Antibiotic Resistance of Microbes Obtained from Sindh, a Glacier Fed River of Sonamarg Kashmir

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Abstract

Antibiotic-resistant microbes find their way into aquatic ecosystems from human and animal sources. These microbes have potential to spread their genes into water-indigenous microbes, which also contain resistance genes. Resistance to several different antibiotics at the same time is even more significant problem. It is because of the acquired resistance that microbe's particularly bacterial isolates must be subjected to antibiotic susceptibility testing. Awareness of antibiotic resistant microbes in aquatic ecosystems is growing. In the present study bacteria cultured from the river waters were tested for resistance against eight antibiotics [Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (G), Vancomycin (Va)] and Amphotericin-B was used for fungi. The results reveal that, all the strains showed high resistance to almost all the drugs tested against except Gentamycin and Ofloxacin that showed 100% susceptibility. For fungal species it was found that *Asperigillus* spp. and *Candida* spp. were susceptible while *Pencillium* spp. was found to be resistant. Our findings from this preliminary research have indicated that microbes in our aquatic systems are resistant to the antibiotics and hence pose a potential threat to ecosystem function and potentially human health.

Keywords: Aquatic ecosystem, bacteria, antibiotics, pathogens.

Introduction

The microbial quality of aquatic ecosystems particularly of those which are important for drinking purposes, have focused on the occurrence of pathogens in drinking water distribution systems (Berry *et al*., 2006; Szewzyk *et al*., 2000). Environmental contamination affects microbial communities in a myriad of ways. Source and amount of pollutants together with ecosystem dynamics modulate the responses of microorganisms to anthropogenic impacts (Cardoso *et al*., 2012). Microbe communities react with drastic changes in ecosystem functioning, species composition and abundance (Nogales *et al*., 2011; Vieira *et al*., 2008). Several consequences may arise from aquatic pollution. The connection between these impacts and potentially pathogenic bacteria is of particular relevance for human welfare (Nogales *et al*., 2011). There are various ways to estimate the anthropogenic load on an ecosystem, including chemical analysis, bioindication, bioassays, etc. (Doust *et al*., 1994; Ostroumov, 2000, Lindstron-Seppa *et al*., 2001; Samecka-Cymerman and Kempers, 2001). Bacteria are often used to monitor the state of the environment (Jacobs *et al*., 1995; Schwedt *et al*., 1997; Backhaus and Grimme, 1999), as their evolution rate is very high, and under the effect of environmental factors they can acquire various specific features, and thus, serve as good indicators for the presence of pollutants. One of the properties of bacteria used to assess the anthropogenic load is their antibiotic resistance (Hagedorn *et al*., 1999; Goni-Urriza *et al*., 2000; Mary *et al*.,

2000; McArthur and Tuckfield, 2000). Thus, the presence of antibiotic resistant microbes in aquatic ecosystems can greatly affect public health and hence is an emerging issue for the public in general and the drinking water industry in particular (Armstrong *et al*., 1981; Schwartz *et al*., 2003). Although several studies have detected antibiotic resistant bacteria in drinking water systems (Armstrong, 1982; Armstrong *et al*., 1981; Pavlov *et al*., 2004; Schwartz, 2003; Zhang *et al*., 2009), most previous studies focused on cultivable bacteria and/or indicator organisms. The aim of this work was to investigate the dynamics of antibiotic resistance of microbes in aquatic system against the selected antibiotics.

Materials and Methods

Sampling site

Sonamarg – the Meadow of Gold is situated at an altitude of 2730m. The Sindh River that meanders through the valley is locally known as "SENDH" originates from the Panjtarni glacial fields at an altitude of 4,250 m (a.s.l) at the base of Saskut, a peak (4,693 m a.s.l) in the Ogput Range running parallel to the North-West to South-East. On its descend, the Sindh receives glacial melt waters from the glaciers like Nicchang, Mashram Bal and Kolhai (The largest glacier of Kashmir) in addition to the glaciers of the Nilgrar region, Thajwas glaciers and Harmukh glaciers. From Saskut, River Sindh drops steeply north-westward to reach the main strike valley. Gathering momentum, the river runs towards Sonamarg between steeply towering mountain areas, over a boulder streambed, emerging into the pleasant upland serenity of the Sonamarg, as if to rest before it plunges roaring headlong torrent sharply to the Southwest through the Gangagir gorge, 4000 ft (1,230 m) deep. The climate of Sonamarg is very bracing, with average temperature around 14^{o}C. Winters (November to April) are chilly with temperature goes down to subzero levels. Two sites selected for the present study were Yousmarg (Site I) renowned for its green pastures, pines and fir with geographical co-ordinates lying between 34^{0} 17′ 0″N and 75^{0} 19′ 0″E and an elevation of 2,712 m (a.s.l) and Thajwas Grar (Site II) known for the glaciers, the miniature plateaus, snowfields, pines and islets with geographical co-ordinates between 34^{0} 17′ 50″N and 75^{0} 12′ 52″ E and an elevation of 2,617 m (a.s.l).

Sample collection

Samples of water were collected from the selected sites for six months from July 2010 to December 2010 in suitable plastic bottles, which were previously carefully cleaned, rinsed three to four times with distilled water (A.P.H.A, 1998). During collection of samples, extreme care was exercised to avoid contamination. The collected samples were later processed for microbial analysis. Water samples obtained from different sites were serially diluted five folds and then spread plate technique was followed for isolation of bacteria and fungi, spreading 0.1ml inoculum from the serial dilution tubes on the Petri dishes containing nutrient agar and Rose- Bengal, Streptomycin, Agar medium for bacteria and fungi respectively. In case of bacterial isolation inoculum from the serial dilution tubes was spread onto the Petri dishes containing Nutrient agar medium by two different techniques which are Serial dilution (Clesceri *et al*., 1998) and Spread plate (Sharp and Lyles, 1969) and were incubated at a temperature of 37 °C for 24-48 hours. For provisional identification of bacteria important Gram staining were performed and then Antibiotic sensitivity tests were done.

Antibiotic sensitivity test of the isolated strains was carried out using Kirby-Bauer Method (Bauer *et al*., 1966) using antibiotic octa-combi discs from Hi-media. The media used for test are Sabouraud dextrose agar and Mueller Hinton Hi-Veg Broth. In this method the standardized bacterial isolate is spread on an agar plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37^{o}C overnight. The bacterial strains were tested for sensitivity against eight antibiotics namely Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (G), Vancomycin (Va). Fungal strains were tested against amphotericin-B Isolates susceptible to the antibiotic, does not grow around the disk thus

forming a zone of inhibition. Strains resistant to an antibiotic grow up to the margin of disk. The diameter of zone of inhibition was measured and results were compared from the Kirby Bauer chart as sensitive, intermediate or resistant.

Results and Discussion

During the study period four strains of bacteria were isolated from Site I and six strains were from Site II and were identified on the basis of macro-morphological characters and Gram staining. The strains isolated were given codes ranking from B1 to B7. Most of the colonies were circular, entire and flat in appearance, margin, and elevation respectively and the majority of the strains isolated were Gram negative. About 60% of strains isolated were observed as Gram-negative cocci. Presences of gram negative cocci are of much concern because of their pathogencity resulting in diseases in humans. Wernar *et al*., 1969 has reported that outbreak of gastro enteritis is because of sewage-polluted water containing *E. coli* Pathogenic bacteria has also been isolated from River Tawi in Jammu (Gandotra *et al*., 2009). Similarly *Asperigillus* spp., *Candida* spp. and *Pencillium* spp. were found at Site I while at Site II only two *Asperigillus* spp. and *Pencillium* spp. were found. The relevance of fungi and their activities in water is emphasized by increasing knowledge of their pathogenicity for humans, animals and plants, their role as food for energy, their activity in natural purification processes, their exploitation for science and technological use (Cooke,1954; Castellani, 1963; Curtis,1972; Kishimoto and Baker, 1969; Suzuki, 1962). The comparative study of observation of investigators indicates that some species of fungi especially water moulds show variation in their ecological requirements (Mer *et al*., 1980).

The isolated strains were then tested for sensitivity against eight antibiotics namely Cephalothin (Ch), Clindamycin (Cd), Trimaxozole (Co), Erythromycin (E), Gentamycin (G), Ofloxacin (Of), Penicillin (P), Vancomycin (Va). The results of antibiotic sensitivity test for bacteria (Table 1) reveal that, in general 46.42% of strains were resistant, 35.7% of strains were susceptible, and 17.8% of strains showed intermediate sensitivity. In addition, all the strains showed high resistance to almost all the drugs tested against except Gentamycin and Ofloxacin that showed 100% susceptibility. Resistance of a single bacterial isolates to more than one antimicrobial drug has also been reported (Norelli *et al*., 1991; Sayah *et al*., 2005). The increasing spread of antibiotic resistance among environmental bacteria has led some authors to consider antibiotic resistant bacteria and antibiotic resistance genes (ARG) as emerging pollutants (Kümmerer, 2009; Wright, 2010: Pruden *et al*., 2006). The high level of drug resistance in tested bacterial strains could be related to the production of antimicrobial compounds found in some fish-farm isolates belonging to the genus Bacillus (Sarker *et al*., 2010). Further, the antibiotic sensitivity was also carried out for fungi. The antibiotic used was Amphotericin-B. The results (Table 2) reveal that *Asperigillus* spp. and *Candida* spp. were susceptible while *Pencillium* spp. was found to be resistance.

In conclusion, significant levels of antibiotic re sistance were found among isolated species. The development of new antibiotics as well as their increasing and, occasionally, indiscriminate and irrational use provokes the release of antibiotic and antibiotic-resistant bacteria via sewage or treated wastewater to the environment. The anthropogenic impact on the aquatic system is likely to have been associated with the higher levels of resistance. Unless preventive measures are taken to combat the spread of antibiotic resistance, by reducing pollution and making conscious use of these drugs, humanity may be faced with the end of the antibiotic era.

Table 1. Antibiotic sensitivity behaviour of bacterial isolates.

Bacterial Strain	Antibiotic agent							
	Clindamycin	Trimaxozole	Erythromycin	Gentamicin	Oflaxacin	Pencillin	Vancomycin	Cephalothin
I3	R	R	I	S	S	R	R	R
I4	S	S	R	S	S	S	I	R
I2	S	S	I	S	S	S	R	R
I6	R	R	R	S	S	R	I	I
I1	R	R	I	S	S	I	R	R
I5	R	R	R	S	S	I	R	R
I7	R	I	R	S	S	I	R	R

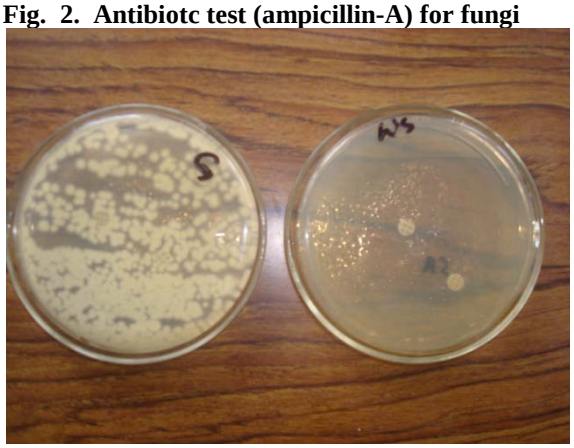
R-Resistant; I-Intermediate; S- Susceptible. (46.42% resistant, 35.7% susceptible, and 17.8% intermediate).

Table 2. Antibiotic sensitivity behaviour of isolates for fungi.

Fungal strain	Antibiotic Agent (AmphotericinB)
Asperigillus spp.	Susceptible
Candida spp.	Susceptible
Pencillum spp.	Resistant

Fig. 1. Antibiotic test (octacombi-discs) for bacteria

Fig. 2. Antibiotc test (ampicillin-A) for fungi



References

American Public Health Association. 1998. ***Standard Methods for the Examination of Water and Wastewater***. (12th Eddition), Amer. Pub. Heal. Ass., New York.

(Andrew D Eaton; Lenore S Clesceri; Arnold E Greenberg; Mary Ann H Franson;)

Armstrong, J. L., Calomiris, J. J. and Seidler. R. J. 1982. Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Appl. Environ. Microbiol.* **44**: 308–316.

Armstrong, J. L., Shigeno, D. S. Calomiris, J. J. and Seidler. R. J. 1981. Antibiotic-resistant bacteria in drinking water. *Appl. Environ. Microbiol*. **42**:277–283.

Backhaus, T. and Grimme, L.H. 1999. The toxicity of antibiotic agents to the luminescent bacterium Vibrio fischeri. *Chemosphere.* **38(14):** 3291–330.

Berry, D., Xi, C. and Raskin. L. 2006. Microbial ecology of drinking water distribution systems. *Curr. Opin. Biotechnol*. **17:**2 97–302.

Cardoso, A. M., Coutinho, F. H., Silveira, C. B., Ignacio, B. L., Vieira, R. P., *et al.*, 2012. Metagenomics in polluted aquatic environments. In: *Water Pollution.* (Nuray Balkis, ed.). In Tech, Rijeka.

Castellani, A. 1963. Further researches on the long viability and growth of many pathogenic fungi and some bacteria in sterile distilled water. *Mycopathol. Mycol. Appl*. **20**:1-6.

Chandrasekaran, S., Venkatesh, B. and Lalithakumari, D. 1998. Transfer and expression of multiple antibiotic resistance plasmid in marine bacteria. *Curr. Microbiol.* **37**:347–35.

Clesceri, L. S., Greenberg, A. E. and Eaton, A. D. 1998. ***Standard Methods for Examination of Water and Wastewater.*** APHA. Washington .D.C. pp 140.

Cooke, W. B. 1954. The use of antibiotics in media for the isolation of fungi from polluted water. A*ntibiotics and Chemotherapy*, **4**: 657-662.

Curtis, C.R. 1972. Action Spectrum of the Photoinduced Sexual Stage in the Fungus *Nectria haematococca* Berk. and Br. var *cucurbitae* (Snyder and Hansen) Dingley. *Plant Physiology. 49*(2): 235-239.

Davison, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid*. **42**:73-91.

Doust, J.L., Schmidt, M. and Doust, L.L. 1994. Biological assessment of aquatic pollution: a review, with emphasis on plants as biomonitors. *Biol. Rev. Camb. Phil. Soc*. **69(2):**147–186.

Gandotra, R. 2009. Evaluation of water quality of river Tawi with reference to physico-chemical parameters Distt. Jammu (J&K) India. *Current World Environment*. **3:** 1-5.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, C. and Quentin, C. 2000. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and *Aeromonas* spp. *Appl. Environ. Microbiol.* **1**:125–132.

Guardabassi, L., Petersen, A., Olsen, J.E. and Dalsgaard, A. 1998. Antibiotic resistance in Acinetobacter spp. isolated from sewers receiving waste effluent from a hospital and pharmaceutical plant. *Appl. Environ. Microbiol.* **9**: 3499–3502.

Hagedorn, C., Robinson, S.L., Filtz, J.F, Grubbs, S.M., Angier, T.A., Raymond, B., Reneau, R.B. 1999. Determining sources of fecal pollution in rural Virginia watershed with antibiotic resistance patterns in fecal Streptococci. *Appl. Environ. Microbiol.* **65(12):** 5522–5531.

Jacobs, M.J, Tynkkynen, S., Sibacov, M. 1995. Highly bioluminescent Streptococcus thermophilius strain for the detection of dairy-relevant antibiotics in milk. *Appl. Microbiol. Biotechnol*. **44**:405–412.

Kishimoto, R. and Baker, G. 1969. Pathogenic and potentially pathogenic fungi isolated from beach sands and selected soils of Oahu, Hawaii. *Mycologia*. **61**: 537–548.

Kümmerer, K. 2009. Antibiotics in the aquatic environment- A review- Part I. *Chemo - sphere.* **75**(4), 417-434.

Lindstron-Seppa, P., Urban, K., Honkaampi-Hamalainen, U. and Roy, S. 2001. Biochemical responses in aquatic plants as markers of environmental contamination. *Altern. Lab. Anim.* **29**(3):277–282.

Mary, P., Defives, C and Hornez, J. P. 2000. Occurrence and multiple antibiotic resistance profiles of non-fermentative gram negative microflora in five brands of non-carbonated french bottled spring water. *Microb. Ecol.* **39(4):** 322–329.

McArthur, J. V. and Tuckfield, R.C. 2000. Spatial patterns in antibiotic resistance among stream bacteria: effect of industrial pollution. *Appl. Environ. Microbiol.* **9**: 3722–3726.

Mer, G.S., Sati, S.C. and Khulbe, R.D. 1980. Occurrence, distribution and seasonal periodicity of some aquatic fungi of Sat Tal (NainiTal), India. *Hydrobiol*. **76**: 200-205.

Nogales, B, Lanfranconi, M. P, Piña-villalonga, J. M, and Bosch, R. 2011. Anthropogenic perturbations in marine microbial communities. *FEMS Microbiol. Rev.* (**35)**, 2-275.

Norelli, J. L., Burr, T.J., Civero, A.M., Gilbert, M.T. and Kartz, B.H. 1991. Homologous Streptomycin Resistance Gene Present among Diverse Gram negative Bacteria in New York State Apple Orchards. *Appl.Environ. Microbial*. 57 (2): 486-491.

Ostroumov, S. A. 2000. A conception of aquatic biota as a labile and vulnerable component of the water self-purification system. *Doclady Akademii Nauk.*. **372**(2):279–282.

Pavlov, D., de Wet, C. M., Grabow, W. O. and Ehlers, M. M. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *Int. J. Food Microbiol*. **92**: 275–287.

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. 2006. Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado. *Env. Sci. and Techno.* **40(23):** 7745-7750.

Samecka-Cymerman A. and Kempers, A.J. 2001. Bioindication of heavy metals with aquatic macrophytes: the case of a stream polluted with power plant sewages in Poland. *Toxicol Environ Health*. **62**(1):57–67.

Sarker, D., Roy, N. and Yeasmin, T. 2010. Isolation and antibiotic sensitivity of Bacillus thuringinesis strain from dump soil. *Malaysian J. Microbiol*., **6**(2): 127-132.

Sayah, R. S., Kaneene, J. B., Johnson, Y. and Miller, R., 2005. Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic and wild animals fecal samples, human septage and surface water. *Applied and Environmental Microbiology*. 71 (3): 1394-1404

Schwartz, T., Kohnen, W., Jansen, B. and Obst, U. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in waste-water, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol*. **43**: 325–335.

Schwedt. G., Reiter, C., Uthemann, R. and Grabert, E. 1997. Development of an automated bacterial luminescence test for Biomonitoring of environmental contaminants Fresenius. *J Anal Chem*. **359**: 155–160.

Sharp, M. S. and Lyles, S. T. 1969***. Laboratory Instruction in Biology of Microorganisms***. Saint Louis the C. V. Masby Company, St. Louis, USA 23-25.

Suzuki, S. 1962. Ecological studies on aquatic fungi in the Matsubara lake group. *J. Jpn. Bot.*, **37**: 60–64.

Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer, K. H. 2000. Microbiological safety of drinking water. *Annu. Rev. Microbiol*. **54**: 81–127.

Vieira, R. P., Gonzalez, A. M., Cardoso, A. M., Oliveira, D. N., Albano, R. M., *et al.*, 2008. Relationships between bacterial diversity and environmental variables in a tropical marine environment, Rio de Janeiro. *Environ. Micro*., **10**(**1):** 189-199.

Wernar, S. B., Jones, P.H. and McCormack, W.M. 1969. Gasteroenterities following ingestion of Sewage polluted water on outbreak at a logging camp on the Olympic peninsula. *J. Epridemicil*. **89 (3):** 277-285.

Wright, G. D. 2010. Antibiotic resistance in the environment: a link to the clinic? *Current Opinion in Microbiology*. **13(5):** 589-594

Zhang, X. X., Zhang, T. and Fang, H. H. 2009. Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol*. **82**: 397–414.