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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

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