Research Article

Genetic Variability Studies of Clinical Isolates of Lower Respiratory Pathogen *Klebsiella pneumoniae*

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Accepted on: 05-10-2014; **Finalized on:** 30-11-2014.

ABSTRACT

Klebsiella is one of the important opportunistic pathogens involved in respiratory tract infections. They cause various types of human infections leading to morbidity and mortality. Of the *Klebsiella* species, *Klebsiella pneumoniae* is the most prevalent in clinical specimens causing pneumonia. Around hundred sputum samples were collected from patients visiting various hospitals of Kanyakumari District, Tamil Nadu. From this clinical specimen, twenty five were identified preliminarily as *Klebsiella pneumoniae* by various morphological and biochemical tests. The identities of the bacterial isolates were confirmed by 16S rRNA sequence analyses. Antibiotic susceptibility testing was performed by the agar disc diffusion method. Variations were observed in the resistance pattern of the bacterial isolates towards various commercial antibiotics. The genetic variability of *Klebsiella pneumoniae isolates* were detected using RAPD assay. The RAPD assay using different primers showed distinct molecular variation among the twenty five isolates suggesting the emergence of new resistant strains among the clinical isolates.

Keywords: Antibiotic susceptibility, Genetic variability, *Klebsiella pneumoniae,* RAPD.

INTRODUCTION

Klebsiella species have been reported as opportunistic, worrisome nosocomial and community-associated pathogens.[1] These bacteria have become important pathogens in nosocomial infections[2] which have been well documented in United States and India.[3] Epidemic and endemic nosocomial infections caused by *Klebsiella* species are leading causes of morbidity and mortality.[4] It is very common among inpatients admitted in ICUs for various ailments. The widespread emergence of multidrug resistance among bacterial pathogens has become one of the most serious challenges in clinical therapy.[5] RAPD-PCR is one of the most rapid and simple methods that generate fingerprints and it can be applied to detect polymorphism in a wide variety of organisms. In RAPD-PCR, random primer sequences may be used in organisms where a specific genome sequence is not known. Random parts of the organism genome are produced, which are expected to be identical among related species, and so similar banding patterns should be produced in gel electrophoresis. This technology is proving to be quite useful in typing strains of bacteria involved in respiratory tract diseases.[6] The aim of the present study was isolation, biochemical characterization and identifying antibiotic sensitivity pattern of the clinical isolates of *Klebsiella pneumoniae* and to study its genetic variability patterns.

MATERIALS AND METHODS

Sample Collection

A total of 100 throat swab samples were aseptically collected from different patients visiting various multispecialty hospitals in Tamil Nadu using sterile cotton swabs. Immediately after collection the samples were inoculated into nutrient broth. These were then transferred to the Microbiology laboratory, Noorul Islam University, Kumaracoil, Tamil Nadu.

Isolation and Identification of *Klebsiella pneumoniae*

In the laboratory under aseptic conditions, the collected specimens were streaked directly on blood agar and MacConkey agar and incubated for 24 hrs at 37°C. Characteristic colonies from the plates were isolated and then sub cultured to obtain a pure culture. The isolated organisms were identified based on colonial morphology, and various biochemical tests according to standard laboratory methods.[7] Stock cultures were maintained in both agar slant and 20% sterile buffered glycerin. The non hemolytic opaque creamy colonies on blood agar and non lactose fermenting colonies on MacConkey agar were sub cultured on MacConkey agar and incubated for 24 hours at 37°C[8] for further use.

Genomic DNA of the bacterial species was isolated and the 16S rRNA region of the DNA was amplified using universal 16SrRNA primers in thermal cycler. The 16S rDNA sequence of the bacterial isolate was subjected to BLAST analysis using NCBI BLAST tool at www.ncbi.nlm.nih.gov.

Antibiotic sensitivity tests

Antibiotic sensitivity tests were performed using disc diffusion method.[9] Commercially available antibiotic discs of Imipenem, Ceftriaxone, Refampicin, Gentamycin and Amikacin were used for the determination of drug sensitivity. Inhibition zones developed around the discs

were measured in millimeter (mm) using a metric ruler according to Clinical Laboratories Standards Institute.[10]

DNA extraction and PCR amplification

Amplification of genomic DNA was made on an Agilent cycler 2200 (Germany), using the arbitrary decamers.[11] RAPD primers were purchased from Eurofins, Germany (Table 1.0); these primers included OPA-1, OPA-2, OPA-3 and OPA-4. Amplifications of genomic DNA were performed in 25-µl reaction mix containing 1.2 units of Taq polymerase (Sangon, Shanghai, China), 10 mM Tris-HCl (pH 9.0), 25 mM KCl, 2 mM MgCl 2, 0.2 mM of each dNTP, 24 ng each of random primer and 40 ng of template DNA. RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera. DNA from each band was amplified with the same primer more than once, and the banding patterns were compared.[12]

Table 1: RAPD primers

Primer Name	Sequence
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG

Cluster Analysis and Construction of Phylogenetic tree

The presence or absence of each individual band of the DNA from RAPD analysis was recorded for each lane on the gel representing a given sample. The data thus obtained was analyzed by Neighbour – joining method and the binary output was used to generate a phylogenetic tree for the sixteen bacterial isolates.[13]

RESULTS AND DISCUSSION

Isolation and Identification of *Klebsiella Pneumoniae*

Of the 100 bacterial isolates collected from patients only 25 strains were identified as *Klebsiella pneumoniae* in the preliminary biochemical analysis were taken for further studies. Morphology of *Klebsiella pneumoniae* identified were large, rod-shaped, mucoid colonies on blood agar and lactose fermenting colonies on Mac Conkey agar. Non motile, Gram negative, short plump, straight rods were seen. The biochemical tests such as indole production, methyl red, voges-proskauer oxidase, Acid and gas production from glucose, lactose sugar fermentation tests, citrate utilization, urease, nitrate reduction and catalase revealed the identity of the Klebsiella isolates.[14] The results of biochemical tests were presented in table 2.

The 16SrRNA sequence of the one isolates among the twenty-five isolates revealed 798 base pairs and the BLAST analysis of the sequence confirmed the identity of the bacterial pathogen as *K.pneumoniae.*

Table 2: Biochemical tests for the strains of *Klebsiella pneumoniae*

Biochemical tests	Result
Lactose	+
Glucose	+
Indole	-
Methyl red	-
Vogus proskauer	+
Citrate	+
Urease	+
Nitrate	+
Oxidase	-
Catalase	+

Antibiotic Sensitivity tests

Antibiotic resistance is a major clinical problem in treating infections caused by these microorganisms. The resistance to the antimicrobials has increased over the years. Resistance rates very from country to countries.[15,16] Antibiotic sensitivity testing was done for all the isolates on Muller – Hinton agar by modified Kirby – bauer disc diffusion technique. The antimicrobial agents that were tested against the pathogen include the Imipenem, Ceftriaxone, Refampicin, Gentamycin and Amikacin. Aminoglycosides are prently considered to be the alternative antimicrobial agents for better treatment of *Klebsiella pneumoniae* infection in this part of the country.[17] In the present study, *K. pneumoniae* isolates were sensitive towards Refampicin followed by Ceftriaxone, Amikacin Gentamycin and Imipenem. *Klebsiella pneumoniae* showed least resistance to Rifampicin. The antibiotic susceptibility of *Klebsiella pneumoniae* isolates are listed in table 3.

RAPD Analysis

Genomic DNA was isolated from twenty five clinical isolates of *Klebsiella pneumoniae* and the DNA was separated in Gel electrophoresis to assess their purity. The isolated DNA samples of the twenty five clinical isolates of *Klebsiella pneumoniae* were amplified in PCR and the PCR amplified products of the DNA of the twenty five isolates were subjected to RAPD analysis using the four different primers (Figure 1). RAPD is a new tool that is being used in such studies. The simplicity and wide applicability of the method are dependent of on the use of short nucleotide primers which are not related to known DNA sequences of the target organism. Genetic mapping and determination of the degree of relatedness between strains have been performed, with validation by ribotyping.[18,19]

Cluster Analysis

Phylogenetic diversity between the twenty five clinical isolates of *Klebsiella pneumoniae* were determined by converting RAPD data into similar matrix and analyzed by

Neighbour – joining method to produce a phylogenetic tree (Figure 2). However, differences evident through the visual examination of the polymorphic bands of the DNA were further clarified by cluster analysis of the RAPD data. The cluster analysis revealed four primary clusters. The isolates 25, 2 and 3 were distantly related to isolates 1 and 15, whereas isolates 4, 16 and 6 and 17, 24 and 14 showed definite patterns. The distance matrix similarity ranged from 0.32 – 0.96.

Table 3: Antibiotic Susceptibility of *Klebsiella pneumoniae*

Isolate of *Klebsiella pneumoniae*	Zone of Inhibition in mm				
	Imipenem	Ceftriaxone	Rifampicin	Gentamycin	Amikacin
K1	13	18	19	13	16
K2	20	22	23	22	23
K3	19	21	25	21	22
K4	17	22	22	20	21
K5	17	22	25	20	23
K6	16	25	25	21	23
K7	10	24	24	20	20
K8	9	21	22	21	23
K9	8	17	25	19	20
K10	9	21	23	20	22
K11	8	18	26	21	24
K12	9	21	24	19	20
K13	10	22	25	20	22
K14	10	18	21	15	11
K15	13	20	24	13	16
K16	21	22	25	22	24
K17	8	17	25	20	22
K18	11	16	19	15	12
K19	10	15	24	12	22
K20	9	17	26	19	23
K21	10	20	25	20	19
K22	9	20	23	21	22
K23	8	16	25	23	24
K24	10	18	22	15	11
K25	18	22	24	21	20

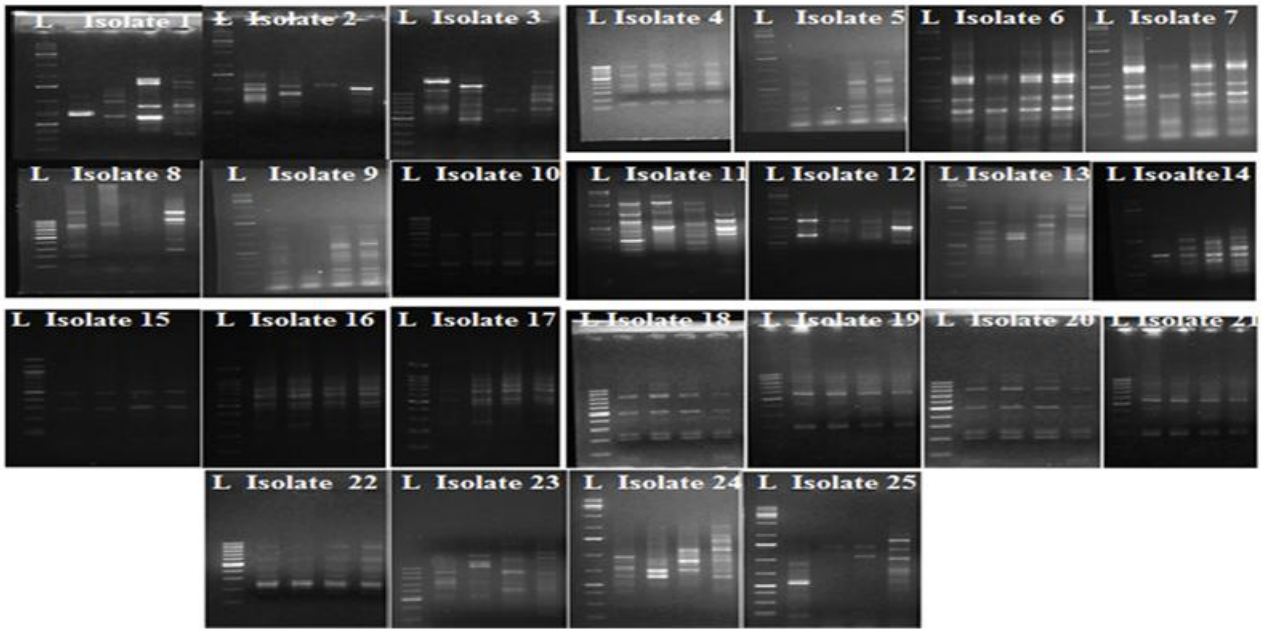

Figure 1: RAPD profile of clinical isolates *of Klebsiella pneumoniae*

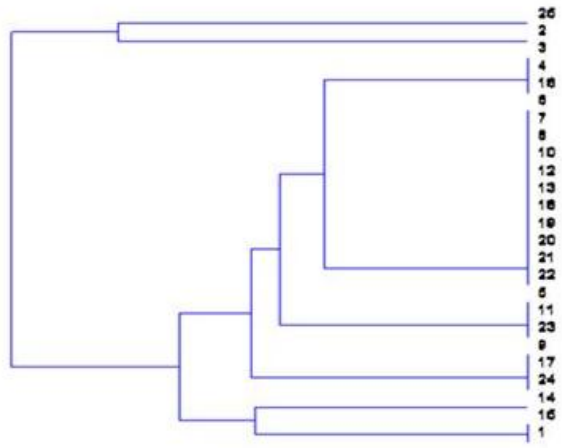

Figure 2: Dendrogram showing the phylogenetic diversity of twenty five clinical isolates of *Klebsiella pneumoniae*

The epidemiological typing of twenty five *K. pneumoniae* isolates was carried out by phenotypic and molecular methods RAPD analysis indicated that pathogenic *K. pneumoniae* isolates comprise a genetically high variable group of organisms.[21] The pathogenic *Klebsiella pneumoniae* strains were highly heterogenous, based on the distribution of different nucleotide sequences.[22] The high number of serotypes in *K.pneumoniae*[23] could also explain the relevant degree of genetic diversity highlighted by RAPD. RAPD analysis revealed genetic heterogeneity in different isolates of the test bacteria. This can be useful to understand the distribution of these pathogens in nosocomial infections.[20]

CONCLUSION

Klebsiella pneumoniae is one of the most serious lower respiratory and nosocomial pathogen and it is resistant to many of the existing antibiotics. Emergence of drug resistant strains has posed serious concerns to the civil society. Hence ascertaining such drug resistant strains and desigining new drugs is need of the hour. Hence in the present investigation, an attempt has been made to isolate and to study the antibiotic sensitivity pattern of *Klebsiella pneumoniae.* The study concludes that there exists genetic polymorphism among the isolates and there are chances of emergence of new strains in the future which may be resistant to the Aminoglycosides which are presently used for the treatment of the pathogenic bacteria. Hence, the scientific community should have a watchful eye on the genotypic variants of the this lower respiratory tract bacteria and should attempt to design a drug using insilico tools which can control the existing and new strains of this pathogenic bacteria.

Acknowledgement: The authors thank the Principal, Mr.Government Arts College and the Chairman of Noorul Islam University for their technical support.

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Source of Support: Nil, **Conflict of Interest:** None.