Isolation of *Streptomyces* from the sediments of selected thermal springs of Northern Pakistan and its intrinsic susceptibility and resistance

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K E Y W O R D S

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A B S T R A C T

This investigation aimed to examine the intrinsic antimicrobial susceptibility and resistance of Streptomyces species, isolated from the sediments of selected thermal springs located in northern Pakistan. Isolated species were subjected to morphological, physiological; biochemical and microscopic analyses. The isolates were examined for the consumption of various carbon sources, deprivation of complex compounds, susceptibility and resistance to antimicrobials and inhibitory compounds. The results indicated that all isolates demonstrated complete resistances to penicillin and ampicillin and weak resistance to Cotrimoxazole, Cephazolin, Deoxycline and Lincomycine. These isolates were moderately susceptible to Erythromycin, Cefaparazone, Cefapime, Amoxicillin and Cephradine while completely susceptible to Tetracycline, Vancomycin, Amikacin Gentamicin and Imipenem. In addition, Streptomyces isolates were more susceptible to Vancomycin and Gentamicin as compared to other antimicrobials and inhibitory compounds. This potential vulnerability of Streptomyces to Vancomycin and Gentamicin in particular, as therapeutic agents against infections by Streptomyces species is discussed.

Introduction

Filamentous soil bacteria belonging to the genus *Streptomyces* are globally accepted as significant due to their potential to produce variety of novel antibiotics as well as secondary metabolites (Williams *et al*., 1983; Crandall & Hamil, 1986; Williams *et al*., 1989; Korn-Wendisch & Kutzner, 1992). Different *Streptomyces species* produce important antibiotics, infact 70 % have been isolated from *Streptomyces* are used in medicine and agriculture (Miyadoh, 1993; Tanaka & Mura, 1993). Some *Streptomyces species* may infect lesions and scratches and instigate abscesses similar to those caused by *Nocardia species* (Huang & Chen, 1989). Severe illness caused by microorganisms have modified resistant to frequently used antibiotics has turn out to be a key universal healthcare problem in the 21st century (Alanis, 2005). Diminutive numbers of *Streptomyces species* ensure infections in humans, animals and plants (Williams *et al*., 1989). It is evident that prior to the use of the drugs, pathogens were susceptible to antibiotics and antimicrobial agents and hence treatment of infections were simple; but in most cases, the antibiotic resistance genes have instigated in the natural microbiota (Davies, 1994; Merson-Davies & Cundliffe 1994). Additionally resistance genes might be responsible for antibiotic resistance in their original organisms (Martinez, 2009; Martinez and Baquero, 2000). *Streptomyces* bacteria are usually present in the soil in the form of grains. *Streptomyces* is a medically important bacterium by producing antibiotics but at the same time it also causes diseases which are a major issue in rural areas. The infecting agent is implanted into the host tissue through a breach in the skin produced by trauma caused by sharp objects such as thorn pricks, stone or splinters. The disease is usually acquired while performing agricultural work and it generally afflicts men between 20 and 40 years old. The disease is acquired by contacting grains of bacterial that have been discharged onto the soil.

Thermal springs in the high altitude region are a special ecosystem, where probability of occurrence of species not found elsewhere is higher than other such places. *Streptomyces* being a unique species in terms of their habitat requirements, thermal springs in the northern Pakistan is hypothesised to be one of their key habitats. Since, conservation of biodiversity is one of the major aims of WWF - Pakistan, an investigation of the occurrence of this unique species in a sole habitat is critical to the goal of biodiversity complication and conservation. Keeping in view its significance for bioactive potential, and pathogenic nature at the same time, the current study was conducted to investigate the occurrence of *Streptomyces* in sediments of thermal springs in northern Pakistan in order to identify intrinsic resistance of the species against key antimicrobial agents. The study further aimed at identifying effective antimicrobial agents to cure the infections which are caused by *Streptomyces* itself.

Material and Methods

Study area

The thermal springs located in the northern part of the country has the suitable water temperature ranging between 28^{0}C and 40^{0}C, which is appropriate for growth of *Streptomyces* bacteria. This provided basis for selection of the site for this study (Fig. 1). This site forms part of the Northern Alpine Wetlands Complex of the Pakistan Wetlands Programme where one of the goals of the project is to compile its biodiversity resources. This provided the basis for selection of the sites for this study (Fig. 1). A total of six sediment samples from a depth of 20 cm were collected from all six study sites: Hajira (HJ), Garam Chasma Upper (GU), Garam Chasma Lower (GL), Chinar Bagh (CB), Tatta Pani (TP) and Hunza Nagar (HN). The samples were kept in properly labelled polyethylene bags, refrigerated and aseptically transported to laboratory for further analysis.

Data Collection

Isolation of Streptomyces species

One gram of soil samples were pre-treated with 0.1 gm of Calcium Carbonate ($CaCO_3$) and incubated at 37°C for 4 days and then suspended in 99 ml sterile solution of distilled water (Laidi *et al.,* 2006). 500 ml of Arginine Glycerol Salt medium (AGS) solution was prepared and sterilised at 121°C for 15 minutes. Laminar flow hood was pre-treated with methylated spirit and Ultraviolet (UV) light and then AGS liquid media was poured in

glass plates and kept it open for 10 minutes to solidify. Serially diluted suspensions of the soil samples were uniformly spread on the surface of AGS medium with the help of a dropper next to a spirit lamp in order to avoid any potential contamination by air borne micro-organisms (International *Streptomyces* Project - ISP).

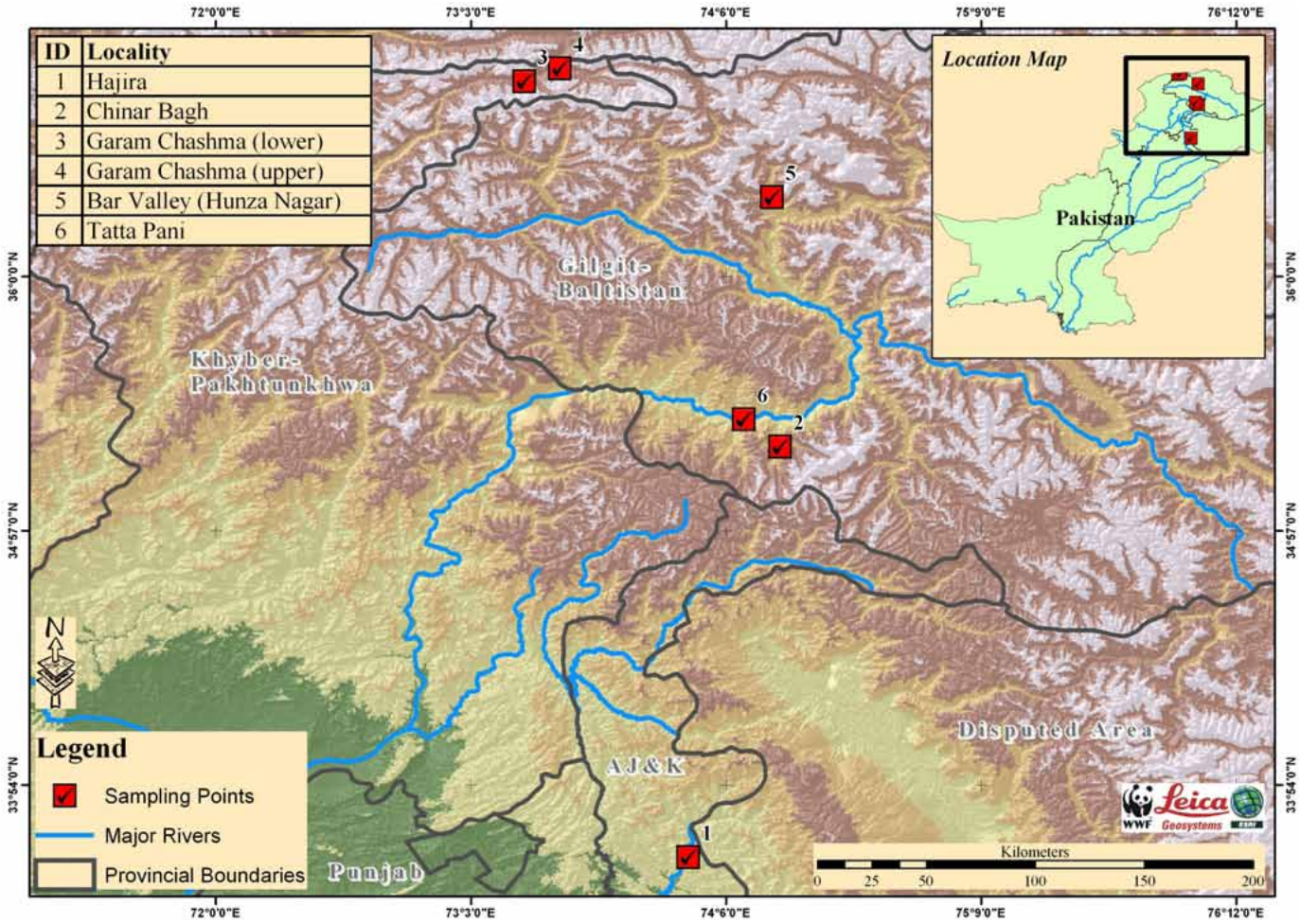

Figure 1: Soil sampling sites in thermal springs, northern areas of Pakistan

The plates were incubated at 37°C for 48 hours. After incubation period, the growth was clearly visible on plates.

Characterisation of the isolates

Gram staining, taxonomic, physiological, morphologic, and biochemical characterisation were carried out by using the methods recommended by the International *Streptomyces* Project (ISP) for characterising *Streptomyces* species (Shirling and Gottlieb, 1966). General morphology was determined using direct light microscopic examination of the surface of the cross-hatched cultures. Colours were determined according to the scale adopted by Prauser (1964). Different culture media such as Blood Agar, Chocolate Agar, Nutrient Agar, Mackcony Agar, SDA (Sabouraud Dextrose Agar) Agar and Cled (Cystine lactose electrolyte deficient) Agar were used to identify and differentiate characteristics of the species. Cells taken from the pure cultures were inoculated in flasks kept at 37°C for 48 hours in shaker incubator at a speed of 150 rotations per minute (rpm) to obtain uniform growth in AGS broth. Testing for antibiotic sensitivity was carried out following the Kirby-Bauer method. Antibiotic disks were placed onto plates and microbial interactions were analysed by determining the size of the inhibition zone.

Results and Discussion

Characteristics of *Streptomyces Species*

The results of physiological and morphological characteristics of *Streptomyces species* investigated are shown in Table 1 and Table 2. The results demonstrate that selective isolates from HJ, GU, GL, CB, TP and HN of *Streptomyces species* used all the carbon sources. Microscopic examination showed that surface of spore was smooth without adherent materials, the chain of spore was linear while slide cultures illustrated that it has aerial mycelia. These examinations are comparable with the investigations of Diab (1982) and Hongjuan *et al.* (2005). The susceptibility and resistance of all the *Streptomyces* isolated *species* was checked against the inhibitory compounds which indicated that all the *Streptomyces* isolated *species* were completely resistant to crystal violet but completely susceptible against Sodium Azide, Phenol and Potassium Tellurite (Deepika & Kannabiran, 2009). Results indicated that the range of temperature for the growth of *Streptomyces* species was 28-40°C while its NaCl tolerance was 0.025 to 0.15 g/L and pH range was 6 to 8 (Table 1), which are comparable results with the study carried out by Ibrahim (2006) and Laidi *et al.,* (2006).

Antimicrobial susceptibility and resistance of *Streptomyces species*

The mean percentage (%) of antimicrobial sensitivity and resistance of *Streptomyces* revealed that isolates from all six sites were (100 %) susceptible to Vancomycin, (99 %) to Amikacin, (98 %) to Gentamicin, (98 %) to Imipenem and (97 %) to Tetracycline. *Streptomyces species* showed modest resistance of (57 %) to Erythromycin, (56 %) to Amoxicillin, (53 %) to Cefaparazone, (51 %) to Cefapime and Cephradine respectively. The results also indicated poor growth against Lincomycine (41 %), Cephazolin

Table 1: Physiological and morphological characteristics of *Streptomyces*. *Positive (+ve)* indicates that the carbon sources have been utilised by *Streptomyces*. HJ*=Hajira, GU*=Garam Chashma Upper, GL*=Garam Chashma Lower, CB*=, Chinar Bagh, TP*=Tatta Pani, HN*=Hunza Nagar represents the sampling sites for the isolation of *Streptomyces*.

Carbon consumption	HJ*	GU*	GL*	CB*	TP*	HN*
Glucose	+ve	+ve	+ve	+ve	+ve	+ve
Xylose	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve
Raffinose	+ve	+ve	+ve	+ve	+ve	+ve
Starch	+ve	+ve	+ve	+ve	+ve	+ve
Galactose	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve
Arabinose	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve	+ve	+ve
Inositol	+ve	+ve	+ve	+ve	+ve	+ve
Glycerol	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve
Rhamnose	+ve	+ve	+ve	+ve	+ve	+ve
Appearance	Mycelia	Mycelia	Mycelia	Mycelia	Mycelia	Mycelia
Range of temperature	28-40°C	28-4°C	28-40°C	28-40°C	28-40°C	28-40°C
pH Range	5.5 - 7.5	5.5 - 7.5	5.5 - 7.5	5.5 - 7.5	5.5 - 7.5	5.5 - 7.5
NaCl tolerance	0.025 - 0.15m	0.025 - 0.15m	0.025- 0.15m	0.025 - 0.15m	0.025 - 0.15m	0.025 -0.15m
Cell wall analysis	Yellow green	Yellow green	Yellow green	Yellow green	Yellow green	Yellow green

Table 2: Effect of inhibitory Compounds on *Streptomyces* Growth. **Res (Resistance)* indicates resistance of *Streptomyces* while **Val (vulnerable)* shows its vulnerability against the inhibitory compounds. HJ*=Hajira, GU*=Garam Chashma Upper, GL*=Garam Chashma Lower, CB*=, Chinar Bagh, TP*=Tatta Pani, HN*=Hunza Nagar represents the sampling sites for the isolation of *Streptomyces*.

Inhibitory Compounds	HJ*	GU*	GL*	CB*	TP*	HN*
Crystal Violet	Res	Res	Res	Res	Res	Res
Sodium Azide	Val	Val	Val	Val	Val	Val
Phenol	Val	Val	Val	Val	Val	Val
Potassium Tellurite	Val	Val	Val	Val	Val	Val

Table 3: Antimicrobial Sensitivity and Resistance of *Streptomyces* isolated species. *Negative *(-ve)* indicates vulnerability while **positive (+ve)* shows resistance of *Streptomyces* to antibiotics. *W* (Week Growth)* indicates weak growth and *M* (Moderate growth)* of *Streptomyces* in the culture media against antibiotics. HJ*=Hajira, GU*=Garam Chashma Upper, GL*=Garam Chashma Lower, CB*=, Chinar Bagh, TP*=Tatta Pani, HN*=Hunza Nagar represents the sampling sites for the isolation of *Streptomyces*.

Antibiotics	HJ*	GU*	GL*	CB*	TP*	HN*
Vancomycin	-ve	-ve	-ve	-ve	-ve	-ve
Penicillin	+ve	+ve	+ve	+ve	+ve	+ve
Ampicillin	+ve	+ve	+ve	+ve	+ve	+ve
Lincomycine	W	W	W	W	W	W
Cephazolin	W	W	W	W	W	W
Deoxycline	W	W	W	W	W	W
Cephradine	M	M	M	M	M	M
Cotrimoxazole	W	W	W	W	W	W
Amoxicillin	M	M	M	M	M	M
Cefapime	M	M	M	M	M	M
Cefaparazone	M	M	M	M	M	M
Erythromycin	M	M	M	M	M	M
Amikacin	-ve	-ve	-ve	-ve	-ve	-ve
Tetracycline	-ve	-ve	-ve	-ve	-ve	-ve
Gentamicin	-ve	-ve	-ve	-ve	-ve	-ve
Imipenem	-ve	-ve	-ve	-ve	-ve	-ve

(39 %), Cotrimoxazole (38 %) and Deoxycline (35 %), while it induced complete resistance to Penicillin and Ampicillin (Fig. 2). These results demonstrated that although *Streptomyces species* have the ability to produce antibiotics, which are noxious to their predators or their contestants but it also protect themselves against their own antibiotics by creating intrinsic antimicrobial resistance (Cundliffe, 1989). Furthermore, results clearly demonstrated that *Streptomyces species* also generated resistant to some extent to other antibacterial agents produced and protected themselves against their own antibiotics. There might be enzymatic defence pathways for their self protection e.g. Nourseothricin producer *Streptomyces noursei* creates resistant to its own antibiotics by inactivating Nourseothricin by enzymatic acetylation (Haupt *et al.,* 1986). Antimicrobial resistance and sensitivity of the *Streptomyces* isolates of different sites were checked against antibiotics, which indicated the following results as shown in (Table 3).

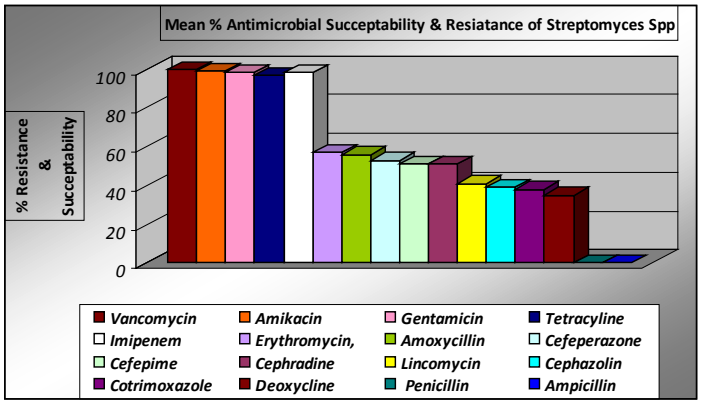

Figure 2: Mean percent, antimicrobial susceptibility and resistance of *Streptomyces* against different antibiotics in the culture media.

These results clearly demonstrate that *Streptomyces* at each location depend on its stress and competitive ecological conditions of their habitat (Walker, 1984), and showing intrinsic modifications of their target sites at each site (Hayes & Wolf, 1984). *Streptomyces erythraeus* which produce Erythromycin protect its ribosomal RNA (Potential site) by methylation to create adaptive change and intrinsic resistance (Cundliffe, 1984).

Conclusion

This study showed that thermal springs located in northern part of the country are sole habitats for *Streptomyces*, as these environments maintain an appropriate temperature regime that support the occurrence and growth of these species. This study also highlighted the range of intrinsic susceptibility and resistance of *Streptomyces* against antimicrobial agents. Thus, humans infected by this species may effectively be treated with Vancomycin, Amikacin, Gentamicin, Imipenem and Tetracycline. As this species occurs in a predominantly un-disturbed wetland region of northern Pakistan, specific protection measures need to be undertaken to protect their habitat from pollution. Their preservation is critical as a source of naturally diverse gene pool of *Streptomyces* that could have significant implications for drugs development and biodiversity conservation.

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