World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Isolation and molecular characterization of urinopathogenic associated *Klebsiella pneumoniae* from urinary tract infected patients

Tariq A. L.

Department of Biotechnology, Government Degree College, Beerwah-193411, Kashmir

Received: 12-03-2017 / Revised: 02-05-2017 / Accepted: 21-05-2017 / Published: 27-05-2017

ABSTRACT

Urinary tract infection is a significant cause of morbidity and mortality. An infection occurs when tiny organisms usually bacteria from the digestive tract, cling to the urethra. A total of 120 urine samples were collected from various hospitals around the Erode district of Tamil Nadu. Out of 120 urine samples were studied, growth of bacteria was observed in 34 urine samples and no growth were seen in 86 samples they found to be healthy. The infected patients were found to be the 15 to 37 years old. The urine samples showed gram negative rod shaped bacteria, non-motile and capsulated bacteria. The respective cultural morphology, biochemical tests and carbohydrate fermentation tests it clear that isolated belongs to be *Klebsiella pneumoniae*. The continuous observation showed that *Klebsiella* has replaced other predominant urinary pathogens. Antibiotic sensitivity test revealed that *Klebsiella pneumoniae* was resistant to six antibiotics by clinical and laboratory standards. Resistance organism has high molecular weight of about 5258bp for *Klebsiella pneumoniae*. Multiple restriction sites of plasmid are analyzed by using EcoR1, Hind III, BamH1 restriction enzymes and found were digested product.

Key Words: Resistant, Plasmid, *Klebsiella pneumoniae,* Restriction Digestion, MDR

INTRODUCTION

Urinary tract infection (UTI) is dangerous and unrecognized indication of systemic sepsis causing morbidity and mortality in all age of groups across the life space [1]. Urinary tract infection is serious health problem affecting millions of people each year and infections tend to arise from a wide range of bacteria [2]. Microorganism develops resistance to antibiotics because of continuously change in their chromosomal structure by acquiring the extra chromosomal genetic material from the resistant organism directly or indirectly [3]. The continuous observation revealed that *Klebsiella* has replaced *Escherichia coli as* the predominant urinary pathogen [4]. *Klebsiella pneumoniae* is frequently causing urinary tract infection and most strains are resistant to antibiotic treatment which poses serious problems [5]. The incidence of bacterial resistance mediated by β-lactamase has been reported in several African countries including Nigeria [6, 7]. In a recent survey the percentage of ESBL production in *Escherichia coli* and *Klebsiella pneumoniae* varies from 4.8% in Korea, 8.5% in Taiwan and 12% in Hong Kong [8]. Nursing home patients are important reservoir of extended spectrum of β - lactamase containing multiple antibiotic resistant *Escherichia coli* and *Klebsiella pneumoniae* wide spread of the plasmid mediated resistance in Iran [9]. The extended spectrum of β - lactamase are most often encoded on plasmids, which can easily be transferred between isolates [10]. The prevalence of *Klebsiella pneumoniae* producing IMP type MBLS in a university outbreak of *Klebsiella pneumoniae* carrying bla_{IMP}-8 in the intensive care units was recognized and thus a retrospective analysis of the cases from which the IMP 8 producing isolates were recovered was also conducted [11]. Extended spectrum of β - lactamase are typically plasmid mediated β-lactamases derived from existing TEM and SHV enzymes, capable of hydrolyzing oxymino-cephalospoorins and Azreonam and are inhibited by clavlanic acid are most often found in *Escherichia coli* and *Klebsiella pneumonia* are less commonly in *Proteus* species [12]. *Klebsiella pneumoniae* is an opportunistic pathogen and has been associated with various ailments such as urinary tract infection, septicemia, respiratory tract infection and diarrhea [13].

***Corresponding Author Address: TARIQ A. L,** Department of Biotechnology, Government Degree College, Beerwah-193411, Kashmir*
Corresponding author E-mail: tariqtasin@gmail.com

MATERIALS AND METHODS

Sample Collection: This study was carried out in the microbiology laboratory of Sree Amman Arts and Science College Chittode Erode, Tamil Nadu during the period of December 2014 to May 2015. A total of 120 urine samples were collected in sterile containers (5 ml bottle) form female of age 15 to 37 various admitted in the hospitals around the Erode District and brought to Microbiology Laboratory and stored at 4°C for the further study. Then the collected sample were brought into the laboratory immediately and those samples which were not processed were immediately stored in the refrigerator at 4°C.

Physical character of urine samples: Total number of urine samples that were studied was 120 and out of them 34 samples showed bacterial growth. The colour of urine was yellow bearing acidic hydrogen ion concentration.

Processing of urine samples: Urine samples were characterized for the physical appearance and followed by microscopic examinations for the presence of expected isolates.

Culturing methods: After sample collection they were processed on Blood agar and Mac Conkey's agar media. These media were prepared, sterilized and inoculated with urine samples. Then the culture plates were incubated at 37°C for 24 hours. After incubation, colonies obtained on solid media were studied further for identification by microscopic and biochemical method.

Microscopic examination: The microscopic examination such as Hanging drop method, Gram staining, Flagella staining and Capsule staining were performed for identification of isolates.

Biochemical characterization: The isolates were biochemically identified via Indole test, Methyl red test, Voges Proskauer test, Citrate test, Triple sugar iron test, catalase test and urease test.

Carbohydrate fermentation: The fermentation broth (with a specific carbohydrate such as Glucose, Lactose, Manitol, Sucrose, Sorbitol, Fructose and Mannose was prepared and poured in to the fermentation tubes containing Durham's tubes in an inverted position without any air bubble and sterilized at 121°C for 15 minutes. After sterilization the isolates were added to the fermentation broth and incubated at 37°C for 24 hours. After incubation result were noted for the colour changes in the fermentation broth.

Antimicrobial susceptibility test (Kirby-Bauer disk diffusion method): Antimicrobial susceptibility test was determined by Kirby-Bauer's disc diffusion method as per CSLI [14]. For this test, Mueller- Hinton agar plates were prepared. Sterile cotton swabs were dipped in the culture broth and then soaked swabs were rotated against the upper inside wall of the tube to remove excess fluid. The entire agar surface of the plate was streaked with the swab three times, turning plate at 60 degree angle between each streaking. The medium was allowed to dry for 60 minutes. Using antibiotic disc dispense following antibiotic discs Amoxycilinm (30µg/disc), Ampicillin (10µg/disc), Carbenicillin(100µg/disc), Cefotetan (30µg/disc), Cefprozil (30µg/disc), Cepodoxime (10µg/disc), Fosfomycin (200µg/disc), Enoxacin (10µg/disc), Gatifloxacin (5µg/disc), Levofloxacin (5µg/disc), Mecillinam (10µg/disc), Meropenam (10µg/disc), Piperacillin (100µg/disc) and Ticarcillin (75µg/disc) were released on to the surface of medium and gently pressed down with sterile bacteriological loop to get imping to the medium. All plates were incubated at 37°C for 24 hours and observed for zone of inhibition.

Extraction of Plasmid Bacterial DNA: The Plasmid DNA was extracted from the bacterial isolate by taking a single colony of bacterial colony was inoculated into the 50ml of Luria Bertani broth containing Ampicillin at (10mg/100ml) and incubated in rotatory shaker incubator for overnight at 37ºC [15]. Then 1.5 ml of culture was transferred into eppendroff tubes and spin at 8000rpm for 10 minutes. The supernatant were discarded and drained on tissue paper. The pellet was resuspended into 400ml of TE buffer, to this 32ml of lysosome was added and incubated at 370C for 30 minutes. After incubation 100ml of 0.5mM EDTA was added followed by 60ml of 10% SDS and 1.5ml of proteinase K (50ml/1ml) respectively then incubated at 500C for 60 minutes. After incubation tubes were brought at room temperature and 250ml of Phenol: Chloroform: Isoamylalchol in the ratio of 25:24:1 were added and centrifuged at 10000rpm for 10 minutes. The aqueous phase was transferred to another eppendroff tubes and RNAase was added at final concentration of 50ml/ml and mixture was incubated at 600C for 1 hour. After incubation DNA was precipitated with ice-cold ethanol and precipitated DNA was collected by centrifugation at 5000rpm for 10 minutes. Finally the pellet was washed with 70% ethanol and completely air dried and resuspended in 350µl of TE buffer at pH 8.0. The DNA samples were run in 0.8% agarose in 1X TEB buffer for electrophoresis were prepared by adding 5µl of gel loading dye in to the prepared plasmid sample and mixed well then the wells were loaded with 10 µl

of sample. Nearby well was loaded with 3µl of DNA markers provided (HIND III Digest). After loading, power pack was switch on and the voltage was adjusted to 50v or 100v. Electrophoresis was continued until the dye reaches to 1/3rd of the gel or above. Bands were observed under UV transilluminators

Restriction Digestion: The restriction digestion is carried out by taking mixture of 22 µg DNA, 2 µl of 10x digestion buffer of a final concentration of 1x, 1µl of restriction endonuclease containing 2 U of mixture of, EcoR I Hind III, Bam HI enzyme were used in a sterile eppendrop tube by using a micropipette and the total volume of the restriction mixture was adjusted to 20µl with TE buffer in a sterile eppendrop tube by using a micropipette. The parallel control was maintained in which only the enzyme has omitted. The DNA was used as the standard marker. All samples setup were incubated at 37°C for 3 hours. The reaction was stopped by adding 0.4 ml of a 0.5M solution of EDTA. After incubation the samples were added with 1/10 volume of loading buffer and then placed at 65°C for15 minutes in order to disrupt any concatamers that might have formed. The samples were run in 0.8% agarose gel by using IX TEB as running buffer. The gel was visualized by transilluminator to observe number as well as the size of the fragments restriction enzyme digestion [16].

RESULT

Sample collection: A total of 120 urine samples were collected from various hospitals around the Erode district of Tamil Nadu. Out of 120 urine samples were studied, growth of bacteria was obtained in 34 (28.33%) urine samples and no growth were found in 86 (71.66%) samples they found to be healthy. The infected patients were found to be the 15 to 37 years old.

Physical character of urine samples: Total number of urine samples that were studied was 120 and out of them 34 samples showed bacterial growth. The colour of urine was yellow bearing acidic hydrogen ion concentration.

Identification of isolates: The urine samples observed for gram staining were found gram negative rod shaped bacteria. Urine isolates were found non motile bacteria in hanging drop method. Under capsule staining it was observed capsulated bacteria.

Colony morphology: Good growth occurred on nutrient agar medium. Colonies were large, thick, grayish white, moist, smooth opaque or partially translucent discs Urine isolates produced lactose fermenting smooth pink colored colonies on Mac Conkey agar (Figure 1) that indicated ability to ferment the lactose in the medium. In Blood agar non hemolytic colonies were seen (Figure 2) that indicated it did not lysis the red blood cells.

Biochemical characterization: The isolates were biochemically identified by showing indole negative, methyl red negative , Voges Proskauer positive, citrate positive, TSI there was acid formation and gas formation in the slant and butt. In catalase test there was no any bubble formation that indicated negative result. In urease test there was colour change in the medium that indicated the positive result. These all biochemical identification supported for *Klebsiella pneumoniae* (Table 1).

Carbohydrate fermentation: The fermentation tests of specific carbohydrate such as Glucose, Lactose, Manitol, Sucrose, Sorbitol, Fructose and Mannose after incubated at 37°C for 24 hours showed colour changes in the fermentation broth in all said carbohydrates (Table 1).

Antimicrobial susceptibility test: Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method with the following set of antibiotics: Amoxycilinm (30µg/disc), Ampicillin (10µg/disc), Carbenicillin(100µg/disc), Cefotetan (30µg/disc), Cefprozil (30µg/disc), Cepodoxime (10µg/disc), Fosfomycin (200µg/disc), Enoxacin (10µg/disc), Gatifloxacin (5µg/disc), Levofloxacin (5µg/disc), Mecillinam (10µg/disc), Meropenam (10µg/disc), Piperacillin (100µg/disc) and Ticarcillin (75µg/disc) to detect extended spectrum beta lactamase in all 34 *Klebsiella pneumoniae*. Of the 34 *Klebsiella pneumoniae*, 06 (17.64%) were positive for extended spectrum beta lactamase production As per CLSI guidelines that extended spectrum beta lactamase production for *Klebsiella pneumoniae* (Table 2.)

Extraction of Plasmid Bacterial DNA: The plasmid DNA from the *Klebsiella pneumoniae* was extracted by alkaline lysis method and run through agarose gel electrophoresis. The bands were visualized under UV transilluminator which showed molecular weight of 5256bP for *Klebsiella pneumoniae* when compared with DNA marker. The plasmid profiles analysis have been shown a relationship between carriage of plasmids and antimicrobial resistance; the number and the size of plasmid in antibiotic resistance, isolates appeared to be higher than in susceptible isolates [Figure 3].

Restriction Digestion Analysis: The *Klebsiella pneumoniae* plasmid was digested with mixture of enzymes EcoR1, Bam Hl, Hind III yields

Fragments of DNA were observed under UV transilluminator for in the presence of multiple sites of these enzymes give rise to a ladder with the bands of following sizes Hind III restricted DNA 23.1, 9.4, 6.5, 4.3, 2.3, 2.0, 0.56kb, Hind III and EcoR I restricted DNA 2.2, 5.1, 4.9, 4.3, 3.5, 2.0, 0.56 kb.

DISCUSSION

In the present study I have identified *Klebsiella pneumoniae* a causative agent of urinary tract infection. The pervious literature shown prevalence of urinary pathogens and their susceptibility to certain antimicrobial drugs has been covered major regions of the country that of all positive urine cultures was mostly Enterobacteriaceae community [17]. The identified *Klebsiella pneumoniae* showed antibiotic resistance which poses a major clinical and public health challenge and spread of carbapenem-resistant Enterobacteriaceae [18].

After the initial report in 2001, *Klebsiella pneumoniae* first spread in the Northeastern United States, then spread worldwide, causing outbreaks in hospitals [19, 20]. At present, *Klebsiella pneumoniae* carbapenem (KPC) producing Enterobacteriaceae have been reported from at least 10 countries in 4 continents [21]. KPC producing organisms are typically resistant to multiple classes of antibiotics, including carbapenems, cephalosporins, fluoroquinolones, and aminoglycosides. Infections due to these pathogens have been associated with high mortality rates [22].

Although the majority of cases that have been reported were due to KPC type β-lactamase has also been identified in various species of Enterobacteriaceae, *Escherichia coli* and also *Pseudomonas* species [23]. The KPC gene is located on transposon *Tn*4401 on a transferable plasmid [24]. These genetic attributes likely facilitate dissemination of the KPC gene to the aforementioned species. The global spread of carbapenem resistant eenterobacteriaceae is increasingly recognized as an developing threat to public health an alternative approach are used to control on the antibiotic resistance shown by urinary pathogenic bacteria [25]. Of these pathogens, *Klebsiella pneumoniae* that produce KPC type β-lactamase has spread worldwide within a decade after its discovery. Most recently, KPC producing isolates that are resistant to colistin and tigecycline have also been reported [26]. On the other hand, under selective pressure from the use of broad-spectrum antibiotics, the KPC gene has spread not only geographically but also horizontally into other species of Enterobacteriaceae has been identified in multiple nursing homes [27].

The comparison of antibiotic resistance patterns of urinary pathogenic isolates revealed that gentamicin resistance increased significantly, from 13% to 48% nalidixic acid resistance increased from 16% to 47% and resistance rates to cotrimoxazole, ampicillin and tetracycline were very high (48% - 83%) in 1978 and remained high throughout the 23 year period [28, 29]. In early there was a slight increase in the incidence of *Escherichia coli* resistance to ESBL drugs over a period of three years, but there was a significant increase the resistance of *Klebsiella pneumonia* [30]. *Klebsiella pneumonia* strains showed 90% resistance to antibiotics and also reported that a particular plasmid is responsible for drug resistance and ESBL production [31]. The incidence of ESBL producing strains among clinical *Klebsiella* isolates has steadily increased over the years and account for 6 to 12% of all nosocomial UTIs [32, 33].

The multidrug resistant *Klebsiella* strain is unfortunately accompanied by a relatively high stability of the plasmids [34, 35]. Unlike multi drug resistant (MDR) strains, the sensitive strains did not contain any plasmid [36]. The resistant isolates harbor plasmids whole number and size are higher than those of plasmids of susceptible isolates; this shows that the resistance was acquired by genetic exchanges between bacteria in hospital environments and patients under antibiotics selective pressure [37, 38]. Thirteen different plasmid profiles associated with 12 resistance phenotypes and plasmid sizes ranges from 2kb to 59kb and common large plasmid (28kb) with similar digest patterns to Hind III restriction enzyme in 10 of 15 *Escherichia coli* with mostly similar antimicrobial resistance pattern [39].

CONCLUSION

The most striking finding from our study was the high prevalence of *Klebsiella pneumonia* resistance to almost all antimicrobial antibiotics Amoxycilinm, Ampicillin, Carbenicillin, Cefotetan, Cefprozil, Cepodoxime, Fosfomycin, Enoxacin, Gatifloxacin, Levofloxacin, Mecillinam, Meropenam, Piperacillin and Ticarcillin.

Table 1. Biochemical and carbohydrate fermentation tests for the urine isolates

Biochemical Test	Result	Carbohydrate Test	Result
Indole	Negative	Glucose	Positive
Methyl Red	Negative	Lactose	Positive
Voges Proskauer	Positive	Manitol	Positive
Citrate	Positive	Sucrose	Positive
TSI	Positive	Sorbitol	Positive
Urease	Positive	Fructose	Positive
Catalase	Negative	Mannose	Positive
Identified as	*Klebsiella pneumoniae*		

Table 2: Antibiotic sensitivity was done using the following antibiotics for the UTI Isolate *Klebsiella pneumoniae* as per CLSI guidelines

Antibiotics	Zone Diameter	Antibiotics	Zone Diameter
Amoxycilinm	>18	Enoxacin	>18
Ampcillin	>17	Gatifloxacin	>18
Carbenicillin	>23	Levofloxacin	>17
Cefotetan	>16	Mecillinam	>15
Cefprozil	>18	Meropenam	>16
Cepodoxime	>21	Piperacillin	>21
Fosfomycin	>16	Ticarcillin	>20

Figure 1. Mac Conkey agar medium showed lactose fermenting colonies of *Klebsiella pneumoniae*

Figure 2 Blood agar medium agar medium showed non haemolytic colonies of *Klebsiella pneumoniae*

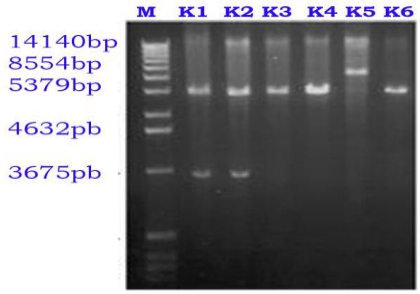

Figure 3. Plasmid DNA of the resistant isolates of *Klebsiella pneumoniae* where M-marks and K1, K2, K3, K4, K5, K6 are extended spectrum beta lactamase producing

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