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Isolation and Characterization of New Anti-MRSA Producing Actinomycete

KEYWORDS: Methicillin resistant Staphylococcus aureus; Actinomycete Isolates; Streptomyces species.

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ABSTRACT *The present study was aimed at isolation of anti-MRSA (Methicillin resistant Staphylococcus aureus) producing actinomycetes and its characterization. A total of 98 actinomycetes were isolated from soil samples collected from various regions and were screened for anti-MRSA activity. The selected strain RS 2 which gave highest antibiotic activity against MRSA was selected for further studies. A combination of morphological studies, biochemical characterization and phylogenetic analysis based on 16S rRNA genes provided strong evidence that the strain belongs to Streptomyces sp. which showed 99% similarity with Streptomyces sp. TRM 46626.*

Introduction:
S. aureus is the most important infectious agent causing a variety of diseases, ranging from relatively benign skin infections to life threatening illnesses. It is one of the most common causes of both endemic nosocomial infections and epidemic of hospital acquired infection (Kakru 2003, V. Lakshmi 2008). The MRSA is a specific strain of the Staphylococcus aureus bacterium that has developed antibiotic resistance to all penicillins, including methicillin and other narrow-spectrum β-lactamase-resistant penicillin antibiotics (Ahmed 2008). For more than two decades, clinicians and public health officials have faced hospital acquired MRSA, which also bears resistance to too many antibiotics. At that time, vancomycin had been the therapeutic answer to MRSA, but now vancomycin resistant strains emerged clinically. Fortunately, newer therapeutic agents like daptomycin, linezolid have entered the clinical arena in the past few years but the undesirable side effects and the spread of pathogens with this new antimicrobial drug resistance emphasize the need for the development of the other newer antimicrobial agents against MRSA (Ceylan 2008).

Actinomycetes are the most widely distributed group of microorganisms in nature. They have provided many important bioactive compounds of high commercial value (N. Kumar 2010; R. Usha 2010). Most of the antibiotics in use today are derivatives of natural products of actinomycetes. Although soils have been screened by the pharmaceutical industries for the past five decades, only a small fraction of actinomycetes have been discovered. The search for novel natural products with useful pharmacological activities often includes the isolation of actinomycetes from soil samples (H. Atta 2012; M. Oskay 2011).

In the present investigation, a new actinomycete strain designated as Act RS-2 was isolated and characterized which showed antibacterial activity against MRSA.

Materials and Methods:
Microorganisms, Media and Growth Conditions:

As described previously, prevalence and antimicrobial susceptibility pattern of Methicillin-resistant **Staphylococci** from a referral hospital in Aurangabad (M.S.), India was studied (Padalkar & Peshwe, 2011). For isolation of actinomycetes, the soil samples were collected from different regions of Maharashtra and Nepal and air dried separately for 2 hrs at 45^{0}C in hot air oven to reduce the bacterial flora. Then the soil samples were separately mixed with calcium carbonate (10:1 w/w) and incubated at 26^{0}C for 7 days in a water saturated atmosphere. It was then suspended in sterile saline solution (0.9%). Serial dilutions up to 10^{-7} were prepared using sterile saline and agitated with the vortex at maximum speed. Mixtures were allowed to settle. An aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-7} was poured in sterile petriplates and then the molten starch casein agar (containing cycloheximide, as an antifungal agent, at concentration of 50 µg/ml of the medium) cooled at 45^{0}C was poured into the petriplates (T. Gurung, 2009). The plates were incubated at 35^{0}C for five days. The isolated colonies of actinomycetes were maintained on starch casein agar slants.

Screening of Actinomycete Cultures for Antibiotic production against MRSA:
For Primary screening of actinomycete isolates, the modified cross streak method was used (J. Siva Kumar, 2010). Single streak of actinomycetes was made on the surface of Muller-Hinton agar and incubated at 35^{0}C. After observing a good, ribbon like growth of the actinomycetes on the plates, the overnight culture of MRSA was streaked at right angles to the original streak of actinomycetes and the plate was again incubated at 37^{0}C. The inhibition distance was measured after 24 hours.

In the Secondary screening the spore suspension of the selected actinomycete isolate was inoculated in starch casein broth (containing cycloheximide, at concentration of 50 µg/ml of the medium) and incubated at 35^{0}C for four days. After incubation the broth was centrifuged at 10,000 rpm for 10 minutes and 100 µl of it was loaded into well bored in Muller Hinton agar plates swabbed with MRSA (adjusted 0.5 O.D.). The plates were incubated at 37^{0}C for 24 hrs. The diameter of the zones was recorded (J. Siva Kumar, 2010; N. Hemashenpagam, 2011). The actinomycete strain which showed efficient antibacterial activity was selected for further studies.

Characterization of Selected Actinomycete:
The potent actinomycete was characterized by morphological and biochemical methods. Morphological characterization consisted of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (C. Manjula, 2009). The morphology was observed by using Scanning Electron Microscope. The mycelium structure, color, and the arrangement of conidiophores and arthrospore structures on the mycelium were compared with Bergey's Manual of Determinative Bacteriology (A. Malibari, 1991).

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Various biochemical tests were performed for the identification of the potent isolate are as follows: Sugar utilization, Temperature tolerance, NaCl resistance, pH tolerance, Starch hydrolysis, Urea hydrolysis, Catalase production, H_2S production, Nitrate reduction test, Oxidase test, IMViC test, Resistance to antibiotics.

Genotypic characterization was done according to standard procedures with the help of Xcelris Laboratories, Ahemadabad, India.

Antimicrobial activity of the selected actinomycete was checked against Bacillus subtilis, Eschretia coli, Pseudomonas aerugenosa, Salmonella abony, Aspergillus niger, Aspergillus flavus alomg with MRSA by cross streak method.

Results and Discussion:
The effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections. MRSA is one of such bacteria which causes a wide range of syndromes, from minor skin infections to life threatening pneumonia and toxinoses (S. K. Nawaz, 2008). In the previous study we highlighted the prevalence and antimicrobial susceptibility pattern of Methicillin-resistant Staphylococci from a referral hospital in Aurangabad (M.S.), India (Padalkar & Peshwe, 2011). Methicillin-resistant Staphylococcal isolates subjected to antibiotic sensitivity pattern showed that the isolates have also developed the resistance to other antibiotics tested. 45% of the isolates showed resistance towards oxacillin while 20% of them exhibited resistance towards vancomycin & teicoplanin. But in the recent reports not even a single isolate showed resistance to vancomycin even though they showed 100 percent penicillin resistance and around 90 percent tetracycline resistance (Mehta A., 1996; Kakru D., 2003; S. Anupurba, 2003). In the present study, it is realizable that 20% of the isolates showed resistance to vancomycin but 100% susceptibility was observed against gentamicin.

Filamentous soil bacteria belonging to the genus Streptomyces are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (P. Sujatha, 2004). In the present study, total 98 actinomycete cultures were isolated from different soils at various localities in Maharashtra (India) and Nepal (Fig. 1).

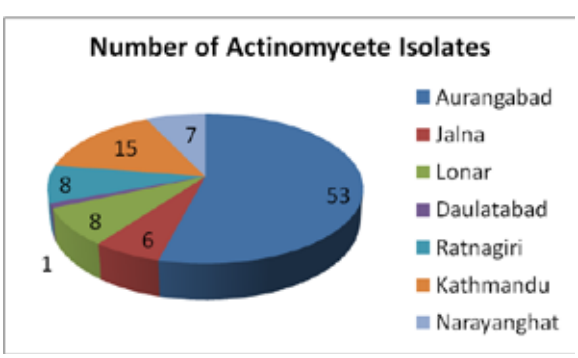
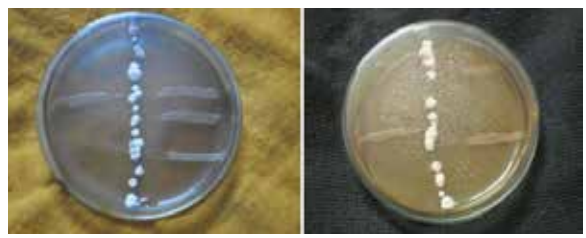


Fig. 1: Number of actinomycetes isolates from different soil samples

Screening of Actinomycete Cultures for Antibiotic production against MRSA:
Out of 98 actinomycetes isolates, four strains showed antibacterial activity against MRSA in the primary screening (Fig. 2) The figures 2A, 2B, 2C and 2D show antibacterial activity of the actinomycete isolate Act R5, Act R6, Act R13 and Act RS 2 respectively against MRSA.

(A: Actinomycete Isolate – Act R5), (B: Actinomycete Isolate – Act R6)



(C: Actinomycete Isolate – Act R13) (D: Actinomycete Isolate – Act RS 2)
Fig. 2: (A to D) Primary screening of actinomycetes showing antibacterial activity against MRSA

These four actinomycetes were then subjected to secondary screening by agar well method for selecting the actinomycete isolate which would show the most efficient anti MRSA activity. The figure 3 shows that the actinomycete isolate Act RS 2 has the maximum anti MRSA activity as it showed the largest diameter of zone of inhibition amongst the four isolated strains.

Fig 3: Secondary screening of the selected actinomycete isolates. Well no. 1, 2, 3 and 4 represent the crude antibiotic extract of Actinomycete isolate Act R13, Act RS2, Act R5 and Act R6 respectively.

Characterization of Selected Actinomycete:
The anti-MRSA producing isolate Act RS2 was characterized first by morphological identification method. Figure 4 shows the purified culture of the actinomycete isolate Act RS 2 and the scanning electron micrograph of the same isolate after doing the coverslip culture technique. Secondly the biochemical characteristics of Act RS 2 were studied. The isolate showed excellent growth in the presence of D-glucose and starch and poor growth in the presence of mannitol and sucrose as carbon source. Further Act RS 2 utilized glycine and not utilized cysteine, valine, histidine, glutamate and tryptophan as a nitrogen source. Under the decomposition/ enzymatic studies, Act RS 2 could produce amylase and oxidase but negative for catalase, urease and nitrate reductase. It could not produce H_2S and negative for Indole test, Methyl red test and Voges Prouskar test. Table 1 given below illustrates the data of morphological and biochemical characteristics of Act RS 2.

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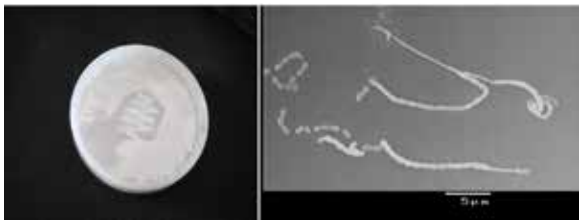

Fig. 4: (A) Purified culture of Act RS 2; (B) Scanning Electron Micrograph of Act RS 2.

Table 1: Morphological and Biochemical Characterization of Act RS 2.

Characteristics	Result	Characteristics	Result
Morphological Characteristics			
Appearance of colony	Powdery	Color of substrate mycelium	Light brown
Shape of colony	Round	Gram's staining	Gram positive
Margin	Entire	Diffusible pigment	Negative
Elevation	Raised	Motile spores	Negative
Color of spore mass	Grayish brown	Spore chain	Looped (Rectinaculiperti)
Color of aerial mycelium	White	Spore surface	Smooth
Utilization of Carbon Sources			
D-glucose	++++	Glycerol	+++
Sucrose	+	Mannitol	+
Fructose	++	Lactose	++
Maltose	+++	Starch	++++
Utilization of Nitrogen Sources			
KNO_3	+++	Histidine	-
Asparagine	+++	Glutamate	-
Cysteine	-	Arginine	++
Casein	+++	Proline	++
Valine	-	Tryptophan	-
Glycine	++++	Cystine	++
Alanine	+++	Lysine	+
Temperature Tolerance			
4^0C	-	37^0C	+++
20^0C	+	42^0C	+++
25^0C	+	50^0C	-
30^0C	++	60^0C	-
pH Tolerance			
4	++	8	+++
5	+++	9	+++
6	++++	10	++
7	++++		
NaCl Tolerance			
0.2%	++++	5.0%	++
0.5%	++++	7.0%	+
1.0%	++++	10.0%	-
2.0%	++++		
Decomposition/Enzymatic Studies			
Starch	Positive	Indole test	Negative
Urea	Negative	Methyl Red test	Weakly positive
Catalase	Positive	Voges Prouskar test	Negative
H_2S production	Negative	Citrate Utilization test	Negative
Nitrate Reductase	Negative	Oxidase test	Positive
Resistance to Antibiotics			
Vancomycin	Sensitive	Amoxycillin	Resistant
Gentamicin	Sensitive	Penicillin	Resistant
Methicillin	Sensitive	Cefpodoxime	Resistant
Oxacillin	Resistant	Ampicillin	Resistant
Teicoplanin	Sensitive	Streptomycin	Sensitive
Rifampicin	Resistant		

'-': No growth; '+': Poor growth; '++': Good growth; '+++': Moderate growth; '++++': Luxuriant growth

Genotypic characterization was done by 16s rDNA based molecular technique by Xcelris Laboratories, Ahemadabad, India. Based on nucleotide homology and phylogenetic analysis, the culture, Act RS 2 (labeled as Sample 1) was found to be Streptomyces sp. which showed 99% similarity with Streptomyces sp. TRM46626 (GenBank Accession Number: JX244140.1). Figure 5 shows the phylogenetic tree of Act RS 2.

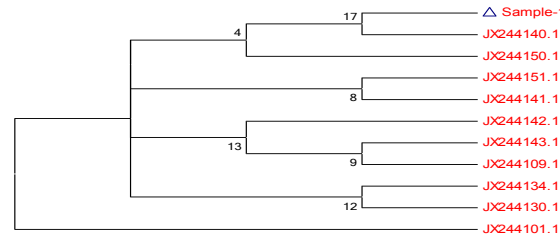

Fig. 5: Phylogenetic Tree of Act RS 2 (labeled as 'Sample 1')

Figure 6 shows antimicrobial activity of Act RS 2 against Bacillus subtilis, Escherichia coli, Pseudomonas aerugenosa, Salmonella abony, Aspergillus niger, Aspergillus flavus along with MRSA.

Fig. 6: Antimicrobial activity of Act RS 2 against Bacillus subtilis, Eschretia coli, Pseudomonas aerugenosa, Salmonella abony, Aspergillus niger, Aspergillus flavus along with MRSA.

From figure 6 it is evident that Act RS 2 i.e. a novel Streptomyces sp. has efficient antibacterial activity, not only against MRSA but also against Bacillus subtilis, Escherichia coli, Salmonella abony. In the figure it is also visible that Pseudomonas aerugenosa is partially inhibited by Act RS 2. The actinomycete isolate also shows antifungal activity against Aspergillus niger. Thus, the antimicrobial compound produced by Act RS 2 has broad spectrum antimicrobial activity.

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