic discs were performed by disc diffusion assay [17,18]. The antibiotic discs used in this study were ampicilin (10 μg/disk), kanamycin (30 μg/disk), erythromycin (15 μg/disk), chloramphenicol (30 μg/disk), levofloxacin (5μg/disk), ciprofloxacin (30 μg/disk), cefradine (25 μg/disk), gentamicin (10 μg/disk), streptomycin (10 μg/disk), and sulphamethoxazole (25 μg/disk). Overnight bacterial culture (30 μl) was inoculated on Tryptocasein Soy Agar plates

Table 1
Isolates with their isolation history.

Isolates	Age	Physical status of the patient	Infection type	Hospital
K1	55	Healthy	First time	Sylhet MAG Osmani Medical College
K2	35	Fever, stomach pain	First time	Sylhet MAG Osmani Medical College
K3	18	Malnutrition	First time	Jalalabad Ragib Rabeya Medical College and Hospitals
K4	22	Secondary bacterial infection by mycoplasma	Re-current	Popular Hospital and Diagnostic Centre, Sylhet
K5	32	Stomach pain, flatulence	Re-current	Popular Hospital and Diagnostic Centre, Sylhet
K6	28	Healthy	First time	Sylhet MAG Osmani Medical College
K7	25	Stomach pain ketosis (Pregnant)	First time	Popular Hospital and Diagnostic Centre, Sylhet
K8	38	Secondary bacterial infection by chlamydia	Re-current	Popular Hospital and Diagnostic Centre, Sylhet
K9	19	Stomach pain, flatulence	First time	Popular Hospital and Diagnostic Centre, Sylhet
K10	52	Healthy	First time	Jalalabad Ragib Rabeya Medical College and Hospitals
K11	36	Secondary bacterial infection	First time	Jalalabad Ragib Rabeya Medical College and Hospitals

Table 2
Primers and restriction enzymes used for the present study.

Target	Primer name	Sequence	Size	Reference
Klebsiella pneumoniae 16s-23s ITS primer	*K. pneumoniae* Pf *K. pneumoniae* PrA	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130 bp	[11]
Universal sequence	27F 1540R	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-AGGAGGTGATCCAACCGCA-3′	1513 bp	[28]
Target	Restriction enzyme	Recognition sequence		Reference
PCR amplified *K. pneumoniae* 16s rRNA	*Pst1* *Alu1*	5′-CTGCAG-3′ 5′-AGCT-3′		[16]

(Micromaster, India) by spreading and discs were placed aseptically onto the culture. After 24 h of incubation at 37 °C, the zone of inhibition was measured according to Sharmin et al. [31].

3. Results

3.1. Identification of K. pneumoniae isolates

All the bacterial isolates from three different hospitals were initially supplied as *K. pneumoniae* grown on selective agar media supplemented with ornithine, raffinose and koser citrate [21]. However, for further confirmation, bacterial isolates were assayed for their morphological, physiological and biochemical properties according to Burgey's manual for the identification of *K. pneumoniae* [22]. All of the isolates were confirmed belong to *K. pneumoniae* after biochemical tests. The isolates were also confirmed by amplification in PCR with the 16s-23s internal transcribed spacers (Fig. 1).

3.2. PCR-RFLP analysis

Among the 11 isolates, seven (K3, K4, K5, K6, K7, K8 and K9) were digested by *Pst1* after 2 h incubation, two isolates (K2 and K11) were amplified in PCR but not digested by *Pst1* and two others were negative in PCR amplification (Fig. 2). In other experiment, six isolates (K2, K4, K5, K7, K8 and K10) were fragmented by *Alu1*, two

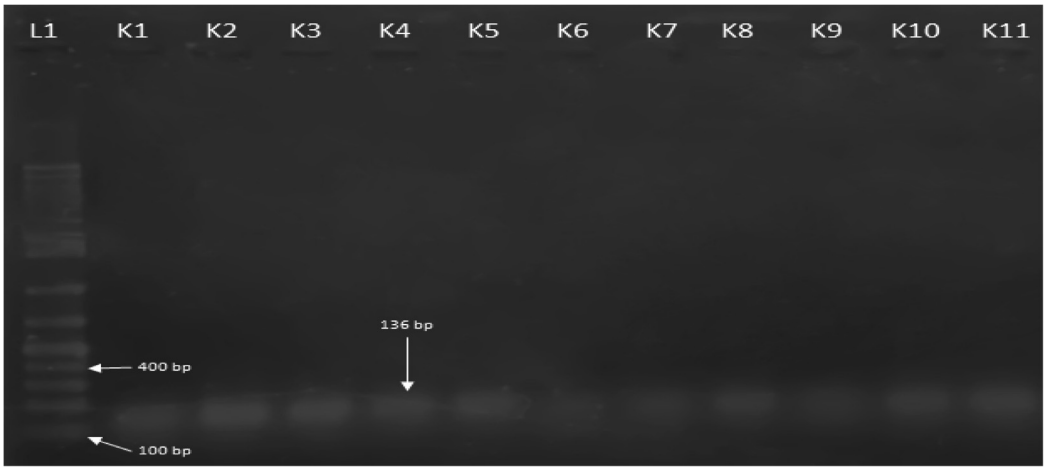

Fig. 1. PCR amplification of *K. pneumoniae* from women with UTI by 16s-23s ITS primer. L1: 1 kb DNA ladder, K1-K11 amplified *K. pneumoniae* isolates.

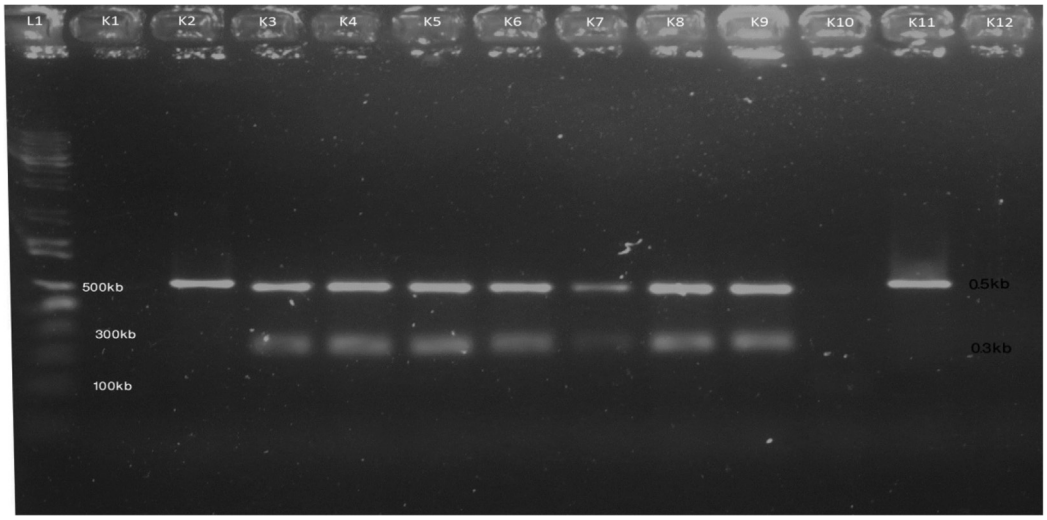

Fig. 2. PCR-RFLP digestion pattern of 16s rDNA sequence of *K. pneumoniae* by *Pst1*. L1: 1 kb DNA ladder, K1-negative control, K2-K12 digested *K. pneumoniae* isolates after incubation.

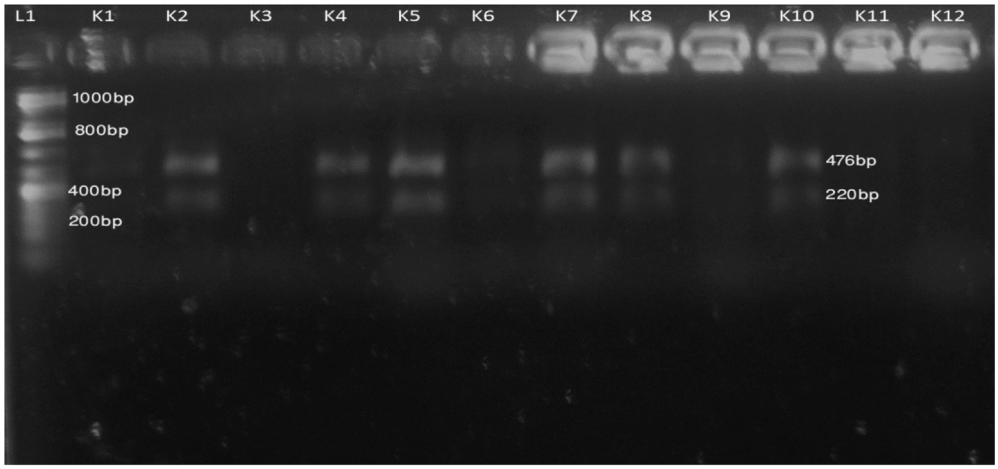

Fig. 3. PCR-RFLP digestion pattern of 16s rDNA sequence of *K. pneumoniae* by *Alu1*. L1: 1 kb DNA ladder, K1-negative control, K2-K12 digested *K. pneumoniae* isolates after incubation.

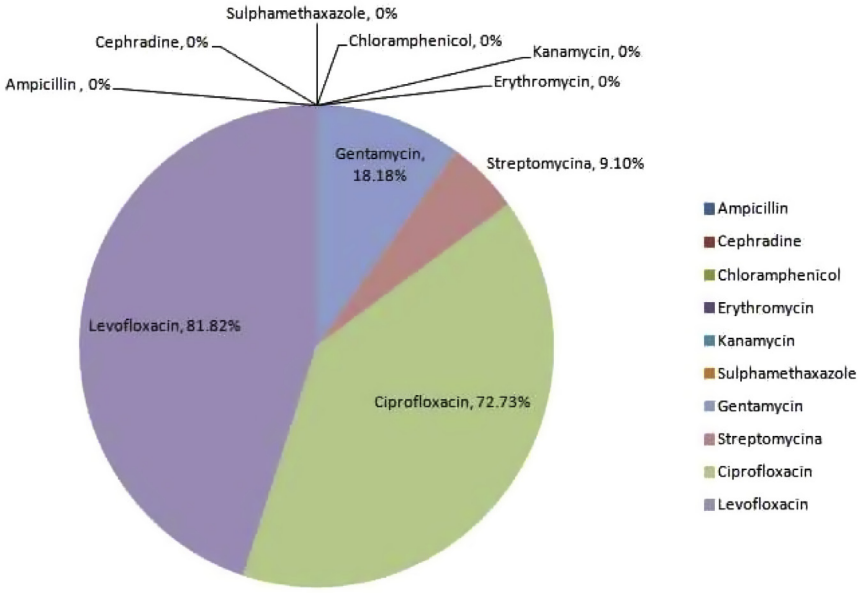

Fig. 4. Antibiotic sensitivity pattern of *K. pneumoniae* isolates.

isolates (K1 and K6) gave negative *Alu1* digestion while three others were negative in PCR amplification (Fig. 3).

3.3. Antibiotic sensitivity of K. pneumoniae isolates

To know the antibiogram profile of the isolates, we screened ten antibiotics representing different antibiotic groups. All of the isolates showed resistant to ampicillin, erythromycin, chloramphenicol, cephradine, kanamycin and sulphamethaxazole. Sensitivity (approximately 80%) only observed for two antibiotics, ciprofloxacin and levofloxacin. Streptomycin and gentamycin showed sensitivity to only K3 isolate (younger patient), but neither showed 100% sensitivity to all of the tested isolates. Therefore, all the *K. pneumoniae* isolates were resistant to multiple antibiotics tested in this study (Fig. 4).

4. Discussion

K. pneumoniae cause a wide variety of diseases in both humans and animals. Among these diseases, urinary tract infection is one of the common that cause serious health threat to women, especially to the pregnant and immunocompromised person [23,24]. In recent years, the prevalence of UTI infection caused by *K. pneumoniae* has been increased in Asia including Bangladesh [6]. Data results also suggest that women during pregnancy witnessed recurrent UTI infection are also suffered from other bacterial infections (Chlamydia and Mycoplasma) [5]. Although pronounce effects, no experimental data available for molecular detection of UTI causing *K. pneumoniae* and its 16s rRNA restriction digestion analysis in infected women in Bangladesh. Therefore, this study could be used as a platform for rapid detection, virulence properties, and drug sensitivity pattern of *K. pneumoniae* associated UTI in women.

Biochemical characterization and other media based identification of *K. pneumoniae* often give false positive results and required considerable amount of time for confirmation [25]. Therefore PCR method has been widely used for precious detection of pathogens and analysis of their genetic diversity [26,27]. Previous PCR based researches for the detection of *K. pneumoniae* by 16s-23s internal transcribed spacer although successful for most of the isolates but didn't produce perfection [2,11,26]. The PCR in this study

was outstanding in amplification of all the 11 isolates and was so sensitive that it produced reproducible results in repetitive experiments.

Sequence specific enzymatic cleavage of amplified 16s rRNA allows precise and early diagnosis of diseases [28]. In this study, RFLP digestion of 16 rRNA produced a distinct pattern of cleavage, size ranging from 0.5 kb to 0.75 kb. Restriction enzyme *Pst1* developed three types of banding pattern; seven isolates were fragmented and produced bands of the same sizes. Enzyme *Alu1* produced digestion pattern had close proximity to *Pst1*; six isolates were cleaved consistently at the same length. Sharma et al. conducted a study on the detection of *E. coli* and *K. pneumoniae* from tertiary care hospital of India and performed restriction digestion analysis with *EcoR1* and *Pst1*, where 60% of isolates were positive for *Pst1*, and gave bands at molecular weight of 150 bp to 750 bp [28]. In present study, we found 64% digestion of *K. pneumoniae* 16s rRNA, a slightly more sensitivity to *Pst1* digestion than the Sharma's study in 2010. This is probably due to similar circulating strains spreading over the South-East Asia [28]. For *Alu1*, Kalghatgi et al. performed an experiment to differentiate *K. pneumoniae* from other pathogenic bacteria using some restriction enzymes where 60% of *K. pneumoniae* isolates were digested by *Alu1* and showed band at 476 bp, 220 bp and 65 bp [29]. In this study, 63.4% of isolates were sensitive to *Alu1* and displayed similar banding pattern like earlier study (476 bp and 220 bp) [29].

Another significant finding of this study was the homogenous banding pattern of isolates from pregnant (K7) women and recurrent UTI (K4, K5 and K8) patients, suggesting a common evolutionary origin for all of these isolates [28]. In addition, patient's data reveals that these samples came from the same hospital (Popular Hospitals and Diagnostic Centre) and community (slum). Moreover, the isolates were resistant to all of the commercial antibiotics tested. Therefore, environmental factors and food habit might play some role for recurrent infection and drug resistant pattern [27,31].

The growth curve for antibiotic resistant pattern has been constantly increased at an alarming rate in Bangladesh. In 2012, 60% of UTI causing *K. pneumoniae* were resistant to commercial drugs [31]. However, in 2016, resistancy pattern increased by 20% to common antibiotics used to treat UTI [27]. Present study found over 90% of the isolates were resistant to multiple antibiotics tested. The availability and frequent use of antibiotics possibly responsible for this upward resistance pattern in Bangladesh [7].

Finally, rather treating *K. pneumoniae* associated UTI by antibiotics, we have to put more emphasis on early detection methods. Antibiotics based common treatment strategy add further complicacy to UTI patients [31]. Therefore, a rapid and precise molecular method needed to address the problem, and PCR-RFLP here could be a simple, selective, and cost effective alternative of the traditional culture based method in Bangladesh.

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Conflict of interest

The author declares no conflict of interest.

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