

Isolation of Antimicrobial Compound Producing Bacterium from Pine Forest, Kodaikanal and Lake Water

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Antibiotics are heterogeneous group of small organic molecules obtained from microbial origin. At low concentrations they are effective against most of the microbial pathogens that cause infections in animals and humans. Over past two decades numerous antibiotics, like acidophilin, bulgaricin, colicins, natamycin, reuterin etc. have been produced by bacteria. Certain members of bacteria can develop drug resistance, which has been a major drawback in the field. This has paved way for the present study on isolation of antimicrobial compound producing bacteria. The bacterial cultures were obtained from soil of pine forest, Western ghats (Kodaikanal) and lake water. The isolate VITRAS3 was effective against all the tested pathogens. The isolates were mass multiplied for the production of antimicrobial compounds. Further the compound produced by the isolate was extracted using polar and non-polar solvents. The compound was identified using gas chromatography-mass spectrometry (GCMS). The bacteria obtained were capable of producing antimicrobial compound and effectively inhibited bacterial pathogens.

Keywords: Antibiotics, GCMS, Multidrug resistance.

Antibiotics are the chemical substances produced by microorganisms which have antagonistic effect on growth of other microorganisms (Grossart *et al.* 2013). There are more than 4000 antibiotics developed in which around 50 has gained commercial importance. They are used therapeutically as well as prophylactically to control infectious diseases. Others failed as they were found to have toxic effects on humans and other life forms, ineffectiveness or high production cost (Helen *et al.* 2012). Some common examples of antibiotic producers are *Bacillus subtilis* (polymyxin, defidicin, subtilin, micobacillin,

bacitracin) (Pervez *et al.* 2010), *Pseudomonas aeruginosa* (1-hydroxy phenazine) (Saosong *et al.* 2009) and *Staphylococcus sp.* (Staphylococcins, epicidin) (Carnio *et al.* 2000).

However recently, more emphasis is laid on aquatic environment as it has useful novel antibiotic producing bacteria (Tawiah *et al.* 2012). The presence of certain antibiotic producing bacteria was reported by Rohini *et al.* 2012 from Nilgiri pine forest. Some common examples of microorganisms found in aquatic environment includes *Pseudomonas sp.*, *Pseudoalteromonas sp.*, *Bacillus sp.*, *Streptomyces sp.* (Ardakanil *et al.* 2012) and *Lysinibacillus sp.* (Praveen *et al.* 2012). Some antibiotics obtained from bacteria isolated from aquatic source include sulfadimethoxile, sunfamethoxazole, oxytetracycline (Loper *et al.* 2006).

The bacteria develop various forms of resistance towards the antimicrobial compounds. The increase in application of antimicrobial drugs, the complexity maybe the factor involved in drug

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resistance. Bacterial resistance is a matter of concern because it often results in treatment failure which can have serious consequences on critically ill patients. Susceptible bacterial populations become resistant by acquiring genes encoding resistance from other bacteria or through mutation and selection. There are several other mechanism of antibiotic resistance like, destruction of antimicrobial agent by β -lactamase, extrusion of antibacterial agents by efflux pumps, mutation that causes resistance, altering bacterial cell walls which do not contain antimicrobial binding sites (Tenover *et al.* 2006).

In the present scenario, antibiotics are produced either by microbial fermentation or are semi-synthetic derivatives using pre-existing antibiotic backbone structure. Antimicrobial compounds target the bacterial cell causing cell death or cessation of growth by inhibition of cell wall or membrane synthesis (eg. glycopeptides and β -lactam), nucleic acid synthesis (eg. fluoroquinolones (FQ) and rifampin), disrupting membrane structure (eg. Daptomycin and polymyxins), effecting metabolic pathways (eg. Folic acid analog and sulphonamides) and targeting protein synthesis (eg. tetracyclin, chloramphenicol, aminoglycosides (Grossart *et al.* 2013).

Hence, the advancement in discovery of antibiotics accompanied with semi synthetic drug generation will lead us to successfully control of infectious diseases using antibiotics. The present study was focussed on the isolation and screening of antimicrobial compound producing bacteria and its effect against various pathogens. Study also involves the extraction of antimicrobial compounds and analyse its effect against various pathogens. The analysis of antimicrobial compounds was performed using GC-MS and molecular characterisation by 16S rRNA sequencing showed that effective culture isolated belonged to the genus *Pseudomonas sp.*

MATERIALS AND METHOD

Sample collection

The samples were collected using a sterile bag from both soil and water sources by random sampling method prescribed by EPA standard protocol. The soil sample was collected from Pine forest, Western Ghats (Kodaikanal) and the water

sample was collected from lake water. The samples were transported in cold condition and were processed within 10h of collection.

Isolation of antibacterial compound producing bacteria

The soil and water samples were serially diluted and were inoculated onto nutrient agar plates. The agar plates were incubated at $28^{\circ}\text{C} \pm 2$ for 24-48h and were assessed for the presence of various bacterial diversity present in the sample.

Screening for antibacterial activity

The isolates obtained were screened for antibacterial activity against gram positive bacteria (*Staphylococcus aureus*), gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*) and fungi (*Candida albicans*). The test pathogens were inoculated in 250 ml conical flask containing 50ml nutrient broth and was kept overnight at 37°C in shaking condition at 120 rpm. An absorbance was measured and 0.5 OD was maintained. The overnight cultures of the corresponding test strains were inoculated by swabbing evenly on to the surface of nutrient agar so as to obtain confluent growth. Further all the isolates were inoculated across (4 isolates in a plate) the test isolates. A clear zone formation around the streak indicated antibacterial activity (Todkar *et al.* 2012).

Mass Multiplication of the effective strain

Mass multiplication of the effective culture was performed by adding 2% inoculum from overnight broth of VITRAS3 onto 400ml sterile nutrient broth. The cultures were inoculated and were kept in shaker incubator at 120rpm for 7d (Khunajakr *et al.* 2008).

Extraction of antimicrobial compounds

The antimicrobial compounds were obtained by solvent extraction, ethyl acetate (polar solvent) and Hexane (non-polar solvent) were used. About 200ml of each solvent was added to 400ml mass multiplied culture for both isolates, and kept on shaker at 120 rpm for 2h at room temperature. Solvent phase was then separated and was concentrated. The weight of crude extract was assessed (Sathya *et al.* 2012).

Confirmation of anti-bacterial activity

Isolate having the potential of producing antimicrobial compounds were tested against the test organisms in order to confirm antibacterial activity. This was performed using agar well diffusion method (Sethy *et al.* 2012).

Morphological and biochemical characterization

Effective isolates were characterized morphologically by gram staining and hanging drop and biochemically by oxidase test, catalase test, indole test, methyl red test, Voges Proskauer test, Triple sugar ion test and citrate utilization test (Cappucino and Sherman 1992).

Gas chromatography-mass spectrometry

The methanolic extract was injected in a Perkin Elmer Gas chromatography-mass spectrometry (GC-MS) analyses were conducted with a mass selective detector. An Agilent 6890 gas chromatograph coupled with a Clarus 600 C mass spectrometer was used to perform the aroma analysis. An HP-5MS capillary column (30 m×0.25 mm×0.25 μm; Agilent Technologies, USA) was equipped with an ion source temperature of 200°C and an interface temperature of 250°. The event time was 0.2 seconds with the scan speed of 5000u/se. The Clarus 680 GC used purified helium as the carrier gas, at a constant flow rate of 1 mL/min. After extraction, the fibre was desorbed in the injector port of the GC at 250! for 20min. The oven temperature was held at 80 ! for 2 min and then increased to 300 ! at a rate of 15!/min. Ion source temperature was at 200 ! and spectra was produced in the electron impact (EI) mode at 70 eV (Gibbs *et al.* 2004).

Identification by 16S rRNA technique

Bacterial strains were characterized using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTGTGTTACGACTT-3'). DNA was extracted from cells and the 16S rRNA sequence was determined by the fluorescent dye terminator method using sequencing kit (ABI Prison Big dye terminator cycle sequencing ready reaction kit v: 3.1). Products were run on an

ABI13730XL capillary DNA sequencer (ABI Prison 310 genetic Analyser, Tokyo Japan). The aligned sequences were computed using ClustalW software, and sequence homologies were determined using BLASTn search to create and evolutionary distance matrix (Poongezali *et al.* 2009).

RESULT AND DISCUSSION

Isolation of antibiotic producing bacteria

Different types of colonies were observed after incubation as shown in Fig. 1. From the lake water sample five isolates and from pine forest sample four isolates were obtained. The isolates were further purified and maintained in slants and glycerol stock. Among all the isolates, one isolate from lake water had the capability of antibiotic production and was named as VITRAS3 as shown in Fig. 2.

Screening for antibacterial activity

The antibacterial activity was tested against five pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans*). VITRAS3 showed positive results against all the pathogens whereas for the other isolates no zone of inhibition was observed as shown in Graph 1. Several bacteria isolated from soil and water have showed an antagonistic activity against pathogens. *Bacillus lentis* and *Bacillus alvei* could inhibit *Staphylococcus aureus*, *E coli* proved to be resistant to *Enterobacter aerogene*, *Bacillus pumillus*, *Micrococcus roseu* and *Pseudomonas* proved to be resistant against *Bacillus alvei* (Abdulkadir *et al.* 2012).

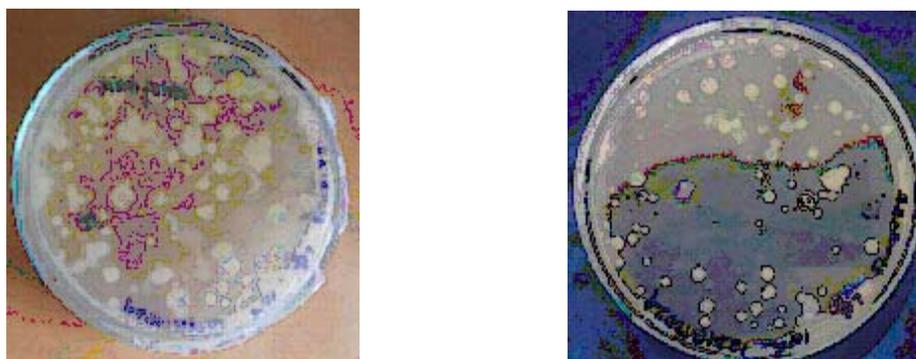


Fig. 1. Samples of agar plates showing (a) Soil isolates and (b) lake water isolate



Fig.2. Subcultured isolates (a) Soil bacterial colonies and (b) lake water bacterial colonies



Fig.3. Sample of agar plates showing zone of growth inhibition; (a) VITRAS3 against pathogen *Candida albicans* and (b) VITRAS3 against pathogen *Pseudomonas aeruginosa*

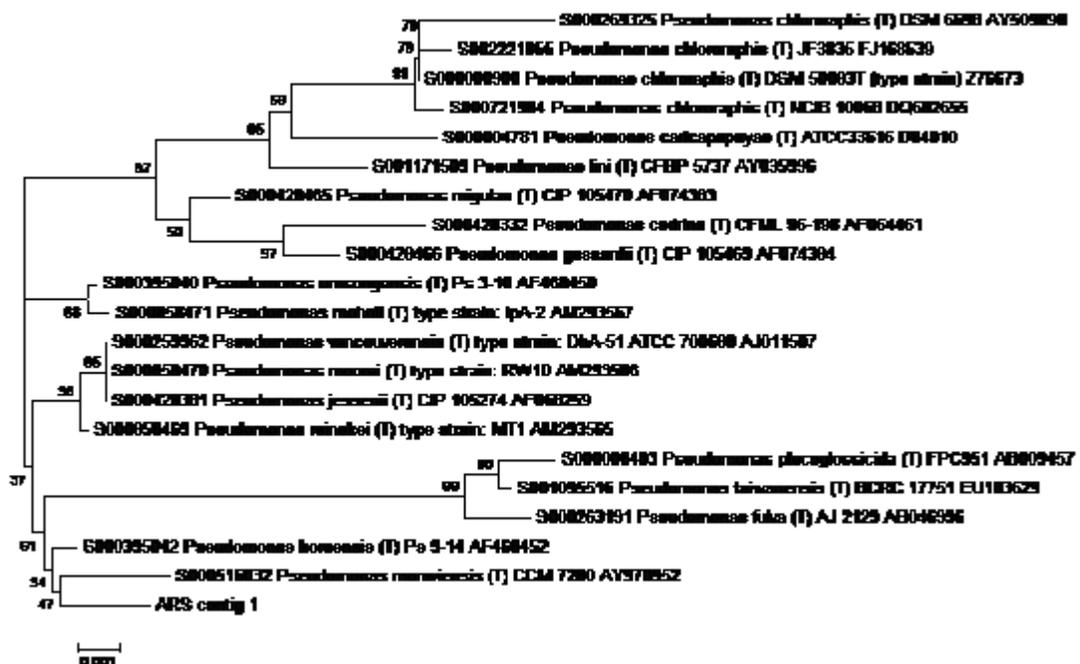


Fig. 4. Neighbour-joining tree based on 16S rRNA gene sequence analysis showing the phylogenetic position of *Pkoorensis*

Morphological and Biochemical characterization

The isolate VITRAS3 was morphologically and biochemically characterized and the results are tabulated as shown in Table 1. The isolate VITRAS3 was found to be gram negative.

Gas chromatography mass spectroscopy:

GC-MS analysis was carried out for identifying of chemical compounds present in the

sample. The chromatogram for sample VITRAS3 dissolved in ethyl acetate and hexane respectively was obtained (as shown in Table 2). The identified antimicrobial compound is Bromocriptine (cycloset). Previously, it was proved Bromocriptine inhibits seasonal fattening and improves insulin resistance (Shuqin Luo *et al.* 2000). It restores normal gonadal function and is highly effective for reducing and normalizing prolactin levels (Fereshtehnejad S. *et al.* 2008).

Identification by 16S rRNA technique

The molecular characterization by 16S rRNA sequencing shows that the antibiotic compound producing bacteria is *Pseudomonas koorensis*. The phylogenetic tree showed sequence similarity of VITRAS3 with *Pseudomonas moraviensis* (as shown in figure 4) (Schumann *et al.* 2006). This is a novel report on the production of antimicrobial compounds by this bacterium.

CONCLUSION

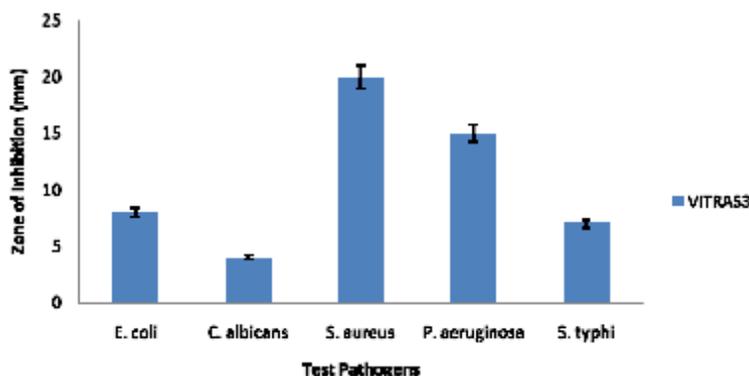
The results of our study strongly supports that the bacterial metabolite plays a vital role in the treatment of microbial infection. The antimicrobial activity shown by the strain VITRAS3 recovered from lake water proves to be unexploited bacteria in water capable of synthesising novel antimicrobial compounds. Further the present study proves the fact that the strain VITRAS3 could be effectively used for the emergence of a novel antibiotic for the treatment of various diseases.

Table 1. Morphological and biochemical characterization

Sr.No.	TESTS	VITRAS3
1	Gram status	-
2	Indole Test	-
3	Methyl Red Test (MR)	-
4	Voges Proskauer Test (VP)	-
5	Catalase Test	+
6	Oxidase test	+
7	Citrate Utilization Test	+
8	Acid Production	+
9	Alkaline Production	+
10	Gas Production	-
11	H ₂ S Production	+

Table 2. Compounds detected in the sample by GC-MS

Sr No	Molecular weight	Compound	Molecular formula
1	654	Bromocriptine	C ₃₂ H ₄₀ BrN ₅ O ₅
2	583	Dihydro-ergosin	C ₃₃ H ₃₇ O ₅ N ₅
3	390	Diocetyl phthalate	C ₂₄ H ₃₈ O ₄
4	278	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄

Zone of Inhibition of Isolate VITRAS3 against test pathogens**Graph 1.** Zone inhibition of isolates against test pathogens

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