Anti-infective Potential of Hot-spring Bacteria

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ABSTRACT

Aim and Background: Antibiotic resistance currently spans most of the known classes of natural and synthetic antibiotics; limiting our options for treatment of infections and demanding discovery of new classes of antibiotics. Much effort is being directed towards developing new antibiotics to overcome this problem. Success in getting novel chemical entities from microbial sources depends essentially on novelty of its habitat. The diversity of geographical location decides the type of micro-flora. In the past various terrestrial and aqueous microorganisms have provided several novel bioactive secondary metabolites of pharmaceutical importance. Hot-springs have not been as extensively exploited as other terrestrial resources. However, perseverance with such microbes augment the probability of getting novel bioactive compounds. **Materials and Methods:** Hot-springs soil samples were collected from Hot-springs in Maharashtra. Actinomycetes and other eubacteria were isolated from these soil samples by selective methods and purified. They were classified based on gram's nature and morphology. Six representative morphological strains were screened for their anti-infective potential by agar well diffusion method as reported by Nathan P. *et al* (1974). The bioactivity of the active microbes was confirmed. **Results:** Seventy three strains of bacteria encompassing eight actinomycetes, and 65 eubacteria were isolated and purified. Among the actives eubacteria PPVWK106001 showed broad spectrum antibacterial activity encompassing both gram positive and gram negative bacterial test models. The extract was active against resistant bacteria such as MRSA and VREs. Activity was very specific as there was no activity against fungi even at 100 fold concentration. The active principle was extractable in butanol. **Conclusions:** The study showed that Hot-springs exhibit diverse bacteria and it serves as potential reservoirs for bacteria of antimicrobial importance with diverse facet of activities. Thus Hot-springs microbes have ability to address issue of resistant bugs.

Key words: Antibacterial activity, Drug-resistant strains, Hot-springs

INTRODUCTION

There is a growing awareness of public health concerns associated with the emergence of drug-resistant strains of bacteria.[1] The occurrence of multiple antibiotic-resistant bacteria has become a major challenge in the treatment of infectious diseases.[2,3] This has created an urgent need for antibiotics with novel scaffolds and mechanism of actions. To achieve metabolite structural diversity, it is generally agreed that a diverse and novel repertoire of microbes is needed.[4] This can be accomplished by isolating microbes from diverse natural ecological units. Hot-springs have been less explored ecological sects for discovery of novel microbial bioactive compounds as compared to other terrestrial samples.[5]

Access this article online

Quick Response Code:

Website: www.jgid.org

DOI: 10.4103/0974-777X.83529

It demonstrates extreme environmental conditions (higher temperature and alkaline pH).[6] Many microbial strains of following major groups/genera have been reported from Hot-springs, viz. sporulating and non-sporulating bacilli, actinomycetes and cyanobacteria, within it thermophilic actinomycetes comprise of the genera Streptomyces, Micromonospora, Actinomadura, Saccharomonospora and Thermoactinomyces.[7] A new strain, Exiguobacterium aurantiacum Colo.Road (BAA -333), has been reported from Yellowstone National Park.[8] Bacilli like Thermus aquaticus, Thermus brockianus, from Hot-springs have gained commercial significance as source of thermostable enzymes.[9,10] On the other hand, there is hardly any report of bioactive compounds from the micro-flora of Hot-springs till date, which opens a window for exploring this resource as potential cache of bioactive compounds. In this paper, we report on the microbial profiling of and antibiotic production potential of microbes isolated from Vajreshwari-Ganeshpuri Hot-Springs, in Western part of India as part of our exploration for new antinfective agents.

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MATERIALS AND METHODS

Sampling

The Hot-springs stretching about 7-km in the bed of the river Tansa are mainly situated at Akoli, Vajreshwari, Ganeshpuri, and Satvalli, in the Western part of Maharashtra state in India. The temperature of the water in the springs ranged from 40°C to 65°C. During the month of May 2006, water from Hot-springs vase, inner wall scrapings of the vents, bottom sediment samples from four sampling points at Vajreshwari and Ganeshpuri Hot-springs were collected. The samples were collected from origin (referred to as face) of the Hot-springs, which are the area known to be not tampered by human activities as such. The samples were collected in sterile polycarbate containers. All the samples were transported to Piramal Life Sciences Limited for further work. They were processed within 24 hours after collection.

Isolation of micro-organisms

The Hot-springs source samples were suitably diluted with N-saline (0.85% sodium chloride in demineralised water) and 100 µl of diluted samples were spread on agarified isolation media. Initially optimization studies were carried out to decide a suitable media for isolation of Hot-spring bacteria (data not published). However, Actinomycetes Isolation Agar (AIA) and Soybean Casein Digest Agar (SCDA) were found to be most suitable, but with enhanced incubation period and incubation temperature (37−39°C instead of 28−30°C). For isolation of actinomycetes, AIA[11] and for other Eubacterial isolation SCDA[12,13] were used. Amphotercine B was incorporated in both media at 20µg/ml concentration to limit the fungal growth, which often overgrow the isolation plates. The media were incubated at 37−39°C and observed regularly up to 30 days. Colonies of bacteria or actinomycetes growing on isolation plates were purified and the pure cultures were streaked on corresponding isolation media and incubated at 37−39°C. Well grown culture slants were used for shake flask fermentation.

Fermentation conditions and sample preparation

LPM-1 [Glucose 1.5%, Corn Steep Liquor; (CSL) 0.5%, Peptone 0.75%, Yeast extract 0.75%, $CaCO_3$ 0.2%, NaCl 0.5%, pH 7.0] was used as seed medium for all isolates. Slant cultures were inoculated into LPM-1 medium and incubated at 30−32°C, 200 rpm for 72 hrs. The production medium LPM-2 [20 ml 5X M9 salts (6.4% $Na_2HPO_4.7H_2O$, 1.5% KH_2PO_4, 0.25% NaCl), 0.2 ml 1M $MgSO_4$, 10 µl 1M $CaCl_2$, NH_4Cl 0.1%, Glucose 0.4%, pH 7.3] was inoculated with 2% (v/v) seed and grown 30−32°C, 200 rpm for 7 days. Acetone extract of the whole broth was prepared using acetone and whole broth at 1:1 (v/v). The lyophilized extract was used further for anti-infective testings. The extracts were dissolved in sterile demineralized water and methanol (1:1, v/v) and used for anti-infective screening assays at 0.01, 0.1, 1.0 mg/ml concentration.

In vitro anti-infective screening

Bacterial test organisms

Staphylococcus aureus 209P (Methicillin Sensitive *Staphylococcus aureus* "MSSA"), *Staphylococcus aureus* E710 (Methicillin-resistant *Staphylococcus aureus* "MRSA"), *Enterococcus faecium* R-2 (Vancomycin Resistant Enterococci "VRE"), E*nterococcus faecium* (Vancomycin Sensitive Enterococci "VSE"), and *Escherichia coli*-2231-SS.

Test culture from the slant was inoculated into sterile 4.0 ml Soybean casein dextrose broth (SCDB). The tubes were incubated at 35−37°C for 18 hrs. About 100 µl of the culture suspension [~5 × 108 colony forming units (CFU)/ml] was added to sterile 4.0 ml SCDB and used as stock suspension.

Fungal test organisms

Candida albicans ATCC 14503, *Candida krusei, Candida glabrata,* and *Aspergillus fumigatus*.

The fungal culture from the slant was inoculated into sterile 4.0 ml Sabouraud dextrose broth (SDB). The tubes were incubated at 35−37°C for 18 hrs. 100 µl of this suspension [~10^8 colony forming units (CFU)/ml] was added to sterile 4.0 ml SDB and used as stock suspension.

The assay was performed by the method followed by Nathan *et al*.[14,15] Predefined volume of stock test culture was put into 40 ml melted (maintained at 40°C) medium. The seeded butt was poured into the petriplate. It was allowed to set for about 30 mins. Required numbers of wells (of diameter 6 mm) were punched into the set medium. 50 µl of test sample was added in each well. The bioassay plates were pre-incubated at 4°C for 30 mins to allow diffusion of the compounds around the agar well. Later the plates were incubated at 35−37°C for 24 hrs.

The results were recorded as diameter of zone (expressed in mm) of clearance around each well. The values in the results are average of values obtained in duplicate testings.

RESULTS

Seventy three strains of bacteria encompassing eight actinomycetes, and 65 eubacteria were isolated and purified from the four sampling points of Hot-springs. The isolates reflected the diversity with respect to macroscopic and microscopic characteristics. Twenty four were Gram-positive and 49 were of Gram-negative bacteria. About 11% of the isolates were actinomycetes. Within 24 Gram-negative bacilli, nine were coccobacilli [Table 1]. Six representative morphological strains were screened for their anti-bacterial or anti-fungal potential. These six strains included five Gram-positive and one Gram-negative bacteria. All were distinct with respect to their colony characteristics [Table 2].

The extracts were tested at concentration of 0.01, 0.1, 1.0 mg/ml. Among the six strains, the culture PPV-WK106001 showed better spectrum of inhibition at 1.0 mg/ml. It showed activity in both Gram-positive and Gram-negative cultures[(a)] [Table 3 and 4]. Strain PPV-WK106003, at 1.0mg/ml showed moderate activity against only *Enterococci faecium* R-II 323 (VRE). Strain PPV-SK206032; an actinomycete also showed broad-spectrum antibacterial activity. None of these three isolate showed antifungal activity. It indicated the specific antibacterial activity [Table 3].

Based on the results of the screening the active extract of PPV-WK106001 was processed so as to find the polarity of the active compound. The active component was extractable in butanol (Refer Fraction[(c)]) as it showed inhibition zones on 12 mm or greater against all four bacterial strains [Table 4]. There is no activity in EA extracts[(b)] or spent. It indicated that the active component in PPV-WK106001 was having intermediate polarity.

DISCUSSION

When the antibacterial activity of six bacteria was observed against test bacteria, Gram-negative test bacteria showed limited susceptibility to the extracts while the Gram-positive bacteria were more susceptible. All six isolates used were Gram-positive aerobic rods and some among these were filamentous. Our findings confirm that majority of antibacterial agents reported from bacilli, paenibacillus and streptomycetes, which are major representatives of antibiotic producing Gram-positive aerobic rods, are anti-gram positive.[16] Isolate PPV-WK106001 a Gram-positive, non-motile, non-spore bearing bacillus was distinct among the Hot-Spring isolates with respect to its activity. It showed a broad-spectrum activity encompassing Gram-positive bacteria such as *Staphylococcus aureus*

Table 1: Distribution of cultures isolated from Hot-springs

Site of sampling	Gram's nature					Total
	Gram-positive			Gram-negative		
	Cocci	Rods	Actinomycetes	Rods	Coccobacilli	
Hot-springs of Vajreshwari	19	11	4	7	5	46
Hot-springs of Ganeshpuri	8	3	4	8	4	27
Total	27	14	8	15	9	73

Table 2: Colony characteristics of six strains isolated from Hot-springs of Vajreshwari-ganeshpuri

Maintenance media	Culture ID	Colony characteristics							Microscopic characteristics Gram nature, shape and motility	Other observations
		Size (diameter in cm)	Shape	Color	Margin	Elevation	Opacity	Consistency		
SCDA	PPV-WK106001	2.1	Circular	Off white	Erose	Effuse	Opaque	Mucoid	Gram +ve rods, NM	Coarsely granular
SCDA	PPV-WK106003	0.7	Circular	Off white	Slightly crenate	Raised	Opaque	Moist	Gram -ve terminal sporulating rods, NM	-
SCDA	PPV-SK206032	0.6	Circular	Light orange	Curled	Umbonate	Opaque	Dry	Gram +ve filaments, branched, septate, NM	Wrinkled
AIA	PPG-SK106024	<0.1	Circular	Dark orange	Entire	Raised	Opaque	Very dry	Gram +ve dense filaments, branched, non-septate, NM	Embedded in agar
AIA	PPG-SK106028	0.6	Circular	Off white	Slightly undulate	Convex	Opaque	Very dry	Gram +ve filaments, branched, non-septate, sporulating, NM	Embedded in agar
AIA	PPG-SK106029	1.2	Irregular	Dark brown	Curled	Raised	Opaque	Soft	Gram +ve rods, NM	Wrinkled

-ve=Negative; +ve=Positive; NM=Non-Motile; cm=Centimeter; -=No peculiar observations

Table 3: Anti-infective activity of the six selected isolates determined by agar the well diffusion assay

Culture Identification Code	Concentration in mg/ml.	Zone of inhibition (mm)							
		S.aureus 209 P	MRSA E710	*E. faecium* R-II	ESS 2231	*C. albicans* ATCC 14503	*C. krusei*	*C. glabrata*	*A. fumigatus*
PPV-WK106001	0.01	-	6.50 h	-	-	-	-	-	-
	0.1	-	10.80 h	-	-	-	-	-	-
	1.0	12.41	12.5	12.1	10.9 sl.h	-	-	-	-
PPV-WK106003	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	-	-	11.81 sl.h	-	-	-	-	-
PPV-SK206032	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	8.17 h	7.97 h	9.16 sl.h	9.52	-	-	-	-
PPG-SK106024	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-
PPG-SK106028	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-
PPG-SK106029	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-
(Media Control-1) LPM-2	0.01	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	-	-	-
Standard antibiotic	20	15*	17*	12*	15**	20#	18#	19#	21#

h=hazy; sl.h=slight hazy; -=no zone; Agar well diameter: 6 mm; Standard antibiotics; *=Vancomycin; **=Gentamicin; #=Amphotericine B

Table 4: Anti-infective activity of extracts of PPV-WK106001

Sample	Zone diameter in mm							
	S.aureus 209 P	MRSA E710	*E. faecium* R-II	ESS 2231	*C. albicans* ATCC 14503	*C. krusei*	*C. glabrata*	*A. fumigatus*
Water: MeOH (1:1, w/w) extract of[a]	9/11 sl.h	11 h	12 dif	10 h	-	-	-	-
Ethyl acetate extract of[b]	-	-	-	-	-	-	-	-
BuOH extract of[c]	12	12.5	12	12	-	-	-	-
Filtered aqueous layer	-	-	-	-	-	-	-	-
Demineralized water	-	-	-	-	-	-	-	-
Only BuOH	-	s	-	-	-	-	-	-
Standard antibiotic (20µg/ml)	15*	17*	12*	15**	20#	18#	19#	21#

h=hazy; sl.h=slight hazy; diff=diffused; s=slight; -=no zone; *=activity confirmed twice. Standard antibiotics; *=Vancomycin; **=Gentamicin; #=Amphotericine B

209P, Methicillin-resistant *Staphylococcus aureus* E710, *E. faecium* R-II (VRE), and Gram-negative bacteria such as *Escherichia coli*-2231 (Super Sensitive Strain). Its anti-gram positive activity is better than anti-gram negative activity. Moreover the strain PPV-WK106001 had shown no antifungal activity, which indicates that the antibacterial activity shown by this culture, was specific. The activity is maintained after 35 days at 4−6ºC, reflecting its stability. This active compound being butanol extractable, indicates that it is more polar compound.

The potential of Hot-springs microbes as source of antibiotic drugs seems to be promising. Within three actives two had shown broad spectrum antibacterial activity including infections caused by pharmaceutical bad bugs like VREs[17,18] and the MRSA.[19] This study has shown that Hot-springs exhibits diverse bacteria and it serves as potential reservoirs for bacteria of antimicrobial importance with diverse facet of activities. A detailed characterization of the active principle of the antibacterial extract is the subject of ongoing investigation in our team.

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How to cite this article: Pednekar P, Jain R, Mahajan G. Anti-infective potential of hot-spring bacteria. J Global Infect Dis 2011;3:241-5.

Source of Support: Nil. **Conflict of Interest:** None declared.