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Anti-*Klebsiella pneumoniae* Activity of Actinomycetes Isolated from Soil

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ABSTRACT

Klebsiella pneumoniae is a gram negative bacteria belongs to Enterobacteriaceae family and a common cause of hospital acquired,blood borne,respiratory and urinary tract infections. The hyper-virulent strains of Klebsiella pneumoniae are emerging due to increased drug resistance and thus posing a serious threat and challenge in treatment of associated infections intensifying the need for novel bioactive molecules. In view of this, in present study 20 actinomycete isolates from rhizospheric soil were screened against clinical isolate of Klebsiella pneumoniae using agar well diffusion assay. The potential isolate AT15 showing highest zone of inhibition (14mm) was selected and identified at molecular level as Streptomyces ferralitis MMS8. The ethyl acetate extract of culture broth of Streptomyces ferralitis MM8 obtained after seven days of incubation at 30°C and 120 rpm showed considerable inhibition of Klebsiella pneumoniae,when compared with standard antibiotics available in the market . The study indicated the potential of bioactive extract of Streptomyces ferralitis MMS8 against Klebsiella pneumoniae after further purification and characterization studies.

Key words *– Bioactive molecules, blood –borne infections, UTI, Streptomyces.*

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INTRODUCTION

There are numerous Klebsiella species, and their infections are often recorded. Klebsiella pneumoniae has recently emerged as a significant pathogen in hospital-acquired infections [1]. In the 21st century, critical infections brought on by pathogenic bacteria resistant to widely used antibiotics in the market [2] becoming a severe healthcare issue. Antibiotic resistance has been created in many bacterial infections as a result of overuse and incorrect application of antibiotics, according to the World Health Organization. Today, drug-resistant pathogenic microorganisms appear more frequently than new antibiotics are discovered. Many scientists working in the pharmaceutical industry have been actively interested in isolating and screening actinomycetes from various uncharted habitats for the production of bioactive secondary metabolites in an effort to solve this issue [3]. Actinomycetes are significant category of microbes that colonise soil. Numerous bioactive secondary metabolites that are significant for industry are known to be produced by them. Actinomycetes are known to produce about 80% of all antibiotics, with the bulk coming from the genera Streptomyces and Micromonospora [4]. The majority of antibiotics in the market are derived from fungus and actinomycetes' natural compounds [5-6]. Actinomycetes have been isolated from both the sea environment and the soil. Only a limited fraction of the world's surface has been sampled due to the fact that many researchers in the pharmaceutical sector have screened soils, and very few actinomycetes species have been identified [8]. The major goal of the current investigation was to identify possible actinomycetes that could compete with drug-resistant Klebsiella pneumoniae in the rhizopheric soil sample. The World Health Organization recently issued a warning to the public stating that the global rise in bacterial antibiotic resistance poses a significant threat to healthcare. If we didn't move right once to address the issue of antimicrobial drug resistance, antibiotics might stop working as effective medicines to treat illness.

MATERIALS AND METHODS

Isolation of Actinomycetes from Rhizospheric soil of *Ficus religiosa*

Collection of soil sample

Rhizospheric soil sample was collected from rhizospheric region of *Ficus religiosa* cultivated in Nanded, M.S. India. The soil was collected from top 10-15 cm region after removing recognizable stones and debries in sterile polythene bags and stored at 4°C until processed for the isolation of actinomycetes.

Isolation of Actinomycetes

Ten g oven dried soil (60°C / 30 min) was suspended in 90 ml of sterile distilled water and mixed properly. From this tube, 1 ml of dilution was taken and added in a second tube containing 9 ml distilled water (10^{-1}). In this manner, remaining tenfold serial dilutions were prepared up to 10^{-7} · 0.1 ml of soil suspension from dilution tubes 10^{-3}to10^{-6} were spread on the surface of sterile starch casein agar (SCA) plates supplemented with 50µg/ml each of cyclohexamide, nystatin and rifamycin as antifungal and antibacterial agents respectively. Plates were incubated at 30°C up to seven days and observed regularly for appearance of morphologically distinct colonies. The 20well isolated, morphologically distinct colonies were selected and purified by repeated sub culturing on SCA plates. Selected colonies were transferred on SCA slants and incubated under 30°C. The slants containing pure culture were stored at 4°C until processed further. Subsequently, a total of 20 isolates were used for next studies.

Extraction of Bioactive compounds

The actinomycetes isolate (AT-1 to AT-20) was taken in 50 ml of soyabean casein digest broth supplemented with 1% dextrose in 250 ml capacity conical flask under sterile condition and incubated at 30°C for 7 days 130 rpm on rotary shaker. After incubation, the fermented broth was centrifuged at ten thousand rpm for 30 min to separate biomass and the supernatant was used for extraction of biologically active metabolites, Ethyl acetate was added to the cell free supernatant in 1:1 ratio in separating funnel and shaken vigorously for 30 min, The organic layers were collected, evaporated the organic solvent and dissolved dried crude extract in Dimethyl sulfoxide.

Microorganism and Media

Bacterial Pathogen (*Klebsiella pneumonia*) used in this study were procured from Department of Microbiology, Dr. Shankarrao Chavan Govt Medical College, Vishnupuri, Nanded.*Klebsiella pneumonia* was cultivated and maintained on nutrient agar slant. selected test pathogen checked for antibiotic resistance in laboratory.

Inoculum preparation

Colonies of *Klebsiella pneumoneae* were inoculated into 10 ml of Nutrient broth.

Antimicrobial susceptibility testing

The antibiotic susceptibility pattern of*klebsiella pneumoniae* was evaluated by Kirby-Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) based on the zone of inhibition. The active culture of *klebsiella pneumoniae* was prepared by inoculating small growth in Nutrient broth. The culture was grown at 37°C for 24 hours and 0.5 McFarland Standards tube was used as reference for adjusting cell density. The culture was spread on Mueller Hinton agar(MHA) plates and standard antibiotic discs(conc.30mcg/ml) were kept aseptically on nutrient agar surface and incubated for 24hrs at 37 °C. After 24hrs of incubation zone of inhibition was measured in millimeter.

Antibacterial activity of actinomycetes

Agar well diffusion assay

Cell density of *klebsiella pneumoniae* was adjusted by comparing 0.5 McFarland turbidity standards and inoculated 10µL culture on MHA plates by using sterilized glass spreader ,agar well were prepared by using sterilized cork borer of 5mm , and 100µL of each crude extract (conc. 30mcg/ml) was poured into wells and plates were incubated for 10 min at 4°C for diffusion of extract, after that Plates were incubated at 37°C for 24h ,inhibition zones were measured in millimeter.

RESULT AND DISCUSSION

Out of twenty actinomycetes isolated on starch casein agar,one ethyl acetate extract of culture broth of *Streptomyces ferralitis MMS8*(AT15) was inhibiting maximum growth of drug resistant *Klebsiella pneumoniae*,when compared with other ethyl acetate extract of culture broth of *Streptomyces ferralitis MMS8*.

Table1 : Antibacterial activity of actinomycetes against *Klebsiella pneumoniae*

Sr. No.	Antibiotics	Zone of inhibition in mm Conc.30mcg/ml	Actinomycetes Isolate(extract)	Zone of inhibition in mm
1	Ceftazidime (CA)	R	AT 1	11mm
2	Erythromycin (E)	R	AT 2	8mm

3	Ampicillin (AMP)	R	AT 3	NI
4	Amoxicillin (AM)	R	AT 4	NI
5	Oxacillin (OX)	R	AT 5	NI
6	Cefotaxime (CTX)	R	AT 6	NI
7	Carbencillin (CB)	R	AT 7	10mm
			AT 8	10mm
			AT 9	10mm
			AT 10	10mm
			AT 11	1mm
			AT 12	10mm
			AT 13	9mm
			AT14	8mm
			AT15	14mm
			AT16	9mm
			AT17	9mm
			AT18	10mm
			AT19	11mm
			AT20	12mm

(Note : NI stand for- no inhibition , R stand for - resistant)

Identification of the promising isolates, PCR Amplification and sequencing of 16*S* rRNA gene:-

National Center for Cell Sciences in Pune provided confirmation of the 16S rRNA. The genomic DNA of a promising isolate was extracted using a DNA isolation kit purchased from Qiagen Biopharma, and the experiment was carried out in accordance with the manufacturer's instructions. Using universal oligonucleotide primers hybridising at positions 8–27 and 1488–1511 in relation to the E. coli 16S rRNA numbering system, the 16S rRNA of a promising isolate was amplified using PCR. The PCR reactions were conducted in a PE 9700 thermal cycler for a promising isolate (Perkin Elmer, USA).

>*Streptomyces ferralitis MMS8*(retrieved sequence):

AACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAGCCCTTCGGGGTGGATCAGTGGCGAACGGGTGA
GTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATTACTGTCG
CAGGCATCTGTGATGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGAT
GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA
CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGC
GGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACTCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTGTGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGA
TCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCC
TGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTA
ATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACGCCAGAAAGCCCCAGAGATGGGGTCCCCCTTGTG
GCTGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCCTGTCCTGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACT
CGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGT
ACAAT
GAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCC
CATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGTCGTCGAAGGTG
GGACTGGCG

Further identification of isolated using NCBI (National Centre for Biotechnology Information) tools. Phylogenetic tree was prepared using MEGA 4.0.

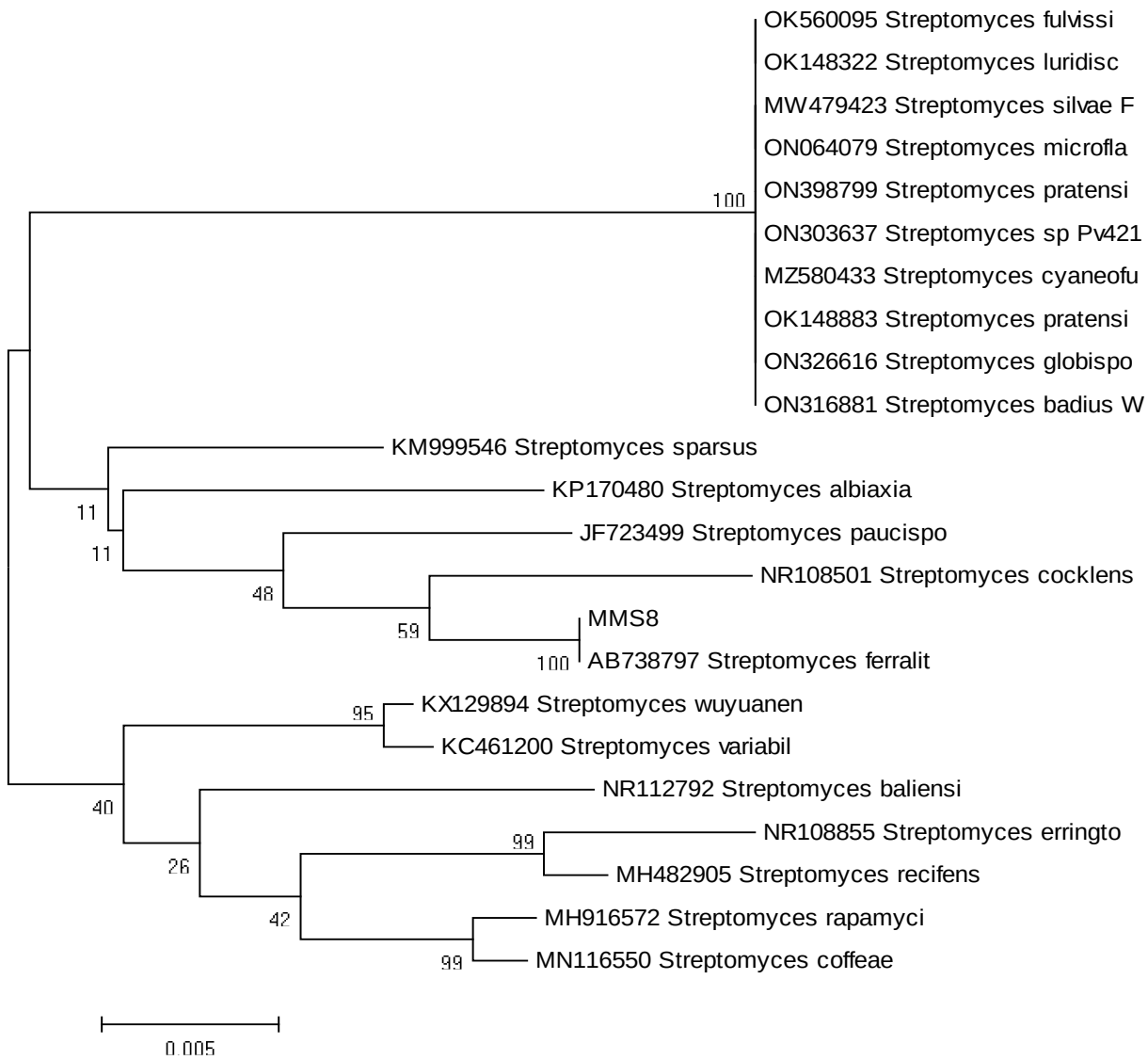

Fig 1. Phylogenetic tree showing the relationship among other *Streptomyces spp.*

After phylogenetic analyses, the sequences were submitted to NCBI and obtained the accession number (LC720128)of *Streptomyces ferralitis MMS8*.

CONCLUSION:

Streptomyces ferralitis MMS8 effectively inhibit the drug resistant *klebsiella pneumoniae* . The study suggest increase in potential of bioactive extract of *Streptomyces ferralitis MMS8*against *Klebsiella pneumoniae* after further purification and characterization studies. Drug resistance in pathogenic bacteria demands that to search for new antibacterial agents active against pathogenic drug resistant bacteria which are resistant to current available antibiotics in market. Due to emerging drug resistant bacterial pathogen world wide it is very essential to screen out potential actinomycetes strains with acceleration from different environment that have not been discovered yet and effective against drug resistant bacterial pathogens.

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