Pakistan J. Zool., vol. 52(2), pp 465-475, 2020. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20190128140128

Antibacterial Activity and Tolerance towards Heavy Metals by Endolithic and Epilithic Bacteria Isolated from Rocks of Nathiagali, Lower Himalaya, Pakistan

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Article Information
Received 28 January 2019
Revised 22 May 2019
Accepted 11 June 2019
Available online 17 January 2020

Authors' Contribution
BA and WS conducted experements. WS interpret data and wrote the manuscript. IK, MR and SZ helped in sampling and statistical analysis. FH and AAS designed the research and assisted in the experiments.

Key words
Endoliths, Epiliths, Antibiotics, Antibiotic resistance, Heavy metal resistance.

ABSTRACT

Designing of new antimicrobial drugs is always needed to solve the problem of continue resistance emerging among microorganisms against antibiotics already in use. Microorganisms from unusual environments, such as reported from the surface and inside of rocks are a repository of certain metabolites that might be able to solve the problem of increasing resistance against antibiotics. The present study was designed to isolate endolithic and epilithic bacterial strains from the rapidly weathering rocks collected from Nathiagali, Pakistan to characterize and screen them for antimicrobial activity. The isolates were identified based on 16S rRNA sequence analysis. Antibacterial activity of the isolates was checked against ATCC strains *E. coli*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa*. Seven different types of antibiotic discs were used to check the intrinsic resistance of all the isolates to various antibiotics. Interestingly, most of the isolates were found resistant while only a few were susceptible, however, the isolates *Lysinibacillus* spp. N40 and *Brevundomonas* spp. P20 showed no activity at all. Increased levels of resistance to heavy metals such as; iron, arsenic, cadmium, chromium, and nickel were shown by strains like; *Alcaligenes* spp. N14 and N21, *Bordetella* spp. N30 and *Streptomyces* spp. N28. *Alcaligenes* spp. N27 and *Lysinibacillus* spp. P17 showed strong activity against all the three ATCC strains. The study concludes that the bacteria isolated from the rocks having substantial resistance to heavy metals are also showing good antibacterial activity as well as, and they are also potential candidates for the applications in pharmaceutical as well as environmental research.

INTRODUCTION

Microorganisms are considered the most diverse, complex and important organisms in biosphere playing a significant role in various biological activities. Microorganisms are being isolated from almost all habitats around the biosphere (Muhammad *et al*., 2009; Jabeen *et al*., 2019). The rocks that are under oligotrophic condition are also inhabited by several species of extremophilic bacteria (Sajjad *et al*., 2015). The study of rock dwellers both endoliths and epiliths have been done in many parts of the world from different habitats (Horath *et al*., 2006; Norris and Castenholz, 2006; Walker and Pace, 2007; Walker *et al*., 2005; Chiellini *et al*., 2018; Yang *et al*., 2019). Endoliths were also reported from cliffs of the Niagara escarpment (Gerrath *et al*., 2000; Matthes-Sears *et al*., 1999) and from streams in the UK (Pentecost, 1992). These microbes were also reported from several harsh and unusual habitats like hot and arid desert environments (Friedmann, 1971; Bell *et al*., 1986; Bell, 1993) from travertine in Turkey (Pentecost *et al*., 1997) from Arctic and Antarctic locations (Ascaso and Wierzchos, 2002; Friedmann *et al*., 1993; Friedmann, 1982; Hughes and Lawley, 2003; Russell *et al*., 1998; Wierzchos and Ascaso, 2001) and also from marine littorals (Mason *et al*., 2007; Whitton and Potts, 1982). Rock-inhabiting microbes have also been isolated from other diverse habitats across the globe such as hydrothermal vents (Daughney *et al*., 2004), meteorite impact crater (Cockell *et al*., 2005, 2002), tsunami deposits (Cockell *et al*., 2007), deep subsurface (Amy *et al*.,

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0030-9923/2020/0002-0465 $ 9.00/0

1992; Pedersen, 1997) and also from the cultural heritage monuments (Scheerer *et al*., 2009). Several types of rocks have been studied for microbial diversity such as igneous rocks (both glassy and crystalline) (Herrera *et al*., 2009; Thorseth *et al*., 1992; Villar *et al*., 2006), sedimentary rocks such as sandstones, salts and limestones (Matthes *et al*., 2001; Weber *et al*., 1996; Wierzchos *et al*., 2006), and metamorphic rocks such as gneisses and granites (Cockell *et al*., 2002; De los Rios and Ascaso, 2005).

Extremophilic microorganisms produce molecules adapted to unusual living conditions and have been recognized as an important source of new biological moieties (Sánchez *et al*., 2009). Accordingly, these microorganisms are an important screening target for a variety of bioactive compounds such as secondary metabolites (Hackl *et al*., 2004). Antimicrobial compound production seems to be the general phenomenon for most bacteria. A commendable array of defense, *i.e.* broad-spectrum antibiotics, lytic agents such as lysozyme and metabolic by products such as organic acids are produced by bacteria. In addition, several other types of bacteriocins and protein exotoxins are also produced which are biologically active peptide moieties with the bactericidal mode of action (Riley and Wertz, 2002; Yeaman and Yount, 2003). Secondary metabolites are produced in the response of exhaustion of nutrients, biosynthesis or addition of an inducer and growth rate decrease (Demain, 1998).

Unwanted microorganism control is essential for life and diseases because microbes must be treated in humans, plants, and animals (Basavaraj *et al*., 2010; Gram *et al*., 2010). However, this leads to rapidly emerged and developed antibiotic-resistant microorganisms, mainly due to the misuse of antibiotics (Muhammad *et al*., 2009; Uzair *et al*., 2009). The problem of multidrug resistance is now recognized as a global health problem (Muhammad *et al*., 2009). The current solution for this problem involves the development of a more rational approach to antibiotic discovery and use of new antimicrobial agents (Bhavnani and Ballow, 2000). Most of the antimicrobial compounds originally isolated from the microbial source are being used in the past several decades (Pathania and Brown, 2008). The present study was aimed to investigate the antibacterial activity of endolithic and epilithic bacteria isolates against ATCC bacterial strains.

MATERIALS AND METHODS

All the chemicals and reagents used in this research were of analytical grade and purchased from Sigma-Aldrich Chemical Co. and Merck.

Sampling procedure

Rock and soil samples were collected aseptically from Nathiagali (34°4'20"N, 73°23'55"E), District Abbottabad, KPK, Pakistan, (elevation 2600 meters), in pre-sterilized polyethylene zipper bags. On return to the laboratory, all the samples were stored at 4°C and preliminary isolation experiments were carried out immediately.

Isolation and purification of endolithic and epilithic bacteria

The outer surface of rocks was swabbed with sterile cotton-tipped swabs in Laminar flow hood. The swab was then immersed and shaken in 1 mL sterile water, the suspensions were then spread on nutrient agar plates for isolation of epilithic bacteria. The rocks were broken down with a hammer under aseptic conditions. Then the inner exposed surface was swabbed with sterile cotton-tipped swab under sterile conditions. The swab was then dipped and shaken in 1 ml sterile water and was then spread on nutrient agar plates for endolithic bacteria. The plates were incubated at 30°C for 3 days. For purification, visible colonies were selected based on morphological differences and cultured on nutrient agar plates separately.

Identification of isolates

All the isolates were identified through biochemical and molecular characterization. For molecular characterization, the bacterial DNA was extracted by CTAB method, and 16S rRNA gene was amplified by using both reverses and forward universal primers 27F' (5'- AGAGTTTGATCCTGGCTCAG-3') and 1494R' (5'-CTACGGCTACCTTGTTACGA-3') bacterial primers (47). 20 mL of PCR reaction mixture contains 1 mL DNA sample, 2 mL PCR buffer, 2 mL deoxynucleotide triphosphate (dNTP) mix, 2 mL each reverse and forward primers, ex Taq DNA polymerase of 0.5 mL and 10.5 mL distil water. Initially, the reaction mixture was incubated at 96°C for 4 min. Then 35 amplification cycles were run at 94°C for 40 sec, 55°C for 55 sec, and 72°C for 60 sec. Further, incubation of reaction was carried out for 7 min at 72°C. Both positive control (*Escherichia coli* genomic DNA) and negative control were run in parallel in the PCR. Sequencing products were resolved on an Applied Bio-Systems model 3100 automated DNA sequencing system (Applied BioSystems, USA) at the Macrogen, Inc., Seoul, Korea and the sequences were submitted to public gene bank NCBI and the accession number has been assigned (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed by the Maximum Likelihood method with the robustness of 1000 bootstrapping value in MEGA 6.0 (47).

Test bacterial strains

ATCC bacterial cultures such as *E. coli* (10536), *Staphylococcus aureus* (6538) and *Pseudomonas*

aeruginosa (15442) were used as test strains to check the antibacterial activity of endolithic and epilithic bacterial isolates.

Antimicrobial assay

A pure test microbial colony was transferred into test tubes having a normal saline solution and adjusting the turbidity comparing with 0.5 McFarland standard solution prepared by adding 0.5 ml of $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) into 99.5 ml of H_2SO_4 (1% w/v) with proper stirring. After adjusting, a sterile cotton swab was dipped into the normal saline suspension having test organisms and swabbed well over the entire surface of the plate containing Mueller Hinton agar (MHA) to ensure uniform distribution of the inoculum. Subsequently, a small portion of each bacterial isolate was point inoculated on plates that contain test microbial colony. These plates were incubated at 30°C for 24 h along with control having an antibiotic disc (ciprofloxacin and rifampicin). After 24 h of incubation, the diameter of the clear zones that showed inhibition of bacterial growth was measured in millimeter (mm). The experiment was done in triplicate and the mean value of zone inhibition was calculated with standard error.

Sensitivity test of isolates against antibiotics

Sensitivity test of isolates was performed to check their resistivity against antibiotics. Seven different types of antibiotic namely Amoxicillin + Clavulanic acid (AMC), Nalidixic Acid (NA), Gentamicin (CN), Piperacillin + Tazobactam (TZP), Cefepime (FEP), Imipenem (IMP), and Fusidic Acid (FD), of 30, 30, 10, 110, 30, 10 and 10 µg/disc, respectively were used for this purpose. A pure bacterial colony was transferred into test tubes having a normal saline solution and adjusted the turbidity comparing it with 0.5 McFarland standard solution. After adjustment, a sterile cotton swab was dipped into the normal saline suspension and swabbed well over the entire surface of the plates containing Mueller Hinton agar (MHA) to ensure uniform distribution of the inoculum. The antibiotic discs were placed on the plate very carefully.

Heavy metal tolerance of isolated strains

To study the metal tolerance of all the strains, 24 h fresh cultures were streaked on nutrient agar plates supplemented with different concentrations (10-1000 ppm) of metal salts. Metal salts used included; $ZnCl_2$, $CrCl_3$, $HgCl_2$, $FeCl_3$ and $CdCl_2$. Cultures were incubated for 24 h at 37°C and cell growth was observed.

RESULTS

Physical characters of the site were recorded during sampling. The pH of the site was 5.0 and the temperature was 20°C. The flora and fauna of the site were recorded. In flora, mostly shrubs and tall trees of pines were present around the sampling site. In fauna, insects, snakes, monkeys and various kinds of birds were seen there. Seventeen different endolithic and epilithic isolates were reported in the present study. Among 17 isolates, eight isolates were epilithic named as P17, P18, P19, P20, P21, P23, P24, and P26 while 9 were endolithic named as N12, N14, N21, N22, N26, N27, N28, N30, and N40. All these different strains were isolated on a morphological basis.

Table I.- Colony morphology and microscopic examination of isolates.

S. No.	Strain	Colony morphology	Microscopic examination
1	P17	Small, circular, cream color, flat, opaque	Gram Negative, long rods
2	P18	Small sized, raised colonies, opaque, pin-pointed colonies	Gram +ve, rods
3	P19	Off white in color, flat in shape, small size, circular	Gram –ve, short rods
4	P20	Grows in net form, off white in color, long threads type	Gram –ve, long rods
5	P21	Off white color, flat colonies, small in size	Gram -ve, rods
6	P23	Large in size, sticky/jelly type, irregular in shape, cream color, raised colonies, after 4 days the colonies become dried having liquid inside and have irregular margins	Gram –ve, short rods
7	P24	Small, circular shape, off white color, flat colonies, opaque	Gram –ve, rods
8	P26	Small in size, shiny appearance, opaque,	Gram –ve, rods
9	N12	Off white color, small in size, flat colonies	Gram –ve, rods
10	N14	Small in size, irregular in shape, cloudy type in appearance, off white in color	Gram +ve, rods
11	N21	Small in size, flat colonies, entire margins, off white color	Gram +ve, short rods
12	N22	Small in size, raised colonies, yellowish in color, circular in shape	Gram -ve, rods
13	N26	Orange color, small in size, flat colonies, irregular in shape	Gram –ve, short rods
14	N27	Transparent type, small in size, irregular in shape, off white in color	Gram –ve, rods
15	N28	Milky white in color, dry, small in size, circular in shape, raised colonies	Gram +ve, rods
16	N30	Large in size, flat colonies, sticky type, off white color	Gram –ve, rod
17	N40	Medium size, yellowish, circular in shape, flat colonies, change the medium color	Gram –ve, rods

Morphological features such as size, shape, color, margins were checked and recorded (Table I). Gram's staining results show that out of 11 endolithic bacteria two of them were Gram-positive rods while 9 were Gram-negative. While out of 10 epilithic bacteria, 2 were Gram-positive and 9 were Gram-negative.

Identification of isolates

The isolates were identified by BLAST search homology in NCBI. 16S rRNA gene sequences of the isolates were assigned the Accession numbers by the NCBI (Table II). Based on microscopic, morphological and biochemical tests the isolates were identified by comparing these characteristics with Bergey's Manual of Determinative Bacteriology (9th Edition). Isolates N22, N21, P21, N14, N27 and P24 were identified as *Alcaligenes* spp., P17 and N40 were identified as *Lysinibacillus* spp., P20 was identified as *Brevundimonas* spp., P23 and N30 were identified *Bordetella* spp., P26 and P19 were identified as *Pseudomonas* spp., N12 as *Pusillimonas* spp.

Table II.- Identified strains with their respective accession numbers.

S. No.	Isolated strains	Homologous species	Accession No. (Assigned)	Query coverage (%)	Identity (%)
1	P17	*Lysinibacillus* spp.	KT004373	100	99
2	N22	*Alcaligenes* spp.	KT004374	100	99
3	N30	*Bordetella* spp.	KT004375	99	89
4	N21	*Alcaligenes* spp.	KT004376	99	86
5	N40	*Lysinibacillus* spp.	KT004377	98	82
6	P21	*Alcaligenes* spp.	KT004378	100	99
7	P23	*Bordetella* spp.	KT004379	99	90
8	P24	*Alcaligenes* spp.	KT004380	100	99
9	P26	*Pseudomonas* spp.	KT004381	100	99
10	N12	*Pusillimonas* spp.	KT004382	100	97
11	N14	*Alcaligenes* spp.	KT004383	100	99
12	N26	*Fluviicola* spp.	KT004384	98	93
13	N27	*Alcaligenes* spp.	KT004385	100	98
14	N28	*Streptomyces* spp.	KT004386	100	100
15	P19	*Pseudomonas* spp.	KT004387	100	98
16	P20	*Brevundomonas* spp.	KT004388	100	100
17	P18	*Parapusillimonas* spp.	KT004389	100	95

Table III.- Antibacterial activity of isolates.

S. No.	Isolates	Activity against ATCC		
		***Pseudomonas aeruginosa* (15442)**	***Staphylococcus aureus* (6538)**	***Escherichia coli* (10536)**
1	*Pusillimonas* spp. N12	+	-	+
2	*Alcaligenes* spp. N14	++	-	++
3	*Alcaligenes* spp. N21	++	+++	-
4	*Alcaligenes* spp. N22	+	-	+
5	*Fluviicola* spp. N26		+++	-
6	*Alcaligenes* spp. N27	+++	+++	+++
7	*Streptomyces* spp. N28	-	+++	-
8	*Bordetella* spp. N30	+	-	-
9	*Lysinibacillus* spp. N40	-	-	-
10	*Lysinibacillus* spp. P17	+++	+++	+++
11	*Parapusillimonas* spp. P18	+++	-	-
12	*Pseudomonas* spp. P19	+++	-	+
13	*Brevundomonas* spp. P20	-	-	-
14	*Alcaligenes* spp. P21	-	-	++
15	*Bordetella* spp. P23	+	-	+
16	*Alcaligenes* spp. P24	-	-	+
17	*Pseudomonas* spp. P26	+	-	++

-, no activity; +, weak activity; ++, moderate activity; +++, strong activity.

N26 as *Fluviicola* spp., N28 as *Streptomyces* spp. and isolate P18 identified as *Parapusillimonas* spp.

Phylogenetic analysis

The phylogenetic tree constructed in MEGA 6.0 revealed that similar bacteria are grouped into the same group. The 1st cluster is of *Proteobacteria* group. Most of the study isolates belong to this group. This group contains bacteria similar to *Advenella* sp., *Alcaligenes* sp., *Pseudomonas* sp. and *Bordetella* sp. These all are gram-negative bacteria. The second cluster of gram-positive bacteria *Actinobacteria*. Isolate N28 is clustered into this group. The 3rd cluster is of gram-positive bacteria *Firmicutes* and isolate P17 belongs to this group (Fig. 1).

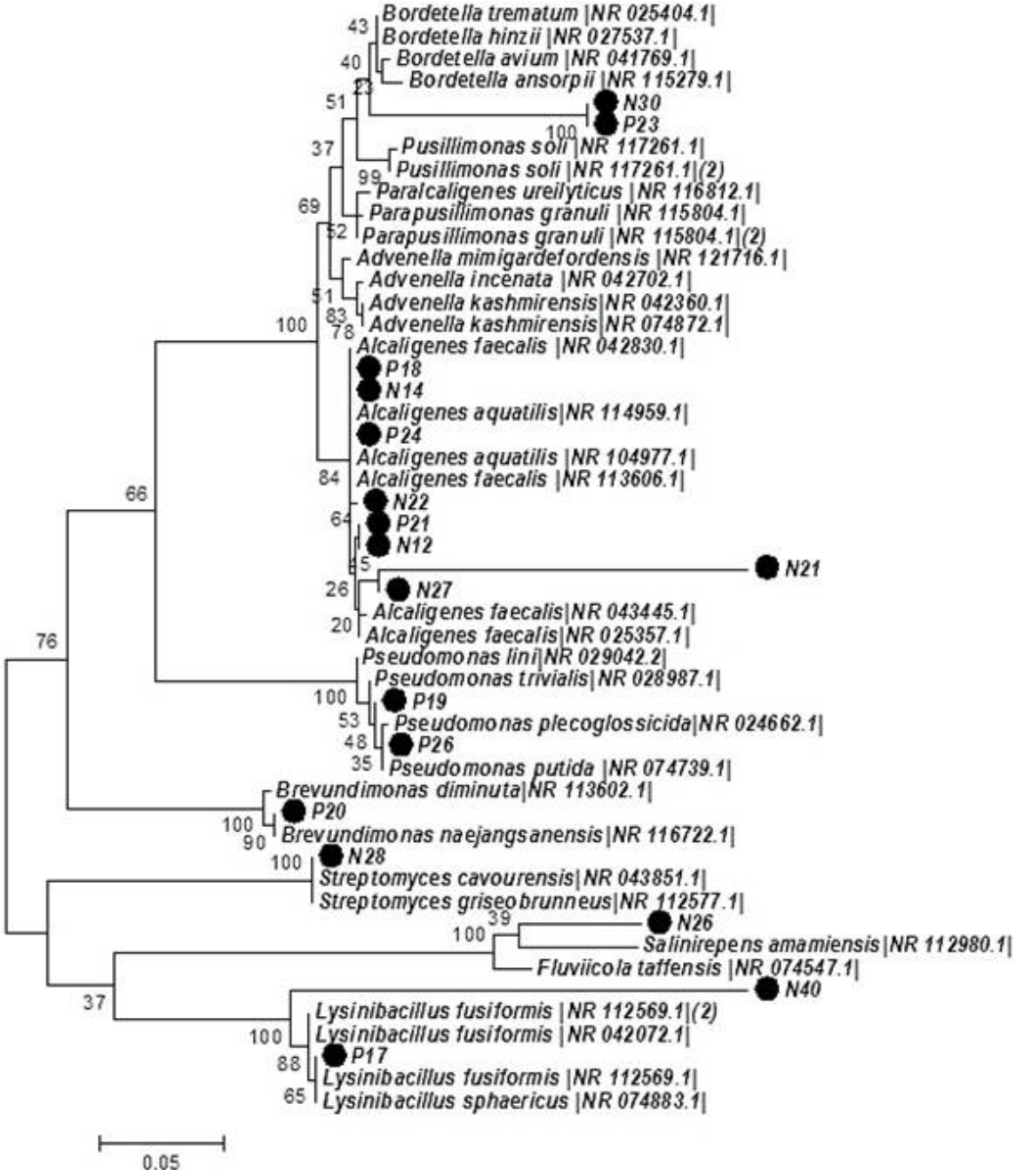

Fig. 1. Phylogenetic tree of isolated bacterial strains.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2757.2947) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 383 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Antimicrobial activity of endolithic and epilithic bacteria

Antimicrobial activity of all 17 isolates was checked against three ATCC strains; *Pseudomonas aeruginosa* (15442), *Staphylococcus aureus* (6538) and *Escherichia coli* (10536). The clear zone around the point inoculation of isolates showed inhibition of test microbes as with control (Table III). *Alcaligenes* spp. N27 and *Lysinibacillus* spp. P17 showed strong activity against all the three ATCC cultures while the strains *Lysinibacillus* spp. N40 and *Brevundomonas* spp. P20 showed no activity.

The sensitivity of isolates against antibiotics

The sensitivity of all the isolates was checked against seven different broad and narrow spectrum antibiotics and the results were interpreted by measuring zones of inhibition according to Clinical and Laboratory Standards Institute (CLSI) protocol (Table IV). All the isolates were found resistant against Amoxicillin + Clavulanic acid and Cefepime except isolate *Bordetella* spp. P23, while *Alcaligenes* spp. N22 was resistant to Amoxicillin + Clavulanic acid and susceptible to Cefepime. Against Piperacillin + Tazobactam (TZP), *Lysinibacillus* spp. P17 and *Alcaligenes* spp. P24 were found resistant, whereas, *Alcaligenes* spp. N14, *Pseudomonas* spp. P26 and *Pusillimonas* spp. N12 were susceptible. It was observed that all strains were susceptible to Imipenem and Gentamicin. Majority of the strains were found susceptible to Nalidixic acid except for *Lysinibacillus* spp. P17, *Streptomyces* spp. N28, *Lysinibacillus* spp. N40 and *Alcaligenes* spp. P24. Against Fusidic acid, the isolates *Parapusillimonas* spp. P18, *Pseudomonas* spp P26, *Alcaligenes* spp. N27, *Alcaligenes* spp. N22, *Lysinibacillus* spp. N40, *Pseudomonas* spp. P19 and *Bordetella* spp. P23 were found resistant, whereas, the remaining were susceptible.

Table IV.- The sensitivity of endolithic and epilithic bacteria against different antibiotics.

Strains	Antibiotics used and zone of inhibition (mm)						
	AMC	FEP	TZP	IMP	NA	CN	FD
Pusillimonas spp. N12	0 (R)	0 (R)	14 (R)	28 (S)	26 (S)	26 (S)	18 (S)
Alcaligenes spp. N14	0 (R)	0 (R)	10 (R)	28 (S)	26 (S)	28 (S)	0 (R)
Alcaligenes spp. N21	0 (R)	10 (R)	13 (R)	15 (I)	14 (I)	20 (S)	0 (R)
Alcaligenes spp. N22	0 (R)	38 (S)	14 (R)	28 (S)	22 (S)	22 (S)	0 (R)
Fluviicola spp. N26	0 (R)	0 (R)	-	24 (S)	24 (S)	20 (S)	30 (S)
Alcaligenes spp. N27	0 (R)	0 (R)	-	32 (S)	18 (I)	22 (S)	0 (R)
Streptomyces spp. N28	14 (I)	0 (R)	-	34 (S)	0 (R)	42 (S)	18 (S)
Bordetella spp. N30	0 (R)	0 (R)	-	28 (S)	26 (S)	32 (S)	27 (S)
Lysinibacillus spp. N40	0 (R)	0 (R)	-	30 (S)	0 (R)	26 (S)	0 (R)
Lysinibacillus spp. P17	0 (R)	0 (R)	0 (R)	34 (S)	0 (R)	28 (S)	12 (R)
Parapusillimonas spp. P18	0 (R)	0 (R)	-	24 (S)	16 (I)	20 (S)	0 (R)
Pseudomonas spp. P19	16 (I)	0 (R)	-	20 (S)	12 (R)	18 (S)	0 (R)
Brevundomonas spp. P20	17 (I)	0 (R)	-	22 (S)	17 (I)	15 (S)	22 (S)
Alcaligenes spp. P21	0 (R)	0 (R)	-	21 (S)	25 (S)	28 (S)	0 (R)
Bordetella spp. P23	10 (R)	12 (R)	-	20 (S)	12 (R)	18 (S)	0 (R)
Alcaligenes spp. P24	0 (R)	0 (R)	0 (R)	18 (S)	0 (R)	26 (S)	0 (R)
Pseudomonas spp. P26	0 (R)	0 (R)	12 (R)	24 (S)	14 (I)	24 (S)	0 (R)

R, no zone produced or resistant; I, intermediate susceptibility; S, susceptible.

Table V.- Tolerance of isolates against different metals.

S. No.	Strain	Concentration (ppm) of metal salts					
		Cd	Cr	Fe	Hg	Ni	As
1	*Lysinibacillus* spp. P17	800	680	880	10	440	780
2	*Parapusillimonas* spp. P18	180	160	200	5	300	300
3	*Pseudomonas* spp. P19	820	280	900	40	120	840
4	*Brevundomonas* spp. P20	810	670	920	10	140	80
5	*Alcaligenes* spp. P21	770	680	880	35	290	820
6	*Bordetella* spp. P23	800	630	840	50	390	760
7	*Alcaligenes* spp. P24	270	600	430	5	360	200
8	*Pseudomonas* spp. P26	800	720	820	40	340	760
9	*Pusillimonas* spp. N12	790	700	920	50	400	820
10	*Alcaligenes* spp. N14	820	220	920	10	420	180
11	*Alcaligenes* spp. N21	780	370	340	20	340	840
12	*Alcaligenes* spp. N22	810	280	780	10	460	800
13	*Fluviicola* spp. N26	760	700	820	40	350	800
14	*Alcaligenes* spp. N27	800	720	820	40	340	760
15	*Streptomyces* spp. N28	790	720	800	10	640	780
16	*Bordetella* spp. N30	800	720	790	20	300	740
17	*Lysinibacillus* spp. N40	760	120	900	25	290	780

Metal resistance

In the current study, five different metals including; Cd^{2+}, Ar^{+2}, Hg^{+2}, $Cr^{+3,}$ and Fe^{+3} were examined for all isolates (Table V). The highest level of resistance was recorded by *Alcaligenes* spp. N14 as 920 ppm to iron, followed by resistance to arsenic by *Alcaligenes* spp. N21, cadmium again by *Alcaligenes* spp. N14, chromium by *Bordetella* spp. N30 and nickel by *Streptomyces* spp. N28. In the case of mercury, less resistivity was observed, yet *Bordetella* spp. P23 and *Pusillimonas* spp. N12 showed maximum resistivity *i.e.* up to 50 ppm. The level of resistance was recorded as; Fe > Ar > Cd > Cr > Ni > Hg.

DISCUSSION

There is a great deal of variation in the degree of antagonistic activity of different cultures (Pandey *et al*., 2002). The antimicrobial chemical compound efficiency has highly affected by its permeability, target site specificity and its effect on the host impact (Pathania and Brown, 2008). Antimicrobial compounds inhibition ability depends on the site of secretion of compounds, *i.e.* compounds are secreted outside the cell or accumulated in the cell (Nofiani, 2009). Increasing resistance pattern and the associated side effects of antibiotics have evolved the importance of some alternative source to be used as an antibacterial agent. One of the options for the solution of this medical problem is the use of microorganisms from unusual habitats. These habitats are beyond the reach of anthropogenic activities, so that can be able to produce some unique types of secondary metabolites that may encounter the pathogenic microbes which is the main issue of the present period. Recently, one such study was conducted from the glacier environment and reported the antimicrobial activity of psychrophilic bacterial isolates (Rafiq *et al*., 2019). Bacteria may have certain genes responsible for encoding metabolites, but it does not express in normal circumstances (Demain, 1998). In this study, isolates showed an efficient antibacterial activity against other ATCC test bacterial species. This kind of environments holds nutrient deficient conditions and the microbes compete for nutritional requirements by producing anti-microbial agents against competing organisms. These antimicrobial compounds may demonstrate weak inhibition because some of them yield it in small amounts. Though, in high amount, these compounds may hold new and beneficial properties. Antimicrobial activity of isolates against *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli* were checked and some isolates showed good activity against these ATCC bacterial cultures. Antibacterial activity of bacterial isolates from different extreme environments has already been done but the activity of endolithic and epilithic isolates is not reported yet. Endolithic and epilithic bacteria inhabit in the oligotrophic environment and possibly having the potential ability to produce antibiotics. Several studies focused on screening secondary metabolites produced by microorganisms that inhabit such extreme habitats as potential sources of useful compounds: antibiotics, immunosuppressant, and statins (Harvey, 2000), exopolysaccharides (Nicolaus *et al*.,

2010), biosurfactants (Banat *et al*., 2010), extremozymes (Singh *et al*., 2011), radiation protective drugs (Singh and Gabani, 2011), antitumoral (Chang *et al*., 2011). Activity against these three strains was also checked by Singh *et al*. (2009), for the screening of antimicrobial activity of bacterial isolates from the soil of stressed ecological niches of eastern Uttar Pradesh, India.

Isolates also showed resistance to several broad and narrow spectrum antibiotics. This property may be adopted due to their survival in harsh conditions where they face unusual conditions by switching on certain genes to face harsh conditions and to encounter the effects of dominant competing microorganism. The sensitivity of isolates was checked against seven different antibiotics, and some isolates were found having resistivity against both broad and narrow spectrum antibiotics. This resistance against the known antibiotics reveals that these isolates can compete for other microorganisms for their nutritional requirements by producing certain secondary metabolites. Resistance against AMC, FD, and FEP was reported by endolithic and epilithic isolates. Similar study was reported by Parvathi *et al*. (2009), performing biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India, and Naphade *et al*. (2012) while performing isolation, characterization and identification of pesticide tolerating bacteria from garden soil in Kalyan, India. The antibiotic resistance of environmental isolates on the other hand also revealed the miss use of the antibiotics that may somehow be transferred from clinical to environmental microflora which is a big alarm for the current medical problems.

Isolates were also found to have resistance against certain metals. This property of these isolates is due to the fact that they are living in the harsh condition inside rocks which is abundant in metal concentration. Tolerance of isolates against six different heavy metals was checked and the results show that most isolates have great potential for metal tolerance. This capability of isolates could be exploited for certain applications such as for the bioremediation of heavy metals affected environments, extraction of metals from low-grade ores and their enzymes are metals tolerated. These isolates from such an unusual environment have several beneficial aspects, which could be exploited for the well-being of mankind.

CONCLUSIONS

Based on our study it was concluded that bacterial isolates from the unusual habitat of rocks not only showed resistance to metals and antimicrobial activity against ATCC isolates but also exhibited resistance towards several antibiotics. Moreover, purification and characterization of each bioactive chemical compound may be pharmaceutically more potential and significant. Further study is required to characterize and purify these antimicrobial compounds.

ACKNOWLEDGMENTS

We are grateful to Quaid-i-Azam University, Islamabad for providing transport facility for the collection of samples and funds in order to accomplish research successfully on time.

Statement of conflict of interest

The authors declare no conflict of interest.

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