



Prevalence and abundance of antibiotic-resistant genes in culturable bacteria inhabiting a non-polar passu glacier, karakorum mountains range, Pakistan

Sabir Nawaz¹ · Muhammad Rafiq² · Ian L. Pepper³ · Walter Q. Betancourt³ · Aamer Ali Shah¹ · Fariha Hasan¹

Received: 27 December 2022 / Accepted: 20 January 2023 / Published online: 9 February 2023
© The Author(s), under exclusive licence to Springer Nature B.V. 2023

Abstract

Natural pristine environments including cold habitats are thought to be the potent reservoirs of antibiotic-resistant genes and have been recurrently reported in polar glaciers' native bacteria, nevertheless, their abundance among the non-polar glaciers' inhabitant bacteria is mostly uncharted. Herein we evaluated antibiotic resistance profile, abundance of antibiotic-resistant genes plus class 1, 2, and 3 *integron integrases* in 65 culturable bacterial isolates retrieved from a non-polar glacier. The *16S rRNA* gene sequencing analysis identified predominantly Gram-negative 43 (66.15%) and Gram-positive 22 (33.84%) isolates. Among the Gram-negative bacteria, *Gammaproteobacteria* were dominant (62.79%), followed by *Betaproteobacteria* (18.60%) and *Alphaproteobacteria* (9.30%), whereas Phyla *Actinobacteria* (50%) and *Firmicutes* (40.90%) were predominant among Gram-positive. The Kirby Bauer disc diffusion method evaluated significant antibiotic resistance among the isolates. PCR amplification revealed phylum *Proteobacteria* predominantly carrying 21 disparate antibiotic-resistant genes like; *bla*_{AmpC} 6 (100%), *bla*_{VIM-1}, *bla*_{SHV} and *bla*_{DHA} 5 (100%) each, *bla*_{OXA-1} 1 (100%), *bla*_{CMY-4} 4 (100%), followed by *Actinobacteria* 14, *Firmicutes* 13 and *Bacteroidetes* 11. Tested isolates were negative for *bla*_{KPC}, *qnrA*, *vanA*, *ermA*, *ermB*, *int12*, and *int13*. Predominant Gram-negative isolates had higher MAR index values, compared to Gram-positive. Alignment of protein homology sequences of antibiotic-resistant genes with references revealed amino acid variations in *bla*_{NDM-1}, *bla*_{OXA-1}, *bla*_{SHV}, *mecA*, *aac(6)-Ib3*, *tetA*, *tetB*, *sul2*, *qnrB*, *gyrA*, and *int11*. Promising antibiotic-resistant bacteria, harbored with numerous antibiotic-resistant genes and class 1 *integron integrase* with some amino acid variations detected, accentuating the mandatory focus to evaluate the intricate transcriptome analysis of glaciated bacteria conferring antibiotic resistance.

Keywords Antibiotic resistance · Antibiotic-resistant genes · Glacial sediment · Integron integrase · Multiple antibiotic resistance index · β -lactamase

✉ Muhammad Rafiq
mr14311@my.bristol.ac.uk

✉ Fariha Hasan
fariha.hasan@yahoo.com

Sabir Nawaz
sabir.nawaz@bs.qau.edu.pk

Ian L. Pepper
ipepper@ag.arizona.edu

Walter Q. Betancourt
wbetancourt@arizona.edu

Aamer Ali Shah
alishah@qau.edu.pk

¹ Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan

² Department of Microbiology, Balochistan University of Information Technology, Engineering and Management Sciences, Quetta, Pakistan

³ Water & Energy Sustainable Technology (WEST) Center, University of Arizona, 2959 W. Calle Agua Nueva, 85745 Tucson, AZ, USA

Introduction

The planet Earth's surface is unevenly distributed with environments and some of them are termed "Extreme Environments", which may be detrimental to the biosphere. Extreme environments once thought to be lethal for life existence, be brimming with organisms (Gilichinsky et al. 2007). Extremes exist in physical (temperature, pressure, or radiation) and geochemical (salinity, desiccation, pH, redox potential, and oxygen species) forms, and occupants include Archaea, Bacteria, and some Eukaryotes (Rothschild and Mancinelli 2001). MacElroy (1974) coined the word "Extremophile" for organisms that thrive in a such hostile environment (MacElroy 1974). Extremophiles have adapted themselves to get through and retain their existence in extreme environments through evolution. Based on such environmental conditions for bacteria to thrive in extreme environments, extremophiles are described as psychrophilic (0 to 20 °C), thermophilic (41 to 122 °C), hyperthermophilic (80 °C to > 122 °C), acidophilic (pH 1–5), alkaliphilic (\geq pH 9), piezophilic (elevated hydrostatic pressure, up to 50 MPa), halophilic (salt concentration up to 5 M), oligotrophic (grow in trace nutrients environment), endolithic (nourish within mineral pores and rock) and xerophilic (low availability of free water) (Gupta et al. 2014; Kamekura 1998; Kato et al. 1998).

Considering cold extreme environments on planet earth, approximately 90% of oceans and 26% of terrestrial ecosystems are exposed to low temperatures (Zhang et al. 2009), which stretch across the Arctic to Antarctic plates, as well as from high mountain regions to cavernous of oceans. An utmost portion of the deep sea (90% oceans) have < 5 °C, snow covers 35% of the land surface, permafrost comprises 24% of the land surface, sea ice 13% of the earth's surface and glaciers occupy 10% of the land surface (Margesin and Miteva 2011; Cowan et al. 2007). Glaciers are moving gigantic masses of stable ice bodies that consist of highly pressurized and re-crystallized snow which displays oblique slope topology under the influence of gravitational force (Takeuchi et al. 2006). Besides the north and south poles, a massive reservoir of tropical glaciers lies in the region of Karakoram and Himalaya (third pole of the world), with more than 54,252 glaciers that occupy an area of 60,000 km², mainly with < 0 °C annual temperature (Rafiq et al. 2019). Like other extreme environments, glaciers also harbor all life forms and microorganisms that have successfully colonized permanently frozen habitats favorable for their survival (Anesio and Laybourn, 2012; Morita, 1975).

A diverse range of bacterial flora from cold environments such as glaciers, cold soil, subsoil, frozen lakes, and cold caves have been explored across the globe (Rafiq et al. 2019, 2017; Anesio et al. 2017; Anesio and Laybourn, 2012; Margesin and

Miteva 2011; Amato et al. 2007; Takeuchi et al. 2006). From glaciers located in the Himalaya region, researchers have reported bacterial species such as *Pedobacter himalayensis* (Shivaji et al. 2005), *Exiguobacterium indicum* (Chaturvedi and Shivaji 2006), *Dyadobacter hamtensis* (Chaturvedi et al. 2005), *Leifsonia pindariensis*, *Bacillus cecembensis*, *Cryobacterium roopkundense*, *Cryobacterium pindariense* (Reddy et al. 2010, 2008), *Paenibacillus glacialis* (Kishore et al. 2010), psychrotrophic proteolytic bacteria such as *Bacillus liceniformis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Baghel et al. 2005). On the contrary, algae and fungi are reported in low abundance in cold environments (Lutz et al. 2015; Margesin and Miteva 2011). Globally, glaciers have been found with drastic variations in form of microbial ecology, community structure, and biomass (Liu et al. 2009; Zhang et al. 2007, 2009), which is under the main influences of environmental and climatic factors like geographic location (Takeuchi et al. 2004; Mueller and Pollard 2004), sunlight intensity, winds speed and direction, nutrients and liquid water availability (Bhatia et al. 2006).

Organisms inhabiting the earth's cryosphere thrive in extremely inhospitable environments for existence and are therefore exposed to nutrient-deficient conditions, characterized by low temperatures, free water availability, freeze-thaw cycles, crystallization, and increased salinity. To endure such challenges, microorganisms adopt intracellular mechanisms in the form of elevated expression of exopolysaccharides, unsaturated fatty acids, transcription and translation of cold-active enzymes, ice nucleating and antifreeze proteins (Margesin and Miteva 2011; Casanueva et al. 2010; Stibal et al. 2008; Sakamoto and Murata 2002). For life continuity, the glacier microbiota performs several requisite roles in biogeochemical cycling, subglacial weathering, mineralization, sustaining food web, and immobilization of numerous xenobiotic compounds (Anesio et al. 2017; Montross et al. 2013; Dong et al. 2006; Takeuchi and Koshima 2004). Moreover, to cope with other competitors for survival, indigenous microbiota produces antimicrobial compounds and violet pigments that exert selective pressure on resident microbes dwelling in cold environments (Allen et al. 2010; Giudice et al. 2007; Nakamura et al. 2003).

Antibiotics production and resistance are natural phenomena with primordial origins (D'Costa et al. 2011; Allen et al. 2010; Martinez 2008; Hall and Barlow 2004) and presently the expeditious spread of antibiotic resistance among bacteria is the preeminent global threat, imposing an emerging problem on public and animal's health and this occurrence is not only confined to health care settings but has also been majorly observed in cold pristine environments (Van Goethem et al. 2018; Perron et al. 2015; Segawa et al. 2013; Ushida et al. 2010; Wright 2010). Previous research

has notably highlighted the phenomenon of antibiotic resistance in clinical settings, but the surge in community-acquired infections by antibiotic-resistant bacteria has called the attention of scientists to search for antibiotic-resistant determinants in natural environments (Gibson et al. 2015). Environmental bacteria inhabiting soil, glacier, and marine water are thought to be the vigorous reservoir of antibiotic-resistant genes (ARGs) (Van Goethem et al. 2018; Perron et al. 2015; Segawa et al. 2013; Ushida et al. 2010; Allen et al. 2009; De Souza et al. 2006) that are massively circulating in clinical settings since bacteria in the natural environment have evolved mechanisms of resistance against antimicrobial compounds for billions of years in the absence of anthropogenic effect (Blair et al. 2015; Forsberg et al. 2012; D'Costa et al. 2006). The discovery of natural reservoirs of antibiotic-resistant determinants manifests the vital role of the natural environment in the dissemination of ARGs (Whitman et al. 1998). Bacteria can effectively resist intrinsically to antibiotics in a natural environment under stressful conditions as shown for *Escherichia coli* which evolved innate resistance against vancomycin drug (Russell 2003) and innate efflux pump mechanism present in other Gram-negative bacteria (Blair et al. 2015). In addition to acquired mechanisms of antibiotic resistance via mutation and exchange of plasmids, horizontal gene transfer (HGT) of integrons and transposons represent additional acquired resistance mechanisms (Blair et al. 2015; Martinez et al. 2008).

Anthropogenic impact on natural environments, due to misuse of antibiotics for medical and agricultural purposes is mainly responsible for the origination and dissemination of antibiotic resistance in natural environments (Hawkey 2008; Cabello 2006; Chee-Sanford et al. 2001; Goni-Urriza et al. 2000). Bacteria harboring ARGs usually thrive in antibiotic dense habitats such as human and livestock gut, agricultural soil and antibiotic-resistant bacteria (ARB) resulting from atmospheric deposition and aquatic circulation. The dissemination of ARGs occurs across adjoining environments (Gibbs et al. 2006; Levy and Marshall 2004; Lighthart and Shaffer 1995). Such adjacent environments are found to be abundant with ARB compared to remote sites (Malik et al. 2008; Furushita et al. 2003). In contrast, several studies revealed the presence of ARB-carrying ARGs in geographically secluded habitats free of anthropogenic effect (Van Goethem et al. 2018; Perron et al. 2015; Ushida et al. 2010; Allen et al. 2009).

Worldwide, researchers have reported numerous ARGs among bacterial communities thriving in remote cold habitats like; the Gulkana glacier (Eastern Alaska, USA), Ürümqi glacier (Xinjiang, China) (Ushida et al. 2010), Central Asia (China, Tajikistan, and Kyrgyzstan), Himalayas (Bhutan and Nepal), Africa (Uganda), South America (Chile), North

America (Alaska), Arctic (Greenland), Antarctica (Segawa et al. 2013) and Mackay glacier (Antarctica) (Van Goethem et al. 2018). The existence of AR bacteria harbored with ARGs in a natural antibiotic-free environment like a glacier is a curious and quintessential case for ARGs (Ushida et al. 2010). Globally, polar cold habitats are extensively searched for microbial biodiversity, antibiotic resistance, biogeochemical processes, and the effect of climate changes (Glaring et al. 2015; Lanoil et al. 2009; Segawa et al. 2010; Christner et al. 2002). On the contrary, the non-polar cold habitats have not been addressed properly. A decade ago, scientists has started to explore these regions (Karakorum Himalaya) for microbial diversity and antibiotic resistance (Rafiq et al. 2017; Shen et al. 2012; Reddy et al. 2010; Zhang et al. 2010; Liu et al. 2009; Chaturvedi et al. 2005). Considering the culturable bacterial diversity and antibiotic resistance in glaciers and other pristine cold environments, the main objectives of this research were to screen the unexplored non-polar glacier of Pakistan for bacterial diversity and antibiotic resistance genes. In this study, bacterial isolates from Passu glacier were isolated and evaluated for culturable diversity, antibiotic susceptibility, and abundance of ARGs.

Materials and methods

Site description of study isolates

The Passu glacier is located in Gilgit-Baltistan, Northern Pakistan, corresponding to the most largely populated non-polar glacier's region of the Karakorum mountains range covering an area of 72,971 square kilometers that lies at latitude 36° 27' 59.99" N and longitude 74° 53' 59.99" E (Fig. 1).

Sample collection

The bacterial isolates were collected from glacial ice, meltwater, and sediment from three random distant locations (250–300 m apart) during March and April 2017. With the help of a ceramic knife sterilized by ethanol and UV exposure, more than 0.01-meter dept samples were taken to avoid the influence of mammals, birds, and humans. All the samples from three sources were collected and placed in sterile Whirl-Pak Bags (Nasco, USA) with extremely careful measurements taken during sample collection and packaging to avoid any sort of contamination by gloves, masks, and suits. Finally, the collected samples were transferred to the Applied, Environmental and Geomicrobiology Lab, Department of Microbiology, Quaid-i-Azam University Islamabad, and stored at -70°C for subsequent uses.

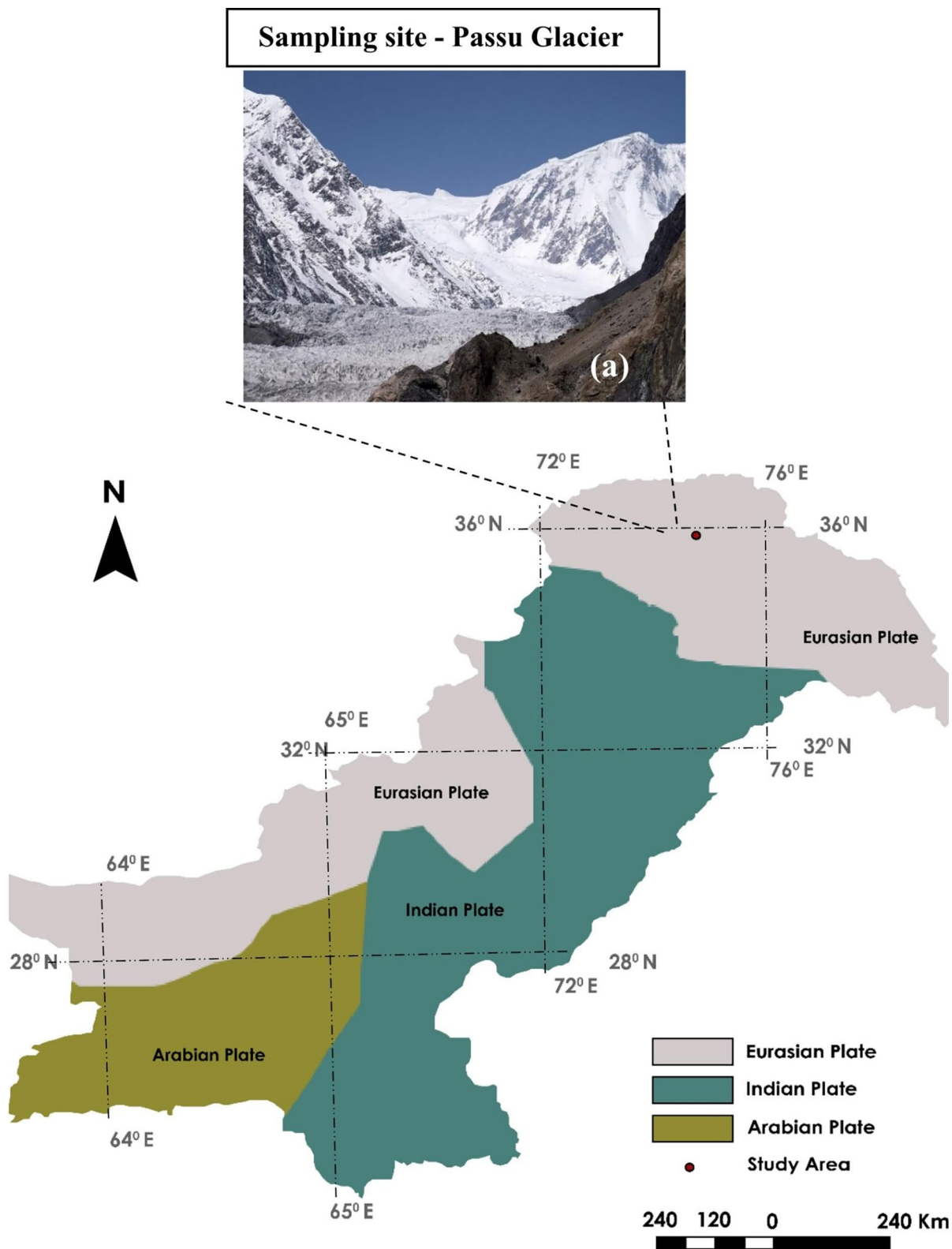


Fig. 1 Sampling site location: Passu glacier, Northern Pakistan, Karakorum Mountains Range (a) Photograph of Passu glacier from where samples were collected (Latitude 36° 27' 59.99" North and longitude 74° 53' 59.99" East)

Chemicals and reagents

Media (R2A medium, Nutrient Agar, Nutrient Broth, Lauria-Bertani Broth, Mueller Hinton Agar), glycerol, and normal saline (0.85%NaCl) from Sigma Chemicals (St. Louis, MO, USA), were used for bacterial isolation while antibiotic discs were obtained from Liofilchem, Italy.

Total viable count, isolation, and morphological identification of bacteria

Both aqueous and sediment samples were diluted 1:10 (1 mL sample and 9 mL of sterile saline solution or 1 g of sediment in 9 mL of sterile saline solution). Volumes of 200 μ L were spread over R2A medium agar plates and incubated at 4 and 15 $^{\circ}$ C for 40 to 60 days to isolate both psychrophilic and psychrotrophic isolates. Afterward, examining different colonies on R2A plates, the selection, isolation, and characterization of culturable bacteria were performed by following Zhang et al. criteria (Zhang et al. 2013). The total viable count of isolates was calculated by evaluating colony-forming units (CFU) per gram (g) or milliliter (mL) of the corresponding sample. Isolates were identified based on colony morphology, growth requirements, and *16S rRNA* gene sequencing. All isolates were constantly cultured on Lauria-Bertani agar and R2A agar media and were preserved in glycerol and LB broth at -20° C for further study.

Antibiotic susceptibility testing

The antibiotic susceptibility profile of the isolated bacteria was determined in vivo by using the Kirby Bauer agar disc diffusion method (Bauer et al. 1996) and standardized by guidelines of the Clinical and Laboratory Standards Institute (CLSI 2018). A total of 29 antibiotic groups from 10 classes were used for antibiotic susceptibility evaluation. Gram-positive bacteria were tested against 28 antibiotics while Gram-negative bacteria were against 21 antibiotics. The antibiotics panel included; Fluoroquinolones (levofloxacin 5 μ g, ciprofloxacin 5 μ g, ofloxacin 5 μ g, norfloxacin 10 μ g and nalidixic acid 30 μ g), Penicillins (amoxicillin 10 μ g, penicillin G 10 μ g, carbenicillin 100 μ g, ticarcillin 75 μ g, oxacillin 1 μ g and piperacillin 100 μ g), Cephalosporins (ceftriaxone 30 μ g, cefotetan 30 μ g, cephalixin 30 μ g, cephalothin 30 μ g, cefepime 30 μ g, ceftazidime 30 μ g, cephalozin 30 μ g, cefpodoxime 10 μ g and ceftaroline 30 μ g), Carbapenems (meropenem 10 μ g, imipenem 10 μ g and ertapenem 10 μ g), Monobactam (aztreonam 30 μ g), Oxazolidinone (linezolid 30 μ g), Macrolide (erythromycin 5 μ g), Glycopeptide (vancomycin 30 μ g) Glycylcine (tigecycline 15 μ g) and Nitroimidazole (metronidazole 5 μ g).

Multiple antibiotic resistance index

Multiple antibiotic resistance (MAR) indexes of antibiotic-resistant bacteria were determined by utilizing the following formula equation, suggested by Krumperman (1983).

$$\text{MAR index} = a/b$$

'a' shows number of resistant antibiotics

'b' refers to total number of tested antibiotics

Bacteria with MAR index values ≥ 0.2 are thought to be originated from contaminated sources with a high-risk (Krumperman 1983).

Genomic DNA extraction and PCR amplification

Chromosomal DNA was extracted using DNA Purification Kit # Ko721 (Gene JET Genomic, Thermo Scientific) by following the manufacturer's guidelines. PCR amplification of *16S rRNA* gene, ARGs, and class 1, 2 and 3 *integron integrase* genes were carried out in Eppendorf Gradient Thermocycler in a final volume of 30 μ L reaction which contained 15 μ L of Dream Taq green PCR 2x Master Mix (Thermo Scientific), followed by 1 μ L of each oligonucleotide primer (1 μ M) (Integrated DNA Technologies), 8 μ L of PCR grade nuclease-free water (Thermo Scientific) and 5 μ L of template DNA. The forward and reverse primers sequences of *16S rRNA* gene, ARGs, and *integron integrase* class 1, 2 and 3, expected amplicon size, and PCR cycling conditions are summarized in (Supplementary File 1). For visualization of desired amplified products, electrophoresis was carried out on agarose gel (1.5%), stained with ethidium bromide, and combined with GeneRuler 100 bp *DNA Ladder* (Thermo Scientific) as a standard marker, followed by band analysis through gel documentation system (Alpha Innotech, Biometra).

Sanger sequencing

PCR products of *16S rRNA*, ARGs, and class 1 *integron integrase* were purified and quantified using PCR Purification Kit (Gene JET, Thermo Scientific) and Qubit 3 Fluorometer (Invitrogen), respectively. Qubit Fluorometer dsDNA HS Assay Kit was used to quantify the final purified products. Bi-directional Sequencing of *16S rRNA* (27 F, 1492R), ARGs, and class 1 *integron integrase* was performed through the University of Arizona Genetic Core (<https://uagc.arl.arizona.edu/services/services/dna-sequencing>), on Applied Biosystems 3730XL DNA Analyzer, with 600 bases sequence per reading in one direction. The *16S rRNA* sequences of the bacterial isolates obtained in this study were compared for sequence homology search through the Basic Local Alignment Search Tool (BLAST) program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for further evaluation.

Table 1 Total viable count of retrieved isolates in term of CFU mL⁻¹ or g⁻¹

Sample/Source	pH	Temp (°C)	CFU mL ⁻¹ or g ⁻¹		No. of colonies
			4 °C	15 °C	
Glacial Sediment	7	1	3.73 × 10 ⁸	1.53 × 10 ⁹	43
Glacial Ice	7	-2	9.92 × 10 ⁵	3.65 × 10 ⁶	14
Glacial melt water	7	2	2.34 × 10 ⁵	7.01 × 10 ⁵	8

The gene sequences were submitted to the GeneBank Nucleotide Sequence Database, NCI (National Center for Biotechnology Information), and the corresponding accession numbers are provided in (Supplementary File 2).

ARGs and class 1 *integron integrase* sequence analysis

The nucleotide sequences of ARGs and *integron integrase* class1 were further scrutinized through BLAST search and compared to the relative available sequences in the NCBI database. Different variants of ARGs were determined from The Comprehensive antibiotic Resistant Database (CARD) (<https://card.mcmaster.ca/analyze/blast>). For variant calling, each gene nucleotide homology sequences were downloaded from relative reference genes available in the NCBI database and translated to amino acids with the ExPASy Translate Tool (<https://web.expasy.org/tools/translate/dna.html>). Clustal W was used to align the sequences. To evaluate the amino acid variation, alignment was performed with CLC Main Workbench 8 software. To evaluate the evolutionary relationship of ARGs and *intI1* MEGA X software was used (Kumar et al. 2018).

Results

Description and retrieval of bacteria from glacier

In this study, a total of 65 bacterial isolates were collected from Passu glacier, of which 43 (66.1%) were retrieved from glacial sediment, followed by 14 (21.5%) from ice and 8 (12.3%) from meltwater, respectively. Based on the R2A agar plate examination, in terms of CFU g⁻¹ and CFU mL⁻¹, bacteria-enriched colonies were obtained from glacial sediment at 4 and 15 °C (Table 1). Among isolated species, 43 (66.15%) were Gram-negative bacteria whereas 22 (33.84%) were Gram-positive bacteria. Out of the total retrieved isolates, only 5 (7.69%) retained their growth at 37 °C. Morphological and growth characteristics of retrieved bacterial isolates are documented in (Supplementary File 3).

Antibiotic susceptibility testing

The tested bacterial isolates showed significantly varying phenotypic resistance to a panel of 29 antibiotics representing 10 classes. The susceptibility profile revealed that Gram-positive and Gram-negative bacteria both exhibited significant resistance to the majority of antibiotics. More precisely, Gram-positive bacteria exhibited more resistance to Fluoroquinolones than Gram-negative bacteria, whereas Gram-negative bacteria were more resistant to other antibiotic classes. Among Gram-positive bacteria that exhibited resistance to Fluoroquinolones, resistance was maximum against norfloxacin, while levofloxacin was a more effective drug with the least resistance. The penicillin group showed the least effective against tested bacteria except for piperacillin with only 1 isolate showing resistance to this antibiotic. Out of the Cephalosporins class, the isolated bacteria showed high sensitivity to ceftriaxone and resistance to cephazolin. Resistance to Carbapenems was maximum against ertapenem and minimum against imipenem. The resistance against linezolid, erythromycin, vancomycin, and metronidazole was also significant among Gram-positive bacteria.

Among the Gram-negative isolates, Fluoroquinolone resistance was also prevalent but to a lesser extent against levofloxacin and more against norfloxacin and nalidixic acid. Gram-negative bacteria also revealed prevalent resistance to Cephalosporins with a maximum against cephalothin and a minimum against ceftaroline and ceftriaxone. Gram-negative bacteria were slightly resistant to Carbapenem group antibiotic imipenem while this group exhibited significant resistance against meropenem and ertapenem. Considering Monobactam, Macrolide, Glycylglycine, and Nitroimidazole, the most effective drug was tigecycline, whereas 100% of the Gram-negative isolates were resistant to metronidazole. The antibiotic susceptibility profile of Gram-negative and Gram-positive bacterial isolates is illustrated in (Table 2).

Multiple antibiotic resistance (MAR) index

MAR indexes for AR bacteria were calculated through the formula designed by Krumperman (Krumperman 1983). When MAR indices were calculated, 87.9% of tested isolates yielded MAR index value >0.2. Among Gram-positive isolates, 86.04% have >0.2 MAR index whereas 90.9% Gram-negative isolates exhibited >0.2 MAR index. Gram-positive species showing these patterns of MAR included *Staphylococcus equorum* (HP19), *Leucobacter aridicollis* (HP22), *Leucobacter komagatae* (HP51) and *Arthrobacter* sp. (LP2) while Gram-negative bacteria included *Brevundimonas diminuta* (HP21), *Rahnella inusitata*

Table 2 Antibiotic susceptibility profile of Gram-positive and Gram-negative bacteria isolated from glacier

Antibiotics	Gram Positive (n=22)			Gram Negative (n=43)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
FLUOROQUINOLONES						
Levofloxacin	15 (68.18)	3 (13.63)	4 (18.18)	39 (90.69)	1 (2.32)	3 (6.97)
Ciprofloxacin	15 (68.18)	2 (9.09)	5 (22.72)	35 (81.39)	4 (9.30)	4 (9.30)
Ofloxacin	14 (63.63)	2 (9.09)	6 (27.27)	33 (76.74)	6 (13.95)	4 (9.30)
Norfloxacin	11 (50)	2 (9.09)	9 (40.90)	30 (69.76)	2 (4.65)	11 (25.58)
Nalidixic acid	10 (45.45)	4 (18.18)	8 (36.36)	30 (69.76)	5 (11.62)	8 (18.60)
PENICILLINS						
Amoxicillin	1 (4.54)	1 (4.54)	20 (90.90)	NA	NA	NA
Penicillin G	3 (13.63)	0 (0)	19 (86.36)	NA	NA	NA
Carbenicillin	9 (40.90)	1 (4.54)	12 (54.54)	NA	NA	NA
Ticarcillin	7(31.81)	3 (13.63)	12(54.54)	NA	NA	NA
Oxacillin	3 (13.63)	0 (0)	19 (86.36)	NA	NA	NA
Piperacillin	20 (90.90)	1 (4.54)	1 (4.54)	NA	NA	NA
CEPHALOSPORINS						
Ceftriaxone	13 (59.09)	2 (9.09)	7 (31.81)	29 (67.44)	2 (4.65)	12 (27.90)
Cefotetan	7 (31.81)	0 (0)	15 (68.18)	21 (48.83)	1 (2.32)	21 (48.83)
Cephalexin	7 (31.81)	1 (4.54)	14 (63.63)	5 (11.62)	1 (2.32)	37 (86.04)
Cephalothin	5 (22.72)	0 (0)	17 (77.27)	2 (4.65)	0 (0)	41 (95.34)
Cefepime	11 (50)	0 (0)	11 (50)	23 (53.48)	5 (11.62)	15 (34.88)
Ceftazidime	13 (59.09)	0 (0)	9 (40.90)	23 (53.48)	2 (4.65)	18 (41.86)
Cephazolin	4 (18.18)	0 (0)	18 (81.81)	3 (6.97)	0 (0)	40 (93.02)
Cefpodoxime	4 (18.18)	1 (4.54)	17 (77.27)	15 (34.88)	1 (2.32)	27 (62.79)
Ceftaroline	14 (63.63)	0 (0)	8 (36.36)	29 (67.44)	2 (4.65)	12 (27.90)
CARBAPENEMS						
Meropenem	13 (59.09)	0 (0)	9 (40.90)	21 (48.83)	2 (4.65)	20 (46.51)
Imipenem	20 (90.90)	0 (0)	2 (9.09)	37 (86.04)	1 (2.32)	5 (11.62)
Ertapenem	10 (45.45)	0 (0)	12 (54.54)	22 (51.16)	2 (4.65)	19 (44.18)
MONOBACTEM						
Aztreonam	NA	NA	NA	19 (44.18)	1 (2.32)	23 (53.48)
OXAZOLIDINONES						
Linezolid	5 (22.72)	0 (0)	17 (77.27)	NA	NA	NA
MACROLIDE						
Erythromycin	5 (22.72)	1 (4.54)	16 (72.72)	6 (13.95)	1 (2.32)	36 (83.72)
GLYCOPEPTIDE						
Vancomycin	4 (18.18)	0 (0)	18 (81.81)	NA	NA	NA
GLYCYCLINE						
Tigecycline	10 (45.45)	9 (40.90)	3 (13.63)	25 (58.13)	11 (25.58)	7 (16.27)
NITROIMIDAZOLE						
Metronidazole	0 (0)	1 (4.54)	21 (95.45)	0 (0)	0 (0)	43 (100)

Abbreviations: S: Sensitive; I: Intermediate; R: Resistant; NA: Not applicable

(HP3) and *Alcaligenes faecalis* (HP55) that showed highest MAR index value corresponding to 0.7. Multiple antibiotic resistance indexes and resistance patterns of bacteria are recorded in (Table 3).

PCR amplification and sequencing of 16S rRNA

The amplification of specific 1350 bp bands was visualized under the UV trans-illuminator (Fig. 2). Based on *16S rRNA* gene sequencing, the bacterial isolates detected belong to the phyla *Proteobacteria* 40 (62%), *Actinobacteria* 12

(18%), *Firmicutes* 9 (14%) and *Bacteroidetes* 4 (6%). The relative abundances of bacterial phyla in three different sample sources isolated from the glacier are represented in (Fig. 3). Bacterial communities and their relative abundance, distribution plus structural community in glacial sediment, ice, and meltwater are shown in (Fig. 4).

Table 3 Antibiotics resistance pattern and MAR indexes of bacteria isolated from glacier

Isolate code Gram-Negative	Antibiotic Resistance Profile	No. of resistant antibiotics (%)	MAR Index	Isolate code Gram-Positive	Antibiotic Resistance Profile	No. of resistant antibiotics (%)	MAR Index
HP2	OFX,NOR,NA,CTT,CL,KF,KZ,CPD,E,TGC,MET	11 (52.3)	0.5	HP1	NOR,AML,P,CAR,TIC,OX,CTT,KF,FE,P,KZ,CPT,LZD,E,VA,TGC,MET	16 (57.1)	0.5
HP3	LEV,CTT,CL,KF,FEP,KZ,CPT,E,TGC,MET	10 (47.6)	0.4	HP5	NA,AML,P,CAR,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	14 (50)	0.5
HP4	CTT,CL,KF,CAZ,KZ,ATM,E,TGC,MET	9 (42.8)	0.4	HP7	LEV,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	15 (53.5)	0.5
HP6	NA,CL,KF,KZ,CPD,E,TGC,MET	8 (38)	0.3	HP8	AML,P,OX,PRL,CTT,KF,KZ,LZD,E,VA,MET	11 (39.2)	0.3
HP10	NOR,CL,KF,KZ,CPD,E,MET	7 (33.3)	0.3	HP13	P,CTT,FEP,CAZ,CPD,CPT,MRP,ETP,MET	9 (32.1)	0.3
HP11	NA,CL,KF,FEP,KZ,CPD,E,MET	8 (38)	0.3	HP14	LEV,AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,KZ,MRP,ETP,LZD,E,VA,MET	17 (60.7)	0.6
HP12	CIP,NOR,NA,CTT,CL,KF,KZ,CPD,E,MET	10 (47.6)	0.4	HP17	NOR,NA,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5
HP15	CTT,CL,KF,KZ,CPD,ATM,E,MET	8 (38)	0.3	HP18	CIP,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	19 (67.8)	0.6
HP16	NOR,NA,CTT,CL,KF,KZ,CPD,E,MET	9 (42.8)	0.4	HP19	CIP,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,CAZ,KZ,CPD,ETP,LZD,E,VA,TGC,MET	22 (78.5)	0.7
HP20	OFX,NOR,CRO,CL,KF,FEP,KZ,CPD,CPT,IMP,ATM,E,TGC,MET	14 (66.6)	0.6	HP22	LEV,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,KZ,CPD,CPT,ETP,LZD,E,VA,MET	21 (75)	0.7
HP21	LEV,CIP,OFX,NOR,NA,CRO,CTT,CL,KF,KZ,CPD,ETP,ATM,E,TGC,MET	16 (76.1)	0.7	HP23	CIP,OFX,NOR,NA,AML,P,CAR,OX,CL,KF,KZ,CPD,ETP,LZD,E,VA,MET	17 (60.7)	0.6
HP50	CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	14 (66.6)	0.6	HP36	FEP,CPD,CPT,MRP,ETP,MET	6 (21.4)	0.2
HP52	NA,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	15 (71.4)	0.7	HP51	AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,LZD,E,VA,MET	20 (71.4)	0.7
HP54	NOR,CTT,CL,KF,FEP,KZ,CPD,MRP,ETP,MET	10 (47.6)	0.4	HP53	AML,P,CAR,TIC,OX,CTT,CL,KF,FE,P,KZ,CPD,MRP,ETP,LZD,E,VA,MET	17 (60.7)	0.6
HP55	NOR,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	15 (71.4)	0.7	LP1	AML,P,CAR,TIC,OX,CTT,CL,KF,FE,P,CAZ,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5
HP56	KF,MRP,MET	3 (14.2)	0.1	LP2	OFX,AML,P,TIC,OX,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,LZD,E,VA,TGC,MET	21 (75)	0.7
HP57	CL,KF,CAZ,KZ,MRP,MET	6 (28.5)	0.2	LP3	AML,P,TIC,OX,CL,KF,FEP,KZ,CPD,LZD,E,VA,MET	13 (46.4)	0.4
HP58	CL,KF,KZ,MRP,ETP,MET	6 (28.5)	0.2	LP4	AML,P,OX,CL,KF,FEP,CAZ,KZ,ETP,LZD,E,VA,MET	13 (46.4)	0.4
LP5	CIP,CL,KF,FEP,CAZ,KZ,CPD,ETP,ATM,E,MET	11 (52.3)	0.5	LP8	CIP,NOR,AML,P,OX,CPD,CPT,MRP,ETP,LZD,VA,MET	12 (42.8)	0.4
LP6	CTT,CL,KF,FEP,KZ,CPD,E,MET	8 (38)	0.3	LPI6	NOR,NA,AML,CRO,CAZ,CPD,ETP	7 (25)	0.2
LP7	CTT,CL,KF,FEP,KZ,CPD,ATM,E,MET	9 (42.8)	0.4	LPI7	NA,AML,P,OX,CRO,CTT,KF,FEP,CAZ,KZ,CPD,CPT,MRP,IMP,VA,MET	16 (57.1)	0.5
LP9	CTT,CL,KF,FEP,KZ,CPD,ATM,E,MET	9 (42.8)	0.4	LPW5	LEV,CIP,OFX,NOR,AML,OX,CAZ,KZ,MRP,IMP,MET	11 (39.2)	0.3
LP10	CIP,OFX,NOR,CAZ,CPD,CPT,MRP,ETP,ATM,E,TGC,MET	12 (57.1)	0.5				
LP11	LEV,CL,KF,FEP,CAZ,KZ,CPD,MRP,ATM,E,MET	11 (52.3)	0.5				

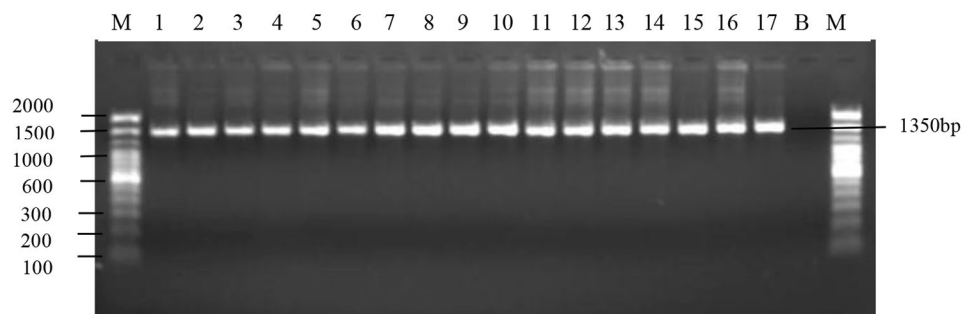
Table 3 (continued)

Isolate code Gram-Negative	Antibiotic Resistance Profile	No. of resistant antibiotics (%)	MAR Index	Isolate code Gram-Positive	Antibiotic Resistance Profile	No. of resistant antibiotics (%)	MAR Index
HPI1	CRO,CTT,CL,KF,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	13 (61.9)	0.6				
HPI2	CL,KF,KZ,MRP,ETP,MET	6 (28.5)	0.2				
HPI3	CRO,CTT,CL,KF,CAZ,KZ,CPT,MRP,ETP,ATM,E,MET	12 (57.1)	0.5				
HPI4	CRO,CTT,CL,KF,CAZ,KZ,CPD,MRP,ETP,ATM,E,MET	12 (57.1)	0.5				
HPI5	CL,KF,KZ,MRP,ETP,E,MET	7 (33.3)	0.3				
HPI6	CL,KF,KZ,MRP,ETP,E,MET	7 (33.3)	0.3				
HPI7	CTT,KF,FEP,CAZ,KZ,CPD,CPT,ATM,MET	9 (42.8)	0.4				
HPI8	CTT,KF,CAZ,KZ,CPD,CPT,IMP,ATM,E,MET	10 (47.6)	0.4				
LPI1	CRO,CL,KF,CAZ,KZ,IMP,ATM,E,MET	9 (42.8)	0.4				
LPI2	CL,KF,KZ,ETP,E,MET	6 (28.5)	0.3				
LPI4	CL,KF,KZ,MRP,E,MET	6 (28.5)	0.3				
LPI5	CL,KF,KZ,MRP,IMP,ETP,E,MET	8 (38)	0.3				
LPW1	CRO,CL,KF,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	12 (57.1)	0.5				
LPW2	CL,KF,KZ,E,MET	5 (23.8)	0.2				
LPW3	CL,KF,KZ,E,MET	5 (23.8)	0.2				
LPW4	KF,FEP,KZ,ETP,ATM,E,MET	7 (33.3)	0.3				
LPW6	CRO,CTT,CL,KF,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	13 (61.9)	0.6				
LPW7	NOR,CRO,CTT,FEP,CAZ,CPD,MRP,IMP,ATM,MET	10 (47.6)	0.4				
LPW8	NOR,NA,CL,KF,CAZ,KZ,CPD,ATM,E,MET	10 (47.6)	0.4				

Key: Gram-negative bacteria was tested against 21 antibiotics while Gram-positive against 28

Abbreviations: LEV: Levofloxacin, CIP: Ciprofloxacin, OFX: Ofloxacin, NOR: Norfloxacin, NA: Nalidixic acid, AML: Amoxicillin, P: Penicillin G, CAR: Carbenicillin, TIC: Ticarcillin, OX: Oxacillin, PRL: Piperacillin, CRO: Ceftriaxone, CTT: Cefotetan, CL: Cephalexin, KF: Cephalothin, FEP: Cefepime, CAZ: Ceftazidime, KZ: Cephazolin, CPD: Cefpodoxime, CPT: Ceftaroline, MRP: Meropenem, IMP: Imipenem, ETP: Ertapenem, ATM: Aztreonam, LZD: Linezolid, E: Erythromycin, VA: Vancomycin, TGC: Tigecycline, MET: Metronidazole
MAR: Multiple Antibiotic Resistance

Fig. 2 PCR amplicons of *16S rRNA* gene on 1.5% agarose gel. **Lane 1** to **17**: 27 F 1492R (1350 bp), **B**: Blank, **M**: 100 bp DNA Ladder (Invitrogen)

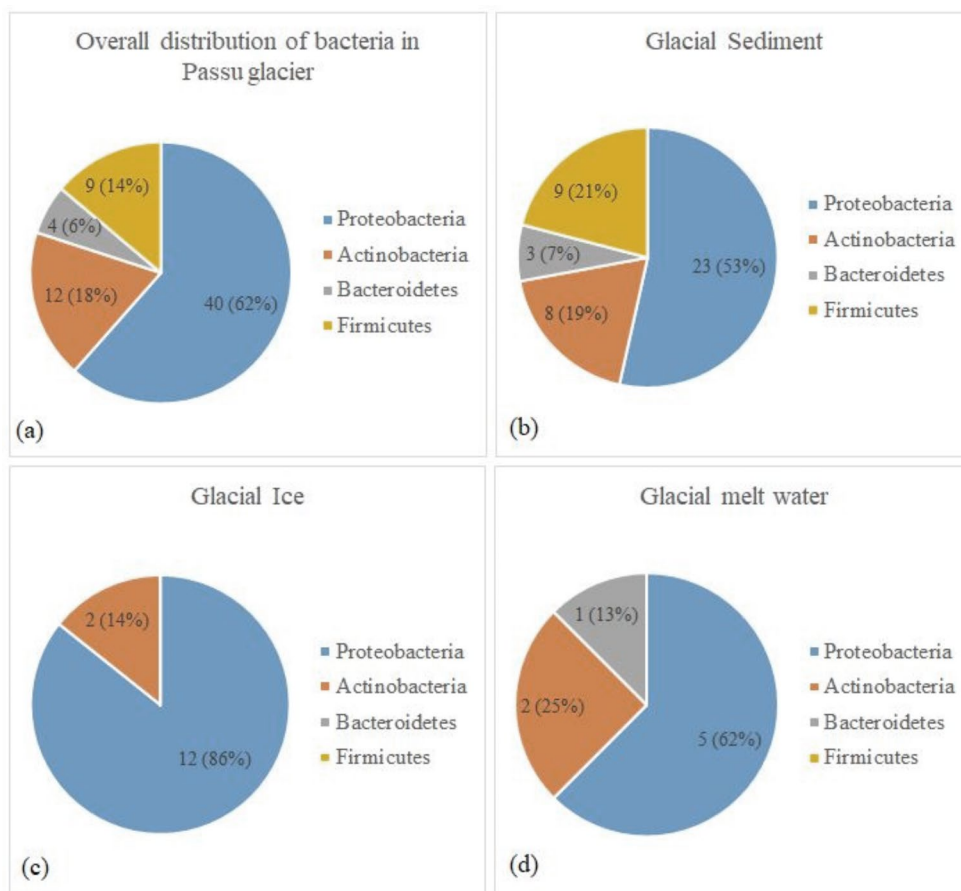


Molecular detection of ARGs and class 1 integron integrase

The presence of ARGs and class1 *integron integrase* was

revealed by PCR amplification using primers and cycling conditions described in the methods. The ARGs detected ratios were *blaCTXM-15* (21.53%), *blaNDM-1* (24.61%), *blaAmpC* (9.23%), *blaVIM-1* (7.69%), *blaSHV* (7.69%),

Fig. 3 Bacterial Phyla relative abundance in glacier samples (a) shows the whole bacteria abundance in glacier (b) bacterial phyla in glacial sediment (c) glacial ice and (d) glacial meltwater



*bla*TEM-1 (18.46%), *bla*OXA-1 (1.53%), *bla*GES (6.15%), *bla*CMY-4 (6.15%), *bla*DHA (7.69%), *sul1* (21.5%), *sul2* (24.6%), *tet(A)* (12.30%), *tet(B)* (7.69%), *qnrB* (15.38%), *aac(6)-Ib3* (29.23%), *mecA* (18.46%), *cat* (15.38%), *qepA* (15.38%), *gyrA* (24.6%) and *intI1* (24.6%), respectively. All tested strains were negative for *bla*KPC, *qnrA*, *vanA*, *ermA*, *ermB*, *intI2*, and *intI3*. The representative amplified bands of desired genes are illustrated in (Fig. 5). The PCR analysis showed that *aac(6)-Ib3*, *bla*CTXM-15, *bla*NDM-1, *sul1*, *sul2*, and *gyrA* genes were consistently present among tested isolates, while single isolate harbored *bla*OXA-1. Moreover, ARGs and *integron integrase* class 1 were more prevalent in Gram-negative bacteria from glacial sediment than Gram-positive bacteria.

Among the Gram-negative bacterial strains, *Brevundimonas diminuta* (HP21) and *Rahnella inusitata* (HP3) harbored *intI1* plus 19 and 17 ARGs, respectively. Thirteen ARGs were identified in *Alcaligenes faecalis* (HP55), however, *intI1* was not associated with this bacterium. Meanwhile, *intI1* plus 11 and 8 ARGs were identified in the Gram-positive bacterial strains *Staphylococcus equorum* (HP19) and *Leucobacter aridicollis* (HP22). *Arthrobacter sp.* (LP2) was found positive for 7 ARGs and negative for *intI1*. The distribution of ARGs and *intI1* among the

tested bacteria are categorized in (Table 4) and the relative abundance of ARGs in 3 different sample sources is summarized in (Fig. 6).

Considering the bacterial Phyla, *Proteobacteria* harbored the largest number of ARGs and *intI1*, followed by *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, respectively. The abundance of ARGs and *intI1* among bacterial phyla is listed in (Table 5).

ARGs and class 1 *integron integrase* sequence analysis and protein alignment

The BLAST sequence homology search was evaluated with >98% similarity for all ARGs and *intI1* except *bla*OXA-1 (97.36%) and *aac(6)-Ib3* (97.86%) and different variants of ARGs were determined through the CARD database. When compared to the reference gene, *bla*OXA-1 amino acids alignment showed variations at terminal positions (*Leu160*→ *Val*, *161Gln*→ *Trp*, *162Asn*→ *Glu*, *163Gly*→ *Asn*, *165Phe*→ *Cys*, *166Glu*→ *Arg*, *170Ile*→ *His*). The *bla*NDM-1 gene was found with single amino acid variation (*61Asp*→ *Tyr*), *bla*SHV with 2 amino acids (*80Val*→ *Leu*, *84Arg*→ *Pro*), whereas, (*tetA*) with 3 amino acids variations (*4Pro*→ *Arg*, *52Leu*→ *Val*, *54Phe*→ *Ile*) and *sul2* with 2

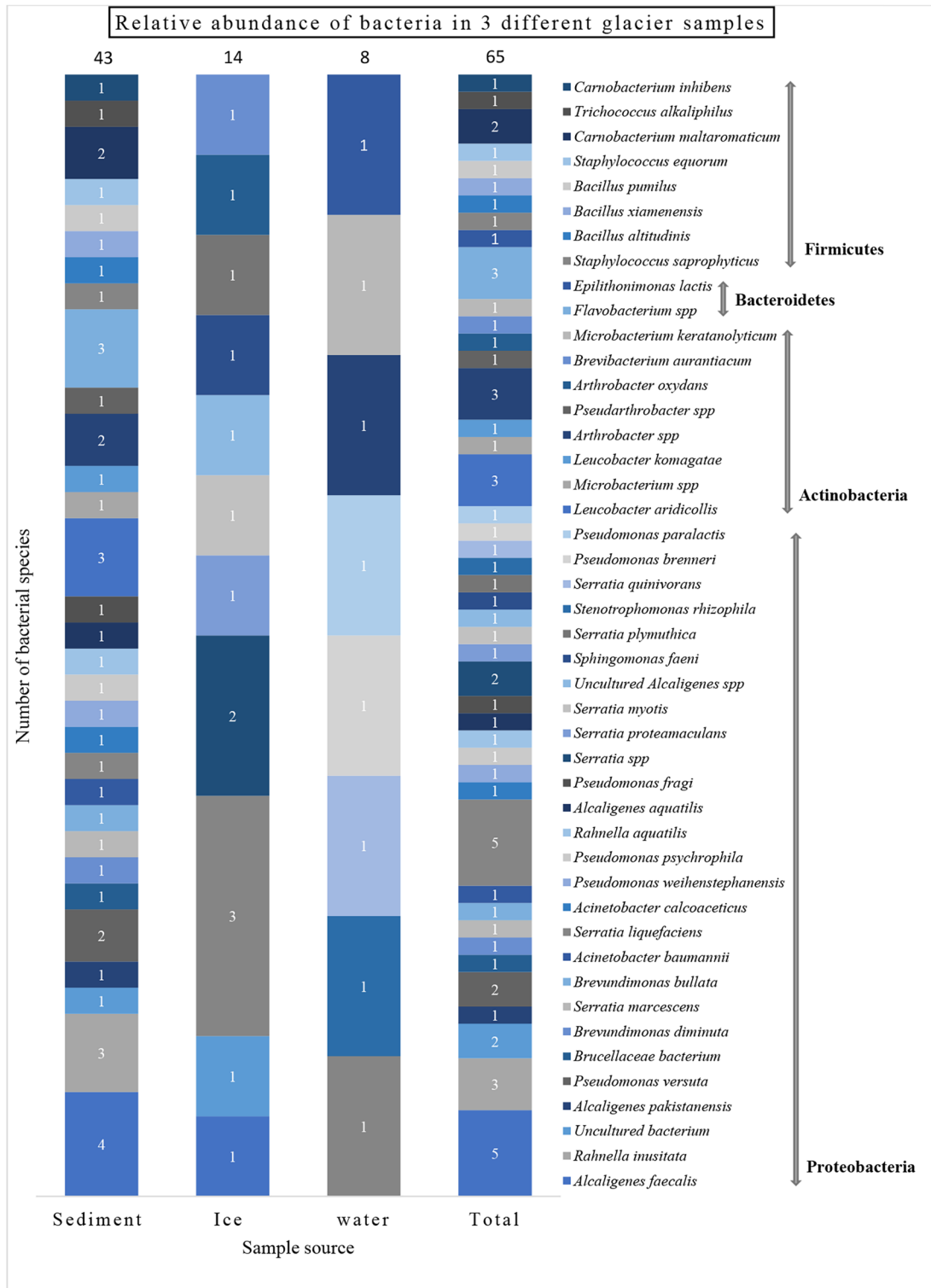


Fig. 4 Bacterial communities and their relative abundance, distribution plus structural community in glacial sediment, ice and meltwater identified by amplicon sequencing of *16S rRNA* gene

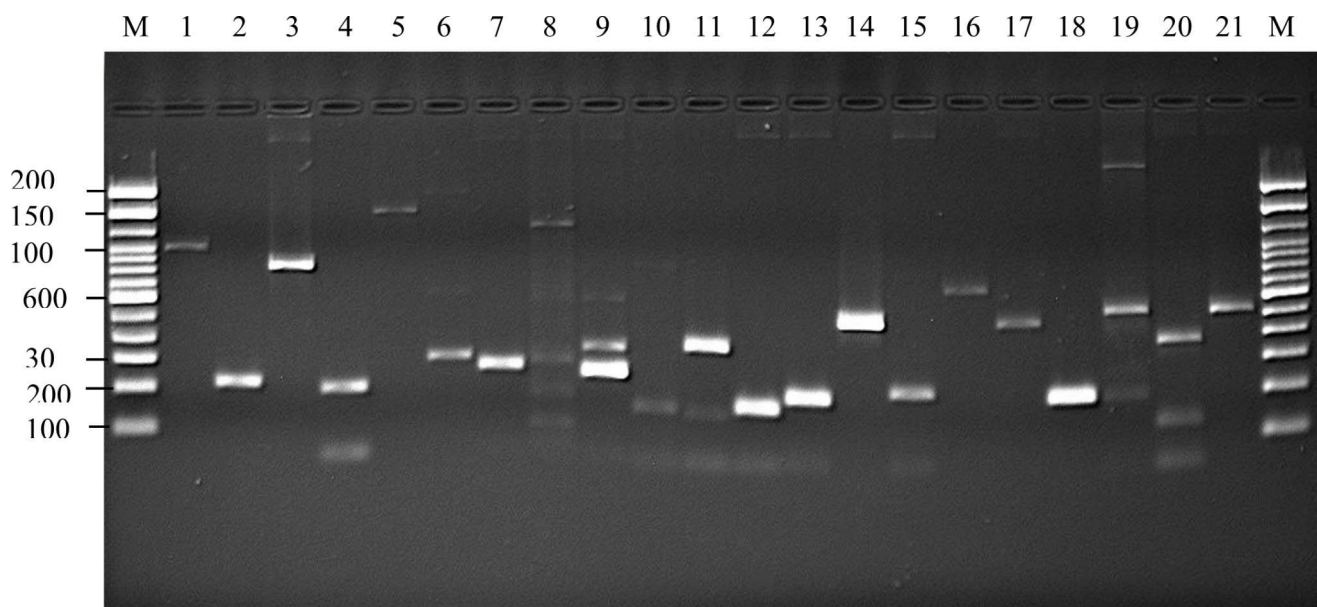


Fig. 5 PCR amplicons of ARGs and *intI1* on 1.5% agarose gel. **Lane 1:** *CTX-M* (1000 bp), **Lane 2:** *bla^{NDM-1}* (214 bp). **Lane 3:** *bla^{OXA}* (813 bp), **Lane 4:** *bla^{AmpC}* (189 bp). **Lane 5:** *bla^{CMY}* (1432 bp), **Lane 6:** *bla^{SHV}* (300 bp), **Lane 7:** *bla^{VIM}* (189 bp), **Lane 8:** *bla^{TEM}* (1150 bp), **Lane 9:** *bla^{DHA}* (404 bp), **Lane 10:** *bla^{GES}* (890 bp),

Lane 11: *gyrA* (344 bp), **Lane 12:** *sulI* (163 bp), **Lane 13:** *sul2* (191 bp), **Lane 14:** *aac(6')* (482 bp), **Lane 15:** *tetA* (210 bp), **Lane 16:** *tetB* (659 bp), **Lane 17:** *qnrB* (476 bp), **Lane 18:** *intI1* (196 bp), **Lane 19:** *qepA* (596 bp), **Lane 20:** *cat* (122 bp), **Lane 21:** *mecA* (527 bp), **M:** 100 bp DNA Ladder (Invitrogen)

amino acids variations (*4Ala*→*Thr* and *62Pro*→*Thr*). Moreover, *qnrB* gene amino acids alignment demonstrated 3 amino acids variations at positions (*6Ile*→*Asn*, *103Thr*→*Ser*, *128Arg*→*Pro*), *gyrA* by 2 amino acids (*5Phe*→*Tyr*, *46Asp*→*Glu*), and *intI1* protein alignment illustrated the amino acids coverage and consensus with 2 amino acids variation at positions (*60Val*→*Asp*, *61Phe*→*Leu*). The complete description and evolutionary relationship details of ARGs and class 1 *integron integrase* are summarized in (Supplementary File 4).

Discussion

This research work includes a comprehensive assessment of antibiotic resistance and prevalence of ARGs and class 1 *integron integrase* among culturable bacteria isolated from a non-polar glacier (Passu, Pakistan) in the Karakoram (Himalaya) region. A total of 65 bacterial species were isolated from glacial sediment, ice, and meltwater. *16S rRNA* gene sequencing identified different bacteria belonging to multiple phyla including *Flavobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. From various cold and frozen habitats worldwide such as glaciers, permafrost, ice sheets, and lakes, bacterial phyla belonging to *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, and *Acidobacteria* have been frequently reported (Margesin and Miteva 2011; Junge

et al. 2011). Early studies from Hindu Kush (Tirich Mir, Chitral, Pakistan) identified *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Flavobacteria*, and *Proteobacteria* in Siachen glaciers and samples collected from multiple sources including glacial ice, sediment, and meltwater (Rafiq et al. 2019, 2017). In our study, (66.1%) of isolates were retrieved from glacial sediment, followed by (21.5%) from ice and (12.3%) from meltwater. Glacial sediment is rich in nutrients with slightly higher temperatures in comparison to glacial ice and meltwater which favors diverse microbial flora (Parnell and McMahon 2016). Our findings revealed a high prevalence of Gram-negative bacteria (66.15%) when compared to Gram-positive (33.84%). These bacterial communities were dominated by *Proteobacteria* (61.53%), followed by *Actinobacteria* (18.46%), *Bacteroidetes* (6.15%), and *Firmicutes* (13.84%), similar to studies conducted previously from Siachen glacier, Greenland ice Sheet and Finnish Lapland (Rafiq et al. 2017; Musilova et al. 2015; Männistö and Häggblom 2006) which reported *Proteobacteria* (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*) and Gram-negative bacteria as most predominant bacteria. Boetius et al. (2015) reported Gram-positive bacteria as the most dominating strains from Arctic ice sheets and Antarctic glaciers which contrasts with our findings. Seasonal variations in cold environments and geographical locations may contribute to bacterial diversity dominance and variation because of the counter-selection

Table 4 Distribution of antibiotic resistant genes and class 1 *integron* among Gram-negative and Gram-positive bacteria isolated from glacier

Isolate code	β-lactams ARGs										Non β-lactams ARGs										Integron	
	<i>CTXM-15</i>	<i>NDM-1</i>	<i>AmpC</i>	<i>VIM-1</i>	<i>SHV</i>	<i>TEM-1</i>	<i>OXA-1</i>	<i>GES</i>	<i>CMY-4</i>	<i>DHA</i>	<i>sul1</i>	<i>sul2</i>	<i>tetA</i>	<i>tetB</i>	<i>qnrB</i>	<i>aac(6′)-Ib3</i>	<i>mecA</i>	<i>cat</i>	<i>qepA</i>	<i>gyrA</i>	<i>intI1</i>	
HP2	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	-	
HP3	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	
HP7	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	
HP18	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	+	-	
HP19	+	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	+	+	+	+	
HP20	+	-	-	-	-	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	+	
HP21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
HP22	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+	+	
HP23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
HP50	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	-	+	-	-	+	
HP51	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	
HP54	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	
HP55	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
HP57	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
HP58	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
LP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LP2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
LP5	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
LP7	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
LP10	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
LP11	-	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	
HP11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
LP17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
LPW6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
LPW7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
LPW8	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	+	-	-	+	-	

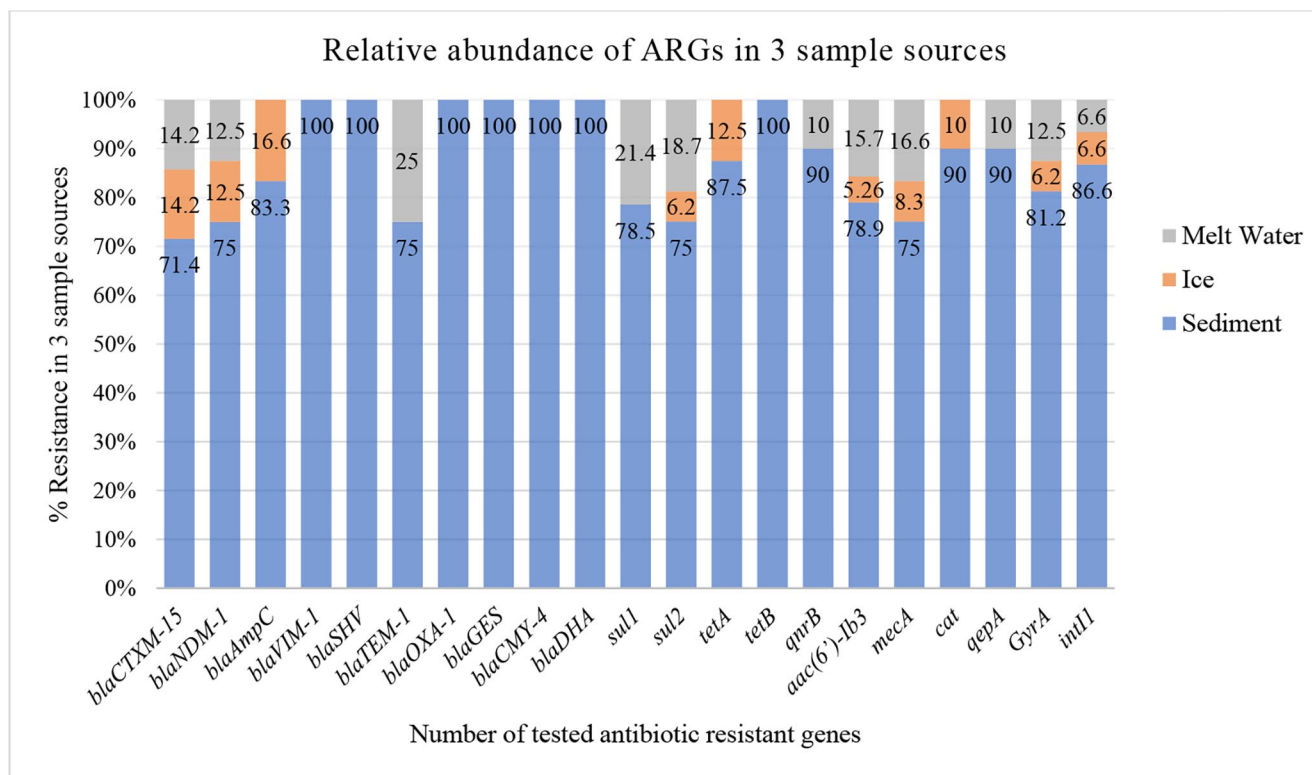


Fig. 6 Relative abundance of ARGs and class 1 *integron integrase* among glacier sediment, ice and meltwater

of more adoptable bacteria (Zhang et al. 2010, 2007; Bhatia et al. 2006).

Bacteria were evaluated for antibiotic resistance for a panel of 29 antibiotics and the results revealed significant antibiotic resistance. The antibiotic susceptibility profile revealed that Gram-positive bacteria were comparatively more resistant to antibiotic class Fluoroquinolones than Gram-negative bacteria, whereas Gram-negative bacteria showed more resistance to the rest of antibiotic classes. This might be due to the inherited less susceptibility of Gram-positive bacteria towards Fluoroquinolones where a single mutation can confer antibiotic resistance. *Staphylococcus equorum* (HP19) was resistant to nearly 78.57% of tested antibiotics (including more than one antibiotic from the same class), followed by *Leucobacter aridicollis* (HP22) and *Arthrobacter sp.* (LP2) that were resistant to 75% antibiotics each, *Leucobacter komagatae* (HP51) to 71.42% and *Bacillus pumilus* (HP18) to 67.85% antibiotics. *Carnobacterium maltaromaticum* (HP36) showed a minimum of 21.42% antibiotic resistance. Among the Gram-negative bacteria, *Brevundimonas diminuta* (HP21) was resistant to a maximum of 76.19% antibiotics, *Rahnella inusitata* (HP52) and *Alcaligenes sp.* (HP55) to 71.42% each, while *Flavobacterium antarcticum* (HP20) and *Serratia marcescens* (HP50) were resistant to 66.66% each. *Alcaligenes faecalis* (HP2) and *Acinetobacter calcoaceticus*

(LP5) were resistant to 52.38% of overall tested antibiotics. Our results were in contradiction to reports by Rafiq et al. (2017) from the Siachen glacier (Karakoram, Pakistan), which reported increased antibiotic resistance among Gram-positive bacteria corresponding to ofloxacin 11.76%, ceftriaxone 58.82%, cefotaxime 76.47%, imipenem, and vancomycin each 64.70% resistance, whereas among Gram-negative bacteria resistance pattern was ofloxacin 24.24%, nalidixic acid 15.15%, cefotaxime 45.45% and imipenem 51.51%, among nearly similar genus isolates (Rafiq et al. 2017). Meanwhile, we reported ofloxacin 27.27%, ceftriaxone 31.81%, nalidixic acid 36.36%, cefpodoxime 77.27%, imipenem 9.09% and vancomycin 81.81% among Gram-positive bacteria while among Gram-negative ofloxacin 9.3%, nalidixic acid 18.6%, cefotaxime 62.79% and imipenem 11.62% were resistant. In another study, conducted by James and Wong (2015), the highest resistance was reported against vancomycin (64.2%), metronidazole (92.8%) and ceftazidime (42.8%) among Antarctic isolates, including *Arthrobacter* and *Pedobacter* species and these are in close association with our isolates' resistance pattern.

In addition, Tomova et al. (2015) have reported higher degrees of antibiotic resistance delineated in bacteria procured from sediment and soil samples of Antarctic islands, with 100% multiple antibiotic resistance among *Proteobacteria* and overall, 79% multi-drug resistance,

Table 5 Abundance of antibiotic resistant genes and class I integron integrase among 4 bacterial phyla

ARGs	Proteobacteria n (%)	Bacteroidetes n (%)	Actinobacteria n (%)	Firmicutes n (%)	Total (n)
<i>bla</i> _{CTXM-15}	6 (42.85%)	1 (7.14%)	5 (35.71%)	2 (14.28%)	14
<i>bla</i> _{NDM-1}	10 (62.50%)	1 (6.25%)	4 (25%)	1 (6.25%)	16
<i>bla</i> _{KPC}	0	0	0	0	0
<i>bla</i> _{AmpC}	6 (100%)	0	0	0	6
<i>bla</i> _{VIM-1}	5 (100%)	0	0	0	5
<i>bla</i> _{SHV}	5 (100%)	0	0	0	5
<i>bla</i> _{TEM-1}	10 (83.33%)	1 (8.33%)	1 (8.33%)	0	12
<i>bla</i> _{OXA-1}	1 (100%)	0	0	0	1
<i>bla</i> _{GES}	3 (75%)	1 (25%)	0	0	4
<i>bla</i> _{CMY-4}	4 (100%)	0	0	0	4
<i>bla</i> _{DHA}	5 (100%)	0	0	0	5
<i>sul1</i>	9 (64.28%)	1 (7.14%)	3 (21.42%)	1 (7.14%)	14
<i>sul2</i>	11 (68.75%)	1 (6.25%)	3 (18.75%)	1 (6.25%)	16
<i>tetA</i>	4 (50%)	0	2 (25%)	2 (25%)	8
<i>tetB</i>	1 (20%)	0	2 (40%)	2 (40%)	5
<i>qnrA</i>	0	0	0	0	0
<i>qnrB</i>	6 (60%)	1 (10%)	1 (10%)	2 (20%)	10
<i>vanA</i>	0	0	0	0	0
<i>ermA</i>	0	0	0	0	0
<i>ermB</i>	0	0	0	0	0
<i>aac(6)-Ib3</i>	13 (68.42%)	1 (5.26%)	4 (21.05%)	1 (5.26%)	19
<i>mecA</i>	7 (58.33%)	0	3 (25%)	2 (16.66%)	12
<i>cat</i>	5 (50%)	1 (10%)	3 (30%)	1 (10%)	10
<i>qepA</i>	5 (50%)	0	3 (30%)	2 (20%)	10
<i>gyrA</i>	7 (43.75%)	1 (6.25%)	5 (31.25%)	3 (18.75%)	16
<i>int11</i>	9 (60%)	1 (6.66%)	3 (20%)	2 (13.33%)	15
<i>int12</i>	0	0	0	0	0
<i>int13</i>	0	0	0	0	0

most frequently towards cephalosporins (75%), erythromycin (62%) and vancomycin (58%), while *Acinetobacteria* were all sensitive to tested antibiotics. There are no major differences in resistance profile in comparison to our study as among our tested isolates, *Proteobacteria* showed a higher degree of multiple antibiotic resistance while strain *Rahnella inusitata* (HP56) was the most sensitive strain. The area of Passu glacier is usually free of anthropogenic effects and still, the resistance pattern of currently tested isolates was comparable to bacterial resistance inhibiting antibiotic-rich environments. The same scenario has been reported by James and Wong (2015) who evaluated that bacteria living in anthropogenic free environment exhibit significant antibiotic resistance same as in habitats exposed to major human activities since pristine Antarctic bacteria were resistant to multiple antibiotics compared to tropical bacteria. On the other hand, Miller et al. (2009) described the lower frequency of multiple antibiotic resistances among native Antarctic bacteria which increased with an increase in anthropogenic activities. Usually, it is believed that the effect of anthropogenic activities on pristine environments plays a vital evolutionary role in the origination of multiple AR bacteria (Ushida et al. 2010; Cabello 2006; Goni-Urriza

et al. 2000). On the contrary, different physiological states like trace nutrients, low-high temperature, pressure, presence of secondary metabolites might be involved to stimulate stress signaling pathways in the upset bacterial genome resulting in resistant traits (antibiotics resistance, heavy metals resistance) in bacteria thriving in hostile environments. Moreover, resistance mechanisms can be acquired by gaining additional resistant determinants (genes, transposons, integrons, gene cassettes). However, the phenomenon of multiple antibiotic resistance among bacteria in a pristine environment like Passu glacier is strange and not clear as these habitats are usually free of anthropogenic effects.

Tropical glaciers are of major interest for researchers to study, regarding antibiotic resistance, as tropical glaciers interact differently with climate changes in comparison to glaciers located in mid and high latitudes (Kaser 1999). Passu glacier is also located in tropical regions of Pakistan and is exposed to drastic seasonal variations. A study on tropical glacier located in Venezuelan Andes, by Ball et al. (2014) reported an elevated degree of multiple resistant strains with maximum resistance against ampicillin (64.44%), followed by chloramphenicol, nalidixic acid, and penicillin (57.77%

each). streptomycin, kanamycin, and tetracycline resistances were (24.44%, 22.22%, and 4.4%), respectively, whereas in the current study resistant ratios for nalidixic acid (36.36% Gram-positive, 18.60% Gram-negative) and penicillin were (86.36% Gram-positive). It is generally reported that antibiotic resistance and heavy metals resistance coexist and are common traits expressed by bacteria trapped in glacier environments (Sherpa et al. 2020). From Antarctic shallow sediments, nearly free of antibiotics, chemicals, and heavy metals pollution, Giudice et al. (2013) reported multiple AR bacteria with remarkable resistance to heavy metals and chemicals, thus confirming the presence of coexistence of antibiotics and metals resistance genes on the same genome and this is not only confined to glaciers environment but observed ubiquitously (De Souza et al. 2006).

MAR index provides useful information to better address the risks associated with exposure to environments loaded with antibiotic-resistant bacteria. An arbitrary value of >0.2 is an indicator of a high-risk source of contamination where antibiotics are frequently used (Krumperman 1983). A significant number of bacterial isolates (87.9%) from this current study showed MAR index value >0.2 . Among Gram-positive bacteria, 86.04% had >0.2 MAR index value, whereas 90.9% Gram-negative isolates were found with >0.2 MAR index value, emphasizing high degrees of risk source of pollution or bacteria are previously exposed to antimicrobials. Among Gram-positive strains, *Staphylococcus equorum* (HP19), *Leucobacter aridicollis* (HP22), *Leucobacter komagatae* (HP51), and *Arthrobacter sp.* (LP2) while *Brevundimonas diminuta* (HP21), *Rahnella inusitata* (HP3) and *Alcaligenes sp.* (HP55) from Gram-negative showed highest MAR index value 0.7. From Siachen glacier, bacteria reported by Rafiq et al. (2017), were found to possess the highest MAR index value of 0.8 for *Bevibacterium sp.*, followed by *Rhodococcus sp.*, *Arthrobacter sp.* and *Bacillus simplex* (0.7 each) which is comparable to our results. Environmental bacteria thriving in cold environments are found to be excessively producing antimicrobial, antibacterial compounds (Giudice et al. 2007) and antibacterial violet pigments (Nakamura et al. 2003). Psychrophilic bacteria have also the potential to biosynthesize extracellularly highly stable silver nanoparticles at low temperatures, with remarkable antimicrobial activity (Shivaji et al. 2011). In natural environments, under the selective pressure of antimicrobial compounds, bacteria may evolve to resist the action of these compounds. Psychrophilic bacteria previously isolated from Pakistan (Karakorum region), Passu glacier (Rafiq et al. 2016), Hindu Kush range glacier (Rafiq et al. 2019), and Siachen glacier (Rafiq et al. 2017) were found to possess strong antimicrobial potential.

In this current study, PCR amplification revealed that tested bacteria were found positive for 21/28 (75%) tested ARGs (β lactam, non- β lactam, and integron integrase). Gram-negative bacteria were found with maximum ARGs (67.85%, 19 ARGs) while among Gram-positive bacteria maximum (39.28%, 11 ARGs) were detected. Scientists from all over the world have reported numerous ARGs from cold environments. A diverse set of 117 ARGs showing resistance to β -lactam antibiotics, aminoglycosides, macrolides, rifampicin, and tetracycline have been reported in Mackay glacier (Antarctica), among which 71% ARGs were detected in Gram-negative bacteria while only 9% were detected in Gram-positive bacteria (Van Goethem et al. 2018), which is following our results. Moreover, Ushida et al. (2010) detected *bla*CTX-M, *ampC*, *cmrA*, *msrA*, *msrB*, *aacC1*, *aph6*, *tetD*, *tetE* and *tetG* from Gulkana glacier in Eastern Alaska, (USA) and Ürumqi glacier in Xinjiang (China). In addition, a massive collection of ARGs including; *bla*IMP, *bla*OXA, *vanA*, *bla*CMY1, *bla*CMY2, *ampC2*, *strA*, *msrA*, *msrB*, *aacC1*, *aacC2*, *aac2'Ic*, *aac3*, *aac(6)*, *ermA*, *ermC*, *ermM*, *ermML*, *ermTR*, *aadA*, *aadB*, *aadE*, *aadK*, *aph6*, *aph3'*, *cat1*, *catA4*, *catB3B4*, *catB5B8*, *catB7*, *tetD*, *tetG*, *tetL*, *tetM*, *tetO1*, *tetO2*, *tetS1*, *tetS2*, *tetX*, *tetW*, *cmlA*, *cmlV*, *cmrA*, *mefA*, *mefE* and *cmx* were detected in remote pristine glaciers of Central Asia (China, Tajikistan and Kyrgyzstan), the Himalayas (Bhutan and Nepal), Africa (Uganda), South America (Chile), North America (Alaska), Arctic (Greenland) and Antarctica regions, thus accentuating the worldwide distribution of ARGs across the cryosphere (Segawa et al. 2013). In our study, 81.81% of Gram-positive bacteria were phenotypically resistant to vancomycin but all remained negative for *vanA* gene PCR amplification, which was the strange thing among our findings. This might be due to the innate active efflux mechanisms in bacteria (Blair et al. 2015).

In a recent study conducted in Greenland, Svalbard, and Caucasus glaciers, Makowska et al. (2020) documented different β -lactamase ARGs (*bla*CTX-M, *bla*SHV, *bla*OXA, *bla*GES, *bla*TEM, *bla*CMY, *bla*VEB, *bla*DHA) plus *intI1* among 138 strains. More precisely, 4.6% corresponded to *bla*CMY, 16.3% to *intI1* from Greenland, 4.6% corresponded to *bla*OXA, and 2.2% each to *bla*SHV and *bla*TEM, plus 14.8% to *intI1* from Georgia. Moreover, from Spitsbergen (High Arctic Island, Norwegian territory), 1.2% were associated with *bla*OXA, and 1.1% with *intI1*, nearly under our findings. Another report by Shen et al. (2019) found a prevalence of 11.3% of β -lactamase from Urumqi glacier China. Allen et al. (2010) reported *bla*NDM-1, *bla*SHV-1, *bla*KPC, and *bla*GES-1 ARGs from the Arctic and sub-Arctic environment. In addition, McCann et al. (2019) reported 8% β -lactamase genes from high Arctic environments. Previous reports highlighted the presence of β -lactamase genes and resistance against

numerous β -lactam antibiotics (ampicillin, tetracyclines, chloramphenicol, sulfamethoxazole, and trimethoprim) among AR bacteria isolated from bird's feces samples from Arctic environment which resolutely involucrated migrating birds in the propagation of ARGs and *integrans* (Literak et al. 2014). Moreover, through clonal expansion, dissemination of AR bacteria, and exchange of mobile genetic elements integrated with resistant determinants via HGT, ARGs can frequently propagate among bacterial communities in a new environment (Van Elsas and Bailey 2002).

The detection of *integrans* in glacier environments regardless of location is associated with biotic pollution in nature (Makowska et al. 2020) and can be used as an indicator of anthropogenic impacts on pristine environments (Ushida et al. 2010). Besides, the plausible modes of transmission of AR bacteria and *integrans* through hydrological systems, wind, migrating birds, vertebrate feces, tourists, and airborne bacteria may induce the geographical dispersal of ARGs and AR bacteria (Makowska et al. 2020; Literak et al. 2014; Segawa et al. 2013). Moreover, the mixing of glacier melt and rainwater induces bacterial dispersion and promotes HGT which contributes to ARGs dissemination (Makowska et al. 2020). Initially, *integrans* were only confined to clinical bacteria but studies conducted in natural pristine environments indicated their ubiquitous presence in environmental microbiomes and their emergence under the influence of selective pressure caused by antibiotics and other pollutants (Cambray et al. 2010). Our *integron*-harbored bacteria showed resistance to multiple antibiotics when compared to *intI1*-negative bacteria which manifest the key role of HGT among diverse glaciated bacteria which is supported by other studies (Makowska et al. 2020; Zhang et al. 2018).

In this current study, we further evaluated ARGs and *intI1* protein alignment with counter-clinical-based references to find out amino acid variations between natural and clinical bacteria. The ARGs and *intI1* showed >97% BLAST similarities and the majority showed aligned identities to Gram-negative bacteria. For *bla*_{OXA-1}, *bla*_{NDM-1}, *bla*_{SHV}, *sul2*, (*tetA*), *qnrB*, *gyrA* and class 1 *integron integrase* amino acid variations were detected, whereas *bla*_{CTX-M15}, *bla*_{CMY-4}, *bla*_{TEM-1}, *aac(6)-Ib3*, *mecA* and *tetB* were negative for amino acid variations (Supplementary File 4). The sequence similarity (99%) for ARGs from glacier bacteria isolated from North Sikkim glaciers has been reported by Sherpa et al. (2020) where aligned identities belonged to Gram-negative bacteria including clinical-based *Escherichia coli* and *Acinetobacter baumannii*. In our current study, *Staphylococcus* species (*S. saprophyticus* (HP7) and *S. equorum* (HP19)) were positive for *mecA* gene beside other ARGs which correlated with the study conducted by Kashuba et al. (2017), where the

ancient *Staphylococcus haemolyticus* strain isolated from Mammoth Mountain permafrost was positive for *mecA* gene and showed 99% homology and 100 query length in both cases. Unfortunately, the available literature data is limited regarding the amino acids-based alignment of ARGs and *integrans* among glaciated bacteria as well with over the counter clinical-based ARGs which need more extensive research for intricate genome-wide comparative analysis.

Conclusion

In an antibiotic-free glacier environment, the occurrence of indigenous AR bacteria with class 1 *integron integrase* is a quintessential scenario and an archetypal case of antibiotic-resistant elements in an antibiotic-free environment. The current study also reflects the same where the culturable glacier bacteria was found with significant antibiotic resistance, integrated with various ARGs plus class 1 *integron integrase* and was more prevalent among *Proteobacteria*, Gram-negative from sediment source. The *Brevundimonas diminuta* (HP21) and *Staphylococcus equorum* (HP19) were the most resistant strains, yielded the highest MAR indexes, and harbored maximum ARGs. The sequence alignment of some ARGs and *intI1* showed amino acids variation and were found in associations with the clinical-based counterparts and it might be inferred that the presence of ARGs and *intI1* in glacier bacteria is an indication of the evolutionary process of opportunistic as well as relevant clinically resistant species. In a glacier environment, native bacteria are exposed to an eternal selective pressure of elevated UV radiation doses, daily freeze-thaw cycles, oligotrophy, radionuclides, and heavy metals which evolves antibiotics production and counter-resistance mechanisms. The existence of AR bacteria in glaciers can be indicated as an adaptation to survive hostile environments. Under the influence of climatic changes like; global warming, the glaciers will favor the expansion of ecosystems and it is crucial to mention the risks that are associated with the dissemination of glacial AR bacteria to new habitats when there is an elimination of the naturally existing ecological filters along with the migration of animals and human activities near non-polar glaciers.

As a final remark, it's worth mentioning that; (1) The existence of AR bacteria possessed with ARGs and class 1 *integron integrase* abundance among non-polar glaciated bacteria, (2) The rapid melting of glaciers with emancipation and reactivation of dormant bacteria, and (3) The promising donor behavior of environmental bacteria in sequential episodes of HGT, is of significant concern regarding public health. Therefore, precautionary measures should be taken to monitor glacier melting water as a potentially hazardous

source, especially in areas where the only source of domestic fresh water is from glaciers.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11274-023-03532-4>.

Acknowledgements The authors did not receive support from any organization for the submitted work.

Author contributions SN, FH, MR and ILP conceived and designed study. SN and WQB performed research. MR, FH and AAS analyzed data. SN and WQB wrote the manuscript. All authors read and approved the manuscript.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication All authors have read and approved the final version.

References

- Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* 8(4):251–259. <https://doi.org/10.1038/nrmicro2312>
- Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J (2009) Functional metagenomics reveals diverse β -lactamases in a remote alaskan soil. *ISME J* 3(2):243–251. <https://doi.org/10.1038/ismej.2008.86>
- Amato P, Hennebelle R, Magand O, Sancelme M, Delort AM, Barbante C, Boutron C, Ferrari C (2007) Bacterial characterization of the snow cover at Spitzberg, Svalbard. *FEMS Microbiol Ecol* 59(2):255–264. <https://doi.org/10.1111/j.1574-6941.2006.00198.x>
- Anesio AM, Laybourn-Parry J (2012) Glaciers and ice sheets as a biome. *Trends Ecol Evol* 27(4):219–225. <https://doi.org/10.1016/j.tree.2011.09.012>
- Anesio AM, Lutz S, Christmas NA, Benning LG (2017) The microbiome of glaciers and ice sheets. *npj Biofilms and Microbiomes* 3(1):1–1. <https://doi.org/10.1038/s41522-017-0019-0>
- Baghel VS, Tripathi RD, Ramteke PW, Gopal K, Dwivedi S, Jain RK, Rai UN, Singh SN (2005) Psychrotrophic proteolytic bacteria from cold environment of Gangotri glacier, western Himalaya, India. *Enzyme Microb Technol* 36(5–6):654–659. <https://doi.org/10.1016/j.enzmictec.2004.09.005>
- Ball MM, Gómez W, Magallanes X, Rosales R, Melfo A, Yarzabal LA (2014) Bacteria recovered from a high-altitude, tropical glacier in Venezuelan Andes. *World J Microbiol Biotechnol* 30(3):931–941. <https://doi.org/10.1007/s11274-013-1511-1>
- Bhatia M, Sharp M, Foght J (2006) Distinct bacterial communities exist beneath a high Arctic polythermal glacier. *Appl Environ Microbiol* 72(9):5838–5845. <https://doi.org/10.1128/AEM.00595-06>
- Blair J, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13(1):42–51. <https://doi.org/10.1038/nrmicro3380>
- Boetius A, Anesio AM, Deming JW, Mikucki JA, Rapp JZ (2015) Microbial ecology of the cryosphere: sea ice and glacial habitats. *Nat Rev Microbiol* 13(11):677–690. <https://doi.org/10.1038/nrmicro3522>
- Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol* 8(7):1137–1144. <https://doi.org/10.1111/j.1462-2920.2006.01054.x>
- Cambray G, Guerout AM, Mazel D (2010) Integrons. *Annu Rev Genet* 44:41–66. <https://doi.org/10.1146/annurev-genet-102209-163504>
- Casanueva A, Tuffin M, Cary C, Cowan DA (2010) Molecular adaptations to psychrophily: the impact of ‘omic’ technologies. *Trends Microbiol* 18(8):374–381. <https://doi.org/10.1016/j.tim.2010.05.002>
- Chaturvedi P, Reddy GS, Shivaji S (2005) *Dyadobacter hamtensis* sp. nov., from Hamta glacier, located in the Himalayas, India. *Int J Syst Evol Microbiol* 55(5):2113–2117. <https://doi.org/10.1099/ijs.0.63806-0>
- Chaturvedi P, Shivaji S (2006) *Exiguobacterium indicum* sp. nov., a psychrophilic bacterium from the Hamta glacier of the Himalayan Mountain ranges of India. *Int J Syst Evol Microbiol* 56(12):2765–2770. <https://doi.org/10.1099/ijs.0.64508-0>
- Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie RI (2001) Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* 67(4):1494–1502. <https://doi.org/10.1128/AEM.67.4.1494-1502.2001>
- Christner BC, Mosley-Thompson E, Thompson LG, Zagorodnov V, Reeve JN (2002) Isolation and Identification of Bacteria from Ancient and Modern Ice Cores. *Patagonian Icefields 9–15*. Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-0645-4_2
- Catalog (2018) Clinical & Laboratory standards institute. https://clsi.org/media/2042/catalog2018_web-unlinked
- Cowan DA, Casanueva A, Stafford W (2007) Ecology and biodiversity of cold-adapted microorganisms. *Physiol Biochem Extremophiles* 117–32. <https://doi.org/10.1128/9781555815813.ch9>
- D’Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB (2011) Antibiotic resistance is ancient. *Nature* 477(7365):457–461. <https://doi.org/10.1038/nature10388>
- D’Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. *Sci* 311(5759):374–377. <https://doi.org/10.1126/science.1120800>
- De Souza MJ, Nair S, Bharathi L, Chandramohan D (2006) Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters *Ecotoxicol* 15(4):379–84. <https://doi.org/10.1007/s10646-006-0068-2>
- Dong H, Zhang G, Jiang H, Yu B, Chapman LR, Lucas CR, Fields MW (2006) Microbial diversity in sediments of saline Qinghai Lake, China: linking geochemical controls to microbial ecology. *Microb Ecol* 51(1):65–82. <https://doi.org/10.1007/s00248-005-0228-6>
- Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Sci* 337(6098):1107–1111. <https://doi.org/10.1126/science.1220>
- Furushita M, Shiba T, Maeda T, Yahata M, Kaneoka A, Takahashi Y, Torii K, Hasegawa T, Ohta M (2003) Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl Environ Microbiol* 69(9):5336–5342. <https://doi.org/10.1128/AEM.69.9.5336-5342.2003>
- Gibbs SG, Green CF, Tarwater PM, Mota LC, Mena KD, Scarpino PV (2006) Isolation of antibiotic-resistant bacteria from the air plume downwind of a swine confined or concentrated animal feeding

- operation. *Environ Health Perspect* 114(7):1032–1037. <https://doi.org/10.1289/ehp.8910>
- Gibson Molly K, Forsberg Kevin J, Dantas G (2015) Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. *ISME J* 9(1):207–216. <https://doi.org/10.1038/ismej.2014.106>
- Gilichinsky DA, Wilson GS, Friedmann EI, McKay CP, Sletten RS, Rivkina EM, Vishnivetskaya TA, Erokhina LG, Ivanushkina NE, Kochkina GA, Sheherbakova VA (2007) Microbial populations in Antarctic permafrost: biodiversity, state, age, and implication for astrobiology. *Astrobiology* 7(2):275–311. <https://doi.org/10.1089/ast.2006.0012>
- Glaring MA, Vester JK, Lylloff JE, Abu Al-Soud W, Sørensen SJ, Stougaard P (2015) Microbial diversity in a permanently cold and alkaline environment in Greenland. *PloS one* 10(4):e0124863. <https://doi.org/10.1371/journal.pone.0124863>
- Goñi-Urriza M, Capdepuy M, Arpin C, Raymond N, Caumette P, Quentin C (2000) Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas spp.* *Appl Environ Microbiol* 66(1):125–132. <https://doi.org/10.1128/AEM.66.1.125-132.2000>
- Gupta GN, Srivastava S, Khare SK, Prakash V (2014) Extremophiles: an overview of microorganism from extreme environment. *Int J Agri Environ Biotechnol* 7(2):371. <https://doi.org/10.5958/2230-732X.2014.00258.7>
- Hall BG, Barlow M (2004) Evolution of the serine β -lactamases: past, present and future. *Drug Resist Updates* 7(2):111–123. <https://doi.org/10.1016/j.drug.2004.02.003>
- Hawkey PM (2008) The growing burden of antimicrobial resistance. *J Antimicrob Chemother* 62(suppl1):i1–9. <https://doi.org/10.1093/jac/dkn241>
- James E, Wong CM (2015) Antibiotic resistance among bacteria from Antarctic and Tropics. *Trans Sci Tech* 2:16–20. <https://doi.org/10.15242/iicbe.c0815032>
- Junge K, Christner B, Staley JT (2011) Diversity of psychrophilic bacteria from sea ice-and glacial ice communities. *Extremophiles Handb.* https://doi.org/10.1007/978-4-431-53898-1_39
- Kamekura M (1998) Diversity of extremely halophilic bacteria. *Extremophiles* 2(3):289–295. <https://doi.org/10.1007/s007920050071>
- Kaser G (1999) A review of the modern fluctuations of tropical glaciers. *Glob Planet Change* 22(1–4):93–103. [https://doi.org/10.1016/S0921-8181\(99\)00028-4](https://doi.org/10.1016/S0921-8181(99)00028-4)
- Kashuba E, Dmitriev AA, Kamal SM, Melefors O, Griva G, Römling U, Ernberg I, Kashuba V, Brouckov A (2017) Ancient permafrost staphylococci carry antibiotic resistance genes. *Microb Ecol Health Dis* 28(1):1345574. <https://doi.org/10.1080/16512235.2017.1345574>
- Kato C, Li L, Nogi Y, Nakamura Y, Tamaoka J, Horikoshi K (1998) Extremely barophilic bacteria isolated from the Mariana Trench, Challenger Deep, at a depth of 11,000 meters. *Appl Environ Microbiol* 64(4):1510–1513. <https://doi.org/10.1128/AEM.64.4.1510-1513.1998>
- Kirby-Bauer A (1996) Antimicrobial sensitivity testing by agar diffusion method. *J Clin Pathol* 44:493
- Kishore KH, Begum Z, Pathan AA, Shivaji S (2010) *Paenibacillus glacialis* sp. nov., isolated from the Kafni glacier of the Himalayas, India. *Int J Syst Evol Microbiol* 60(8):1909–1913. <https://doi.org/10.1099/ijs.0.015271-0>
- Krumperman PH (1983) Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol* 46(1):165–170. <https://doi.org/10.1128/aem.46.1.165-170.1983>
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35(6):1547. <https://doi.org/10.1093/molbev/msy096>
- Lanoil B, Skidmore M, Priscu JC, Han S, Foo W, Vogel SW, Tulaczky S, Engelhardt H (2009) Bacteria beneath the West Antarctic ice sheet. *Environ Microbiol* 11(3):609–615. <https://doi.org/10.1111/j.1462-2920.2008.01831.x>
- Levy SB, Marshall B (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 10(12):S122–S129. <https://doi.org/10.1038/nm1145>
- Lighthart B, Shaffer BT (1995) Airborne bacteria in the atmospheric surface layer: temporal distribution above a grass seed field. *Appl Environ Microbiol* 61(4):1492–1496. <https://doi.org/10.1128/aem.61.4.1492-1496.1995>
- Liu Y, Yao T, Jiao N, Kang S, Xu B, Zeng Y, Huang S, Liu X (2009) Bacterial diversity in the snow over Tibetan Plateau Glaciers. *Extremophiles* 13(3):411–423. <https://doi.org/10.1007/s00792-009-0227-5>
- Lo Giudice A, Bruni V, Michaud L (2007) Characterization of Antarctic psychrotrophic bacteria with antibacterial activities against terrestrial microorganisms. *J Basic Microbiol* 47(6):496–505. <https://doi.org/10.1002/jobm.200700227>
- Lo Giudice A, Casella P, Bruni V, Michaud L (2013) Response of bacterial isolates from Antarctic shallow sediments towards heavy metals, antibiotics and polychlorinated biphenyls. *Ecotoxicology* 22(2):240–250. <https://doi.org/10.1007/s10646-012-1020-2>
- Lutz S, Anesio AM, Edwards A, Benning LG (2015) Microbial diversity on Icelandic glaciers and ice caps. *Front Microbiol* 2015;6:307. <https://doi.org/10.3389/fmicb.2015.00307>
- MacElroy RD (1974) Some comments on the evolution of extremophiles. *BioSystems* 6(1):74–75. [https://doi.org/10.1016/0303-2647\(74\)90026-4](https://doi.org/10.1016/0303-2647(74)90026-4)
- Makowska N, Zawierucha K, Nadobna P, Piątek-Bajan K, Krajewska A, Szwedek J, Iwasieczko P, Mokracka J, Koczura R (2020) Occurrence of integrons and antibiotic resistance genes in cryoconite and ice of Svalbard, Greenland, and the Caucasus glaciers. *Sci Total Environ* 716:137022. <https://doi.org/10.1016/j.scitotenv.2020.137022>
- Malik A, Çelik EK, Bohn C, Böckelmann U, Knobel K, Grohmann E (2008) Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol Lett* 279(2):207–216. <https://doi.org/10.1111/j.1574-6968.2007.01030.x>
- Männistö MK, Häggblom MM (2006) Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Sys Appl Microbiol* 29(3):229–243. <https://doi.org/10.1016/j.syapm.2005.09.001>
- Margesin R, Miteva V (2011) Diversity and ecology of psychrophilic microorganisms. *Res Microbiol* 162(3):346–361. <https://doi.org/10.1016/j.resmic.2010.12.004>
- Martínez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. *Sci* 321(5887):365–367. <https://doi.org/10.1126/science.1159483>
- McCann CM, Christgen B, Roberts JA, Su JQ, Arnold KE, Gray ND, Zhu YG, Graham DW (2019) Understanding drivers of antibiotic resistance genes in high Arctic soil ecosystems. *Environ Int* 125:497–504. <https://doi.org/10.1016/j.envint.2019.01.034>
- Miller RV, Gammon K, Day MJ (2009) Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, Antarctica. *Can J Microbiol* 55(1):37–45. <https://doi.org/10.1139/W08-119>
- Montross SN, Skidmore M, Tranter M, Kivimäki AL, Parkes RJ (2013) A microbial driver of chemical weathering in glaciated systems. *Geology* 41(2):215–218. <https://doi.org/10.1130/G33572.1>
- Morita RY (1975) Psychrophilic bacteria. *Bacteriol Rev* 39(2):144–167. <https://doi.org/10.1128/br.39.2.144-167.1975>

- Mueller DR, Pollard WH (2004) Gradient analysis of cryoconite ecosystems from two polar glaciers. *Polar Biol* 27(2):66–74. <https://doi.org/10.1007/s00300-003-0580-2>
- Musilova M, Tranter M, Bennett SA, Wadham J, Anesio AM (2015) Stable microbial community composition on the Greenland ice sheet. *Front Microbiol* 6:193. <https://doi.org/10.3389/fmicb.2015.00193>
- Nakamura Y, Asada C, Sawada T (2003) Production of antibacterial violet pigment by psychrotropic bacterium RT102 strain. *Biotechnol Bioprocess Eng* 8(1):37–40. <https://doi.org/10.1007/BF02932896>
- Parnell J, McMahon S (2016) Physical and chemical controls on habitats for life in the deep subsurface beneath continents and ice. *Philos Trans R Soc A* 374(2059):20140293. <https://doi.org/10.1098/rsta.2014.0293>
- Perron GG, Whyte L, Turnbaugh PJ, Goordial J, Hanage WP, Dantas G, Desai MM (2015) Functional characterization of bacteria isolated from ancient arctic soil exposes diverse resistance mechanisms to modern antibiotics. *PLoS one* 10(3):e0069533. <https://doi.org/10.1371/journal.pone.0069533>
- Rafiq M, Hayat M, Anesio AM, Jamil SU, Hassan N, Shah AA, Hasan F (2017) Recovery of metallo-tolerant and antibiotic resistant psychrophilic bacteria from Siachen glacier. *Pakistan PLoS one* 12(7):e0178180. <https://doi.org/10.1371/journal.pone.0178180>
- Rafiq M, Hayat M, Hassan N, Ibrar M, Haleem A, Rehman M, Ahmad F, Shah AA, Hasan F (2016) Characterization of antibacterial compounds produced by psychrotrophic *Alcaligenes faecalis* HTP6 isolated from Passu Glacier, Pakistan. *Int J Biosci* 8(5):122–135. <https://doi.org/10.12692/ijb/8.5.122-135>
- Rafiq M, Hayat M, Zada S, Sajjad W, Hassan N, Hasan F (2019) Geochemistry and bacterial recovery from Hindu Kush Range glacier and their potential for metal resistance and antibiotic production. *Geomicrobiol J* 36(4):326–338. <https://doi.org/10.1080/01490451.2018.1551947>
- Reddy GS, Prabakaran SR, Shivaji S (2008) *Leifsonia pindariensis* sp. nov., isolated from the Pindari glacier of the Indian Himalayas, and emended description of the genus *Leifsonia*. *Int J Syst Evol Microbiol* 58(9):2229–2234. <https://doi.org/10.1099/ijs.0.65715-0>
- Reddy GS, Pradhan S, Manorama R, Shivaji S (2010) *Cryobacterium pindariense* sp. nov., a psychrophilic bacterium from a Himalayan glacier. *Int J Syst Evol Microbiol* 60:866–870. <https://doi.org/10.1099/ijs.0.011775-0>
- Rothschild LJ, Mancinelli RL (2001) Life in extreme environments. *Nature* 409(6823):1092–1101. <https://doi.org/10.1038/35059215>
- Russell AD (2003) Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect Dis* 3(12):794–803. [https://doi.org/10.1016/S1473-3099\(03\)00833-8](https://doi.org/10.1016/S1473-3099(03)00833-8)
- Sakamoto T, Murata N (2002) Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. *Curr Opin Microbiol* 5(2):206–210. [https://doi.org/10.1016/S1369-5274\(02\)00306-5](https://doi.org/10.1016/S1369-5274(02)00306-5)
- Segawa T, Takeuchi N, Rivera A, Yamada A, Yoshimura Y, Barcaza G, Shinbori K, Motoyama H, Kohshima S, Ushida K (2013) Distribution of antibiotic resistance genes in glacier environments. *Environ Microbiol Rep* 5(1):127–134. <https://doi.org/10.1111/1758-2229.12011>
- Segawa T, Takeuchi N, Ushida K, Kanda H, Kohshima S (2010) Altitudinal changes in a bacterial community on Gulkana Glacier in Alaska. *Microb Environ* 25(3):171–182. <https://doi.org/10.1264/jsme2.ME10119>
- Shen JP, Li ZM, Hu HW, Zeng J, Zhang LM, Du S, He JZ (2019) Distribution and succession feature of antibiotic resistance genes along a soil development chronosequence in Urumqi No. 1 Glacier of China. *Front Microbiol* 1569. <https://doi.org/10.3389/fmicb.2019.01569>
- Shen L, Yao T, Xu B, Wang H, Jiao N, Kang S, Liu X, Liu Y (2012) Variation of culturable bacteria along depth in the East Rongbuk ice core. *Mt Everest Geosci Front* 3(3):327–334. <https://doi.org/10.1016/j.gsf.2011.12.013>
- Sherpa MT, Najar IN, Das S, Thakur N (2020) Distribution of antibiotic and metal resistance genes in two glaciers of North Sikkim, India. *Ecotoxicol Environ Saf* 203:111037. <https://doi.org/10.1016/j.ecoenv.2020.111037>
- Shivaji S, Chaturvedi P, Reddy GS, Suresh K (2005) *Pedobacter himalayensis* sp. nov., from the Hamta glacier located in the Himalayan mountain ranges of India. *Int J Syst Evol Microbiol* 55(3):1083–1088. <https://doi.org/10.1099/ijs.0.63532-0>
- Shivaji S, Madhu S, Singh S (2011) Extracellular synthesis of antibacterial silver nanoparticles using psychrophilic bacteria. *Process Biochem* 46(9):1800–1807. <https://doi.org/10.1016/j.procbio.2011.06.008>
- Stibal M, Tranter M, Benning LG, Řehák J (2008) Microbial primary production on an Arctic glacier is insignificant in comparison with allochthonous organic carbon input. *Environ Microbiol* 10(8):2172–2178. <https://doi.org/10.1111/j.1462-2920.2008.01620.x>
- Takeuchi N, Kohshima S (2004) A snow algal community on Tyndall Glacier in the Southern Patagonia Icefield, Chile. *Arct Antarct Alp Res* 36(1):92–99. <https://doi.org/10.1657/1523-0430.2004.036.0092:ASACOT.2.0.CO;2>
- Takeuchi N, Uetake J, Fujita K, Aizen VB, Nikitin SD (2006) A snow algal community on Akkem Glacier in the Russian Altai Mountains. *Ann Glaciol* 43:378–384. <https://doi.org/10.3189/172756406781812113>
- Tomova I, Stoilova-Disheva M, Lazarkevich I, Vasileva-Tonkova E (2015) Antimicrobial activity and resistance to heavy metals and antibiotics of heterotrophic bacteria isolated from sediment and soil samples collected from two Antarctic islands. *Front Life Sci* 8(4):348–357. <https://doi.org/10.1080/21553769.2015.1044130>
- Ushida K, Segawa T, Kohshima S, Takeuchi N, Fukui K, Li Z, Kanda H (2010) Application of real-time PCR array to the multiple detection of antibiotic resistant genes in glacier ice samples. *J Gen Appl Microbiol* 56(1):43–52. <https://doi.org/10.2323/jgam.56.43>
- Van Elsas JD, Bailey MJ (2002) The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol* 42(2):187–197. <https://doi.org/10.1111/j.1574-6941.2002.tb01008.x>
- Van Goethem MW, Pierneef R, Bezuidt OK, Van De Peer Y, Cowan DA, Makhalanyane TP (2018) A reservoir of ‘historical’ antibiotic resistance genes in remote pristine Antarctic soils. *Microbiome* 6(1):1–2. <https://doi.org/10.1186/s40168-018-0424-5>
- Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. *Proc Natl Acad Sci* 95(12):6578–6583. <https://doi.org/10.1073/pnas.95.12.6578>
- Wright GD (2010) Antibiotic resistance in the environment: a link to the clinic? *Curr Opin Microbiol* 13(5):589–594. <https://doi.org/10.1016/j.mib.2010.08.005>
- Zhang S, Yang G, Hou S, Zhang T, Li Z, Liang F (2018) Distribution of ARGs and MGEs among glacial soil, permafrost, and sediment using metagenomic analysis. *Environ Pollut* 234:339–346. <https://doi.org/10.1016/j.envpol.2017.11.031>
- Zhang DC, Brouchkov A, Griva G, Schinner F, Margesin R (2013) Isolation and characterization of bacteria from ancient Siberian permafrost sediment. *Biology* 2(1):85–106. <https://doi.org/10.3390/biology2010085>
- Zhang S, Yang G, Wang Y, Hou S (2010) Abundance and community of snow bacteria from three glaciers in the Tibetan Plateau. *J Environ Sci* 22(9):1418–1424. [https://doi.org/10.1016/S1001-0742\(09\)60269-2](https://doi.org/10.1016/S1001-0742(09)60269-2)

- Zhang XX, Zhang T, Fang HH (2009) Antibiotic resistance genes in water environment. *Appl Microbiol Biotechnol* 82(3):397–414. <https://doi.org/10.1007/s00253-008-1829-z>
- Zhang S, Hou S, Ma X, Qin D, Chen T (2007) Culturable bacteria in Himalayan glacial ice in response to atmospheric circulation. *Biogeosciences* 4(1):1–9. <https://doi.org/10.5194/bg-4-1-2007>

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.