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Research Article

Molecular Identification and Analysis of Multi-Drug Resistant *Klebsiella pneumoniae*

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Abstract

Multidrug resistant *Klebsiella pneumoniae* was resistant to various antibiotics which are commonly used to treat against the bacterial infections, and is now emerged as a great risk. Antibiotic susceptibility tests were performed to determine the scope of drug resistance of the bacteria. Further studies regarding the responsible genetic material were performed by Polymerase Chain Reaction and RFLP techniques. The remedial measures for treating these bacteria were studied with the help of metabolites obtained from various strains.

Keywords: *Klebsiella pneumoniae*; Antibiotic susceptibility tests; PCR; RFLP; Remedial measures

Introduction

The bacteria resistant to antibiotics are an increasing threat to public health all over the world. Generally, β-lactam antibiotics such as penicillin (ampicillin, amoxicillin, carbenicillin, methicillin, etc.) cephalosporin (cefepime, cefixime, cefuroxime etc.) prevents the peptidoglycan synthesis to act against the bacteria (Elsayed *et al.,* 2016). But these multi-drug resistant bacteria can grow in the presence of two or more antibiotics, due to the presence of resistance gene in their genomes. A recent estimate in US depicts that Methicillin-resistant *Staphylococcus aureus* (MRSA) contribute to higher death rate than HIV (Bancroft, 2007). The antibiotic resistant bacteria cannot be killed by the commonly used antibiotics. Prolonged use of antibiotics results in the acquisition of antibiotic resistant gene to the bacteria, causing the change in the bacterial membrane, and modifies the receptor sites (Hassan *et al.,* 2014). The main threat of the antimicrobial resistance is in the treatment of Diabetic Foot Infections (DFI). The Multidrug resistant property is due to the inappropriate antibiotic treatment, chronic stage wound infection, frequent exposure to the MDR environment, abuse of antibiotics (Kumar and Shahi, 2016).

The spread of these antibiotic resistant gene is facilitated by the presence of class I integron, plasmids and transposons. Class I integrons are found in many multidrug resistant bacteria, especially Enterobacteriaceae. These genetic elements play an important role in the carriage and dissemination of antibiotic resistant gene by incorporating additional cassettes (Kem *et al*., 2005). These can be seen in gram-negative Enterobacteriaceae such as *Klebsiella*

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pneumoniae, Salmonella, E. coli, Shigella, etc. *Klebsiella pneumoniae* is an important pathogen in nosocomial infections. The virulence effect of *K. pneumoniae* is due to their capsular serotype, lipopolysaccharide, adhesions and iron acquiring systems. The iron chelator siderophore allows the bacteria to gain the protein bound iron from the host organisms. These multidrug *K.pneumoniae* infections are intricate to treat with the limited available antibiotics (Wasfi *et al.*, 2016).

Molecular typing and virulence analysis of clinical isolates are the prevailing methods to disclose the effective methods against the multidrug resistant *K.pneumoniae* infections. In the present investigation the multidrug resistant *Klebsiella pneumoniae* was used to study the molecular characterization of gene mediated characters and an alternate medicine against the pathogens.

Materials and Methods

Source of Pathogens

The pathogens were obtained from clinical laboratory of Sneha clinical laboratory Coimbatore -8, Tamilnadu, India. Aseptically transferred to the research institute and subcultured in nutrient broth for further study.

Hi Comb Assay Test

Hicomb assay method was done to identify the antibiotic susceptibility of the sample against methicillin comb with the concentration of 0.001 to 4µg. To the prepared Mueller hinton agar the culture of *K.pneumoniae* (60µl) was swabbed and followed by the comb was placed and incubated 37ºC for 24hrs. After incubation the zone of inhibition was measured.

Antibiotic Susceptibility Test

Antibiotic susceptibility of *Klebsiella pneumoniae* isolates were determined by disc diffusion method using 10 selected antibiotic discs namely CTX- Cefotaxime (30mcg); CAZ-Ceftazidine (30mcg); E- Erythromycin (15mcg); LE-Levofloxacin (15mcg); AK- Amikacin (30mcg); C-Chloramphenicol (30mcg); CFM- Cefixime (5mcg); AMP-Ampicillin (2mcg); CIP- Ciprofloxacin (5mcg); GEN-Gentamicin (10mcg). The bacterial isolates were inoculated into the medium and swabbed the culture followed by placed the discs onto the surface of the inoculated Mueller hinton agar plates. The plates were incubated for about 24 hrs at 37°C and the zone of inhibition was measured for every individual antibiotic disc.

DNA Isolation

2 ml of the bacterial culture was transferred to an eppendorf tubes and was centrifuged at 5000 rpm for 5 minutes. The pellet was suspended in 500 µl of extraction buffer and 10 µl of lysozyme to lyse the bacterial cell wall. It was then mixed well and incubated at room temperature for 30 minutes. After incubation, 150 µl of 10% SDS was added, mixed well and incubated at 65 °C for 30 minutes. Phenol chloroform isoamyl alcohol was added to it and centrifuged at 10,000 rpm for 10 minutes. The aqueous layer was collected and added 0.2 volume of sodium acetate and 5 volumes of isopropanol, the tube were allowed for centrifugation at 10000 rpm for 10 minutes. After the DNA precipitated, 500 µl of absolute ethanol and 70% ethanol was added and centrifuged at 5000 rpm for 5 minutes. The final pellet obtained was air dried and then diluted using 1X TE buffer, it was then followed by Agarose Gel Electrophoresis.

Plasmid Isolation

2 ml of the bacterial culture was transferred to an eppendorf tubes and was centrifuged at 5000 rpm for 5 minutes. The pellet was suspended in 100 µl of solution A containing 50mM of glucose, 25mM of Tris HCl and 10mM of EDTA along with 20 µl of lysozyme to lyse the bacterial cell wall. It was then mixed well and incubated at room temperature for 30 minutes. After incubation, 200 µl of solution B containing 1% SDS and 0.2N NaOH was added, vortexes for 5-10 minutes and incubated in ice box for 10 minutes. 150µl of 3M Sodium acetate was added and again incubated in icebath for 30 minutes to 1 hour. The cell suspension is centrifuged at 10,000 rpm for 10 minutes to degrade the DNA. 2 volumes of isopropanol was added to the supernatant and centrifuged at 10,000 rpm for 10 minutes to collect the pellet. To the pellet 40 µl of 3M Sodium acetate and 700 µl of absolute ethanol was added and incubated initially at deep freezer for 10-30 minutes and centrifuged at 6000 rpm for 6 minutes. The pellet was collected and 700 µl of 70% ethanol was added and centrifuged at 5000 rpm for 5 minutes to purify the plasmid. The final pellet obtained was air dried and added 30 µl of 1X TE buffer, it was then visualised by loading in Agarose Gel Electrophoresis.

PCR Amplification of SHV Gene

The presence of a particular gene was determined by performing the Polymerase Chain Reaction. 20 µl of reaction mixture containing 2 µl of the DNA, 8 µl of the PCR master mix, 6 µl of nucleus free water, 2 µl of forward primer and 2 µl of reverse primer was taken (Table 1). The PCR master mix contained equal amount dNTP, MgCl and Taq Polymerase. The PCR was performed for the samples with suitable condition and was followed by Agarose Gel Electrophoresis.

Restriction Fragment Length Polymorphism for Plasmid

The presence of unique patterns of the restriction site was determined by Restriction Fragment Length Polymorphism. The reaction mixture contained 2 µl of plasmid DNA, 3 µl of restriction enzymes containing EcoR1, Bam H1, Hind III of each 1 µl, 2 µl of 10X assay buffer. The mixture was incubated for 3 to 4 hours at 37°C. After incubation the reaction mixture was mixed with loading dye and loaded in Agarose Gel Electrophoresis.

Table 1: Primer used for the Polymerase Chain Reaction

Gene	Primer Sequence	Amplicon size (bp)	Reference
SHV	F: 5'-TACCATGAGCGATAACAGCG-3'	450	8
	R: 3'-GATTTGCTGATTTCGCTCGG-5'		
Mec	F: 5'-GTTGTAGTTGTCGGGTTTGG-3'	533	9
	R: 5'-CCACCCAATTTGTCTGCCAGTTTCTCC-3'		

Alternative Remedy by Well Diffusion Method

The alternative remedy for the multidrug resistant *Klebsiella pneumoniae* was studied by using various microbial components and commercial antibiotics. To determine the antibacterial activity of these components, Mueller Hinton Agar medium was prepared by sterilization and poured into sterile petri plates. The plates were allowed to solidify at room temperature and Methicillin resistant *Klebsiella pneumoniae* was swabbed. Mean while, *Bacillus*, *Lactobacillus* and *Actinomycetes* strains were centrifuged and the supernatant were taken to obtain the secondary metabolites. Wells were cut out from the agar plates using a sterile stainless steel bore and filled with the supernatants of the above mentioned organisms in the respective wells. A commercial antibiotic, Levofloxacin disc was also placed on the medium. Then the plates were incubated at 37°C for 24 hours. After incubation, the zone of inhibition was measured using a measuring scale (Susan *et al.*, 2017).

Results and Discussion

HiComb Assay Test

The *Klebsiella pneumoniae* strains were tested against the HiComb - Methicillin comb. 8 concentrations of Methicillin were shown resistance against all the 8 concentrations. This was given in Fig. 1.

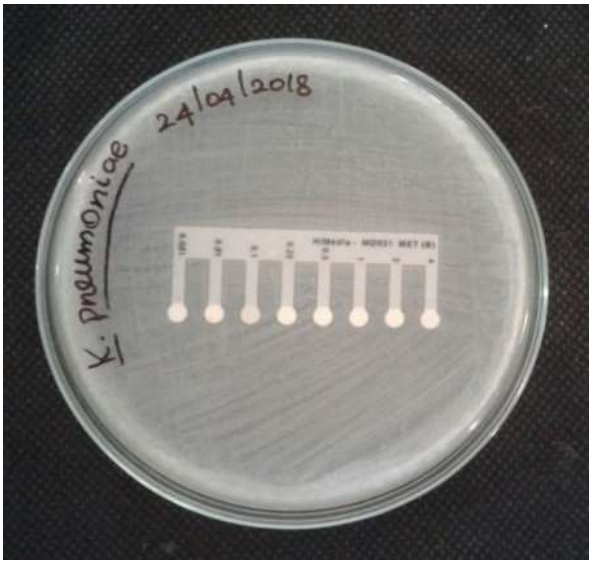

Fig. 1: HiComb assay using Methicillin comb, showing no zone of inhibition against *K.pneumoniae*

Antibiotic Susceptibility Test

Totally ten commercially available antibiotics were tested against the experimental organism of *Klebsiella pneumoniae* and the organism shows resistance against all the antibiotics. No clear zones were formed for any of the antibiotics.

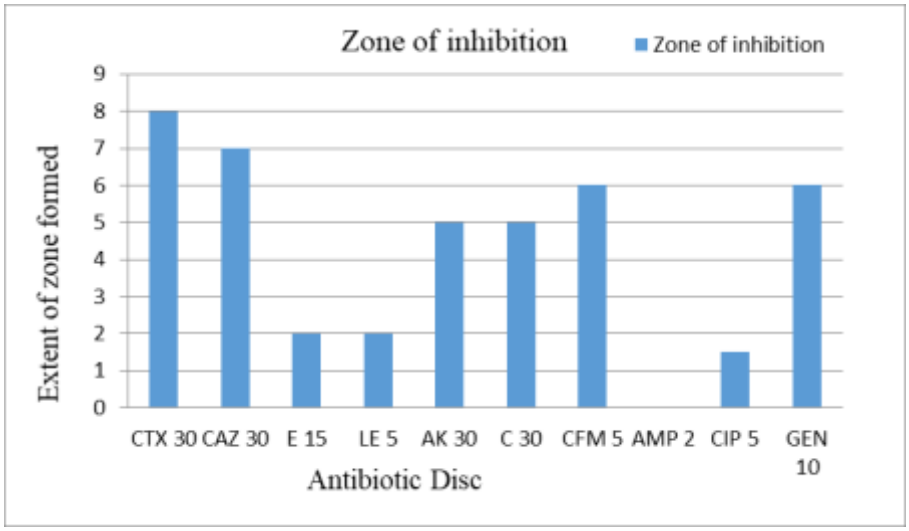

Fig. 2: Response of *Klebsiella pneumoniae* against various antibiotics

Klebsiella pneumoniae is a main causative agent for many severely impaired diseases in middle-aged and old people, causing respiratory infections, bronchitis and bronchopneumonia. Many studies depicts that these *Klebsiella* species are resistant to most of the antibiotics and is found that the antibiotic resistance is due to the bacterial plasmids (Husdon *et al.*, 2014). Many evident show that frequent use of any particular antibiotic, makes the organism resistant against the antibiotic (Falagas *et al.*, 2009).

Fig. 3: Antibiotic Susceptibility assay, using 10 commercial antibiotic discs

In our study, the *Klebsiella* strain shows resistant against various commercial antibiotics, in which many of them are completely resistant. Thus the secondary metabolites from various microbial strains were used and better results were seen and are compared with the response for the commercial antibiotic Levofloxacin, representing in Fig. 2 and 3. In a recent study, secondary metabolites from plants were extracted and were tested for antimicrobial activity against various organisms and found effect against the *Klebsiella* species (Compean *et al.*, 2013).

DNA Profile of the Klebsiella Isolates

The DNA extraction was performed by using Rapid Cell Lysis method. The extracted DNA was examined by using Agarose Gel Electrophoresis. The DNA band formation is depicted in the Fig.4.

Fig. 4: DNA profile of *Klebsiella pneumoniae*

Plasmid Profile of the Klebsiella Isolates

The plasmid isolation was performed for ampicillin resistant *Klebsiella* isolates. The extracted plasmid was loaded into 1% agarose and is electrophoresed. The band formation is shown in the Fig.5.

Fig. 5: Plasmid profile of *K. pneumoniae*

DNA Amplification by PCR

The DNA extracted by rapid cell lysis method was then amplified by Polymerase Chain Reaction using SHV and mecA primers. The Lane 1 was loaded with the PCR marker, the lane 2 and 3 were loaded with PCR product along with the SHV and mecA primer respectively. The molecular weight of the amplified gene was found to be for SHV and for mecA (Fig. 6).

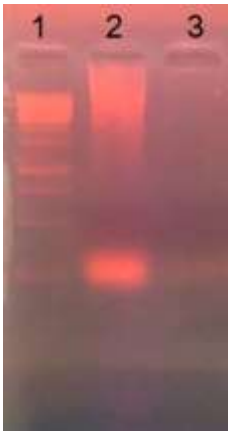

Fig. 6: DNA amplified using Polymerase Chain Reaction

[Lane 1: 100bp DNA ladder : Lane 2 SHV gene : lane 3 : Mec gene]

Plasmid Study by RFLP

The restriction sites present in the bacterial plasmid was studied by RFLP techniques. The Lane 1 is loaded with the marker and the lane 2 is loaded with the extracted plasmid treated with the enzymes EcoR1, Bam H1 and Hind III (Fig.7).

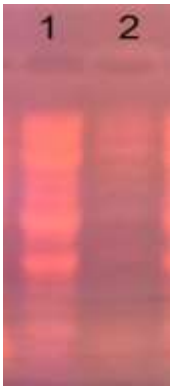

Fig. 7: RFLP - Lane 1 : DNA ladder (1kb) ; Lane 2 ; RFLP products

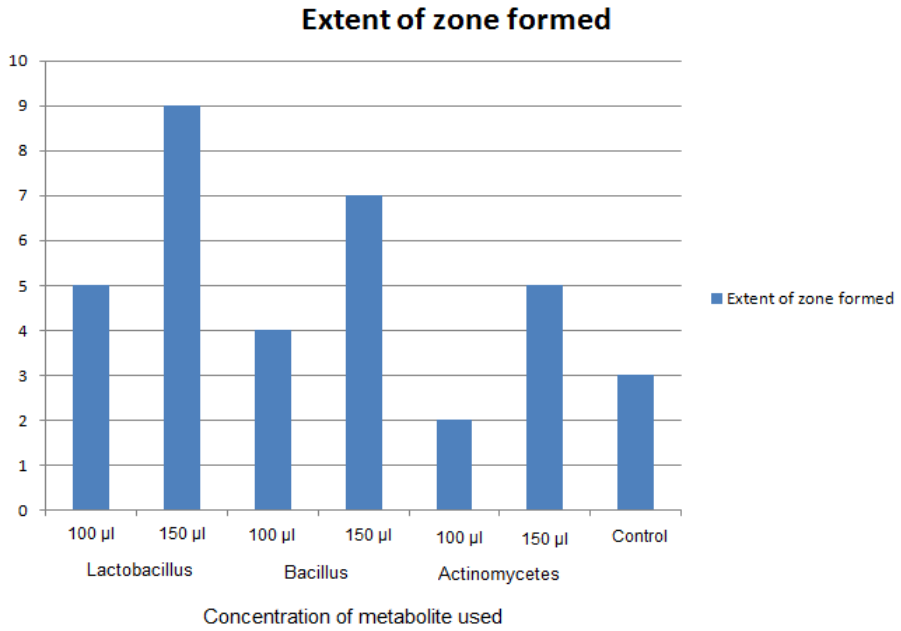

Fig. 8: Activity of *K. pneumoniae* for various secondary metabolites

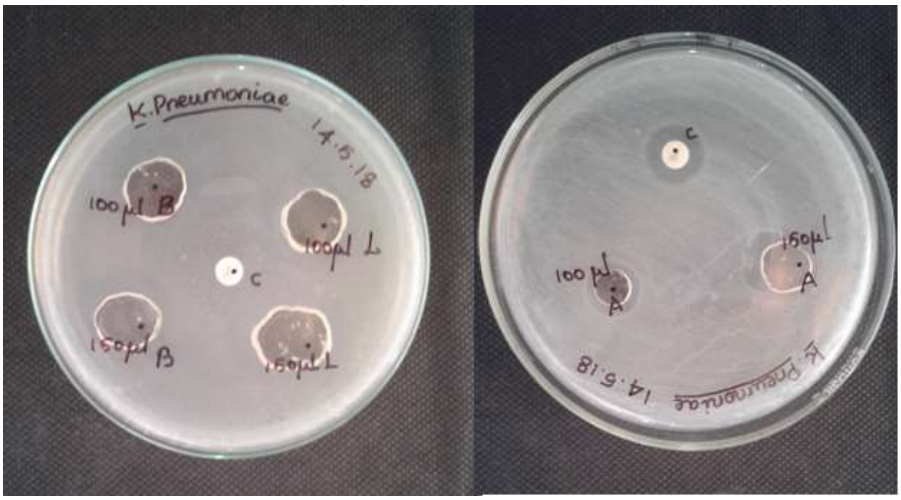

Fig. 9: Antibiotic ability of secondary metabolites against *Klebsiella pneumoniae*

Alternative Remedy

K. pneumoniae shows resistance against all the commercial antibiotics, the secondary metabolites obtained from various organisms were used and tested against *Klebsiella* spp. Initially when it was tested for lower concentrations of the metabolites, it showed no effect against the organism. But when tested with greater concentration of 100 µl and 150 µl, zone of inhibition was formed. Though a clear zone is not formed, a little effect of the secondary metabolite against the *Klebsiella* organism was observed, this was shown in Fig. 8 and 9.

Conclusion

Klebsiella pneumoniae playing a major role worldwide burden of antibiotic resistance. The *Klebsiella* spp. showing different type of resistant character based upon the geological variation and the cultural populations. The present study also mentioning this types of issues with the isolated *K.pneumoniae*, it showing resistant activity against the used antibiotics. Molecular level identification is also giving a clearance about these findings. Alternative source also tried to inhibit the bacteria and showing resistant character.

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