IN VITRO ANTAGONISTIC ACTIVITY OF SOIL ACTINOBACTERIA AGAINST MULTI DRUG RESISTANT BACTERIA

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Summary

The purpose of this study was to explore the antimicrobial properties of actinobacteria isolated from the soil samples of Ranipet, Vellore, Tamil Nadu, India. A total of 13 actinobacteria were isolated and screened for antagonistic property against four multi drug resistant (MDR) bacteria included *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, Non fermenting gram negative bacilli. Among the 13 isolates, two (AR7 and AR12) were found to be effective against *K. pneumoniae* and *E. coli* in primary screening (cross streak method), further the potential isolates were secondary screened by agar well diffusion method, only one isolates (AR 12) was found to be effective against *K. pneumoniae* and *E. coli*. Further AR 12 isolate (potential strain) was characterized by morphological test, biochemical test and Nonomura key. Actinobacteria isolate (AR12) identified as *Streptomyces gougeroti*. This study also reports the antibiogram of the MDR strains used in this study.

Key words: Antibacterial, multi drug resistant, antimicrobial activity, antibiogram

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Introduction

Infectious diseases are one of the major causes of death in early 1900’s. Later, discovery of broad spectrum antibiotics reduced the mortality rate due to infections. Most recently, a number of microorganisms are reported to possess drug resistant towards the commonly used drugs. Drug resistant is defined as the reduction of effectiveness of a drug during a course of the drug treatment for an infectious disease. Multi drug resistance is a condition enabling the microorganism to resist distinct drug or chemical of a wide variety of structure and function targeted at eradicating the organism. These multi drug resistance organisms are highly infectious with high mortality rate and severity of infection by these multi drug resistant organism is very high in immunocompromised patients especially who are suffering with AIDS.

Usually Gram negative microorganisms are more resistant towards antibiotics than Gram positive microorganisms. This is because of the presence of outer membrane in the cell wall of gram negative organisms. This protects or limits the permeability of the antibiotics to their targets in the bacterial cells. Other factor of microbial drug resistance includes resistant plasmids, efflux pump and alteration of metabolic pathways etc.

To combat the multi drug resistance organism, discovery and development of new antimicrobial compounds is very essential, therefore scientist are looking forward to discover novel antimicrobial compounds from alternative sources and here actinobacteria provides an important option for the discovery of new antimicrobial compounds. Actinobacteria are considered as one of the major groups of the soil population. They are very important class of microorganism for drug discovery and development as about 70% of antibiotics available in market obtained from actinobacteria and still there are 300 new antibiotics are being discovered yearly from actinobacteria. Antibiotic produced by actinomycetes includes streptomycin, aminoglycosides, glycopeptides and tetracycline etc.

Focus of this present work was to isolate the native actinobacteria flora from the soil samples of Ranipet, Vellore, TN, India and screen the actinobacteria isolates for their antimicrobial potential against four Gram negative multi drug resistant organisms.

Materials and Methods

Multi drug resistant strains
Four clinical isolates of MDR bacteria were collected form Narayani Hospital, Ariyur, Vellore District, Tamil Nadu, India during December, 2009. Bacterial isolates includes Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Non-fermenting gram negative bacilli (NFGNB).

Reference bacterial cultures
Three standard ATCC cultures were used as reference organisms. Bacterial isolates included E. coli ATCC 25922, P. auruginosa ATCC 27853, K. pneumoniae ATCC 13883.
Antibiogram
All MDR organisms and reference cultures were screened for their sensitivity towards ten standard antibiotics. Antibiotics included ampicillin (10 mcg/disc), Cepodoxime (10 mcg/disc), Chloramphenicol (30 mcg/disc), Ciprofloxacin (5 mcg/disc), Co-trimoxazole (23.75 mcg/disc), Gentamicin (10 mcg/disc), Imipenem (10 mcg/disc), Nalidixic acid (30mcg/disc) and Rifampicin (5mcg/disc). Drug sensitivity test was performed by disc diffusion method on Muller Hinton agar (MHA) plates. Bacterial isolates were inoculated in to nutrient broth for 8 hours. The concentration of the suspensions was adjusted to 0.5 (optical density) by using a spectrophotometer. Isolates were seeded on Mueller Hinton agar plates by using sterilize cotton swabs. The standard antibiotic discs were placed on the agar surface using a sterilize forceps. Plates were incubated at 37°C for 48 hours. Plates were observed for zone of inhibition. The experiment was performed in triplicates. 10

Soil Samples
Soil samples were collected from different parts of Ranipet, Vellore, TN, India during December 2009. Soil samples were collected from the different habitats like lake sediment soil, plant rhizosphere soil and agricultural soil at the depth of 10-25 cm. Samples were collected in sterilized plastic bags and brought to the Molecular and Microbiology Research Laboratory, VIT University, Vellore, TN, India, and stored in a refrigerator at 4°C up to further processing.

Processing of the soil samples
Soil samples were treated with CaCO₃ (10:1 w/w) and then suspended in ringers solution (1/4 strength). The soil samples were heated for 16 hours at 45°C in hot air oven for the separation of spores from the vegetative cells. 11

Isolation of soil actinobacteria
Isolation of soil actinobacteria was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10⁻¹ to 10⁻⁶. A volume of 0.1 ml of each dilution was transferred aseptically to starch casein (SCM) medium, modified nutrient medium (MNA) and Kuster’s medium. The Petri plates were rotated clockwise and anticlockwise to spread the sample uniformly. The plates were incubated at room temperature for 7 days. 12

The actinobacteria isolates were further subcultured on the respective media in order to obtain pure culture. Pure isolates were maintained at 4°C in refrigerator for further studies.

Antimicrobial activity of actinobacteria isolates

Primary screening (cross streak method)
Primary screening of 13 actinobacteria isolates was performed by cross streak method on MNA plates. 13 The actinobacteria isolates were inoculated in straight line on MNA plates and incubated for 7 days. MDR strains were cross streak on the same plate in perpendicular manner. The plates were incubated at 37°C for 24 hours. The plates were examined for the zone of inhibition of the MDR organisms.
Fermentation process
Antagonistic actinobacteria isolates were inoculated in 100 ml of MN broth and SCM in Erlenmeyer flasks. Flasks were lodged on the flask shaker at a speed of 120 rpm at room temperature for 7 days. After fermentation, the medium was harvested and centrifuged to remove growth and debris. Filtrate was collected in a sterilized screw cap bottle and stored in freeze at 4°C for further use.  

Isolation of antibacterial metabolites
The bioactive compounds were recovered from the harvested medium by solvent extraction method. The filtrate was mixed with ethyl acetate, chloroform, butanol (1:1 v/v) and shaken vigorously for 1 hour in a solvent extraction funnel. The solvent phase that contains the extracted compound was separated from the aqueous phase. The solvent extracts were concentrated in rotary evaporator and dried in lyophilizer.

Secondary screening (Agar well diffusion method)
Secondary antimicrobial screening of the actinomycetes isolate was performed by agar well diffusion method. All four MDR strains were inoculated in nutrient broth and incubated for 8 hours at 37°C. The turbidity of the broth was adjusted at 0.5 (optical density) using spectrophotometer. The bacteria cultures were inoculated on MHA plates using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer. Using a micropipette, 100 µl of actinobacteria supernatant was poured into each well. Plates were incubated at 37°C for 48 hours. After the incubation, all plates were examined for the presence of zone of inhibition around the Wells. All Experiments were performed in triplicates.

Minimum inhibitory concentration (MIC)
MIC of the potent actinobacteria isolate was determined by tube dilution method. Actinobacteria extracts were diluted in 10% DMSO to obtain a concentration range of 1000, 500, 250, 125, 62.5 and 31.25 µg/ml. 5ml of sterile Muller Hinton broth was inoculated with 0.05 ml of bacterial culture. 1 ml of different concentration of the extract was added in the tubes. Tubes were incubated for 24 hour and observed for any turbidity. Minimum concentration that doesn’t showed any growth considered as MIC.

Taxonomic Investigation of the Potential Strain
Morphological characteristics
Actinobacteria isolates were inoculated in seven different International Streptomyces Project mediums (ISP 1 to ISP 7) and incubated for 5 days at room temperature. The colonies were observe under a high power magnifying lens and colony morphology was noted with respect to color, aerial mycelium, size, nature of colony, reverse side color and feeling the consistency with a sterile loop.

Microscopic characterization
The actinobacteria isolate was morphologically characterized by Gram staining and spore chain morphology.

Biochemical characterization
The ability of different actinomycetes strains in utilizing various carbon sources viz., arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose as sources of energy was studied following the method recommended by International Streptomyces Project.
Generic investigation
The genus of strains with good antagonistic activity against the MDR Pathogens was identified using by cell wall composition analysis (amino acids analysis and whole cell sugars analysis). \(^{19}\)

Statistical Analysis
All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

Results

Antibiogram
Multi drug resistant strains and reference cultures were screened for the antimicrobial activity by disc diffusion method on MH agar plates. The result is reported with respect to inhibition zone and expressed as mean±standard deviation. Result exhibits that the ten tested drugs did not showed any zone of inhibition against the MDR strains and the zone of inhibition was observed in case of reference cultures. Zone of inhibition was expressed as mean±standard deviation and summarized in Table 1.

Isolation of actinobacteria
A total of three soil samples were collected from Ranipet, Vellore, Tamil Nadu, India. A total of 13 actinobacteria cultures were isolated and differentiated on the bases of colony characters and microscopic appearance. Actinobacteria isolates were named AR 1 to AR 13.

Antimicrobial screening of actinobacteria isolate
Actinobacteria isolates were screened for antimicrobial activity against four multi drug resistant bacteria. Among 13 actinobacteria isolates only 2 isolates (AR 7 and AR 12) were found to be effective in cross streak method (Table 2). Potential isolate (AR 12) was inoculated in SC medium and MN medium, the bioactive compound was extracted in different polarity solvents and the extracts were screened for antimicrobial activity toward MDR by well diffusion method. In this study butanol extract exhibits activity against MDRS E. coli and K. pneumoniae whereas chloroform extract inhibit only K. pneumoniae. Use of two different production mediums did not showed any significant difference in the antimicrobial activity of the microorganism (Table 3). MIC test was performed with the butanol extract against MDR E. coli and K. pneumoniae and found to be 62.5 and 32.25 µg/ml respectively. Results are listed in Table 4.

Characterization of the potential strain
Potential strain was identified by morphological test and biochemical test. The potential strain was identified as S. gougeroti by using nonomura key. The complete data was reported in Table 5. Further generic investigation was made for the isolate. Results are listed in Table 6. Findings of this study conclude that the actinobacteria isolate AR 12 (S. gougeroti) possess high antimicrobial activity against MDR K. pneumoniae and E. coli.
Table 1: Antibiotic Susceptibility test Kirby Bauer Method:

<table>
<thead>
<tr>
<th>Organisms</th>
<th>AM</th>
<th>IM</th>
<th>NA</th>
<th>CO</th>
<th>CI</th>
<th>CE</th>
<th>CF</th>
<th>CH</th>
<th>GE</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MDRS</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Nfgnb MDRS</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>K. pneumoniae MDRS</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P. aeruginosa MDRS</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>16.0±1.0</td>
<td>25.6±0.57</td>
<td>21.3±0.57</td>
<td>23.6±1.15</td>
<td>31.3±1.15</td>
<td>22.3±1.15</td>
<td>30.3±1.52</td>
<td>26±1.0</td>
<td>25.3±0.57</td>
<td>9.3±0.57</td>
</tr>
<tr>
<td>K. pneumoniae ATCC 13883</td>
<td>21.6±0.57</td>
<td>31.6±0.57</td>
<td>27.0±0.57</td>
<td>29.0±1.0</td>
<td>31.3±1.15</td>
<td>27.3±0.75</td>
<td>25.3±1.52</td>
<td>21.6±1.15</td>
<td>18.3±0.57</td>
<td>9.3±1.52</td>
</tr>
<tr>
<td>P. aeruginosa ATCC 27853</td>
<td>21.3±1.15</td>
<td>R</td>
<td>R</td>
<td>24.3±0.57</td>
<td>R</td>
<td>22.6±1.57</td>
<td>R</td>
<td>17.0±1.0</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

Here, AM: Ampicillin, IM: Imipenem, NA: Nalidixic acid, CO: Cotrimoxazole, CI: Ciprofloxacin, CE: Cepodoxime, CF: Ceftazidime, CH: Chloramphenicol, GE: Gentamicin, RF: Rifampicin. R: resistant. All values represent the mean±standard deviation (n = 3 test).
Table 2: Primary screening (Cross Streak Method)

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Zone of inhibition (mm)</th>
<th>AR 7</th>
<th>AR 12</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>14.66±0.57</td>
<td>20.6±0.57</td>
</tr>
<tr>
<td>NFGNB</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>25.0±1.0</td>
<td>24.33±0.57</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

All values represent the mean±standard deviation (n = 3 test).

Table 3: Secondary screening of isolate AR 12 (Well diffusion method)

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Zone of inhibition (mm)</th>
<th>SCM</th>
<th>MN broth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BE</td>
<td>CE</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>13.0±1.0</td>
<td>R</td>
</tr>
<tr>
<td>NFGNB</td>
<td></td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>15.3±0.5</td>
<td>15.3±0.5</td>
<td>R</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Here, BE: butanol extract, CE: chloroform extract, EAE: ethyl acetate extract, R: resistant, All values represent the mean±standard deviation (n = 3 test).

Table 4: Minimum inhibitory concentration for AR 12 isolate

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>62.5</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>31.25</td>
</tr>
</tbody>
</table>
Table 5: Taxonomical investigation

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AR-1 isolate</th>
<th>S. gougeroti</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphological characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colour of aerial mycelium</td>
<td>White-Yellow</td>
<td>White-Yellow</td>
</tr>
<tr>
<td>Melanoid pigment</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reverse side pigment</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spore chain morphology</td>
<td>Recti flexible</td>
<td>Recti flexible</td>
</tr>
<tr>
<td>Spore surface morphology</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
</tbody>
</table>

**Utilization of sole carbon sources**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>AR-1 isolate</th>
<th>S. gougeroti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Manitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Here, +: positive, -: negative, ±: intermediate

Table 6: Cell wall amino acids and whole cell sugars of Ar-10 isolate

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>LL-DAP</th>
<th>Meso-DAP</th>
<th>Glysine</th>
<th>Whole cell Sugars</th>
<th>Wall type</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>I</td>
</tr>
</tbody>
</table>

Here: +: present, -: not present

Discussion

Soil serves as a natural media for the growth of the microorganisms and a variety of microorganisms found in the soil as a native flora. Actinobacteria are a wide group of Gram positive spore forming bacteria. Usually actinobacteria are abundant in nature and reported in soil, fresh water, back water, lake, compost, sewage and marine environment. Actinomycetes are considered as one of the major microbial population in soil. Actinobacteria are saprophytic in nature as they grow easily in the soil rich with dead organic material. Actinobacteria contains high G+C content in their nucleic acid (60-70%). They forms a long multi nuclete hyphae, cell wall is similar with that of gram positive bacteria with the presence of Diaminopimilic Acid (DAP). These actinobacteria are reported to produce several industrially important metabolites such as enzyme, hormone, vitamins, protein and antibiotics. More than 70% of the commercial drugs is being produced from soil actinobacteria.
In this study the drug producing potential of the soil actinobacteria was explored. Actinobacteria samples were isolated from the soil sample of Ranipet, Vellore, TN, India. This place is considered to be a highly contaminated place because of industrial effluents and toxic material from industrial effluents provides the stress conditions for the native flora of soil to produce some novel metabolite with pharmaceutical applications. Earlier studies also reported the antimicrobial activity of soil actinobacteria against MDR organisms.  

In future, bioactive compound can be isolated and characterized by analytical techniques and the bioactive principle can be determined.

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References


